Université de Montréal

Implication of IL-2 and IL-15 in the exhaustion of $CD8^+$ T cells during a chronic viral infection

Par

Jean-Christophe Beltra

Département de Microbiologie, Infectiologie et Immunologie

Faculté de Médecine

Thèse présentée à la Faculté des Études Supérieures

en vue de l'obtention du grade de Ph.D.

en microbiologie et immunologie

Mars 2017

© Jean-Christophe Beltra, 2017

Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse est intitulée

Implication of IL-2 and IL-15 in the exhaustion of CD8 T cells during a chronic viral infection

Présentée par:
Jean-Christophe Beltra

A été évaluée par un jury composé des personnes suivantes:
Dre Sylvie Lesage, president rapporteur
Dre Hélène Decaluwe, directrice de recherche
Dr Nicolas Chomont, member du jury
Dr Martin Richer, examinateur externe
Dr Walid Mourad, représentant du doyen de la FESP

Abstract

CD8$^+$ T cell exhaustion is a unique differentiation pathway which occurs during particular pathological contexts such as chronic viral infections (i.e. HIV, HCV and HBV) and cancers in which antigen (Ag) persists in the host. It appears clear now that this mechanism provokes the failure of adaptive responses against these pathologies and is particularly harmful to humans. The study of this process has led to the discovery of relevant molecules ("immune checkpoints") that can be targeted to prevent and/or reverse exhaustion. Ensuing clinical trials have provided extremely promising results in the treatment of several cancers. However, although these targeted therapies allow a temporary regain of CD8$^+$ T cell functions they still fail at reversing the exhaustion process. It is thus crucial to investigate the causative factors of such process that remain to be identified.

The common gamma-chain (γ_c) family of cytokines which includes IL-2, -4, -7, -9, -15, and -21 are key soluble mediators involved in the development of adaptive immunity. These cytokines are intimately linked to T cell development, homeostasis, differentiation and maintenance. Among them, IL-2 and IL-15 display important functions on CD8$^+$ T cell differentiation during an acute viral infection. However, impact of these cytokines on CD8$^+$ T cell responses during a chronic viral infection remains to be investigated. Based on current knowledge of the functions of IL-2 and IL-15 on CD8$^+$ T cell differentiation during an acute viral infection, we hypothesized that these cytokines promote CD8$^+$ T cell exhaustion during a chronic viral infection.

We first demonstrate in a mouse model of chronic viral infection (LCMV clone 13) and patients with chronic HCV that the IL-2-receptor β chain (IL2Rβ[CD122]) a cytokine receptor chain which binds to both IL-2 and IL-15 is selectively expressed on exhausted CD8$^+$ T cells during a chronic viral infection. The intensity of CD122 expression positively correlates with severe exhaustion of CD8$^+$ T cells in mice and humans. Using a mouse model in which CD8$^+$ T cells lack the expression of the IL2Rβ-chain, we demonstrate that IL-2 and IL-15 control several aspects of exhaustion. IL-2 and

IL-15-dependent signals sustain the expression of several inhibitory receptors (characteristic of exhaustion) on $CD8^+$ T cells and directly control the expression of some of them (e.g. 2B4 and TIM-3). IL-2 and IL-15 also direct the terminal exhaustion of $CD8^+$ T cells and irreversibly abrogate their developmental plasticity toward memory T cell development. Together, we show for the first time key functions of IL-2 and IL-15 in directing $CD8^+$ T cell exhaustion during a chronic viral infection.

Next, we investigated the unique and redundant functions of IL-2 and IL-15 on $CD8^+$ T cell exhaustion. We also determined individual time-frames of these cytokines and intracellular pathways by which they control $CD8^+$ T cell exhaustion. IL-2 and IL-15 cooperate to promote 2B4 and TIM-3 expression on $CD8^+$ T cells, and these cytokines likely collaborate to direct terminal exhaustion. In contrast, IL-2-dependent signals during priming preclude subsequent differentiation into central memory cells (Tcm) while prolonged exposure to IL-15 upon viral persistence likely suppresses effector memory cell (Tem) developmental potential. Finally, we demonstrate that the JAK3/STAT5 pathway is the dominant pathway by which IL-2 and IL-15 direct $CD8^+$ T cell exhaustion.

This thesis provides evidence of novel functions of IL-2 and IL-15 in directing $CD8^+$ T cell exhaustion during a chronic viral infection. These results increase our understanding of the $CD8^+$ T cell exhaustion process and demonstrate for the first time the involvement of cytokines. We hope that this work will contribute to the improvement of actual therapeutic strategies against chronic viral infections and cancers.

Key words: CD8 T cell, chronic viral infection, exhaustion, IL-2, IL-15, memory T cell, STAT5.

Résumé

L'épuisement des lymphocytes T $CD8^+$ (LT CD8) est une voie de différentiation unique survenant lors de contextes pathologiques particuliers ayant en commun la persistance d'antigènes dans l'hôte, tel que les infections virales chroniques (expl : VIH, hépatites B et C) et différents types de cancers. Il apparait aujourd'hui très clairement que ce mécanisme est à l'origine de l'échec de l'immunité adaptative face à ces pathologies particulièrement néfastes pour l'homme. L'étude de ce processus a mené à la découverte de cible thérapeutiques d'un grand intérêt (« immune checkpoints ») pouvant être ciblées pour corriger et/ou reverser l'épuisement. Les essais thérapeutiques ayant découlés de ces découvertes ont donné des résultats extrêmement prometteurs dans le traitement de plusieurs cancers. Cependant, bien que ces thérapies ciblées permettent un regain temporaire de la fonction des LT $CD8^+$, elles ne permettent pas d'inverser le processus d'épuisement. Il est donc crucial aujourd'hui de se tourner vers les agents causateurs de cet état d'épuisement qui restent très méconnues à ce jour.

La famille de cytokines partageant la chaine commune gamma (cytokines γ_c) comprenant l'IL-2 -4 -7 -9 -15 et -21 sont des acteurs solubles clés de l'immunité adaptative. Ces cytokines sont intimement liées aux processus de développement, d'homéostasie, de différenciation et de maintenance des lymphocytes T. Parmi elles, l'IL-2 et l'IL-15 ont un rôle majeur dans le processus de différenciation des LT $CD8^+$ au cours d'une infection virale aigue. Malgré cela, l'implication de ces cytokines dans l'épuisement des LT $CD8^+$ dans un contexte d'infection virale chronique n'a jamais été investiguée. En se basant sur les connaissances actuelles des rôles de l'IL-2 et de l'IL-15 sur la différenciation des LT $CD8^+$ au cours d'une infection virale aigue, nous avons émis l'hypothèse que ces cytokines pourraient promouvoir l'épuisement dans un contexte d'infection virale chronique.

Dans un premier temps, nous avons démontré chez l'homme (patients atteints d'hépatite C chronique) et la souris (modèle LCMV Clone 13) que la chaîne β du récepteur à l'IL-2 (IL2Rβ[CD122]) qui se lie à l'IL-2 et l'IL-15 reste sélectivement

exprimée à la surface des LT $CD8^+$ épuisés au cours d'une infection virale chronique. De plus, une expression élevée de cette chaîne de récepteur corrèle avec un épuisement plus sévère des LT $CD8^+$ chez l'homme et la souris. En développant un modèle murin dans lequel les LT $CD8^+$ sont déficients pour cette chaîne, nous avons démontré que l'IL-2 et IL-15 contrôlent plusieurs aspects clés du processus d'épuisement. Ces cytokines augmentent l'expression de plusieurs récepteurs inhibiteurs (caractéristiques de l'épuisement) et contrôlent même directement l'expression de certains d'entre eux (notamment 2B4 et TIM-3). L'IL-2 et l'IL-15 dirigent également la différenciation terminale des LT $CD8^+$ vers un état d'épuisement extrême et abrogent de manière irréversible leur potentiel de différenciation en cellules mémoires. Nous montrons donc pour la première fois un rôle clé de l'IL-2 et l'IL-15 dans l'épuisement des LT $CD8^+$ au cours d'une infection virale chronique.

Dans un deuxième temps nous avons investigué les fonctions individuelles et redondantes de l'IL-2 et l'IL-15 dans l'épuisement des LT $CD8^+$. Nous avons également déterminé les fenêtres d'actions déterminantes de ces cytokines et les mécanismes intracellulaires clés par lesquels elles contrôlent le processus d'épuisement. L'IL-2 et l'IL-15 coopèrent pour promouvoir l'expression de 2B4 et TIM-3 à la surface des LT $CD8^+$ et ces cytokines semblent collaborer pour diriger leur différenciation terminale. En revanche, les signaux médiés par l'IL-2 pendant la phase de « priming » abrogent sélectivement leur potentiel de différenciation en cellules T centrales mémoires (Tcm) alors que l'IL-15 semble plutôt supprimer celle des T effecteurs mémoires (Tem) pendant la phase chronique. Pour finir, nous avons identifié la voie JAK3/STAT5 comme étant la principale voie intracellulaire par laquelle l'IL-2 et l'IL-15 dirigent l'épuisement des LT $CD8^+$.

Au cours de cette thèse, nous avons donc mis en évidence un nouveau rôle de l'IL-2 et l'IL-15 dans l'épuisement des LT $CD8^+$ au cours d'une infection virale chronique. Nos résultats apportent une meilleure compréhension du processus d'épuisement des LT CD8+ et démontrent pour la première fois une implication des

cytokines. Nous espérons que ces travaux contribueront à améliorer les stratégies thérapeutiques actuelles contre le cancer et les infections virales chroniques.

Mots clés: lymphocyte T CD8, infection virale chronique, épuisement, IL-2, IL-15, cellule T mémoire, STAT5.

Summary

ABSTRACT .... **IV**

RÉSUMÉ .... **VI**

SUMMARY .... **IX**

LIST OF ABBREVIATIONS .... **XV**

ACKNOWLEDGEMENTS .... **XVIII**

INTRODUCTION **1**

I- CD8$^+$ T CELL RESPONSE TO A CHRONIC VIRAL INFECTION: LESSONS FROM INFECTIOUS MOUSE MODELS **1**

1.1- Key mouse models of acute and persistent viral infection 2

1.2- CD8$^+$ T cell response to an acute viral infection 3

1.3- CD8$^+$ T cell response to a chronic viral infection 5

1.3.1- Overview 5

1.3.2- Disruption of immunodominance hierarchy and loss of antiviral CD8$^+$ T cell responses 6

1.3.3- Loss of effector functions 11

1.3.4- Inhibitory receptors 13

1.3.4.1- Programmed cell death 1 (PD-1) *14*

1.3.4.2- Lymphocyte-activation gene 3 (LAG-3) *16*

1.3.4.3- CD160 .... *17*

1.3.4.4- Signaling lymphocyte activation (SLAM) family receptor 2B4 (CD244) *18*

1.3.4.5- T-cell Ig and mucin-domain containing-3 (TIM-3) *21*

1.3.4.6- Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) *22*

1.3.4.7- T-cell Ig and ITIM domain (TIGIT) *23*

1.3.5- Loss of plasticity to memory T cell development 26

II- CD8$^+$ T CELL EXHAUSTION: A UNIQUE DIFFERENTIATION PROGRAM **27**

2.1- A unique transcriptional program 28

2.2- Key transcription factors 31

2.2.1- T-BET (*Tbx21*) 32
2.2.2- EOMESODERMIN (*Eomes*) 34
2.2.3- T Cell Factor 1 (*Tcf7*) 36
2.2.4- BLIMP-1 (*Prdm1*) 38
2.2.5- NFATc1 (*Nfat2*) 40
2.2.6- BATF (*Batf*) 41
2.2.7- FOXO1 (*Foxo1*) 43
2.3- Unique epigenetic landscape 47
2.3.1- Epigenetic remodeling and T cell differentiation 47
2.3.1.1- Pioneer studies on CD4$^+$ T cells 47
2.3.1.2- DNA methylation and CD8$^+$ T cell differentiation 48
2.3.1.3- Histone modifications and CD8$^+$ T cell differentiation 48
2.3.1.4- Lineage-specific TFs: impact on epigenetic remodeling 49
2.3.2- Unique epigenetic features of exhausted CD8$^+$ T cells 49
III- DYNAMIC OF EXHAUSTED CD8$^+$ T CELLS 53
3.1- Lineage origin of exhausted CD8$^+$ T cells 53
3.2- PD-1^{hi} and PD-1$^{int/lo}$ effectors: Distinct but lineage related populations 54
3.2.1- Characteristics of PD-1^{hi} and PD-1$^{int/low}$ effectors 54
3.2.2- Development and lineage relationship between PD-1$^{int/lo}$ and PD-1^{hi} effectors 56
3.3- TCF-1$^+$ CXCR5$^+$ stem cell-like effectors 57
IV- FACTORS UNDERLYING CD8$^+$ T CELL EXHAUSTION AND POTENTIAL NEWCOMERS 60
4.1- Antigen persistence 61
4.2- CD4$^+$ T cell help 62
4.3- Lack of co-stimulation 63
4.3.1- Altered DC priming 63
4.3.2- Tumor Necrosis Factor Receptors (TNFRs) superfamily 64
4.3.2.1- CD27 (Tnfrsf7)/CD70 (Tnfsf7) 64
4.3.2.2- GITR (Tnfrsf18)/GITR-L (Tnfsf18) 66
4.3.2.3- OX40 (Tnfrsf4)/OX40-L (Tnfsf4) 66

4.3.2.4- 4-1BB (Tnfrsf9)/4-1BBL (Tnfsf9) ... *67*
4.4- Regulatory T cells (Tregs) ... 68
4.5- Myeloid-derived suppressor cells (MDSCs) ... 69
V- Cytokines and persistent viral infections: Implication on T cell exhaustion ... 70
5.1- Immunoregulatory cytokines ... 70
5.1.1- IL-10 ... 70
5.1.2- Transforming Growth Factor β (TGFβ) ... 72
5.2- Inflammatory cytokines ... 76
5.2.1- Type I interferons (IFN) ... 76
5.2.2- Glycoprotein (gp130)-dependent cytokines ... 79
5.3- Common gamma (γ_c) chain-dependent cytokines ... 83
5.3.1- IL-21 ... 83
5.3.2- IL-7 ... 84
5.3.3- IL-2 ... 86
5.4- Concluding remarks ... 88
VI- Reasoning and hypothesis ... 89
6.1- Relevance of research project ... 89
6.2- Cytokines: master regulators of T cell differentiation ... 90
6.3- IL-2 and IL-15: instigators of $CD8^+$ T cell exhaustion? ... 90
6.3.1- IL-2 and IL-15: biology and impact on $CD8^+$ T cell differentiation ... 90
6.3.2- IL-2 and IL-15: potential implication on $CD8^+$ T cell exhaustion ... 92
RESULTS ... 95
Article I. IL2Rβ-dependent signals drive terminal exhaustion and suppress memory development during chronic viral infection ... 95
Article II. Cutting Edge: IL-2 and IL-15 cooperate to program CD8 T cell exhaustion through STAT5 ... 143
DISCUSSION ... 169
I- Regulation of inhibitory receptors expression ... 169
1.1- IL-2 and IL-15 direct 2B4 and Tim-3 expression on CD8 T cells ... 170

1.2- Regulation of 2B4 171
1.3- Regulation of Tim-3 172
1.4- 2B4 and Tim-3 expression on secondary effectors 174
1.5- Three classes of inhibitory receptors 175
1.6- Perspectives 177
II- ACQUISITION AND MAINTENANCE OF EFFECTOR FUNCTIONS 177
2.1- Priming phase 178
2.2- Chronic phase 179
III- DEVELOPMENT OF PD-1^{HI} TERMINAL EFFECTORS 181
3.1- IL2Rβ-dependent signals are dispensable for CD8^{+} T cells proliferation and survival during chronicity 182
3.2- IL2Rβ-dependent signals instigate CD8^{+} T cell terminal differentiation during chronicity 184
3.3- A new model of PD-1^{hi} terminal effector development 185
3.4- Therapeutic potential 186
3.5- Perspectives 187
IV- DEVELOPMENT OF MEMORY CD8^{+} T CELLS 188
4.1- IL2Rβ-dependent signals preclude memory CD8^{+} T cell differentiation during a chronic viral infection 189
4.1.1- Persistent antigenic stimulation versus IL2Rβ-dependent signals 189
4.1.2- Tcm development 190
4.1.3- Tem development 192
4.2- Transcriptional control of memory differentiation arrest during a chronic viral infection 193
4.3- Antigen addiction versus IL-7-mediated survival 193
4.4- Therapeutic interests 194
4.5- Perspectives 195
V- CONCLUSION, SIGNIFICANCE AND FUTURE DIRECTIONS 195
REFERENCES 200
APPENDICES 238

List of figures

Figure. 1: Acute model of $CD8^+$ T cell differentiation.

Figure. 2: Acute VS chronic model of viral infection

Figure. 3: Intrinsic and extrinsic apoptosis

Figure. 4: Inhibitory receptors

Figure. 5: Inhibitory receptors-mediated suppression of T-cell functions

Figure. 6: Genes differentially expressed between effector, memory and exhausted $CD8^+$ T cells

Figure. 7: Impact of key TFs in the development of $CD8^+$ T cell responses during acute and chronic viral infections.

Figure. 8: ChARs overlap between acutely and chronically-stimulated effectors

Figure. 9: PD-1 gene methylation dynamic

Figure. 10: Characteristics of PD-1^{hi} and PD-$1^{int/lo}$ effectors

Figure. 11: Gradual erosion of PD-$1^{int/lo}$ cells

Figure. 12: Factors underlying T-cell exhaustion

Figure. 13: Cytokines regulate T-cell responses during chronic viral infections

Figure. 14: Biology of IL-2 and IL-15

Figure. 15: Impact of IL-2 and IL-15 on $CD8^+$ T cell responses

Figure. 16: Regulation of 2B4 and Tim-3 expression by IL-2 and IL-15

Figure. 17: Classification of inhibitory receptors based on their induction requirements

Figure. 18: Regulation of effector functions by IL-2 and IL-15 during a chronic viral infection

Figure. 19: IL-2 and IL-15 direct PD-1^{hi} terminal differentiation

Figure. 20: Gradual loss of memory $CD8^+$ T cell potential

Figure. 21: Conclusion scheme: Impact of IL-2 and IL-15 on $CD8^+$ T cell responses during a chronic viral infection

List of Tables

Table. 1: Characteristics of inhibitory receptors

Table. 2: Characteristics of anergy, senescence and exhaustion

Table. 3: Key features of relevant TFs involved in $CD8^+$ T cell differentiation and exhaustion

List of abbreviations

A
Ab : *Antibody*
Ag *: Antigen*
AICD : *Activation-induced cell death*
AIDS : *Acquired immune deficiency syndrome*
Arm : *Armstrong*
APC : *Antigen presenting cell*
ATAC : *Assay for transposase-accessible chromatin*

B
BAD : *Bcl-2 agonist of cell death*
BAX : *Bcl-2 associated x protein*
BAK : *Bcl-2 agonist/killer*
BATF : *Basic leucine zipper transcription factor*
BBC3 : *Bcl-2-binding component 3*
BCL-2 : *B-cell Lymphoma 2*
BCL-XL : *B-cell Lymphoma-extra Large*
BID : *BH3-interacting domain death agonist*
BIK : *Bcl-2-interacting killer*
BIM : *Bcl-2-interacting mediator of cell death*
BLIMP-1 : *B-lymphocyte-induced maturation protein-1*
BM : *Bone marrow*

C
CAR : *Chimeric antigen receptor*
CCR7 : *C-C chemokine receptor type 7*
CCR5 : *C-C chemokine receptor type 5*
CD : *Cluster of differentiation*
ChAR : *Chromatin accessible region*
Cl-13 : *Clone-13*
CPG : *Cytosine-phosphate-guanine*
CTL : *Cytotoxic T lymphocyte*
CXCR3 : *C-X-C chemokine receptor 3*
CXCR5 : *C-X-C chemokine receptor 5*

D
Dc : *Dendritic cell*
DNMT1 : *DNA methyltransferase 1*
DR4 : *Death receptor 4*

E
EAE : *Experimental autoimmune encephalomyelitis*
EBV : *Epstein-Barr virus*
EE : *Early effector*
ER : *Endoplasmic reticulum*
EZH2 : *Enhancer of zest homolog 2*

F
FASL : *Fas ligand*
FOXO1 : *Forkhead box protein O1*
FOXO3 : *Forkhead box protein O3*
FOXP3 : *Forkhead box P3*

G
GC : *Germinal center*
GITR : *Glucocorticoid-induced TNF-related protein*
GITR-L : *GITR-ligand*
GP : *Glycoprotein*
GPI *: Glycophospatidylinositol*
GRZA : *Granzyme A*
GRZB : *Granzyme B*
GSEA : *Gene set enrichment analysis*

H
HAART : *Highly active antiretroviral therapy*
HDAC2 : *Histone deacetylase 2*
HIV : *Human immunodeficiency virus*
HCV : *Hepatitis C virus*
HBV : *Hepatitis B virus*
HTLV1 : *Human T-lymphotropic virus 1*
HVEM *: Herpes virus entry mediator*

I
ICOS : *Inducible T-cell co-stimulator*
ID2 : *Inhibitor of DNA binding 2*
ID3 : *Inhibitor of DNA binding 3*
IDO : *Indoleamine 2,3-dioxygenase*

IELs : *Intraepithelial lymphocytes*
IFN : *Interferon*
IFNAR : *Interferon alpha/beta receptor*
Ig : *Immunoglobuline*
IL : *Interleukin*
IRF4 : *Interferon regulatory factor 4*
ISG : *Interferon signature gene*
ITIM : *Immunoreceptor tyrosine-based inhibitory motif*
ITSM : *Immunoreceptor tyrosine-based switch motif*

J
JAK : *Janus kinase*

K
KLF2 : *Krüppel-like factor 2*

L
LAG-3 : *Lymphocyte activation gene-3*
LAP : *Latency-associated peptide*
LAT*:* Linker for activation of T cells
LCMV : *Lymphocytic choriomeningitis virus*
LIF : *Leukemia inhibitory factor*
LLC : *Large latent complex*
LTBP : *Latent TGF-β binding protein*

M
MAP : *Mitogen activated protein*
MAPK : *Mitogen activated protein kinases*
MBD2 : *Methyl-CpG Binding Domain Protein 2*
MHC : *Major histocompatibility complex*
MPEC : *Memory precursor effector cell*
mTOR : *Mammalian target of rapamycin*

N
NFAT : *Nuclear factor of activated T-cells*
NK : *Natural killer*
NP : *Nucleoprotein*

O
OSM : *Oncostatin M*
OX40-L : *OX40-Ligand*

P
P13K : *Phosphoinositide 3 kinase*
PBX3 : *PBX homeobox 3*
PD-1 : *Programmed death-1*
PD-L1 : *PD-1 ligand*
pDC: *Plasmacytoid dendritic cell*
PMA : Phorbol myristate acetate
PP2A : Protein phosphatase 2A
PRDM1 : *PR domain zinc finger protein 1*
PTGER4 : *Prostaglandin E receptor 4*
PUMA : *p53-upregulated modulator of apoptosis*

R
RAG : *Recombination activating gene*

S
SAP : *Serum amyloid P protein*
SCA-1 : *Stem-cells antigen-1*
Sh2d1a : *SH2-domain containing 1A*
Sh2d1b : *SH2-domain containing 1B*
SHP-1 : *Src homology-2 domain-containing phosphatase-1*
SHP-2 : *Src homology-2 domain-containing phosphatase-2*
SIV : *Simian immunodeficiency virus*
SLAM : *Signaling lymphocytic activation molecule*
SLC : *Small latent complex*
SLEC : *Short-lived effector cell*
SMAD : *Mothers against decapentaplegic homolog*
SMAD4 : *Mothers against decapentaplegic homolog 4*
SOCS : *Suppressor of cytokine signaling*

STAT : *Signal transducers and activators of transcription*

T

***TCF7*/TCF-1** : *Transcription factor 7*
Tcm : *Central memory T-cell*
TCR : *T-cell receptor*
Tem : *Effector memory T-cell*
Tex: *Exhausted T cell*
TF : *Transcription factor*
Tfh : *T follicular helper*
TGF-β : *Transforming growth factor β*
TGF-β : *Transforming growth factor β receptor II*
Th1: *T helper type 1*
Th2: *T helper type 2*
Th17: *T helper type 17*
TiLs: *Tumor infiltrating lymphocytes*
TIM-3: *T-cell Ig and mucin domain-containing molecule-3*
TLE1: *Transducing-like enhancer protein 1*
TNF: *Tumor necrosis factor*
TNF-α: *Tumor necrosis factor α*
TNF-R: *Tumor necrosis factor receptor*
TRAF1: *TNF-receptor associated factor*
TRAIL: *tumor-necrosis-factor related apoptosis inducing ligand*
Tregs: *Regulatory T-cells*
Trm: *Resident memory T cell*
Tscm: *Stem cell memory T cell*
TYK2: *Tyrosine Kinase 2*

W

WT: *Wild type*

X

XLP: *X-linked lymphoproliferative disease*

Acknowledgements

Undertaking a Ph.D. is not only obtaining a degree, it's also a human adventure. This experience continuously challenges your determination to finally make yourself grow up and build confidence on your own capacities. For that formidable adventure I would first like to thank my lab director and mentor Dre Hélène Decaluwe for allowing me to undertake a Ph.D. in her lab. Her scientific rigor and pointillism made me a better scientist. I have always appreciated the trust she placed in me, her advice and the convergence of our ideas. Over the last years, I will remember the first CIHR of the lab, which submitted a few hours before her childbirth, the two coffees she offered me from her Nespresso machine over the five years of my Ph.D., the midnight emails, and finally, our relief when the first paper was finally accepted. I wish her the best for the future of her lab, and I hope we'll have the chance to work together again.

I had the chance to work with many collaborators throughout my Ph.D. I would like to thank Dr Alain Lamarre and members of his laboratory at "Institut Armand Frappier" notably Tania Charpentier and Esther Tarrab, for their help, technical support, and winter carpooling during the first years of my Ph.D. I am also very grateful to Dre Naglaa H. Shoukry at "Centre Hospitalier de l'Université de Montréal" for her help with human samples. Her kindness, constructive comments, and advice was always appreciated. Within her lab, I would like to thank Nathalie Bédard for her great professionalism and efficiency rendered that collaboration extremely productive.

I had the chance to work with many people at Ste Justine Research Center during my Ph.D. that I would like to thank. First I address special thanks to members of my lab ("the totally spies"). Sara Bourbonnais (alias Sarau/Mom etc..), who is our research assistant, your technical support and helpful discussions were always welcomed. It was a real pleasure to "frenchify" your sense of humor over the years. Josée-Anne Joly (alias, JJ/ Ginette), continue to practice your "paint" art and don't hesitate to send me some. Chloé Berthe (I just realized you don't have a nickname yet), thanks for your help during the last months I wish you success for your doctorate. I also want to thank past members

of the lab, Natacha Cotta Grand and Cédric Mathieu, who were present since the beginning. To finish, two amazing interns I supervised during my Ph.D., Eva Michaud and Moana Boulangé. Your good mood and motivation were really appreciated. I also want to thank Aurélien and William for their help in molecular biology, Anne and Vanja for genetic stuff, Nicolas Montpas for his knowledge on the molecular biology and Irish pubs, Dr. Valérie Abadie for precious advice, Ines Boufaied for long hours of cell-sorting and Véronique Pellerin for animal care.

To finish, I would like to address special thanks to my family in France, particularly my Mom, Dad, and Grandmas. You supported me every time I needed it during the last five years and it is thanks to you I am finally a doctor! To my friends in France and Canada, thank you for your encouragements and all the refreshing moments we shared that fueled my determination. Finally, I dedicate this thesis to my other half and future wife! You followed me in this 5 year-adventure six thousand kilometers from home and encouraged me every day. Your presence, support, and daily little attentions, notably these last months were my inspiration. I hope you are ready to follow me again into a new American adventure that I am sure will be full of new surprises and happiness.

Introduction

I- CD8$^+$ T cell response to a chronic viral infection: lessons from infectious mouse models

Developing new strategies to improve vaccination protocols and immunotherapeutic regimens is the ultimate aim of immunological research. CD8$^+$ T cells are the foot soldiers of adaptive immunity, capable of eliminating virally infected and transformed cells. During the last decade, efforts were made to develop T-cell inducing vaccines with the hope of establishing long-term CD8$^+$ T cell-mediated protection against various infectious diseases and cancers.(1) These T-cell based vaccines showed efficacy at protecting against various infectious diseases including influenza (Flu) or tuberculosis (TB) but failed at eradicating chronic viral infections such as the Human Immunodeficiency Virus (HIV), Hepatitis C (HCV) and B (HBV) Viruses or Epstein- Barr Virus (EBV).(1) This reveals a critical need to further understand the causative factors of such failure and to consequently adapt specific immunotherapeutic strategies to eliminate chronic viral diseases. Two strains of the lymphocytic choriomeningitis virus (LCMV) were extensively used in mice to decipher the CD8$^+$ T cell response to chronic viral infections. Infection of mice with LCMV Armstrong (Arm) generates an acute infection that is rapidly cleared within a week whereas LCMV clone-13 (Cl-13) causes a chronic infection with 2-3-month viremia.(2, 3) Comparative analyses between these two strains of LCMV revealed a unique and chronic specific differentiation program that leads to severe CD8$^+$ T cell dysfunction and loss of plasticity toward memory development. This program common to chronic viral infections and cancers is now referred to as “exhaustion”. Attempts to prevent and/or reverse such immunosuppressive program shows extremely promising results in the treatment of chronic viral infections and several cancers. However, actual approaches still fail to completely reverse CD8$^+$ T cell exhaustion and establish long-term immune protection. A better understanding of the factors implicated in the development of CD8$^+$ T cell

exhaustion is crucial to improve immunotherapeutic regimens. Precise characteristics of $CD8^+$ T cell exhaustion are discussed below.

1.1- Key mouse models of acute and persistent viral infection

Modeling viral infections in mice has been the cornerstone of major breakthroughs in our understanding of innate and adaptive immune responses to pathogenic infections.(4) Notably, LCMV has been extensively used for almost a century as a reference model in the study of immune responses to viral infections.(5-8) LCMV is a group V member of the *Arenaviridae* family composed of two fragments of single-strand RNA (ssRNA). The Parental strain of the virus was originally isolated by Charles Armstrong in 1933 and named LCMV Armstrong in reference to its discoverer as well as its capacity to trigger lymphocytosis and inflammation of the meninges (notably the choroid plexus surrounding the intracerebral ventricles).(9) LCMV Armstrong causes an acute infection in mice characterized by strong CTL and antibody (Ab) responses.(2, 3, 10, 11) Infection is generally cleared within a week in a $CD8^+$ T cell-dependent manner that allows for memory T cell development.(2, 11, 12) LCMV Arm has also been a starting point for the study of persistent viral infections. Mice infected at birth with LCMV Arm become life-long carriers (carrier-mice) of the virus and are characterized by a complete absence of anti-LCMV CTL responses.(13-16) In 1984, R.Ahmed and M.Oldstone discovered that LCMV persistence in these mice was due to the emergence of LCMV variants.(3) One of these variants, the LCMV clone 13 isolated from splenic tissues of carrier-mice, causes a persistent infection in adult mice with detectable viremia for 2-3 months in the serum and for life in "reservoir" organs, including the kidneys and the brain.(2, 3, 10, 17) In such chronic environment, responding $CD8^+$ T cell become dysfunctional and are gradually deprived of memory developmental plasticity.(2, 18-20) Between 1990 and 1993 Matloubian *et al* defined that the persistent nature of LCMV Cl-13 resides in two central amino acid changes within the RNA fragments of the virus compared to the parental Arm strain. The first mutation (U→C) situated at position 260 in the small ssRNA fragment (S) causes a phenylalanine to leucine (F→L) substitution in the viral glycoprotein. The second mutation (A→C) situated at position 1079 in the large

ssRNA fragment (L) causes a lysine to glutamine (K→Q) substitution in the viral polymerase.(10, 17) LCMV Arm and Cl-13 infect and replicate similarly in mouse fibroblasts, but these two mutations provide a selective advantage for the Cl-13 strain to infect and replicate in macrophages.(17) In fact, the F→L substitution in the viral glycoprotein allows LCMV Cl-13 to infect macrophages more efficiently than the parental Arm strain while the K→Q substitution in the viral polymerase provides the replicative advantage.(17) This fundamental difference results in distinct viral tropisms in vivo. The parental Arm strain replicates preferentially in the central nervous system (CNS), while the superior ability of the Cl-13 strain to infect and replicate into macrophages allows its dissemination not only in the CNS but also into lymphoid and non-lymphoid tissues (spleen, liver, kidney).(21, 22) Therefore, the higher capacity of the Cl-13 strain at infecting macrophages is fundamental and forms the basis of its ability to cause a systemic infection and persist in the host.(23) At the molecular level, the neuronal versus macrophage tropism of LCMV can be dictated by the glycosylation level of the GP protein.(24) Hence, one hypothesis would be that the F→L substitution in the viral glycoprotein of the LCMV Cl-13 alters the protein glycosylation level favoring macrophage tropism. Importantly, the two mutations with the Cl-13 strain are not situated in viral sequences recognized by the immune system (viral epitopes), allowing the direct comparison of epitope-specific responses between an acute and persistent infection caused by LCMV Arm and Cl-13 respectively.(10, 17) This powerful comparative tool has been used for almost two decades to decipher the underlying mechanisms behind defective $CD8^+$ T cell responses to persistent viral infection.

1.2- $CD8^+$ T cell response to an acute viral infection

Upon activation, $CD8^+$ T cells integrate several environmental signals that dictate their cellular fate. After Ag encounter, naive $CD8^+$ T cells require three distinct signals for activation. TCR engagement, co-stimulatory signals (CD28:CD80/86) and inflammatory cytokines (IL-12, type I IFN) are the pre-requisite for optimal $CD8^+$ T cell activation (**Figure. 1**).(25) Following the priming phase, activated $CD8^+$ T cells undergo a massive expansion phase associated with a heterogeneous differentiation into two

different effector subtypes with distinct cell-fate referred to as short lived effector cells (SLECs; KLRG1^{lo}CD127^{hi}) and memory precursor effector cells (MPECs; KLRG1^{lo}CD127^{hi}) (**Figure. 1**).(26-28) Initially, high levels of inflammatory signals (IL-12, type I IFNs) but also IL-2 and to a lesser extend IL-15 promote SLECs differentiation through up regulation of key transcription factors (TFs) including T-BET (*Tbx21*) and BLIMP-1 (*Prdm1*) while lower levels of inflammatory signals sustains MPECs development.(28-33) Differentiation toward SLEC is terminal, these effectors lose further developmental plasticity (including into memory T cells), proliferate poorly to additional antigenic stimuli, possess a short lifespan and are incapable to perform homeostatic proliferation.(28, 34) In contrast, MPECs differentiation is not terminal, these cells retain developmental plasticity and proliferate to additional antigenic signals.(28, 34) MPECs also proliferate in response to homeostatic signals and survive longer compared to SLECs.(28) Functionally, the two subsets secrete similar amounts of antiviral and pro-inflammatory cytokines (interferon gamma [INFγ] and tumor necrosis factor alpha [TNFα]), produce equivalent levels of cytotoxic molecules (granzymes) and demonstrate comparable killing potential.(28, 31, 34) Transcriptionally, SLECs express high levels of TFs associated with terminal differentiation and low levels of memory-associated genes (generally T-BET^{hi}, EOMES^{int}, BLIMP-1^{hi}, BCL6^{low}, ID2^{hi}, ID3^{low}). Conversely MPECs rather display a pro-memory transcriptional program (T-BET^{low}, EOMES^{int}, BLIMP-1^{low}, BCL6^{hi}, ID2^{low}, ID3^{hi}).(28, 30, 31, 34-36) Following expansion, a contraction phase follows during which 90-95% of effector CD8^{+} T cells die by apoptosis.(37, 38) SLECs are preferentially deleted during that contraction phase while few MPECs survive to form a pool of memory CD8^{+} T cells (**Figure. 1**). During the memory phase, two main types of memory cells develop referred to as effector memory cells (Tem [CD62L^{lo}CD127^{hi}]) and central memory cells (Tcm [CD62L^{hi}CD127^{hi}]) T cells.(39) Tem cells preferentially home to non-lymphoid tissues, preserve immediate cytotoxic functions but expand poorly upon re-challenge.(40, 41) Tcm cells mainly locate to secondary lymphoid organs and the bone marrow.(42, 43) Importantly, Tcm cells possess long-term survival and self-renewal potential through integration of homeostatic signals provided by IL-7 and IL-15.(44, 45) These cells also rapidly develop effector functions, secrete high amounts of IL-2 and massively expand upon secondary Ag-encounter (**Figure. 2**).(12, 43) Hence, generation

of Tcm after resolution of an acute viral infection provides long-term efficient immune protection to the host.>

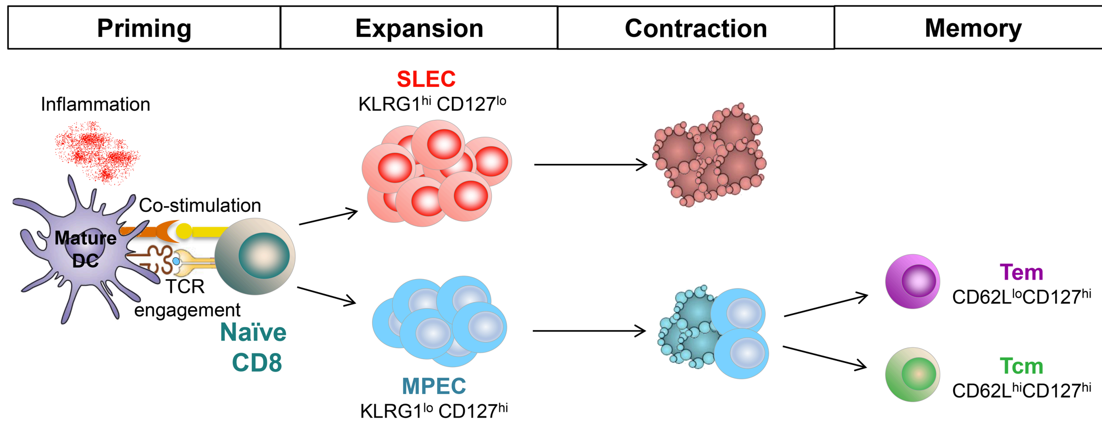


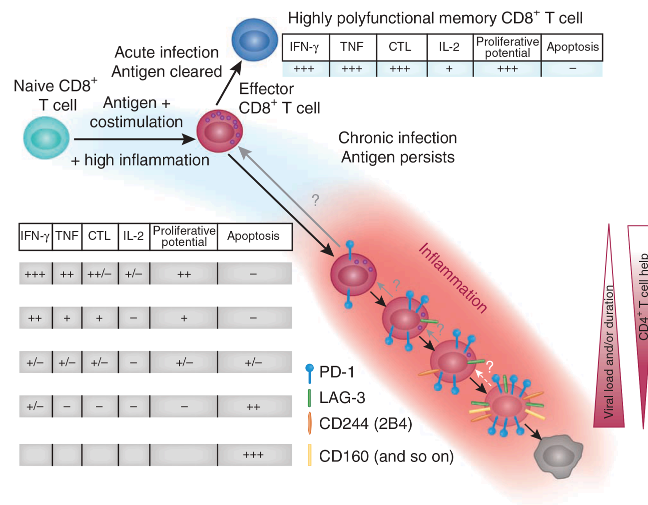
Figure. 1: Acute model of CD8$^+$ T cell differentiation. Upon an acute viral infection, activated CD8$^+$ T cells differentiate into either KLRG1^{hi}CD127^{lo} SLECs or KLRG1^{lo}CD127^{hi} MPECs. Following the expansion phase and virus clearance, a contraction phase follows in which 90-95% of effector CD8$^+$ T cells die by apoptosis. MPEC preferentially survive the contraction phase and generate two types of memory cells: Tem (CD62L^{lo}CD127^{hi}) and Tcm (CD62L^{hi}CD127^{lo}).

1.3- CD8$^+$ T cell response to a chronic viral infection

1.3.1- Overview

During a chronic viral infection, CD8$^+$ T cell responses differ drastically from the prototypical response seen in an acute viral infection (**Figure. 2**). In that particular context, persistent antigenic stimulation, prolonged inflammatory milieu, and several other factors (discussed in sections 4 and 5 of the introduction) hijack the normal development of CD8$^+$ T cell differentiation leading to exhaustion.(46, 47) While antigen (Ag)-specific CD8$^+$ T cells rapidly expand, the normal immunodominance hierarchy of epitope-specific populations is disrupted. Most dominant epitope-specific effectors are selectively lost and only the less dominant epitope-specific CD8$^+$ T cells persist.(2, 18) Persisting CD8$^+$ T cells are subsequently deprived of their principal effector functions

(**Figure. 2**).(2, 18) This loss of function is hierarchical and correlates with an increased expression of numerous inhibitory receptors on CD8$^+$ T cells (**Figure. 2**).(2, 48, 49) These inhibitory receptors use distinct and non-redundant methods to suppress CD8$^+$ T cell functions.(50) As they develop increasingly severe exhaustion, CD8$^+$ T cells are progressively and irreversibly deprived of their plasticity toward memory development (**Figure. 2**).(19, 20, 51) These functional and developmental defects dramatically impair adaptive immune responses to chronic viral infections and form the basis of the CD8$^+$ T cell exhaustion program.



<u>Figure. 2</u>: Acute versus chronic model of viral infection. Resolution of an acute viral infection results in the development of a polyfunctional pool of memory CD8$^+$ T cells. During a chronic viral infection, CD8$^+$ T cells become exhausted. These Ag-specific cells up-regulate several inhibitory receptors, lose their principal effector functions, and are eventually eliminated by apoptosis. *Wherry Nature Reviews Immunology 2011*

1.3.2- Disruption of immunodominance hierarchy and loss of antiviral CD8$^+$ T cell responses

One of the first differences reported during chronic viral infections is a dramatic disruption of the normal immunodominance hierarchy of epitope-specific CD8$^+$ T cells.(2) In acute LCMV infection, the relative abundance of epitope-specific populations

of $CD8^+$ T cells is prioritized from most dominant to least dominant epitope (D^bNP396>K^b/GP34>D^bGP33>D^bGP276).(2, 52, 53) Establishment of that hierarchy is reproducible and maintained at the memory phase after viral elimination.(2) This immunodominance hierarchy is disrupted during a chronic LCMV infection. In the context of chronic viral infections, $CD8^+$ T cells specific for the most immunodominant epitopes (NP396 and GP34 in the LCMV model) are misrepresented at the onset of infection and physically deleted during chronicity. Only $CD8^+$ T cells specific for the less dominant epitopes (GP33 and GP276 in the LCMV model) are maintained and become exhausted.(2, 18, 54, 55) It is noteworthy that GP276-specific $CD8^+$ T cells, which are subdominant during an acute LCMV infection, become the most dominant and numerous population in the chronic model.(2, 18, 54, 55) Hence, chronic viral infections cause a selective deletion of the most dominant virus-specific $CD8^+$ T cell populations while only less dominant epitope-specific populations (GP33 and GP276) survive to form a pool of exhausted cells.

In addition to the disruption of the normal immunodominance hierarchy, chronic viral infections eventually lead to the collapse of antiviral $CD8^+$ T cell responses. This dramatic feature only occurs at late stages of chronic viral infections. For example, in HIV patients, erosion of $CD4^+$ T cells and progression to AIDS ([Acquired Immune Deficiency Syndrome] ultimate stage of HIV infection) is often associated with a loss of $CD8^+$ T cells.(56) Loss of antigen-specific $CD8^+$ T cells also occurs in patients with prolonged episodes of uncontrolled HCV or HBV infection.(57, 58) In LCMV Cl-13-infected mice, lack of $CD4^+$ T cell help (usually achieved by depleting $CD4^+$ T cells with anti-CD4 antibodies) causes an extremely severe infection with high viral titers persisting for the life of animals (discussed in section 4.2 of the introduction).(11, 18, 49, 59) In these extreme conditions designed to model an uncontrolled viremia, antiviral $CD8^+$ T cell responses ultimately fail to maintain mirroring the what happens in patients at late stages of chronic viral infections.(11, 18, 49, 59) Previous studies demonstrated that $CD8^+$ T cells responding to a chronic viral infection are more susceptible to apoptosis, and this sensitivity increases with exhaustion severity.(60) Apoptosis is an important mechanism to maintain T-cell homeostasis and temper immune responses that might

cause severe immunopathologies.(61, 62) Two distinct but eventually converging forms of apoptosis referred to as “intrinsic apoptosis” and “extrinsic apoptosis” occur in mammalian cells (**Figure. 3**).(63, 64) Intrinsic apoptosis (also called mitochondrial pathways) is triggered by several parameters, including cytokine/growth factor deprivation, DNA damage, or endoplasmic reticulum (ER) damage.(64, 65) This form of apoptosis is regulated by a tight balance in the expression of pro- and anti-apoptotic molecules of the BCL-2 family of proteins. Whereas BCL-2-like proteins (BCL-2, BCL-XL, BCL-W, A1 and MCL-1) provide survival signals to naïve and effector $CD8^+$ T cells,(66-70) other members of the family including BAX, BAK and the BH3-only proteins BAD, BID NOXA, HRK/DP5, BIK, BLK/NBK, PUMA/BBC3, and BIM promote apoptosis notably through cytochrome c release from mitochondria and subsequent caspase 9 and 3 activation (**Figure. 3**).(64, 71-74) Interestingly, the relative expression of the antagonist molecules BCL-2 and Bim emerged as a reliable indicator of the survival potential of effector and memory $CD8^+$ T cells.(75, 76) Extrinsic apoptosis (also called “death receptor pathways” or antigen-induced cell death [AICD]) is mediated by engagement of death receptors, notably members of the tumor necrosis factor receptors (TNF-R) (e.g. FAS, TRAIL R1/DR4, TRAIL R2/DR5 and TNF-R1), with their cognate ligands (FAS-L, TRAIL and TNF respectively) at the surface of $CD8^+$ T cells.(64, 65) Engagement of these receptors promotes $CD8^+$ T cell apoptosis through activation of caspase 8 (**Figure. 3**).(62, 77, 78)

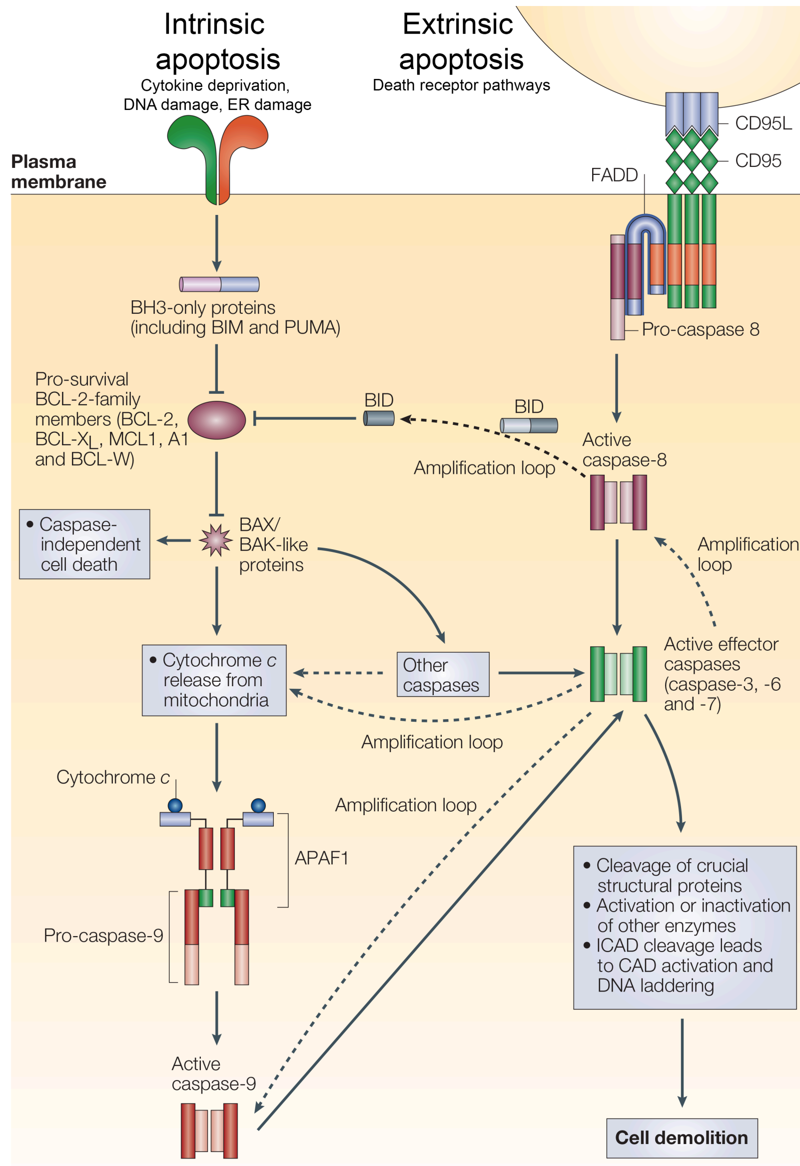


Figure. 3: Intrinsic and extrinsic apoptosis. Intrinsic apoptosis can be triggered by several factors, including cytokine deprivation, and is regulated by members of the Bcl2-familly of proteins. This pathway provokes the release of cytochrome c by mitochondria, activation of caspase 9 and subsequent cell death. Extrinsic apoptosis involves the interaction of death receptors (i.e. CD95) with their cognate ligands, leading to caspase 8 activation and cell death.

Strasser Nature Reviews Immunology 2005

During the contraction phase of an acute viral infection, 90-95% of effector CD8$^+$ T cells succumb while only 5% survive to form a pool of memory cells. This massive elimination of effector CD8$^+$ T cells is mainly caused by cytokine deprivation (intrinsic

apoptosis) and is initiated by BH3-only proteins, notably BIM and PUMA.(76, 79-81) However, both intrinsic and extrinsic apoptosis likely direct the elimination of exhausted $CD8^+$ T cells during chronic viral infections. Indeed, exhausted $CD8^+$ T cells display minimal expression of BCL-2,(19) whereas high levels of its pro-apoptotic counterpart Bim was reported on $CD8^+$ T cells from patients with HIV, chronic HCV, and HBV as well as SIV-infected macaques, and mice chronically infected with LCMV.(58, 82-84) Remarkably, Bim-deficiency rescues NP396 epitope-specific $CD8^+$ T cells from clonal deletion in mice chronically infected with LCMV.(85) This underscores a critical role for Bim-mediated apoptosis in the clonal deletion of immunodominant epitope-specific populations during chronic viral infections. Similarly, expression of FAS and FAS-L (extrinsic apoptotic factors) are upregulated on exhausted $CD4^+$ and $CD8^+$ T cells from HIV patients, SIV-infected macaques, and LCMV-infected mice, and these cells are highly susceptible to FAS-mediated apoptosis.(48, 82, 86, 87) Mirroring the results obtained with $Bim^{-/-}$ mice, FAS-deficiency resulted in sustained NP396-specific responses on LCMV chronically-infected mice.(54) Further studies determined that physical deletion of $CD8^+$ T cells during a chronic viral infection was mediated by a cooperation between intrinsic and extrinsic apoptosis mediated by BIM and FAS respectively.(88) Together, these results indicate that the loss of immunodominant epitope-specific populations during a chronic viral infection is dictated by the cooperative action of intrinsic and extrinsic apoptosis.

Factors underlying $CD8^+$ T cell deletion during chronic viral infections are many. Several studies point to repetitive TCR signaling as a culprit. Indeed, limiting Ag-presentation by parenchymal cells in LCMV Cl-13 infected mice (deletion of MHC class 1 molecules specifically on parenchymal cells) leads to a dramatic increase in the number of polyfunctional Ag-specific $CD8^+$ T cells at lymphoid and non-lymphoid locations despite decayed viral control.(46) Numbers of polyfunctional NP396-specific $CD8^+$ T cells are also augmented demonstrating that excessive Ag-presentation actually erodes that population. Previous studies on the LCMV model demonstrated that NP levels are superior to GP levels in infected cells and that the NP396 epitope is presented at higher levels compared to the GP33 or GP276 epitopes.(2, 89, 90) Further, binding affinity of

the NP396 peptide to MHC class I molecules is superior compared to the GP33 and GP276 peptides.(53) Increased and sustained TCR signaling was shown to promote FAS-L expression and subsequent autocrine/paracrine engagement of FAS. Expression of BIM is also enhanced by repetitive TCR signal-induced calcium flux.(38, 91, 92) Thus, repetitive TCR signals likely promote extrinsic and intrinsic apoptosis of exhausted $CD8^+$ T cells, rendering populations specific for a dominant and over-presented epitope (e.g. NP396) more susceptible to physical deletion during a chronic viral infection.

Other factors including cytokines and co-inhibitory molecules likely contribute to the deletion of $CD8^+$ T cells during chronic viral infections. For example, TGF-β signals trigger BIM expression in $CD8^+$ T cells during chronic viral infection. Genetic ablation of TGF-β-receptor II (TGFβRII) on $CD8^+$ T cells restrains apoptosis and promotes the maintenance of a functional NP396-specific CTL pool.(84) The common gamma-chain (γ_c) cytokine IL-2 positively regulates AICD on lymphocytes through its capacity to trigger high levels of FAS and FAS-L expression.(93-99) However, a direct involvement of IL-2 in the deletion of Ag-specific $CD8^+$ T cells during a chronic viral infection remains to be determined. Inhibitory receptor signals provided by PD-1 potently decrease the expression of anti-apoptotic molecules (e.g. BCL-XL) on $CD8^+$ T cells.(60, 100) Increased PD-1 expression correlates with a higher rate of apoptosis and blocking its interaction with PD-L1 reduces $CD8^+$ T cell apoptosis.(60) De facto, deletion of $CD8^+$ T cells during chronic viral infection is multifactorial, and further studies are needed to clarify the exact contribution of each causative factor.

1.3.3- Loss of effector functions

Development of an efficient $CD8^+$ T cell response is indispensable for the clearance of viral infections. During an acute viral infection, activated $CD8^+$ T cells undergo massive clonal expansion and acquire several effector functions indispensable for the rapid elimination of invading pathogens. However, some viruses (e.g. HIV, HCV, HBV, EBV in humans and LCMV [Cl-13] in mouse) have developed strategies to escape that first wave of adaptive immune response. These viruses eventually persist in the host

and cause a chronic viral infection. In this scenario, a substantial antiviral $CD8^+$ T cell response is maintained during chronicity but these Ag-specific cells gradually lose their principal effector functions as they are pushed towards exhaustion and fail to acquire the typical protective capacities of memory cells (**Figure. 2**).(101, 102) One of the most characteristic signs of $CD8^+$ T cell exhaustion is a dramatic loss of the capacity to produce antiviral and immuno-stimulatory cytokines (e.g. IFNγ, TNFα and IL-2).(2, 18, 48, 103) Such disability occurs gradually and in a hierarchical fashion. During the first week of a LCMV Cl-13 infection, $CD8^+$ T cells almost cease to produce TNFα and IL-2 (partial exhaustion stage I). IFNγ secretion is unaffected at early time points but gradually decline over chronicity (partial exhaustion stage II). Complete loss of IFNγ secretion capacity occurs only in the most severe scenario in which $CD4^+$ T cells are removed (full exhaustion).(2, 18) Importantly, as $CD8^+$ T cells are pushed towards severe exhaustion, this loss of capacity to produce cytokines becomes irreversible and cannot be re-acquired even when $CD8^+$ T cells are removed from the chronic environment.(51) Heritable and "exhaustion-exclusive" epigenetic remodeling at the locus of effector genes (i.e. IFNγ gene locus) likely account for such irreversible loss of function.(104, 105) Killing abilities of chronically stimulated $CD8^+$ T cells is also rapidly diminished. As soon as day-8 post LCMV infection, the cytolytic potential of $CD8^+$ T cells from mice infected with the chronic strain Cl-13 dramatically falls compared to potent effectors generated during an acute LCMV infection.(2) Depletion of $CD4^+$ T cells further accentuates this loss of cytolytic potential.(11, 18) Perforin/granzyme is a master mechanism for $CD8^+$ T cell killing. Interestingly, exhausted $CD8^+$ T cells actually express granzyme B whereas a critical reduction in perforin mRNA was reported.(48) Alteration in the expression of genes involved in vesicle transport and cytoskeleton rearrangement was also observed.(48) This suggests that the loss of killing potential by exhausted $CD8^+$ T cells might stem from a combined lack of perforin expression and defective formation/secretion of lytic granules. Memory cells that develop after resolution of an acute viral infection acquire the capacity to rapidly proliferate in response to a secondary Ag-encounter. In contrast, exhausted $CD8^+$ T cells fail to acquire this cardinal property of memory cells even after viral control and poorly proliferate to Ag-stimulation.(19, 106) This inability to respond correlates with altered expression of several genes involved in

cell cycle progression.(48) Unlike conventional memory cells, exhausted $CD8^+$ T cells are not maintained through IL-7 and IL-15-mediated homeostatic signals.(107-109) Rather, these cells develop an Ag-addiction for survival and are maintained by extensive Ag-driven proliferative events.(19, 20) Together, during a chronic viral infection $CD8^+$ T cells are pushed toward exhaustion. Establishment of such program gradually and irreversibly hampers $CD8^+$ T cell functions and precudes the acquisition of cardinal memory properties. Nevertheless, exhausted $CD8^+$ T cells are not inert and continue to mediate substantial antiviral functions.(11)

1.3.4- Inhibitory receptors

During the last decades, efforts were made to identify molecular factors responsible for $CD8^+$ T cell loss of function during chronic viral infections. Whole genome comparative analysis between naive, effector, memory and exhausted $CD8^+$ T cells allowed for the identification of a set of genes encoding several inhibitory receptors (including PD-1, LAG-3, CD160, 2B4 and TIM-3) that are more particularly up-regulated on exhausted T cells (**Figure. 4 and Table. 1**).(48, 106) Further studies revealed that the number and diversity of inhibitory receptors expressed on $CD8^+$ T cells was a reliable indicator of exhaustion severity.(49) Interestingly, *in vivo* blockade of these receptors rescues a fraction of less severely exhausted $CD8^+$ T cells and improves the clearance of a chronic viral infection.(49, 60, 106, 110, 111) This observation demonstrated that inhibitory receptors are the main culprit of $CD8^+$ T cell loss of functions during chronic viral infections. It is clear that these receptors use distinct and non-redundant ways to shut-down $CD8^+$ T cell responses, (**Figure. 5**) and the co-blockade of several receptors leads to a better reinvigoration compared to the blockade of one receptors alone.(49, 50, 110) Precise characteristics of individual inhibitory receptor are discussed below.

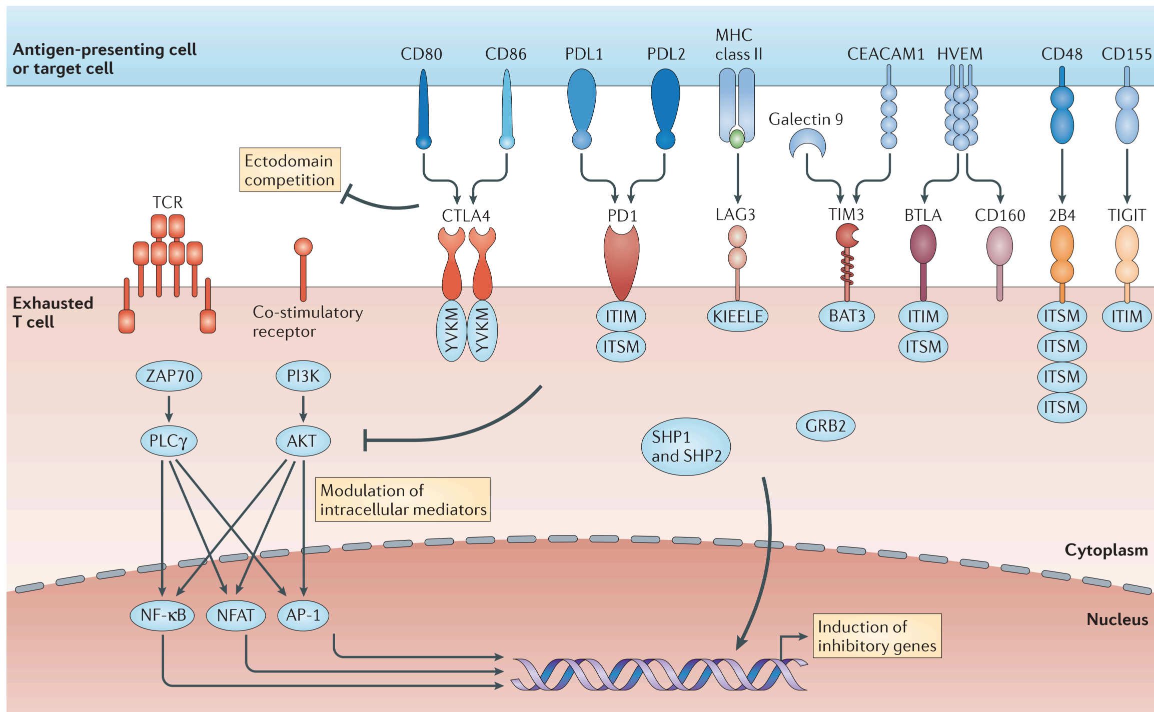


Figure. 4: **Inhibitory receptors**. Schematic representation of known inhibitory receptors expressed by exhausted CD8$^+$ T cells and their cognate ligands on APCs.

Wherry Nature Reviews Immunology 2015

1.3.4.1- Programmed cell death 1 (PD-1)

PD-1 is the first inhibitory receptor evidenced on exhausted CD8$^+$ T cells.(106) Among others, PD-1 is the best described and probably the most important inhibitory receptor implicated in CD8$^+$ T cell exhaustion. PD-1 expression is mainly reported on T cells, but this receptor is also expressed by B cells, NKT cells, and monocytes.(50) While conventional effector and memory CD8$^+$ T cells express substantial levels of PD-1, its expression is far more elevated and sustained on exhausted CD8$^+$ T cells.(48, 49, 106) First evidenced in a mouse model of chronic viral infection (LCMV Cl-13),(106) PD-1 expression was subsequently detected on CD8$^+$ T cells from patients with HIV, HCV, HBV,(112-114) persistent bacterial and parasitic infections (*Mycobacterium tuberculosis, Leishmania donovani and Toxoplasma gondii*),(115-117) cancers(102) and also SIV-infected macaques.(118) PD-1 is an Ig-like type I membrane protein that belongs to the CD28/CTLA-4 family of T cell regulators.(119) PD-1 expression is mainly triggered by

TCR-dependent signals (and downstream NFATc1) and can be upregulated by cytokines *in vitro* including IL-6, IL-12, and members of the common γ_c family of cytokines (e.g. IL-2, -7, -15 and -21).(120-122) Certain transcription factors (TFs), notably T-BET and BLIMP-1, are known to limit PD-1 expression on $CD8^+$ T cells.(123, 124) PD-1 possesses an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic domain (**Figure. 4 and Table. 1**).(125) Upon binding to the B7-family members PD-L1 and/or PD-L2 (mostly expressed by DCs and macrophages)(126), ITIM and ITSM motifs are phosphorylated, allowing the recruitment of the Src homology region 2 domain-containing phosphatase-1 and -2 (SHP-1 and SHP-2).(125, 127-130) These tyrosine phosphatases can then dephosphorylate activating molecules downstream of TCR/CD3 and CD28 signaling, notably ZAP70, and PKCθ but also the canonical PI3K-AKT-mTOR and RAS-MEK-ERK pathways triggered by cytokine signals.(125, 127, 130) A recent study also demonstrates that CD28 is a preferred target over TCR for PD-1/SHP-2-mediated dephosphorylation.(131) This supports the notion that PD-1 primarily functions by inhibiting B7-CD28-mediated costimulation in $CD8^+$ T cells. In addition to inhibiting proximal TCR and co-stimulatory receptor-signaling, PD-1 also promotes the transcription of genes involved in T cell dysfunction (i.e. *Batf*).(132)

Disrupting the interaction between PD-1 and its ligand PD-L1 using αPD-L1, efficiently reinvigorates exhausted $CD8^+$ T cells. In a mouse model of chronic viral infection, αPD-L1 treatment sustained $CD8^+$ T cell proliferation (and/or survival) and restored some killing ability and cytokine secretion. This resulted in the accumulation of a greater number of functional $CD8^+$ T cells and subsequent decrease in viral load and tumor burden. (49, 106, 133-135) Beneficial effects of PD-L1 blockade on $CD8^+$ T cells reinvigoration are abrogated in the absence of B7-CD28 costimulation and this effect was cell-intrinsic. This observation demonstrates that B7-CD28 costimulation of $CD8^+$ T cells is essential for effective PD-L1 therapy and supports the work by Kamphorst *et al* showing that CD28 is a preferential target of PD-1-mediated inhibition of T-cell function. Of note, combining PD-L1 blockade with other therapies (αLAG-3, αTIM-3, αCTLA-4, IL-10 blockade, 4-1BB agonists) further improves the reinvigoration of exhausted $CD8^+$

T cells.(49, 110, 136, 137) Positive impacts of PD-1/PD-L1 blockade were also extended to humans with chronic viral infections and cancers.(112-114, 138-140) Humanized anti-PD-1 antibodies were developed and approved by the FDA (Nivolumab, Pembrolizumab) for the treatment of late-stage human cancers (**Table. 1**). Although extremely promising, the therapeutic strategy of blocking the PD-1/PD-L1 interaction also has some limitations. Only the less terminally-exhausted $CD8^+$ T cells ($PD\text{-}1^{int/lo}$ cells; see part 3 of the introduction) are rescued after PD-L1 treatment.(60) Such an approach causes a temporary regain of function but fails to overcome the exhaustion program and to restore memory cells development.(135) This is in line with the fact that PD-1 does not by itself program $CD8^+$ T cell exhaustion.(141) Reinvigorated $CD8^+$ T cells appear even more terminally exhausted months after treatment interruption.(135) Finally, a substantial number of cancer patients still relapse after treatment interruption. This demonstrates that PD-1/PD-L1 blockade must be combined with other agents to achieve long-term protective immunity in patients with chronic diseases.

1.3.4.2- Lymphocyte-activation gene 3 (LAG-3)

Similar to PD-1, LAG-3 is a member of the Ig superfamily (SF) of receptors.(50) The gene encoding LAG-3 is adjacent to that of CD4 and possesses 20% homology with the latter.(142, 143) LAG-3 binds to MHC class II molecules but with a greater affinity and at a distinct region compared to CD4.(50, 144) Numerous hematopoietic cell types express LAG-3 including plasmacytoid dendritic cells (pDCs), B cells, NK cells, NKT cells, γδ T cells, regulatory T cells (Tregs), T cells, and tumor-infiltrating T cells (TILs).(145) Elevated and sustained expression of LAG-3 was reported on $CD8^+$ T cells in mice with chronic LCMV(48, 49) and humans with HIV,(146) HCV,(147) and cancer.(148) Naive $CD8^+$ T cells express low levels of LAG-3 but expression increases with T cell activation. On its intracellular domain, LAG-3 is devoid of ITIM or ITSM motifs. Instead, LAG-3 possesses a unique KIEELE motif essential to mediate its inhibitory functions (**Figure. 4 and Table. 1**).(149-151) However, little is known on how LAG-3 exerts its inhibitory functions on $CD8^+$ T cells.

LAG-3 was classified as an inhibitory receptor because of its capacity to limit T-cell activation and proliferation *in vitro*.(151, 152) Numerous studies subsequently linked LAG-3 expression to damped T-cell proliferation.(150, 153-155) In the LCMV model, LAG-3-deficiency or blocking antibodies against LAG-3 had little impact on $CD8^+$ T cell effector function and viral load despite increased proliferation.(49, 156) A similar increase in Ag-driven proliferation was also observed in HIV and HCV-specific $CD8^+$ T cells *ex vivo* in the absence of LAG-3.(146, 147) Hence, LAG-3 mainly functions as an anti-proliferative factor for $CD8^+$ T cells during chronic viral infections. However, LAG-3 blockade synergizes with PD-L1 therapy to reinvigorate $CD8^+$ T cell functions. Blockade of both receptors leads to a greater improvement of $CD8^+$ T cell effector function and lower viral loads compared to PD-L1 treatment alone.(49) In addition, LAG-3 appears to mediate its greatest inhibitory functions on antitumor $CD8^+$ T cells.(157) Phase I and II clinical trials are ongoing to evaluate the potency of disrupting the LAG-3 axis in several types of cancers.(158)

1.3.4.3- CD160

CD160 is an Ig-like glycosylphosphatidylinositol (GPI)-anchored membrane receptor expressed by a variety of immune cell types including NK cells, NKT cells, T cells and intraepithelial lymphocytes IELs.(159-161) On T cells, CD160 is induced upon activation by TCR-dependent signals in vitro, and its expression is sustained in vivo by the exhaustion-associated TF BLIMP-1.(162, 163) CD160 has binding affinities to classical and non-classical MHC class I molecules (low affinity) and most notably with the Herpes Virus Entry Mediator (HVEM) (**Figure. 4 and Table. 1**).(50, 160, 164-167) Levels of CD160 are elevated on $CD8^+$ T cells responding to chronic viral infections in mice (LCMV) and humans (HIV, HCV).(48, 49, 163, 168-171) However, due to the absence of an intracellular domain (**Figure. 4 and Table. 1**), little is known about how CD160 mediates its inhibitory functions on $CD8^+$ T cells.

Conflicting reports performed on $CD8^+$ T cells from HIV patients evidenced activating and inhibitory functions of CD160. In some studies, CD160 expression on

HIV-specific $CD8^+$ T cells defined a population with higher cytolytic potential (higher GrzB, perforin and specific lysis).(168, 169) The presence of $CD160^+$ HIV-specific $CD8^+$ T cells was also correlated with greater response to HAART, and this population was more frequent in elite controllers.(168, 169) Lastly, triggering CD160 on virus-specific $CD8^+$ T cells with activating Abs increased IFNγ secretion and complemented αPD-L1 blockade at rescuing HIV-specific $CD8^+$ T cells proliferation.(168, 172) In another study, functional analysis of $CD160^+$ and $CD160^-$ HIV-specific $CD8^+$ T cells correlated CD160 expression to higher dysfunction.(170) In the same study, transcriptional analysis revealed that $CD160^+$ cells are transcriptionally more similar to exhausted cells compared to $CD160^-$ cells.(170) A number of genes differentially regulated in $CD160^+$ HIV-specific $CD8^+$ T cells involved inhibition of T cell survival and functions. Consistently, the authors found higher proliferation rates and cytokine secretion by re-stimulated HIV-specific $CD8^+$ T cells after blockade of CD160/HVEM interaction.(170) Similarly, in the LCMV mouse model, targeting CD160 increased exhausted $CD8^+$ T cell lysis and survival potential.(49) Further studies on human $CD4^+$ T cells confirmed the inhibitory functions of CD160 by demonstrating that this receptor inhibits TCR-dependent signaling, notably the phosphorylation of CD3ζ.(159)

1.3.4.4- Signaling lymphocyte activation (SLAM) family receptor 2B4 (CD244)

2B4 (CD244) is a member of the signaling lymphocyte activation molecule (SLAM) subfamily of CD2-related Ig-based receptors which also includes SLAM (CD150), CD48, LY9 (CD229), CD84, NTB-A and CS1.(173, 174) All CD2-related receptors possess two or more extracellular Ig-like domains, and SLAM subfamily members are further characterized by the presence of at least two immuno-receptor tyrosine-based switch motifs (ITSM; TxYxxV/I) within their intracellular domain.(173-176) 2B4 is composed of two Ig-like domains in its extracellular N-terminal domain and four ITSMs in its C-terminal intracellular domain (**Figure. 4 and Table. 1**).(174, 177-179) Like CD2, 2B4 binds to CD48 but with a ten-fold greater affinity than CD2.(180-182) At steady state, 2B4 is expressed on a broad variety of hematopoietic cells including

virtually all NK cells, myeloid cells, basophils, $CD14^+$ monocytes, some TCR γδ T cells, and effector/memory $CD8^+$ T cells ($CD44^{hi}$ $IL2R\beta^+$).(178, 183-188)

Our current knowledge regarding the function of 2B4 was mainly provided by studies on NK cells where both activating and inhibitory functions were reported for 2B4. According to several studies, 2B4 is an activating receptor capable of mediating MHC-unrestricted lysis of target cells. First, antibody cross-linking experiments revealed that engagement of 2B4 increases cytokine secretion (IFNγ, IL-2), granule exocytosis, invasiveness, and cytolytic potential of murine and human NK cells.(173, 183, 185, 189-191) Second, the SLAM-associated protein (SAP; Sh2d1a), which acts as an adaptor molecule for 2B4 and SLAM is mutated in X-linked lymphoproliferative syndrome (XLP).(192-194) In these patients, impaired 2B4 and SLAM, signaling due to SAP-deficiency causes an inability of NK cells to lyse EBV-infected cells.(195-198) These findings first defined 2B4 as an activating receptor for NK cells. However, additional observations indicated that 2B4 biology was more complex, and this receptor could deliver either activating or inhibitory signals depending on several parameters. First, two isoforms of 2B4 were identified on murine NK cells. These two isoforms arise from alternative splicing and display opposing functions on NK cells.(199, 200) The 2B4-long (2B4-L) isoform is composed of an intact intracytoplasmic domain (4 ITSMs) and mainly displays inhibitory function. Conversely, the 2B4-short (2B4-S) isoform possesses a truncated intracellular domain with only one ITSM and can exert activating functions.(200-202) Inhibitory functions of 2B4 were further evidenced with blocking antibody experiments *in vitro* and the development of $2B4^{-/-}$ mice. Indeed, antibody-mediated disruption of the 2B4-CD48 interaction or 2B4-deficiency increased NK cell lysis of CD48-expressing target cells.(201, 202) Additional studies defined that 2B4 inhibitory functions are potentiated by the intensity of 2B4 expression and the degree of cross-linking.(203) High expression and engagement of 2B4 leads to inhibitory signaling, whereas lower expression and engagement activates NK cells.(203) Intracellularly, this phenomenon is explained by the recruitment of distinct adaptor molecules. Engagement of 2B4 induces the phosphorylation of intracellular ITSMs, allowing recruitment of several src homology 2 domain-containing adaptor molecules including SAP, SHP-1, -2

and EWS-FLI1-activated transcript 2 (EAT-2/sh2d1b).(174, 204) While association of SAP to 2B4 ITSMs is associated with NK cell activation, recruitment of SHP-1, -2 or EAT-2 rather leads to inhibition of effector functions.(193, 194, 197, 200, 205-207) It is now commonly accepted that 2B4 inhibitory properties mainly occur in the condition of elevated expression of the receptor and limited SAP availability.

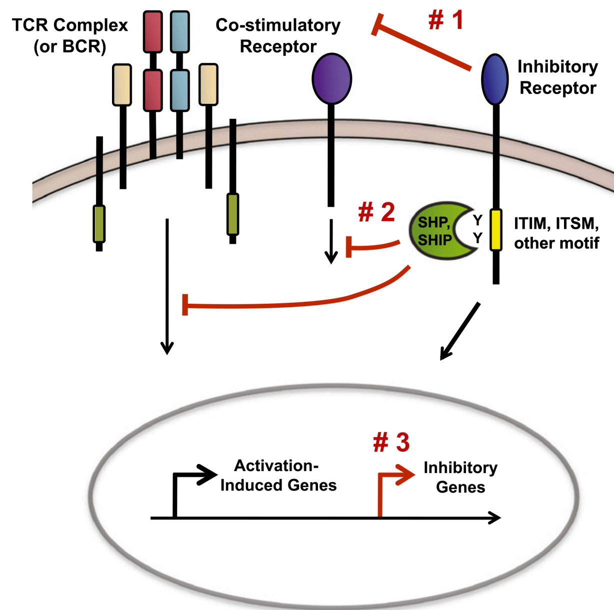
Unlike NK cells, 2B4 expression on $CD8^+$ T cells is not constitutive but can be triggered in vitro in the presence of cytokines (notably IL-2, IL-4 and IL-15) with or without additional stimulation.(188, 208) Interestingly, 2B4 expression on $CD8^+$ T cells increases during viral infection with the highest levels reported on exhausted $CD8^+$ T cells responding to a chronic viral infection.(49) Indeed, elevated levels of 2B4 were detected on $CD8^+$ T cells in mice chronically infected with LCMV (Cl-13) and patients with HIV, HCV, HBV, EBV, and HTLV1.(48, 49, 171, 194, 209-212) In these particular contexts, 2B4 mainly exerts inhibitory functions on $CD8^+$ T cells. Indeed, in vitro blockade of 2B4 or its cognate receptor CD48 increased the proliferation, cytotoxicity and cytokine secretion of antigen-specific $CD8^+$ T cells isolated from mice infected with LCMV (Cl-13) and patients with HCV, HBV and HTLV1.(49, 210, 211, 213) In mice, 2B4 selectively causes the deletion of conventional memory $CD8^+$ T cells challenged with a chronic viral infection (LCMV Cl-13).(214) Importantly, engagement of 2B4 also suppressed PDL1 blockade–mediated enhancement of HCV-specific $CD8^+$ T cell proliferation *in vitro*.(213) Taken together, these data demonstrate that during chronic viral infections, 2B4 mainly exerts inhibitory functions on $CD8^+$ T cells. However, one study in HCV patients also showed that 2B4 could mediate activating properties on $CD8^+$ T cells when expressed at low density and in the presence of high levels of intracellular SAP.(213) This is reminiscent of the dual functions of 2B4 previously observed on NK cell function that are regulated by these same characteristics (discussed above). As mentioned above, 2B4 expression is elevated on $CD8^+$ T cells during chronic viral infection and levels of intracellular SAP expression decrease with commitment to severe exhaustion.(48, 49, 213) These context-dependent parameters likely explain the overall inhibitory role of 2B4 on $CD8^+$ T cells during chronic viral infection.

1.3.4.5- T-cell Ig and mucin-domain containing-3 (TIM-3)

TIM-3 is an Ig-like transmembrane glycoprotein and is a member of the TIM family of genes including TIM-1 to 8 in mice (8 genes) and TIM-1, -3 and -4 in humans (3 genes).(215) Like other members of the TIM family of proteins, TIM-3 possesses two extracellular domains, an Ig-like domain and a mucin-like domain.(215) Within its cytoplasmic tail, TIM-3 carries a highly conserved tyrosine residue that is phosphorylated upon engagement of TIM-3 with its ligand and a src-homology (SH2)-domain binding motif.(215, 216) TIM-3 expression was reported on activated $CD4^+$ and $CD8^+$ T cells but also on DCs.(217, 218) Ligands for TIM-3 include phosphatidylserine, collagen, and most notably galectin-9 which is expressed on lymphocytes and other non-hematopoietic cell types (**Figure. 4 and Table. 1**).(218) Of note, the interaction between galectin-9 and TIM-3 on human and murine Th1 cells remains controversial as two conflicting reports obtained different results.(218, 219) However, the two studies used distinct recombinant forms of galectin-9 in their experiments which may account for the divergent results. Additional studies are needed to fully uncover this point. TIM-3 was first described on Th1 cells that express the highest levels of this receptor compared to other subtypes of effector $CD4^+$ T cells.(220, 221) In the study by Zhu *et al* (showing an interaction between galectin-9 and TIM-3), binding of TIM-3 to galectin-9 promotes a massive calcium influx that leads to the death of Th1 cells.(218) Therefore, TIM-3 has been implicated in the termination of Th1 cell responses as well as the maintenance of peripheral tolerance.(221, 222) Moreover, TIM-3 was also implicated in the regulation of autoimmune diseases, notably the experimental autoimmune encephalomyelitis (EAE) in mice and rheumatoid arthritis in humans.(223-225) De facto, TIM-3 was classified as an inhibitory receptor.

TIM-3 expression is extremely high on exhausted $CD8^+$ T cells during chronic viral infections. This observation was reported in mice with chronic viral infection (LCMV) and patients with HIV, HCV and HBV.(48, 110, 226-229) In these studies, TIM-3 expression defines a more dysfunctional population of $CD8^+$ T cells. In humans with chronic viral infections, blockade of TIM-3 on exhausted $CD8^+$ T cells *ex vivo*

improves several functions including Ag-driven proliferation, cytokine production (notably IFNγ), and killing potential.(226-230) In the LCMV mouse model, TIM-3 blockade alone has little impact but synergizes with PD-L1 blockade to reinvigorate $CD8^+$ T cell functions leading to better viral control compared to PD-L1 blockade alone.(110) TIM-3 is also of great interest in cancer. A substantial fraction of dysfunctional tumor infiltrating lymphocytes (TiLs) express TIM-3, and blockade of this receptor reinvigorates exhausted $CD8^+$ T cells, particularly when combined with PD-L1 blockade, leading to greater tumor regression.(231, 232) De facto, TIM-3 displays important inhibitory functions during chronic viral infections and cancer and represents an attractive target to rescue exhausted $CD8^+$ T cells in these contexts.



<u>Figure. 5</u>: Inhibitory receptor-mediated suppression of T cell functions. Inhibitory receptors use several methods to suppress T-cell functions including #1 ectodomain competition for co-stimulatory receptor ligands, #2 regulation of intracellular pathways (e.g. PI3K/AKT pathways), and #3 transcription of inhibitory genes. *Odorizzi JI 2012*

1.3.4.6- Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)

CTLA-4 is another member of the Ig superfamily of receptor encoded by *Ctla4*. Constitutively expressed on Tregs, CTLA-4 is also upregulated on activated $CD4^+$ and $CD8^+$ T cells upon TCR engagement.(233, 234) Structurally, this receptor is composed of an extracellular V domain, a transmembrane domain and a cytoplasmic tail. The intracellular domain of CTLA-4 does not contains ITIMs or ITSMs but a YvKM motif

allowing recruitment of the intracellular adaptor molecules SHP-2 and PP2A (protein phosphatase 2A) (**Figure. 4 and Table. 1**).(50, 235) Membrane CTLA-4 forms homodimers and like its co-stimulatory homolog CD28, binds to CD80 (B7.1) and CD86 (B7.2) on antigen presenting cells (APCs) but with a 10-fold superior affinity and a higher avidity compared to CD28.(50, 235-237) Inhibitory functions of CTLA-4 were first evidenced with its implication in several autoimmune diseases including type I diabetes, rheumatoid arthritis and the lymphoproliferative Chediak-Higashi syndrome.(238-240) Further, mice carrying a specific deletion of *Ctla4* (*Ctla4*$^{-/-}$) develop fatal autoimmune syndrome by 3-weeks of age associated with massive T-cell infiltration into several tissues and organs.(241, 242) In T cells, CTLA-4 mediates its inhibitory effects through two main mechanisms. First, CTLA-4 competes with CD28 for binding to CD80 and CD86 on APCs and this receptor even impairs the stability of the immune synapse.(235, 243) Second, by recruiting SHP-2 and PP2A via its YvKM motif, CTLA-4 promotes the dephosphorylation of activatory molecules downstream of TCR-signaling including CD3ζ, LAT [linker for activation of T cells and ZAP70.(243) Importantly, ipilimumab, a CTLA-4 monoclonal blocking antibody was the first immune checkpoint therapy approved by the FDA for the treatment of cancer patients (**Table. 1**). Ipilimumab notably showed impressive results in the treatment of late-stage melanoma and demonstrated synergistic effects in combination with nivolumimab (an anti-PD-1 monoclonal antibody).(244, 245) Ipilimumab is now ongoing clinical trials for the treatment of several cancers (lung, bladder and prostate cancers). Tremelimumab, another CTLA-4 blocking antibody also recently received FDA approval for the treatment of malignant mesothelioma.

1.3.4.7- T-cell Ig and ITIM domain (TIGIT)

TIGIT (*Tigit*) is the most recently described inhibitory receptor.(246) Member of the Ig-superfamily of receptors, TIGIT is expressed on a large variety of hematopoietic cells including on NK cells, NK T cells, Treg cells and activated T cells.(246) Structurally, TIGIT is composed of an Ig variable extracellular domain, a transmembrane domain and an intracellular domain containing two noteworthy sequences: an Ig tail

tyrosine (ITT)-like phosphorylation motif and an ITIM domain.(246) TIGIT possesses two ligands, CD112 and CD155, both expressed on monocytes, DCs, fibroblasts, endothelial cells, platelets and activated T and B cells.(246-250) Importantly, CD112 and CD155 are also expressed by a large variety of tumor cells.(251) Activation of TIGIT triggers SHIP-1 recruitment to its intracellular ITT-like domain.(252) The presence of an ITIM motif on TIGIT cytoplasmic tail also suggests potential recruitment of SHP-1, SHP-2 and SHIP-2 (**Figure. 4 and Table. 1**). Several studies evidenced the inhibitory functions of TIGIT on T cells. TIGIT expression is elevated on tumor infiltrating lymphocytes (TILs) in humans and TIGIT-positive $CD8^+$ T cells are less functional in several cancer mouse models.(253, 254) TIGIT blockade exerts therapeutic benefits in animal models of several cancers.(251) Furthermore, TIGIT was shown to act in synergy with TIM-3 to restraining antitumor responses.(254) In vitro, TIGIT suppresses CD3/CD28-induced T-cell proliferation, IFNγ secretion but increases IL-10.(255) TIGIT uses three distinct methods to inhibit T cell functions. Like CTLA-4 and CD28, TIGIT competes with CD226 and other costimulatory molecules for binding to CD112 and CD155.(251) But, TIGIT binds CD155 with a higher affinity compared to CD226.(255, 256) In addition, TIGIT can interact in *cis* with its antagonist CD226 and likely disrupts its homodimerization.(257) Finally, TIGIT directly decreases mRNA levels of TCRα, CD3ε, and PLCγ1, suggesting that this receptor directly restrains the transcription of molecules involved in T cell activation.(258)

	PD-1	LAG-3	CD160	2B4	TIM-3	CTLA-4	TIGIT
Ligands	-PD-L1 -PD-L2	MHC I	-HVEM -MHC I	CD48	-Galectin-9 (?) -Phosphatidyl-serine - Collagen	-B7-1 -B7-2	-CD112 -CD155
Super family	IgSF	IgSF	IgSF	IgSF	IgSF	IgSF	IgSF
Inhibitory sequence motifs	-ITIM -ITSM	KIEELE	No	4 ITSMs	-Y235 -Y242	YvKM	-ITT -ITIM
Effector molecules	-SHP-1 -SHP-2	N/A	N/A	-SHP-1 -SHP-2 -EAT-2	N/A	-SHP-2 -PP2A	-SHIP-1
Induced by	-TCR Signals -Support by IL6 and IL12	-TCR Signals ?	-TCR Signals -BLIMP-1	-IL-2 -IL-15 -BLIMP-1	-IL-2 -IL-15 -T-BET	Activation	N/A
Repressed by	-T-BET -BLIMP-1	N/A	N/A	N/A	N/A	N/A	N/A
Inhibitory effect on ($CD8^+$ Tcell)	-TCR Signaling -Costimulatory-signals -Proliferation -Cytokine secretion -Killing	-Proliferation	-Survival -Proliferation -Cytokine secretion	-Proliferation -Cytotoxicity -Cytokine secretion	-Proliferation -Cytokine secretion -Killing	-TCR signaling -CD28 mediated Costimulatory signals	Proliferation - Synthesis of activating molecules -IFNγ
FDA Approved	-Nivolumab -Pembrolizumab	N/A	N/A	N/A	N/A	-Ipilimumab -Tremelimumab	N/A

Table. 1: Characteristics of inhibitory receptors

1.3.5- Loss of plasticity to memory T cell development

Another dramatic consequence of exhaustion is a complete loss of developmental plasticity towards the effective development of memory T cells. $CD8^+$ T cells that have experienced persistent antigenic stimulation are incapable of differentiating into memory T cells and also fail to acquire key properties required for long-term maintenance and protective ability.(51, 101) Even after control of a chronic LCMV Cl-13 infection or after transfer into Ag-free animals, fully exhausted $CD8^+$ T cells fail to re-acquire phenotypic traits of conventional memory T cells. This includes high expression of IL7R (CD127), IL2Rβ (CD122), the L-selectin CD62L and the chemokine receptor CCR7.(19, 20, 51) Such inability to develop memory gradually emerges and becomes definitive and irreversible as $CD8^+$ T cells commit to severe exhaustion.(51) This incapacity of exhausted $CD8^+$ T cells to re-acquire cytokine receptor expression has dramatic functional consequences. Due to the absence of IL7Rα and IL2Rβ at the cell surface, these cells are unable to integrate homeostatic signals provided by IL-7 and IL-15, which are indispensable for homeostatic proliferation and survival of conventional memory cells.(19, 107-109, 259-261) Instead, exhausted $CD8^+$ T cells are maintained through extensive Ag-driven proliferation *in vivo* and develop an Ag-addiction for survival. This is demonstrated by the fact that exhausted $CD8^+$ T cells do not proliferate and rapidly die without Ag even in conditions where the chronic inflammatory context is preserved.(19, 20) Furthermore, exhausted $CD8^+$ T cells express extremely low levels of BCL2, a pro-survival protein triggered by IL-7 and IL-15, which is critical for the survival of conventional memory T cells.(19, 260) This Ag-dependent survival mode of exhausted $CD8^+$ T cells is particularly problematic in HIV patients as epitope escape mutations or decline in viral loads triggered by highly active antiretroviral therapy (HAART) often cause a drop in the number of effector $CD8^+$ T cells.(262-264)

Ineffective de novo expression of the adhesion molecule CD62L and the chemokine receptor CCR7 likely precludes the redistribution of exhausted $CD8^+$ T cells to memory T cell niches, a privileged environment for survival and homeostasis of memory cells.(265, 266) Unlike conventional memory T cells that efficiently populate

niches in secondary lymphoid organs (spleen, lymph nodes), exhausted $CD8^+$ T cells preferentially accumulate in peripheral tissues (liver, lung, brain) and the bone marrow and such unconventional distribution persists even after local viral control.(2) A similar preferential accumulation of exhausted $CD8^+$ T cells at non-lymphoid sites (liver) was reported in patients with HCV.(267) Hence, chronic viral infections gradually instigate intrinsic defects in $CD8^+$ T cells precluding their differentiation into memory cells and their re-localization to memory niches.

Concomitant with their lack of differentiation to memory cells, exhausted $CD8^+$ T cells also fail to acquire functional properties of memory cells that are required for the rapid elimination of secondary infections. This includes an altered capacity to produce IL-2, to produce antiviral cytokines (IFNγ, TNFα), and to proliferate upon secondary Ag-encounter.(2, 19, 51, 106) Further, even after viral control, chronically-stimulated $CD8^+$ T cells retain substantial expression of PD-1 and rapidly up-regulate this inhibitory receptor upon Ag-challenge.(51, 60, 106, 268) This demonstrates that exhausted $CD8^+$ T cells are not only incapable of acquiring protective functions of conventional memory cells but are also marked with exhaustion-associated “scars” that further inhibit their protective potential. Factors underlying the loss of plasticity toward memory during chronic viral infections remain elusive and immuno-modulatory methods (i.e. PD1/PD-L1 blockade) that are aimed at reinvigorating exhausted $CD8^+$ T cells fail to overcome the developmental defect.(104)

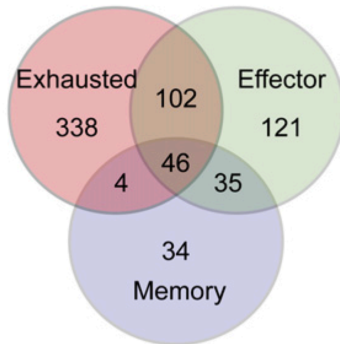
II- $CD8^+$ T cell exhaustion: a unique differentiation program

$CD8^+$, and to a greater extent $CD4^+$ T cells are characterized by their incredible developmental plasticity. One major goal of studying T cell responses to pathogens has been to distinguish and classify the multiplicity of effector T cell subtypes based on their selective expression of lineage-specific transcription factors (TFs). Transcriptional specificities of exhausted $CD8^+$ T cells are discussed below.

2.1- A unique transcriptional program

Comparative transcriptional analysis between naïve, effector, memory (acute settings) and exhausted (chronic settings) $CD8^+$ T cells provided genetic evidence for the uniqueness of the exhaustion differentiation program.(48) Approximately 490 genes are differentially expressed between exhausted and naïve $CD8^+$ T cells. Most of these genes (338) are exhaustion-specific as they are not differentially expressed within effector or memory T cells compared to naive cells. Clustering analysis revealed a set of genes specifically up-regulated (123 genes) or down-regulated (135 genes) in exhausted $CD8^+$ T cells. This transcriptional signature further defines exhaustion as a unique differentiation program distinct from typical effector and memory T cells (**Figure. 6**). Interestingly, exhausted $CD8^+$ T cells show more similarities with effector cells compared to memory cells. Among the 490 genes significantly differentially expressed between exhausted and naive CD8 T cells, 102 are shared with effector cells but only 4 with memory cells. Similarities between effector and exhausted cells gradually vanish as effectors transition to memory and also conversely increase after PD-L1 blockade.(48, 104) This result reflects the previously described inability of exhausted $CD8^+$ T cells to differentiate into memory cells.(19, 20) Of note, a direct comparison of exhausted $CD8^+$ T cells with SLEC and MPEC generated during an acute infection was not provided. As exhausted $CD8^+$ T cells undergo terminal differentiation similar to SLEC, one would predict that exhausted cells share more similarities with SLEC compared to MPEC.(27, 28, 34, 269) Genes specifically modulated in exhausted $CD8^+$ T cells encode several inhibitory receptors (e.g., PD-1, 2B4, KLRA7, CD160, PTGER4, LAG-3, CTLA-4), signaling pathway molecules, cytokine receptors (e.g., TNFR [p55 and p75], IL-4Rα [CD124], IL17R and IL-18R1), cytokine signaling molecules (the janus tyrosine kinases 1 and 3 [JAK1 and 3] and the signal transducers and activators of transcription b [STAT5b]), effector functions (IFNγ, Perforin), vesicle transport, cytoskeleton regulation, chemokines and migration (e.g., CCR7, CD62L), metabolism, and transcription factors (15 upregulated [e.g., PBX3, PRDM1, EOMES, NFATC1, JAK3, NURR1, MAF] and 11 downregulated [e.g., FOS, FOSB, JUNB, MYB, MYC, KLF2]). A lineage-specific

transcription factor linked to the development of exhaustion development remains to be determined.



<u>Figure. 6</u>: Genes differentially expressed between effector, memory, and exhausted CD8$^+$ T cells. This Venn diagram shows the distribution of genes differentially or similarly modulated in effector, memory, and exhausted CD8$^+$ T cells.
Wherry Immunity 2007

Another concern regarding exhaustion as a unique differentiation program was close similarity of exhaustion with anergy (**Table. 2**). Anergy is a state of hyporesponsiveness caused by sub-optimal activation of CD8$^+$ T cells which received TCR signals in the absence of optimal co-stimulation in vivo.(270) These CD8$^+$ T cells persist *in vivo* but are deprived of key effector functions including the capacity to proliferate to Ag or to produce cytokines (IL-2, TNFα).(270) Some authors also observed inhibition of TCR signaling (defective ZAP-70 phosphorylation) as well as defective MAP kinase and tyrosine kinase activation.(270) The inhibitory receptor CTLA-4 was also involved in the process.(270) Further, the transcription factor NFAT which is essential for anergy establishment on T cells, promotes both anergy and exhaustion during persistent viral infection in mice.(271, 272) This similarity tightens the gap between anergy and exhaustion. However, additional studies defined a cluster of 14 anergy-associated genes. This set of genes was extremely powerful as it was consistent between both *in vitro*-induced anergy (induced by blocking Ca2$^+$/calcineurin signals after PMA/ionomycin stimulation) and the more physiologic *in vivo* anergy (also called “adaptive tolerance”).(271) Few of these anergy-related genes were upregulated in exhausted CD8$^+$ T cells, and gene set enrichment analysis (GSEA) further excluded a possible enrichment of these genes in exhausted CD8$^+$ T cells.(48) In addition, while anergy is rapidly established after TCR stimulation, establishment of the exhaustion process is slower.(2, 51, 270) This underlines a fundamental difference between anergy

and exhaustion, the later requiring persistent Ag-stimulation while the former does not. During the initiation of a chronic viral infection, $CD8^+$ T cells are efficiently stimulated, unlike anergic cells.(51) This evidence demonstrated that, despite clear overlapping similarities, exhaustion and anergy are distinct developmental programs. However, most of our knowledge about anergy comes from $CD4^+$ T cell studies. A more accurate analysis of anergy on $CD8^+$ T cells is necessary to definitively separate these developmental lineages.

Senescence has long been tied with exhaustion.(273) This process (also known as replicative senescence) occurs in every cell type and causes a drastic growth arrest after extensive replicative events.(273) Senescence is triggered by two main factors: telomere shortening or unfixable non-telomeric DNA damage. These factors set off a DNA damage response (DDR) that immediately stops cellular replication until the repair is over. If DNA replication cannot be completed successfully, senescent cells are irreversibly maintained in an unresponsive state.(273) In many examples of chronic viral infection in humans (e.g., EBV, HIV, HCV, HBV), highly differentiated $CD8^+$ T cells present characteristic features of senescence, including lower telomerase activity and telomere length. This was correlated with decreased $CD8^+$ T cell function and poor clinical outcome in HIV patients.(58, 274-276) As mentioned above, senescence arrests the cell-cycle, a feature also observed during exhaustion.(19, 106) Lastly, both senescence and exhaustion are progressive processes that require persistent antigenic stimulation.(101, 273) This suggested that senescence and exhaustion might represent overlapping processes. However, several factors indicate a clear distinction between these processes (**Table. 2**). First, it is commonly accepted that in mice and humans, pre-senescent cells can be identified by the expression of KLRG1 and CD57 respectively.(25, 112, 277) Typical markers of exhaustion (e.g. PD-1) do not correlate with KLRG1 in mice or CD57 in humans.(48, 49, 112) Second, replication arrest in senescent cells is driven by specific DDR-associated kinases, including ataxia telangiectasia mutated (ATM) and RAD3 (ATR), that subsequently promote P53 and P21-mediated cell cycle arrest.(273) Conversely, exhaustion-induced proliferation arrest is caused by the direct impact of inhibitory receptors on G1-S cell cycle progression.(49, 104, 106) This

demonstrates that exhaustion and senescence are distinct processes ultimately converging to cell growth arrest. However, PD-L1 blockade in HIV-specific CD8$^+$ T cells was shown to increase telomerase activity.(276) The transcriptional profile of senescent cells also presents some similarities with that of exhausted CD8$^+$ T cells.(278, 279) Finally, enhanced telomerase activity on HIV-specific CD8$^+$ T cells improves effector function and proliferation.(280) This suggests an interplay between exhaustion and senescence which remains to be elucidated.

	Anergy	**Senescence**	**Exhaustion**
Triggered by	-Suboptimal activation	-Telomere shortening -Unfixable DNA damage -Growth factor deprivation	-TCR-signals -Cytokines
Require persistent antigenic stimulation	NO	YES	YES
Establishment	Rapid	Progressive	Progressive
Characteristics common to exhaustion	-Low proliferation -Low cytokine secretion (IFNγ, TNFα) -Impaired TCR signalling -NFAT, CTLA-4	-Diminished telomerase length -Impaired cell-cycle progression	
Characteristics distinct from exhaustion	-14 anergy-specific genes (e.g. *Egr2*, *Egr3*, *Rnf128*)	-Senescence-specific markers (KLRG1 in mouse, CD57 in humans)	

Table. 2: Characteristics of anergy, senescence and exhaustion

2.2- Key transcription factors

Since the molecular profiling of exhausted CD8$^+$ T cells was performed by Wherry *and colleagues*,(48) several transcription factors were shown to be critical for the establishment of CD8$^+$ T cells (**Table. 3**). One intriguing observation is that certain of these transcription factors display opposing functions during acute and chronic viral infections. This underlines a context-specific role of these TFs during viral infections.

Listed below are relevant TFs associated with $CD8^+$ T cell exhaustion, and when pertinent, a comparison of their role during acute and chronic infection is made.

TFs	Expression/Activity				Regulation		
	Naïve	*Effector (acute)*	*Memory (acute)*	*Exhausted (chronic)*	*Triggered by*	*Repressed by*	*Impact on other TFs*
T-BET	-	+++	+/-	+	TCR, IL-12 -27, Type I IFNs	N/A	EOMES (-)
EOMES	+	++	+++	++++	IL-2 -4 -15	IL-12	T-BET (-)
TCF1	+++	+	++	+/-	Wnt-β-catenin pathways	N/A	EOMES (+)
BLIMP-1	-	+++	+/-	++++	IL-2 -12	N/A	BCL6 (-) EOMES (-) ID3 (-) T-BET (+) ID2 (+)
NFATC1	+	+	N/A	++	$Ca2^+$ influx	N/A	N/A
BATF	+	++	+	+++	TCR, IL-12 -21, PD-1		T-BET (+) BLIMP-1(+)
FOXO1	++	-	++	+++	PD-1 (indirect)	TCR, co-stim, IL-2 -12, type I IFNs	T-BET (-) KLF2 (+) EOMES (+) TCF1 (+)

Table. 3: Key features of relevant TFs involved in $CD8^+$ T cell differentiation and exhaustion

2.2.1- T-BET (*Tbx21*)

The T-box transcription factor T-BET is a critical determinant for T cell fate decision during infection. Naïve T cells have low levels of T-BET, but its expression is triggered by TCR signals and magnified by multiple pro-inflammatory cytokines including IL-12, IL-27, Type I (α,β) and II (γ) IFNs (but not IL-2) through the adaptor molecules STAT1 but also STAT3 and likely STAT4 (IL-12).(28, 281, 282) T-BET was first described as a critical TF instigating CD4 T-cell lineage development toward IFNγ-producing Th1 effectors.(283, 284) Subsequent studies also uncovered a role for T-BET in the lineage commitment of $CD8^+$ T cells toward SLECs development and terminal differentiation during an acute viral infection (**Figure. 7**).(28) Indeed, SLECs express

higher levels of T-BET compared to MPECs.(28) T-BET-deficient $CD8^+$ T cells generate few SLEC while T-BET overexpression forces SLEC differentiation during an acute viral infection.(28) Such impact of T-BET on SLECs development is cell-intrinsic as the authors used adoptive transfer experiments of T-$BET^{-/-}$ LCMV-specific P14 $CD8^+$ T cells into WT mice infected with LCMV to draw their conclusions.(28) Concomitant with its role in SLEC differentiation, T-BET represses IL7Rα-expression on $CD8^+$ T cells, and restrains central memory development but is critical for effector memory development (**Figure. 7**).(285, 286) T-BET conjointly with its paralogue EOMESODERMIN (EOMES encoded by *Eomes*) also promotes CTL effector function notably IFNγ, granzyme B and perforin, increasing their cytotoxic potential.(286-288) IL-2 production is hindered by T-BET which is consistent with the fact that SLECs ("T-bet^{hi}") produce less IL-2 than MPECs (T-bet^{low}).(27, 28, 285, 286) Together, T-BET promotes both $CD8^+$ T cell terminal differentiation and acquisition of effector functions during an acute viral infection.

As soon as day 8 p.i., T-bet expression is reduced in effector $CD8^+$ T cells responding to a chronic viral infection (LCMV Cl-13) compared to their equivalent in acutely infected LCMV Arm mice. Its expression further decreases in extreme conditions in which $CD4^+$ T cells are removed from LCMV Cl-13 mice.(123) This demonstrates that during a chronic viral infection, Ag-persistence and/or prolonged inflammation reduce T-BET expression in $CD8^+$ T cells.(289, 290) This result is inconsistent with the known role of TCR signaling and inflammatory cytokines in promoting T-BET expression (see above). Yet unresolved chronic infection-exclusive mechanisms likely act to temper T-BET expression during exhaustion establishment. Of most importance, T-BET deficiency dramatically reduces the number and functionality (IFNγ, MIP-1α) of Ag-specific $CD8^+$ T cells and concomitantly increased viral loads.(123) Therefore, T-BET is critical in sustaining $CD8^+$ T cell responses during a chronic viral infection (**Figure. 7**). T-BET directly represses PD-1 (*Pdcd1*) expression, restrains LAG-3, CD160, and BTLA, but has no effect on 2B4 expression. Conversely, this TF was critical for the early induction of TIM-3 in accordance with previous observations.(123, 291) These functions of T-BET on $CD8^+$ T cells during a chronic viral infection are cell-intrinsic as experiments were

conducted in bone marrow chimeras (50%WT/50%T-BET$^{-/-}$) and further confirmed using adoptive transfer experiments of T-BET$^{-/-}$ CD8^{+} T cells into mice subsequently infected with LCMV Cl-13.(123) Importantly, T-BET overexpression lends to a less differentiated PD-1$^{int/low}$ state while T-BET-deficient CD8^{+} T cells rapidly turn to a PD-1^{hi} terminally exhausted state.(123, 269) T-BET deficiency also restores substantial CD127 expression at early time points p.i. (day 8). Together, these results ascribe a context-dependent role for T-BET on CD8^{+} T cell differentiation. This TF promotes SLEC terminal differentiation during an acute viral infection. However, during Ag-persistence, T-bet restrains terminal differentiation to PD-1^{hi} cells and is critical to maintaining a pool of less differentiated PD-1$^{int/lo}$ progenitors.

2.2.2- EOMESODERMIN (*Eomes*)

EOMESODERMIN (EOMES), encoded by *Eomes* is another T-box transcription factor that functions as a paralogue of T-BET in CD8^{+} T cells.(292) While devoid of T-bet, naïve CD8^{+} T cells express substantial levels of EOMES.(282, 285, 288, 293) Upon viral infection, the EOMES expression kinetic opposes that of T-BET. EOMES expression only slightly increases in developing effectors and further intensifies during effector to memory transition, reaching maximum levels on memory cells.(286, 288, 294) Key requirements for EOMES induction in CD8^{+} T cells are also distinct to that of T-BET. Indeed, several members of the common gamma-chain family of cytokines, mostly IL-2 but also IL-4 and IL-15, control EOMES induction in CD8^{+} T cells.(282, 293, 295) However, TCR-signals do not increase EOMES levels, as opposed to T-BET, and its expression is repressed by IL-12.(282, 293) In the absence of T-BET, EOMES expression increases in effector and memory CD8^{+} T cells suggesting a restrictive role of T-BET on EOMES expression.(285) Unlike T-BET, EOMES has little impact in the cell fate decision toward SLECs and MPECs during an acute viral infection, and both effector subtypes present equivalent levels of the TF.(28, 285, 294) However, while T-BET favors terminal differentiation of SLECs,(28) cell-intrinsic EOMES is crucial for proper development and maintenance of Tcm cells (verified using BM chimeras and adoptive transfer experiments of EOMES$^{-/-}$ P14 CD8^{+} T cells) (**Figure. 7**).(294) Despite this

antagonism, T-BET and EOMES collaborate to promote IFNγ, perforin, and granzyme B expression in effector CD8$^+$ T cells.(286-288) EOMES even binds directly to the perforin promoter region in CD8$^+$ T cells.(282) T-BET and EOMES are also both required for the maintenance and homeostasis of memory cells, given their shared capacity to induce IL2Rβ-expression on CD8$^+$ T cells which enables IL-15-mediated homeostatic proliferation.(286) Together, T-BET and EOMES have reciprocal but also common functions during CD8$^+$ T cell differentiation.

During a chronic viral infection, EOMES expression in exhausted CD8$^+$ T cells largely exceeds expression levels found in typical effector or memory cells and remains extremely high with viral persistence.(269) EOMES levels positively correlate with inhibitory receptors expression on CD8$^+$ T cells, and EOMES haploinsufficient effectors appear more functional.(269) Reciprocity between T-BET and EOMES is even more pronounced in the chronic context as the expression of these TFs is almost exclusively seen during chronicity. While T-bet^{hi} effectors (PD-1$^{int/lo}$) identify less terminally exhausted effectors, Eomes^{hi} cells referred to as PD-1^{hi} effectors are the most terminally differentiated and severely exhausted population.(269) Importantly, EOMES is critical for the development and maintenance of these PD-1^{hi} terminal effectors in striking contrast to its role in promoting Tcm cells development during an acute viral infection (**Figure. 7**).(269, 294) A cell-intrinsic role for EOMES in the development of PD-1^{hi} cells is strongly suggested by temporal deletion of this TF in CD8$^+$ T cells using a Tat-Cre recombinase system.(269) Eomes^{hi} (PD-1^{hi}) effectors are more severely exhausted but retain higher cytolytic potential and granzyme B expression compared to the less exhausted T-bet^{hi} (PD-1$^{int/lo}$) progenitors.(269) This suggests that EOMES supports cytolytic function of PD-1^{hi} effectors which is reminiscent of its role during an acute viral infection. Importantly, deletion of either T-BET or EOMES impairs CD8$^+$ T cell maintenance during chronicity leading to uncontrolled viremia.(123, 269) Thus, despite being associated with severe and terminal exhaustion, EOMES is critically required to sustain effector CD8$^+$ T cell responses to chronic viral infection. The molecular pathways by which EOMES sustains PD-1^{hi} cell development and other TF potentially involved in the process remain to be determined. The critical impact of EOMES on Tcm development

in acute settings contrasts with its role in promoting terminal exhaustion during a chronic viral infection. This is another example of the context-specific functions potently achieved by certain TFs on $CD8^+$ T cell differentiation.

2.2.3- T Cell Factor 1 (*Tcf7*)

T Cell Factor 1 (TCF-1) is a downstream TF of the canonical WNT-β-catenin signaling, an evolutionarily conserved pathway directing important functions in the immune system.(296) This pathway controls hematopoietic stem cells (HSCs) quiescence and is essential for their self-renewal.(296) The WNT-β-catenin signalling also greatly influences T-cell development, maturation and differentiation.(296) Importantly, WNT-signaling in $CD8^+$ T cells direct the development of a highly functional population of memory cells referred to as memory stem cells (Tscm) with enhanced antitumor properties.(297) Activation of this pathway follows the engagement of WNT proteins with their cognate receptor (Frizzled) at the surface of target cells. Of note, the human genome contains 19 *Wnt* genes each encoding secreted glycoproteins.(296) Activation of WNT-signalling stabilizes intra-cytoplasmic β-catenin molecules that subsequently reach the nucleus to act as key co-activators for TCF-1, enabling efficient transcription of WNT-dependent genes. In the absence of WNT-signaling, β-catenins are degraded by the proteasome, and TCF-1 preferentially associates with its co-repressor TLE1 to inhibit gene expression.(296) Hence, TCF-1 displays both activating and inhibitory functions dependent on the activation status of the WNT-β-catenin pathway and subsequent availability of binding partners. TCF-1 plays a critical role in normal thymic development,(298) and recently, it was shown to influence effector and memory $CD8^+$ T cell differentiation during acute infections.(299, 300) Naïve $CD8^+$ T cells express the highest levels of TCF-1, and its expression declines during the effector phase while it increases de novo during memory formation.(297) TCF-1 alone has little impact during primary effector $CD8^+$ T cell development. The absence of TCF-1 in $CD8^+$ T cells slightly induces SLEC development and terminal differentiation traits, including low IL-2 production.(300) Proliferation and granzyme B expression are also decreased in the absence of TCF-1.(300) Of note, combined absence of TCF-1 and LEF-1, another Wnt-

dependent TF, completely abrogates MPEC development.(299) TCF-1 has a greater impact on the development of memory cells. Constitutive TCF-1 expression in $CD8^+$ T cells increases memory cell generation,(301) and inversely, TCF-1-deficiency abrogates Tcm development (**Figure. 7**).(300) Such impact of TCF-1 on Tcm development is cell-intrinsic as the authors used adoptive transfer experiments of $Tcf7^{-/-}$ OT-1 $CD8^+$ T cells into WT animals infected with *Listeria monocytogenes* (LM-OVA) to draw their conclusions.(300) Phenotypically, TCF-1-deficient memory cells fail to re-express canonical Tcm markers, including CD62L and CCR7, efficiently re-express CD127, and remain mostly KLRG1-positive. These altered memory cells fail to re-acquire IL2Rβ-expression, fail to perform homeostatic proliferation, and have low Bcl-2 protein levels resulting in ineffective long-term maintenance.(300, 302) Thus TCF-1 is not only a critical factor for Tcm differentiation but also for their long-term maintenance, a role shared with Eomes (see above). Interestingly, the WNT-signaling pathway induces Eomes expression in $CD8^+$ T cells partly through a direct interaction of TCF-1 in the Eomes promoter region, and Eomes overexpression in TCF-1-deficient cells restores IL2Rβ-expression and memory cell longevity.(300) Together, TCF-1-induced Eomes largely contributes to memory $CD8^+$ T cell maintenance.

TCF-1 recently received attention regarding its function in the differentiation of chronically-stimulated $CD8^+$ T cells. A stable population of Ag-specific $CD8^+$ T cells expresses TCF-1 in mice and patients with chronic viral infections.(303, 304) That small population is also identifiable by selective expression of the chemokine receptor CXCR5 (a chronic-specific marker of $CD8^+$ T cells) and preferentially locates in “virus-free” zones within the secondary lymphoid tissues (T cell zone in the spleen).(303) Computational analysis also revealed that $TCF\text{-}1^+$ Ag-specific $CD8^+$ T cells (or $CXCR5^+$) share transcriptional similarities with overall exhausted cells and express characteristic markers of exhaustion including PD-1 and LAG-3. But unlike exhausted $CD8^+$ T cell, $TCF\text{-}1^+$ effectors also exhibit transcriptional features of central memory cells and hematopoietic stem cells but not effector cells.(303, 304) $TCF\text{-}1^+$ effectors express substantial levels of central memory markers, including CD62L and CD127 and present low basal proliferation in vivo, reminiscent of stem cell quiescence. Adoptive transfer

experiments revealed that TCF-1^{+} (CXCR5^{+}) cells serve as progenitors and replenish terminally exhausted TCF-1^{-} (CXCR5^{-}) effectors.(303, 304) Of note, TCF-1^{+} cells are a novel population distinct from PD-1$^{int/lo}$ progenitors as they express high levels of EOMES but low T-BET.(303, 304) Importantly, TCF-1-deficiency abrogates the development of this memory/stem cell-like population, ultimately leading to ineffective maintenance of CD8^{+} T cell responses to chronic viral infections.(303, 304) Adoptive transfer experiments of TCF7$^{-/-}$ P14 CD8^{+} T cells into LCMV Cl-13 infected mice evidenced a the cell-intrinsic impact of TCF-1 in the development of this memory/stem cell population.(303, 304) Interestingly, TCF-1^{+} cells exclusively respond and proliferate to PD-L1 blockade as opposed to TCF-1^{-} cells.(303, 304) Thus, TCF-1 is a critical TF essential for the differentiation of a novel chronic exclusive CXCR5^{+} memory/stem cell-like population capable of continuous replenishment of terminally exhausted CD8^{+} T cells, and these cells are highly responsive to PD-L1 therapy.

2.2.4- BLIMP-1 (*Prdm1*)

BLIMP-1, encoded by *Prdm1,* is a transcriptional repressor that displays critical functions in adaptive immune cells homeostasis, development, and differentiation.(305, 306) Upon infection, BLIMP-1 is critical for the terminal differentiation of B cells into Ig-producing plasma cells.(307-309) This TF also impedes Tfh development by competing with its antagonist BCL6.(310) In CD8^{+} T cells, BLIMP-1 is essential for the development and terminal differentiation of SLECs but represses memory T cell establishment (**Figure. 7**).(30, 35) Accordingly, BLIMP-1 expression is low in naïve CD8^{+} T cells but increases in effectors and gradually declines as the cells mature into memory.(30, 34) SLECs express higher levels of BLIMP-1 compared to MPECs.(30, 34) After Ag clearance, BLIMP-1 expression decreases on maturing memory cells, particularly on Tcm, but remains elevated in persisting KLRG1^{+} terminal effectors.(30) IL-2 is the primary inducer of BLIMP-1 in CD8^{+} T cells but recent work also suggested a role for IL-12 in the process.(311, 312) This is in accordance with the well-known function of these cytokines in driving lineage cell-fate decision toward SLEC development.(28, 67, 282, 313, 314) Mouse models deficient for BLIMP-1 which

includes the use of BM-chimeras (50%WT-50%BLIMP-$1^{-/-}$) and adoptive transfer of Blimp-$1^{-/-}$ P14 $CD8^+$ T cells into WT animals, unraveled a cell intrinsic function of this TF in driving SLEC development and restraining memory $CD8^+$ T cells differentiation.(30, 35, 312) In the absence of BLIMP-1, SLEC development is nearly abrogated, and $CD8^+$ T cells converge more rapidly to a central memory phenotype. Trafficking of effector $CD8^+$ T cells to non-lymphoid locations is also altered, as is the expression of key chemokine receptors (CCR7[up], CCR5[down]).(30, 35, 312) Functions of BLIMP-1 are not limited to primary infection as SLEC development is also abrogated on secondary effectors in the absence of this TF.(30, 35) In fact, BLIMP-1 modifies the transcriptional program of developing effectors to foster terminal differentiation. Transcriptional analysis revealed 128 genes differentially expressed between WT and Blimp-$1^{-/-}$ effectors.(30) Several TFs involved in $CD8^+$ T cell terminal differentiation (T-BET, ID2) are downregulated whereas memory-development associated TFs (EOMES, BCL6, ID3) are upregulated.(30, 35, 312) BLIMP-1 deficient effectors also lack granzyme and perforin expression which eventually affects cytolytic potential.(30, 35, 312) Of note, Blimp-1 restrains the proliferation and survival of effector $CD8^+$ T cells.(30, 35) That phenomenon likely stem from the direct repression of IL-2 transcription by Blimp-1 which in turn likely causes cytokine deprivation-cell death.(315) BLIMP-1 also recruits histone modifying enzymes (G9a, HDAC2) to the CD25 locus, further restraining IL-2 signaling.(316) Together, BLIMP-1 is a key TF that programs $CD8^+$ T cell fate decision toward terminal differentiation at the expense of memory development during acute infections.

$CD8^+$ T cells isolated from LCMV Arm or Cl-13 infected mice express similar amounts of BLIMP-1 at day 8 post LCMV infection.(162) Thereafter, BLIMP-1 expression strongly rises in exhausted $CD8^+$ T cells during chronicity while it slowly declines in acute effectors.(162) Most terminally exhausted $CD8^+$ T cells (PD-1^{hi}Eomes ^{hi}) express the highest levels of BLIMP-1 during a chronic viral infection, consistent with the pro-terminal differentiation functions of this TF.(30, 35, 162, 269) However, this observation contrasts with the known role of BLIMP-1 in repressing PD-1 and EOMES expression in $CD8^+$ T cells during acute viral infections, suggesting context-dependent

functions of this TF.(35, 124) Conditional deletion of BLIMP-1 (in activated T cells) increases the accumulation of virus-specific $CD8^+$ T cells, decreases inhibitory receptor expression (PD-1, LAG-3, CD160 and particularly 2B4), and restores memory features (CD62L, CD127, IL-2 production) .(162) This implies a central role for BLIMP-1in exhaustion establishment and associated loss of memory development and survival potential (**Figure. 7**). BLIMP-1-deficiency has little impact on antiviral cytokine secretion but dramatically affects granzyme B expression and cytolytic potential of $CD8^+$ T cells which leads to uncontrolled viremia.(162) Consequently, BLIMP-1 promotes $CD8^+$ T cell exhaustion but also supports important effector functions essential to control a chronic viral infection. Interestingly, deletion of a single allele of BLIMP-1 efficiently diminishes inhibitory receptor expression while keeping intact granzyme B expression and elevated killing potential.(162) Mice carrying such haploinsufficiency can control an LCMV Cl-13 infection more rapidly than WT mice.(162) A limit of the study by Shin *and colleagues* is the use of conditional knock-out mice in which BLIMP-1 is deleted not only in $CD8^+$ T cells but also in a fraction of $CD4^+$ T cells and NK cells (GrzB-Cre *Prdm1*$^{fl/fl}$ system). However, additional experiments using BM-chimeras (50%WT-50%GrzB-Cre *Prdm1*$^{fl/fl}$) strongly indicate a cell-intrinsic role for BLIMP-1 in the development of $CD8^+$ T cell exhaustion.(162) Together, this ascribes an equivocal role for BLIMP-1 on $CD8^+$ T cell responses to chronic viral infection.

2.2.5- NFATc1 (*Nfat2*)

Nuclear Factor of Activated T cells (NFAT) proteins are a Ca^{2+}-dependent family of TFs composed of 5 isoforms. Among them, NFAT1 (NFATc2) and NFAT2 (NFATc1) are constitutively expressed on mature T cells and greatly impact their activation and differentiation.(317-321) NFAT proteins possess numerous conserved serine residues that are heavily phosphorylated in naïve T cells, constraining their localization to the cytoplasm.(322-325) Upon TCR and co-stimulatory molecule engagement, calcium influx activates the protein phosphatase calcineurin which dephosphorylates NFAT proteins, allowing their transposition to the nucleus.(317, 322, 326) Nuclear NFAT triggers several genes involved in T cell activation (IL-2, IFNγ), particularly when

complexed with AP-1 (FOS-JUN).(327-330) NFAT proteins also promote T cell tolerance and anergy, demonstrating the contradictory nature of that family of TFs.(271, 331, 332)

Recent studies demonstrated an elevated level of NFATc1 (NFAT2) in exhausted $CD8^+$ T cells.(48) Although Ca^{2+} influx is conserved in these cells, a selective impairment of NFATc1 nuclear translocation was observed, resulting in reduced cytokine secretion.(333) NFATc1, -2, or double deficiency, further decreases cytokine secretion from exhausted $CD8^+$ T cells but lowers inhibitory receptor expression including PD-1, LAG-3, and TIM-3.(272) Consistently, retroviraly enforced expression of a constitutive form of NFATc1 increases PD-1, LAG-3 and TIM-3 levels on $CD8^+$ T cells. Adoptive transfer experiments of NFATc1, -2 and double deficient $CD8^+$ T cells but also retroviraly transduced $CD8^+$ T cells (with a constitutive form of NFATc1) evidenced a cell-intrinsic role of NFATc1 in promoting exhaustion traits.(272) This is consistent with the direct induction of PD-1 by NFATc1 and further underlines the contradictory functions of that TF on $CD8^+$ T cell exhaustion, which involves promoting cytokine secretion but also inhibitory receptor expression (**Figure. 7**).(120) In fact, NFATc1 forms a cooperative complex with its partner AP-1 supporting the transcription of genes involved in T cell activation. But NFATc1 alone (not complexed to AP-1) promote the expression of a pattern of genes associated with T cell exhaustion.(272) Together, NFATc1 promotes the establishment of a pro- or an anti-exhaustion transcriptional program depending on the presence of AP-1. However, the fact that NFATc1 nuclear translocation is blocked in exhausted $CD8^+$ T cells raises the question of how this TF mediates these functions.

2.2.6- BATF (*Batf*)

Basic leucine zipper transcription factor ATF-like (BATF) is a member of the AP-1/ATF superfamily of TFs. BATF achieves its transcriptional activity by forming heterodimers with binding partners including other members of the AP-1/ATF family (notably c-Jun) but also with the interferon regulatory factor 4 (IRF4).(334-337) This TF

regulates the differentiation and functions of many lymphocyte lineages, including Th17 and Tfh cells.(338-340) BATF is detectable at steady state on peripheral $CD8^+$ T cells, and its expression (potentiated by TCR signals and IL-12) increases with activation and slightly declines during memory maturation.(132, 336, 341, 342) Mice deficient for BATF (or IRF4) fail to control an acute LCMV infection. This was associated with a lower magnitude of $CD8^+$ T cell responses, evidencing a crucial role for BATF in sustaining adaptive immunity to viral infection.(341, 343) Adoptive transfer experiments of P14 $CD8^+$ T cells deficient or not for BATF into LCMV infected mice demonstrate that this TF intrinsically acts in $CD8^+$ T cells to sustain their proliferation and survival potential in vivo.(341) BATF also acts as an early checkpoint molecule shaping the cell-fate decision toward SLEC development (**Figure. 7**). In association with its partners IRF4 and c-Jun, BATF directly binds to and promotes the transcription of several lineage-specific TFs including T-BET and BLIMP-1.(341) BATF also directly promotes cytokine receptor expression that is known to function in directing SLEC differentiation (IL12R, IL2R, IFNAR).(28, 29, 67, 341) Despite these pro-CTL functions, BATF represses genes associated with effector function (IFNγ and perforin).(341) Together, BATF is a critical early checkpoint for SLEC commitment. This TF also increases the magnitude of $CD8^+$ T cell responses but concurrently restrains some effector functions.

$CD8^+$ T cells from LCMV Cl-13 infected mice express higher levels of BATF compared to their LCMV Arm counterpart.(132) BATF levels are also higher in virus-specific $CD8^+$ T cells from patients with progressive HIV compared to asymptomatic HIV-infected patients also known as "elite controllers" or "long-term nonprogressors (refers to a small fraction of HIV patients capable of handling the infection without treatment).(132) Interestingly, PD-1 ligation triggers BATF expression in primary human $CD4^+$ and $CD8^+$ T cells.(132) BATF also restrains TCR-induced AP-1 (c-FOS/c-JUN complex) transcriptional activity by forming heterodimers with c-JUN while displacing c-FOS.(336) Overexpression of BATF on T-cell lines (Jurkat) and knockdown experiments in primary T cells emphasize a role of this TF in restraining effector functions (IL-2, IFNγ) (**Figure. 7**).(132) Thus, induction of BATF in $CD8^+$ T cells was considered as a novel pathway by which PD-1 inhibits TCR-signaling during chronic viral

infections.(101) Rather, a study using BM-chimeras (50%WT-50%BATF$^{-/-}$) evidences a critical and intrinsic role for BATF in sustaining CD8^{+} T cell responses to a chronic viral infection (**Figure. 7**). In their model, IL-21 provided by CD4^{+} T cells augments BATF expression in CD8^{+} T cells, and BATF in association with IRF4 sustains virus-specific CD8^{+} T cell maintenance.(344) In support of this model, IL-21$^{-/-}$ CD8^{+} T cells express lower BATF, and this cytokine triggers BATF expression in CD8^{+} T cells *in vitro*.(344) Further, BATF deletion recapitulates the loss of CD8^{+} T cell responses observed after CD4-depletion or in the absence of IL-21 signaling, and retrovirally expressed BATF rescues these "unhelped" CD8^{+} T cells.(11, 18, 59, 345-347) BATF and IRF4 appear to mediate their impact by promoting BLIMP-1 expression.(344) However, BLIMP-1 is a pivotal TF for exhaustion establishment and associated dysfunctions.(162) Further studies are required to ascertain the functions of BATF in CD8^{+} T cell responses to chronic viral infections.

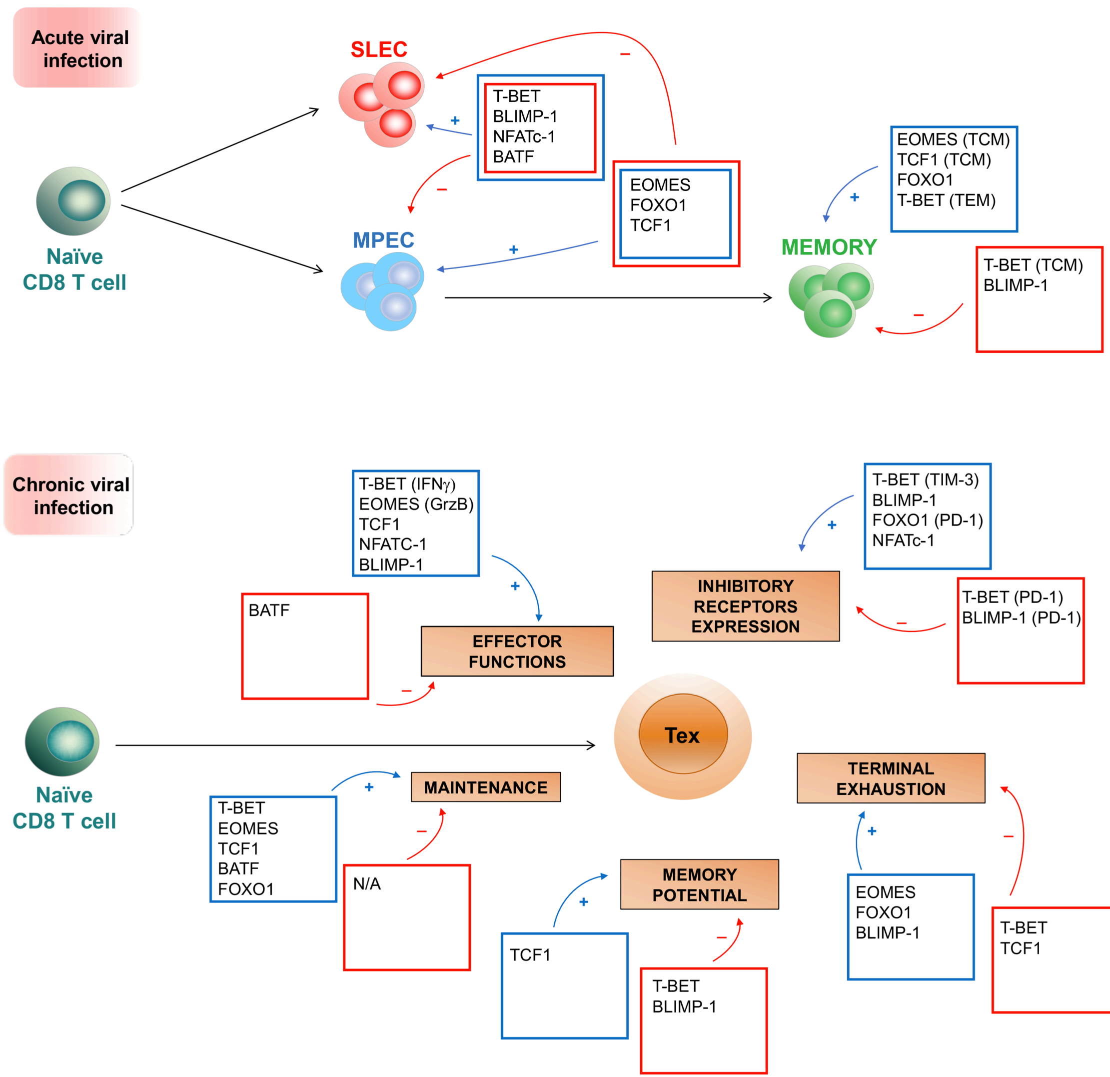
2.2.7- FOXO1 (*Foxo1*)

FOXO1 is a member of the FOXO gene family that also includes FOXO3, -4 and -6 in mammals.(348, 349) This family of TFs regulates several key cellular processes including cell cycle progression, survival, and differentiation.(348, 349) FOXO1 activity in CD8^{+} T cells depends on the activation status of the cell and is regulated by phosphorylation events downstream of the PI3K/AKT pathways. In naïve CD8^{+} T cells, FOXO1 is located in the nucleus and transcribes genes involved in trafficking and homeostasis, including KLF2 and IL7Rα.(350-352) Upon CD8^{+} T cell activation and effector differentiation, multiple stimuli (including TCR-signals, co-stimulation, IL-2, IL-12 or type I IFNs) trigger PI3K/AKT pathways that in turn phosphorylate FOXO1 at three conserved site, provoking its exclusion from the nucleus.(349, 353) Alternatively, PI3K/AKT activation triggers T-BET induction through mTOR/pS6 which favors CD8^{+} T cell terminal differentiation.(353-355) Of note, FOXO1 represses T-BET and increases EOMES expression in CD8^{+} T cell, favoring memory development over terminal differentiation (**Figure. 7**).(353, 356) After resolution of an acute viral infection and PI3K/AKT down-modulation, FOXO1 relocates to the nucleus and transcribes several

genes involved in memory development. FOXO1-deficiency has little impact in primary effectors accumulation but restrains MPEC development in some settings and profoundly modifies their transcriptional program which is in line with the heightened expression of this TF in MPECs over SLECs.(353, 354, 357, 358) FOXO1-deficiency favors the accumulation of terminal effector traits in MPECs (T-BET, granzyme B) but restrains the expression of a set of genes involved in memory development which is either directly (EOMES, TCF1, KLF2, IL7Rα) or indirectly (CD62L, CCR7) controlled by FOXO1.(353, 354, 357, 358) Subsequently, FOXO1-deficiency readily alters memory development (notably Tcm development) and maintenance, and remaining $FOXO1^{-/-}$ memory cells display poor protective potential consistent with an ineffective memory differentiation.(353, 354, 357, 358) The impact of FOXO1 during infections was studied using mouse models carrying a conditional deletion of FOXO1 in T cells (CD4-Cre *Foxo1*$^{fl/fl}$) or cytotoxic cells (GrzB-Cre *Foxo1*$^{fl/fl}$) which affects both $CD4^+$ and $CD8^+$ T cells but also some NK cells in the later.(354, 357) However, as mentioned above, FOXO1 directly promotes the expression of memory-associated genes in $CD8^+$ T cells.(353, 354, 357, 358) This strongly argues towards cell-intrinsic functions of FOXO1 in the development of memory $CD8^+$ T cells during acute viral infections.

Exhausted $CD8^+$ T cells generated during a chronic viral infection have higher FOXO1 protein levels compared to their acute counterparts.(359) In this particular context, elevated and sustained PD-1 expression on $CD8^+$ T cells suppresses proximal induction of PI3K/AKT by TCR and cytokine-mediated signals.(359) This results in decreased FOXO1-phosphorylation allowing its retention to the nucleus where it directly transcribes PD-1. Hence, by regulating PI3K/AKT activity, PD-1 increases FOXO1 activity which in turn sustains PD-1 expression, establishing a positive feedback loop.(359) Using mice carrying a conditional *Foxo1* gene knock-out on activated T cells (GrzB-Cre *Foxo1*$^{fl/fl}$) and adoptive transfer experiments of $FOXO1^{-/-}$ P14 $CD8^+$ T cells into WT animals infected with LCMV Cl-13, Staron and colleagues evidenced cell-intrinsic functions of FOXO1 in the development of $CD8^+$ T cell exhaustion.(359) They found that terminally exhausted $CD8^+$ T cells (defined as $PD\text{-}1^{hi}Eomes^{hi}T\text{-}bet^{low}$) express higher FOXO1 protein levels, and FOXO1-deficiency nearly abrogates the development

of that population.(269, 359) This is in line with the role that FOXO1 plays in promoting EOMES expression over T-BET in $CD8^+$ T cells but in contrast with its role in sustaining memory development over terminal differentiation during an acute viral infection.(353, 354, 357, 358) Alternatively, FOXO1 is essential to maintain $CD8^+$ T cell responses during a chronic viral infection, and absence of this TF in activated T cells leads to higher viremia.(359) Thus, during a chronic viral infection, FOXO1 activity increases in $CD8^+$ T cells. This TF directly promotes PD-1 expression and drives the development of terminally exhausted $CD8^+$ T cells, likely through its capacity to promote EOMES expression over T-BET. FOXO1 also sustains the proliferation and maintenance of $CD8^+$ T cell responses during a chronic viral infection and helps in controlling viremia.



<u>Figure. 7</u>: Impact of key TFs in the development of CD8^{+} T cell responses during acute and chronic viral infections. Upper panel shows the involvement of TFs in the differentiation of SLECs, MPECs and memory CD8^{+} T cells during an acute viral infection. Lower panel represents the impact of TFs in several aspects of CD8^{+} T cell exhaustion during a chronic viral infection. Blue quadrants and arrows stand for a positive impact on the designed functions and red quadrants and arrows signify a negative impact.

2.3- Unique epigenetic landscape

2.3.1- Epigenetic remodeling and T cell differentiation

2.3.1.1- Pioneer studies on CD4$^+$ T cells

Epigenetic modifications via cytosine methylation of conserved CpG sites or through histone modifications (methylation and/or acetylation) recently emerged as an important mechanism for the development, differentiation, and function of T cells.(356, 360-363) Upon T cell activation, epigenetic modifications imparted by DNA-modifying enzymes allow lineage-specific and other TFs to access the chromatin and transcribe target genes.(362, 364) The first evidence of this concept stemmed from the observation that Th1 versus Th2 CD4$^+$ T cell polarization is at least in part controlled by the establishment of "active" epigenetic marks at the IFNγ locus but and repressive marks at the locus of the Th2-cytokine IL-4.(365-369) Additional studies suggested a stepwise process during which STAT proteins, downstream of cytokine-receptor signaling, first access and open the chromatin allowing the subsequent binding of lineage-specific TFs. For example, in CD4$^+$ T cells IL-12-triggered STAT4 activation remodels chromatin at the Th1 gene locus which facilitates T-BET recruitment.(370, 371) Once engaged on the IFNγ distal regulatory region, T-BET displaces the histone deacetylase SIN3A, accentuating the permissive mark (H4) and subsequent Th1 polarization.(372) This demonstrates that cytokines first shape the epigenetic landscape of CD4$^+$ T cells through STATs and other downstream molecules, opening the way for lineage-specific TFs to further amplify this epigenetic remodeling and transcribe lineage-specific genes. CD4$^+$ T cells deficient for the histone methylase Enhancer of Zeste Homolog 2 (EZH2) do not completely convert to either Th1 or Th2 cells upon polarizing conditions but remain plastic.(373) Thus, epigenetic modifications control the expression of lineage-specific genes (IFNγ) and TFs (T-BET) and are extremely sensitive to polarizing environmental factors. Importantly, these modifications also ensure lineage stability, precluding further plasticity of terminally differentiated effectors.

2.3.1.2- DNA methylation and $CD8^+$ T cell differentiation

Cytosine methylation is a repressive epigenetic mark that is controlled by DNA methyltransferases, notably DNMT1, DNMT3A and DNMT3B.(374, 375) Strong evidence now suggests a critical impact of DNA methylation in the development and differentiation of effector $CD8^+$ T cells. First, DNMT1 is required for proper clonal expansion, survival, and polyfunctionality of effector $CD8^+$ T cells.(363, 376) During clonal expansion and effector differentiation, the methylation status of genes encoding CTL functions (IFNγ, granzyme B) decreases while the methylation status of genes associated with memory development (*Tcf7*) increases.(362) $CD8^+$ T cells deficient for the DNA demethylase methyl-CpG-binding domain protein 2 (MBD2), form potent effectors but are incapable of differentiating into memory cells.(377) Interestingly, memory $CD8^+$ T cells possess the unique ability to rapidly demethylate gene loci associated with effector functions.(378-380) Thus, DNA methylation affects the initial development of $CD8^+$ T cell responses but also controls memory formation and some aspects of memory cell recall responses.

2.3.1.3- Histone modifications and $CD8^+$ T cell differentiation

Histone modifications also readily affect chromatin structure and gene expression. Two main modifications are defined: methylation and acetylation. Both influence $CD8^+$ T cell differentiation and function.(356) The permissive hyper-acetylated mark H3K9 on the Eomes proximal promoter permits the rapid re-expression of this TF by memory $CD8^+$ T cells after re-stimulation *in vitro*.(381, 382) Similarly, H3K9 allows for the rapid re-induction of effector molecules by memory cells (granzyme B, perforin).(381, 382) Decreased H3k27me3 but increased H3k4me3 methylation marks at the *Gcnt1* gene locus in memory $CD8^+$ T cells (compared to naïve cells) allows rapid re-localization to inflamed tissues in a TCR-independent but IL-15-dependent manner.(383) Histone acetylation also influences effector $CD8^+$ T cell fate, between terminal effector and memory precursor, by modulating accessibility to IL7Rα locus.(384) Additional studies also demonstrated that Tem and Tcm cells possess a higher number of genes with

permissive histone methylation (H3K4me3) compared to naïve $CD8^+$ T cells, suggesting a role for methylation in memory development.(364) Further studies are needed to fully understand the impact of histone modifications on $CD8^+$ T cell differentiation and the factors instigating such DNA remodeling.

2.3.1.4- Lineage-specific TFs: impact on epigenetic remodeling

As mentioned above, lineage-specific TFs directly influence the epigenetic landscape of target genes mainly by recruiting histone modifying enzymes to specific gene promoter regions.(356) For example, T-bet directly upregulates permissive histone acetylation at the IFNγ locus which maximizes its transcription.(372) In $CD8^+$ T cells, BLIMP-1 induces a repressive chromatin state on CD25 and CD27 promoter regions by recruiting the histone modifying enzymes G9a and HDAC2.(316) Thus, lineage-specific TFs directly control epigenetic remodeling which likely influence $CD8^+$ T cell differentiation. Additional studies are needed to fully understand the impact of such modifications.

2.3.2- Unique epigenetic features of exhausted CD8 T cells

Growing evidence points to a critical role of epigenetic remodeling in $CD8^+$ T cell differentiation, function and memory development. It raises the question of the impact of epigenetic modifications during persistent viral infections, a context in which differentiation is shifted toward exhaustion and the functionality and developmental plasticity of $CD8^+$ T cells toward a memory phenotype gradually vanishes.(101, 102, 356) Using ATAC-seq experiments,(385) a recent study by *Sen et al* compared the landscape of chromatin accessible regions (ChARs) between naïve, effector, memory and exhausted $CD8^+$ T cells.(105) The authors first noticed that $CD8^+$ T cell activation coincides with a drastic increase in the number of ChARs. Most of these ChARs positively correlate with expression of corresponding genes, demonstrating that the presence of ChARs is stimulatory rather than inhibitory. More than 2000 ChARs are unique to exhausted $CD8^+$ T cells. (**Figure. 8**). Exhaustion-specific ChARs correspond to

genes known to be up-regulated during chronic viral infections, including PD-1, TIM-3 (*Havcr2*) and BATF.(48, 105) Chronically stimulated $CD8^+$ T cells at day 8p.i, shared more ChARs with typical day 8 effectors compared to day 27 exhausted cells (**Figure. 8**). This clearly illustrates the gradual loss of effector function occurring during exhaustion establishment.(2) One striking example is the identical epigenetic landscape of the IFNγ promoter between acute and chronic day 8 effectors.(105) ChARs present at this locus are maintained and even increase during memory development but gradually vanish during exhaustion establishment, mirroring the gradual loss of IFNγ secretion.(2, 105) The frequency of ChARs at memory gene loci (CCR7, Bcl2) decreases during the effector phase in both acute or chronic viral infections but are re-acquired only by conventional memory cells (not exhausted cells). Strikingly, CCR7 ChARs vanish between day 8 and 27 in chronically stimulated cells which is consistent with the gradual loss of memory development that marks exhausted $CD8^+$ T cells.(19, 20, 51) The fact that day 8 chronically-stimulated effectors maintain the ability able to form memory (51) shows that the epigenetic landscape is not definitively locked at this time point but that additional signals beyond day 8 likely act to suppress remaining ChARs at memory gene loci.(105) Further studies are required to define the factors responsible for the establishment of the suppressive epigenetic landscape in exhausted $CD8^+$ T cells.

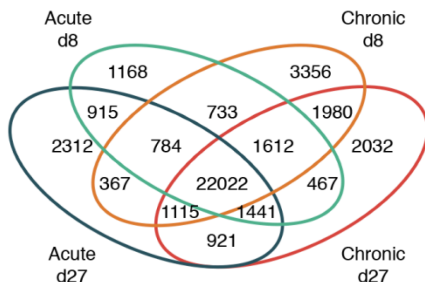


Figure. 8: ChARs overlap between acutely and chronically-stimulated effectors. This diagram shows the number of unique and overlapping ChARs detectable in acute and chronic effectors at indicated time points p.i..
Sen Science 2016

PD-1 is the most-studied inhibitory receptor expressed by exhausted $CD8^+$ T cells, and de facto, the epigenetic regulation of that receptor has recently been of interest. A pioneer study by *Youngblood et al* (268) demonstrates that PD-1 expression is at least partly controlled by DNA methylation. During either an acute or a chronic infection, the PD-1 locus is demethylated in $CD8^+$ T cells at day 8 p.i. compared to naïve cells,

consistent with PD-1 expression on both effector and exhausted cells.(268) The PD-1 locus is re-methylated in memory cells but not in exhausted $CD8^+$ T cells (**Figure. 9**). More precisely, conserved CpG regions around the so-called CR-C and CR-B promoter regions of *Pdcd1* (the gene encoding PD-1) remain unmethylated in exhausted $CD8^+$ T cells even after viral control and a drop in PD-1 protein level.(268) Similar observations were reported in patients with HIV in which the PD-1 locus in Ag-specific $CD8^+$ T cells remains unmethylated even after a HAART-induced drop in viral loads (**Figure. 9**).(268, 386) Such an unmethylated state allows for the rapid re-expression of PD-1 upon de novo activation.(268) Thus, while protective memory cells are epigenetically programmed for rapid re-expansion and activation of effector functions,(378-382) exhausted $CD8^+$ T cells are programmed for rapid inhibitory receptor induction.(214, 268) Analysis of the PD-1 gene locus allows the identification of a single ChAR unique to exhausted $CD8^+$ T cells, and targeting that ChAR with Crispr/Cas9 approaches largely reduces PD-1 expression.(105) This finding opens the gateway for the development of a new generation of CAR T cells deficient for that specific inhibitory region. It is clear that specific epigenetic modifications occur in $CD8^+$ T cells during chronic viral infections. How these modifications are instigated and the factors involved in their establishment remain open questions with critical relevance for the design of new therapeutic avenues.

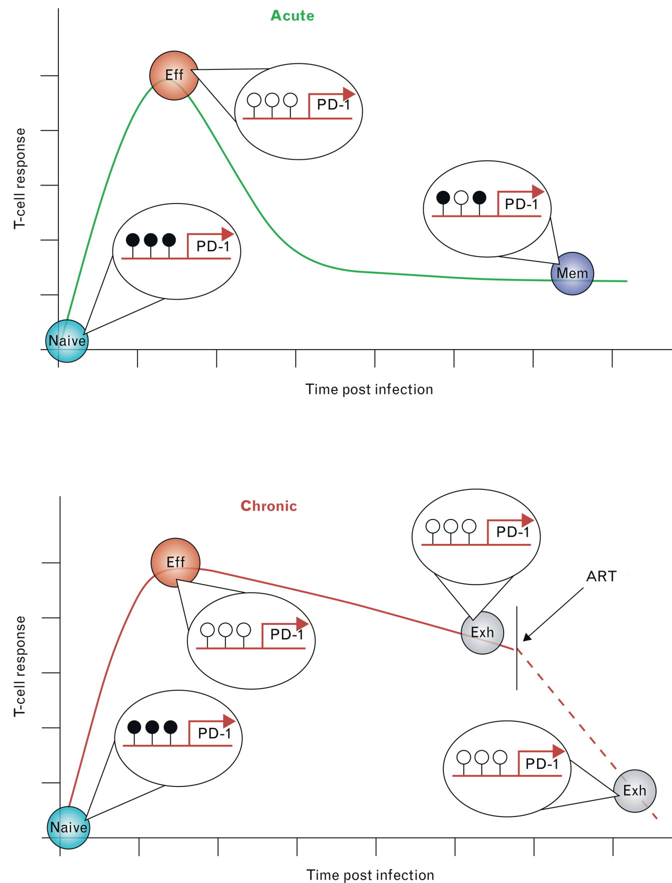


Figure. 9: PD-1 gene methylation dynamic. The PD-1 gene locus is heavily methylated in naïve CD8$^+$ T cells. This locus is demethylated (active transcription) during the effector phase of both acute (upper panel) and chronic (lower panel) viral infections. After resolution of an acute viral infection, the PD-1 gene locus is efficiently remethylated in memory cells (repressed transcription) but remains demethylated in exhausted CD8$^+$ T cells upon viral persistence. In HIV patients, the PD-1 gene locus remains unmethylated in CD8$^+$ T cells even upon ART-induced drop in viral titers. This shows that exhausted CD8$^+$ T cells are poised with a permanently demethylated PD-1 gene locus, a state favoring active transcription that cannot be reversed even by reducing viral titers.

Adapted from Youngblood Curr Opin HIV AIDS 2012

III- Dynamic of exhausted $CD8^+$ T cells

3.1- Lineage origin of exhausted $CD8^+$ T cells

It is well known that during an acute viral infection, effector $CD8^+$ T cells with the potential to survive the contraction phase and form a memory pool can be identified at early time points post-infection based on the expression of distinct phenotypic markers.(387) Indeed, adoptive transfer experiments previously demonstrated that memory cells exclusively arise from $KLRG1^-CD127^+$ MPECs whereas SLECs lose such plasticity and are preferentially eliminated during the contraction phase.(27, 28, 34) *Angelosanto et al*. performed similar experiments in mice chronically infected with LCMV Cl-13 to determine the lineage origin of exhausted $CD8^+$ T cells.(51) In this context, "SLEC like" $KLRG1^+$ effectors formed during the acute phase of infection, although their proportion is drastically lower compared to acute settings.(51) However, due to severe repression of CD127, MPECs fail to develop fully, and most effectors remain $KLRG1^-CD127^-$ early effectors (EEs).(19, 20, 51) *Angelosanto et al*. defined these EEs as the precursors of exhausted $CD8^+$ T cells. Indeed, after adoptive transfer into infection-matched recipients, few $KLRG1^+$ survive while a substantial population of transferred $KLRG1^-$ cells are maintained and develop characteristic features of exhausted cells including high expression of PD-1.(51) The authors concluded that exhausted (chronic) and memory (acute) cells arise from a similar "memory precursor" population. However, it is not exactly the case as MPEC do not develop during a chronic viral infection, and instead exhausted cells rather stem from a population of undifferentiated $KLRG1^-$ EEs. This supports the idea that exhaustion is an alternative differentiation program instigated on EEs exposed to a chronic antigenic stimulation.

3.2- PD-1^{hi} and PD-1$^{int/lo}$ effectors: Distinct but lineage related populations

3.2.1- Characteristics of PD-1^{hi} and PD-1$^{int/low}$ effectors

During an acute viral infection, effector CD8^{+} T cells differentiate into either SLECs or MPECs, two effector subtypes with distinct attributes and cellular fates (**Figure. 1**).(27, 28, 34) In the memory phase, CD8^{+} T cell heterogeneity is even more pronounced as we can distinguish remaining KLRG1^{+} terminal effectors cells (CD62L^{-}CD127^{-}), Tem cells (CD127^{+}CD62L^{-}), Tcm cells (CD127^{+}CD62L^{+}), tissue-resident memory cells (Trm: CD103^{hi}CD69^{hi}CD62L^{lo}CD27^{lo}), Tscm cells (CD44^{low}CD62L^{hi}Sca-1^{hi}CD122^{hi}) and the recently described Tpm cells (peripheral memory T cells; CX3CR1^{int}).(39, 388) Such an observation raises the question of whether similar diversity might exist during chronic viral infections. The first evidence of heterogeneous populations of exhausted CD8^{+} T cells stem from studies investigating the impact of PD-L1 blockade in mice. The authors first identified two sub-populations of exhausted CD8^{+} T cells based on their differential expression of PD-1 and CD44, a PD-1^{hi} population (PD-1^{hi}CD44^{low}) and a PD-1$^{int/lo}$ population (PD-1$^{int/lo}$CD44^{hi}) (**Figure. 10**).(60) Subsequent analysis revealed that PD-1^{hi} and PD-1$^{int/lo}$ effectors can also be distinguished based on their reciprocal expression of T-bet and Eomes. PD-1^{hi} cells have high expression of EOMES (Eomes^{hi}) and low T-BET while PD-1$^{int/lo}$ progenitors conversely express higher T-BET (T-bet^{hi}) and low EOMES.(269) In depth analysis revealed that these two sub-populations have distinct properties and tissue localization. The PD-1$^{int/low}$ subset appears less exhausted and more functional because these effectors express lower levels of inhibitory receptors, secrete more cytokines, show greater survival and proliferative advantage compared to PD-1^{hi} cells, and are more protective against a secondary challenge (**Figure. 10**).(49, 60, 269) The PD-1^{hi} state is more terminal as these effectors are deprived of further developmental plasticity.(269) They are also more severely exhausted (compared to PD-1$^{int/low}$ cells) based on inhibitory receptors levels, cytokine profile, proliferative potential and superior apoptosis (**Figure. 10**).(49, 60, 106, 269) By contrast, PD-1^{hi} effectors produce more granzyme B (a typical feature of

terminal effector $CD8^{+}$ T cells) and are more cytotoxic compared to $PD\text{-}1^{int/lo}$ cells.(269) Hence a suitable qualification for this population would be "terminally exhausted effectors". $PD\text{-}1^{int/low}$ cells preferentially accumulate in the spleen whereas $PD\text{-}1^{hi}$ effectors present at higher levels in non-lymphoid locations and in the bone marrow (BM).(60, 269) Numerical analysis at lymphoid and non-lymphoid areas showed that $PD\text{-}1^{hi}$ cells largely outnumber $PD\text{-}1^{int/lo}$ effectors.(269) Of greatest importance, the less numerous $PD\text{-}1^{int/low}$ population strongly responds and proliferates to PD-L1 blockade while $PD\text{-}1^{hi}$ cells are unresponsive to such treatment.(60, 269)

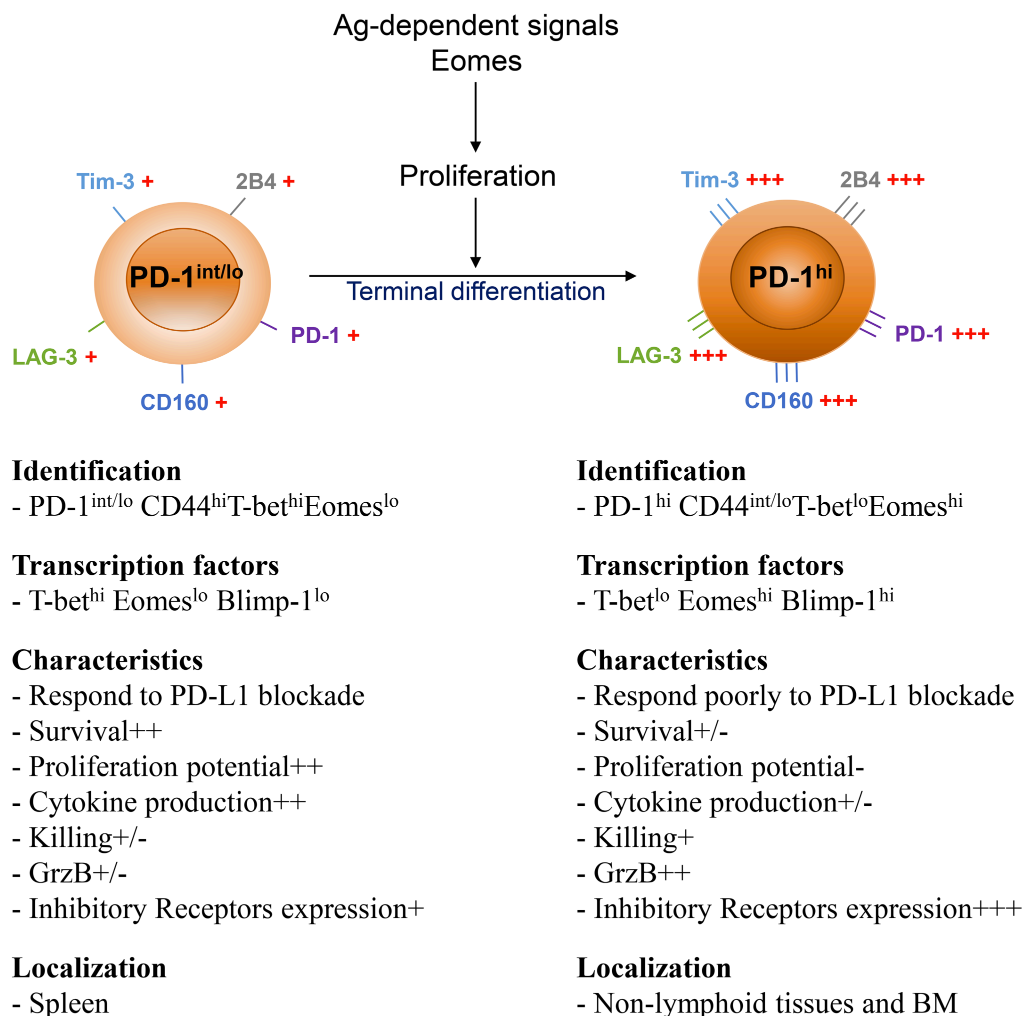


Figure. 10: Characteristics of $PD\text{-}1^{hi}$ and $PD\text{-}1^{int/lo}$ effectors. $PD\text{-}1^{int/lo}$ cells serve as a progenitor population that constitutively replenishes a pool of $PD\text{-}1^{hi}$ terminal effectors.

Conversion of PD-$1^{int/lo}$ cells into PD-1^{hi} terminal effectors is associated with critical changes in terms of expression of cell surface molecules, transcription factors, functional characteristics, and tissue localization.

3.2.2- Development and lineage relationship between PD-$1^{int/lo}$ and PD-1^{hi} effectors

PD-1^{hi}Eomes^{hi} and PD-$1^{int/lo}$T-bet^{low} effectors do not arise from a divergent differentiation program from a common progenitor. Adoptive transfer experiments evidence a lineage relationship between the two effector subtypes. In fact, PD-$1^{int/lo}$ effectors continuously give rise to PD-1^{hi} cells after extensive proliferative events (**Figure. 10**).(269) The PD-1^{hi} state appears to be terminal as these effectors lose their proliferative potential and do not give rise to PD-$1^{int/lo}$ cells.(269) Hence, PD-$1^{int/lo}$ effectors serve as a progenitor pool to continuously replenish a more terminally differentiated and severely-exhausted population of PD-1^{hi} progeny during a chronic viral infection. Of most importance, both populations are essential for the control of a chronic viral infection. Genetic ablation of T-BET or EOMES abrogates PD-$1^{int/lo}$ and PD-1^{hi} cell accumulation respectively, and both scenarios lead to uncontrolled viremia.(269) Dichotomous populations of Eomes^{hi} and T-bet^{hi} effectors were also reported in patients with chronic HCV and HIV.(269, 389) Furthermore, there is evidence in mice and HCV patients that suggests that prolonged periods of heightened viremia gradually erode the PD-$1^{int/lo}$ subset, ultimately leading to a crash of $CD8^+$ T cell responses (**Figure. 11**).(102, 269) Thus, understanding the factors involved in the conversion of PD-$1^{int/lo}$ progenitors into PD-1^{hi} terminal effectors is crucial. Little is known about the factors controlling this conversion. In the absence of T-BET, effector $CD8^+$ T cells massively proliferate and convert rapidly to an Eomes^{hi} phenotype while EOMES KO effectors proliferate less and mainly remain T-bet^{hi}.(269) Absence of Ag-stimulation (but a preserved inflammatory milieu) precludes the proliferation of PD-$1^{int/lo}$ progenitors and subsequent conversion to PD-1^{hi} progeny.(269) Thus, the conversion to PD-1^{hi} cells appears to be at least in part controlled by Ag-stimulation and a balance between T-BET and EOMES expression (**Figure. 10).** Interestingly, PD-1^{hi} terminal effectors express higher levels of the TF

Blimp-1 compared to PD-1$^{int/lo}$ progenitors.(162, 269) BLIMP-1 is an essential component for SLEC terminal differentiation in acute infection settings and a pivotal TF for exhaustion establishment.(30, 35, 162) Furthermore, BLIMP-1$^{-/-}$ CD8^{+} T cells express lower PD-1 during a chronic viral infection.(162) This suggests a role for BLIMP-1 in the terminal differentiation of PD-1^{hi} effectors, but this point remains to be investigated.

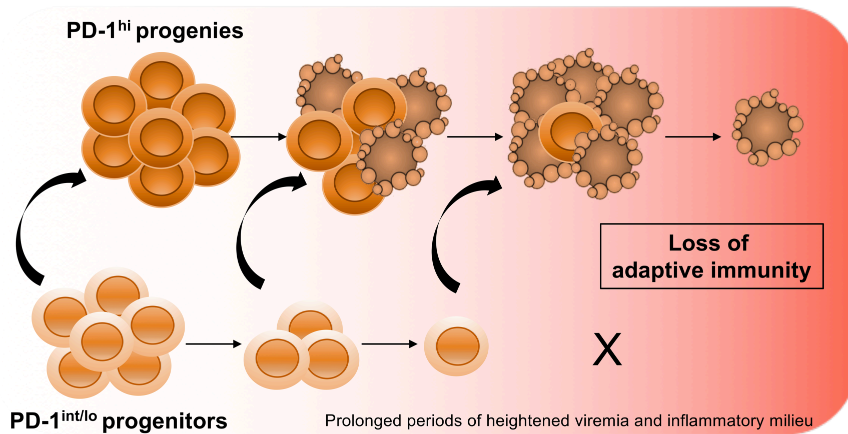


Figure. 11: Gradual erosion of PD-1$^{int/lo}$ cells. Chronic viral infections are often characterized by extended periods of heightened viremia and prolonged inflammatory milieu. In these severe conditions, the PD-1$^{int/lo}$ progenitor pool gradually declines and ultimately fails to replenish PD-1^{hi} terminal effectors that possess a shorter lifespan. A combination of these factors leads to a dramatic loss of adaptive immunity which is often observed in HIV and HCV patients at late stages of infection.

3.3- TCF-1^{+} CXCR5^{+} stem cell-like effectors

Recent studies identified a novel population of exhausted CD8^{+} T cells distinguishable by their selective expression of the chemokine receptor CXCR5.(303) This population only appears during chronic viral infections, and acutely-stimulated CD8^{+} T cells never acquire CXCR5 expression. CXCR5^{+} effectors are detectable as soon as day 8 post-LCMV Cl-13 infection, increase in number until day 15 p.i., and remain stable thereafter.(303) This population represents a small fraction of Ag-specific CD8^{+} T cells (approximately 10%) that preferentially accumulate within lymphoid organs (T-cell

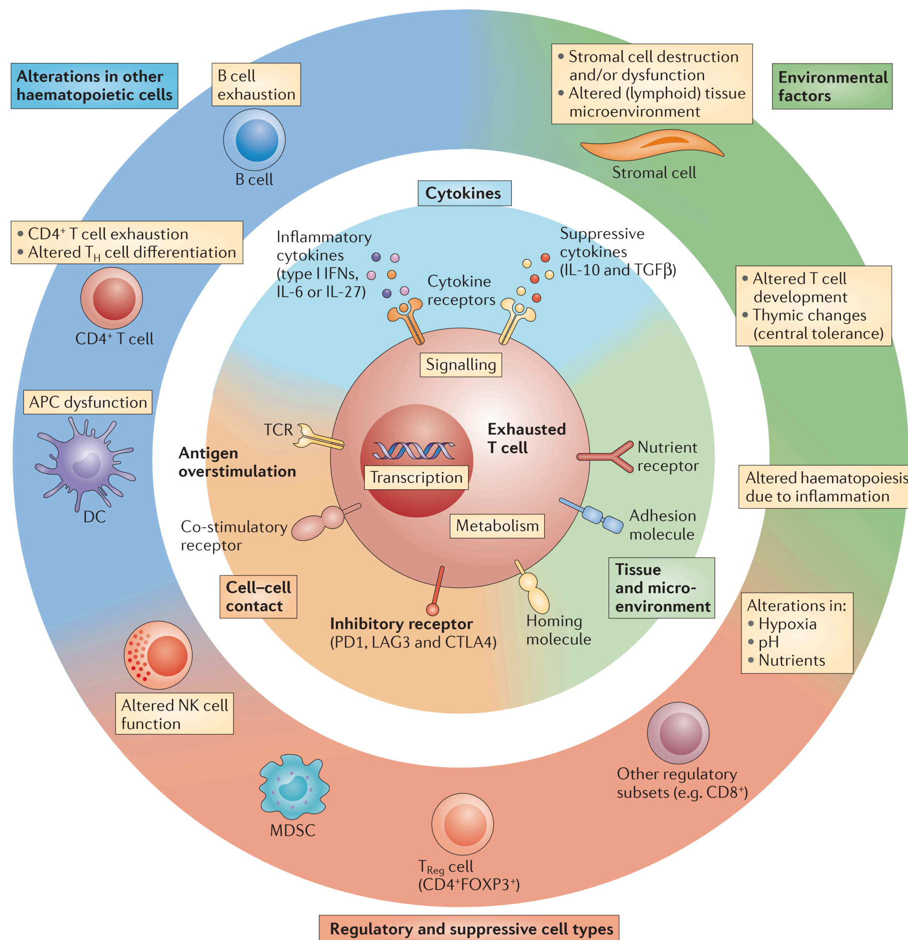
zone of the spleen [white pulp]).(303) $CXCR5^+$ effectors present characteristic features of exhausted cells as they express the inhibitory receptors PD-1 and LAG-3.(303, 304) However, $CXCR5^+$ cells express lower levels of inhibitory receptors (PD-1, LAG-3, CD160) compared to $CXCR5^-$ cells.(303, 304) Strikingly, $CXCR5^+$ effectors lack 2B4 and TIM-3 expression.(303) This suggests that $CXCR5^+$ $CD8^+$ T cells are less exhausted compared to their $CXCR5^-$ counterpart. This point is emphasized by greater cytokine secretion and proliferative potential (upon secondary challenge) of $CXCR5^+$ effectors over $CXCR5^-$ cells.(303, 304) Transcriptional analysis revealed that $CXCR5^+$ $CD8^+$ T cells express higher levels of genes associated with memory development (*Bcl6, Id3, Tcf7*, *Sell* [encoding CD62L], *Cxcr3* and *Ccr7*) and lower levels of genes characteristics of terminal differentiation (*Prdm1, Id2, GrzA, GrzB*) compared to $CXCR5^-$ effectors.(303, 304) More in-depths analysis demonstrated that the transcriptional program of $CXCR5^+$ effectors is unique and closer to that of Tfh cells, memory precursors (MPECs), and also hematopoietic stem cell progenitors whereas $CXCR5^-$ effectors share more similarities with Th1 and terminally differentiated $CD8^+$ T cells.(303) This demonstrates that not only are $CXCR5^+$ effectors less exhausted but these cells also conserve some memory, and perhaps stem cell properties. Consistent with these observations, $CXCR5^+$ $CD8^+$ T cells are able to proliferate, self-renew, and give rise to $CXCR5^-$ terminal effectors that lack proliferative potential, developmental plasticity, and IL-2 production.(303, 304) Thus, similarly to previous observations with $PD\text{-}1^{int/lo}$ and $PD\text{-}1^{hi}$ cells,(269) a progenitor/progeny lineage relationship is present between $CXCR5^+$ progenitors and $CXCR5^-$ terminal effectors during a chronic viral infection.(303, 304) As discussed in section 2.2.3, $CXCR5^+$ progenitors selectively express the Wnt-signaling-dependent TF TCF-1, and is indispensable for the development of this population. TCF-1 deficient $CD8^+$ T cells fail to form a $CXCR5^+$ progenitor population. The absence of the progenitor population dramatically impairs the maintenance of antiviral $CD8^+$ T cell responses and largely compromises viral control.(303, 304) Hence, the TCF-1-dependent $CXCR5^+$ population is essential to replenish and maintain antiviral $CD8^+$ T cell populations during a chronic viral infection. Interestingly, $TCF\text{-}1^+$ Ag-specific $CD8^+$ T cells with advanced memory traits are also detectable in patients with chronic HCV.(304) Lastly, $CXCR5^+$ or $TCF\text{-}1^+$ effectors are selectively able to expand in response to PD-L1

blockade while $CXCR5^-$ or $TCF\text{-}1^-$ terminal effectors do not.(303, 304) Thus, the presence of $CXCR5^+Tcf\text{-}1^+$ progenitors is absolutely required to mount an efficient response to PD-L1 blockade.

Given that $CXCR5^+$ and $CXCR5^-$ effectors possess similar characteristics with $PD\text{-}1^{int/lo}T\text{-}bet^{hi}$ and $PD\text{-}1^{hi}Eomes^{hi}$ cells respectively, one could presume a phenotypic overlap between these populations. $CXCR5^+$ effectors express lower PD-1 and higher CD44 compared to $CXCR5^-$ cells which matches the original identification pattern of $PD\text{-}1^{int/lo}$ progenitors ($PD\text{-}1^{int/lo}CD44^{hi}$) and $PD\text{-}1^{hi}$ progeny ($PD\text{-}1^{hi}CD44^{int}$) respectively.(60, 303, 304) Other positive markers of $PD\text{-}1^{hi}$ terminal effectors, including CD39 and granzyme B, are virtually absent from $CXCR5^+$ effectors, confirming the dichotomy between these effector subtypes.(269, 303, 304, 390) However, $CXCR5^+$ effectors express slightly more Eomes and lower T-bet than $CXCR5^-$ effectors in contrast with the more contemporary definition of $PD\text{-}1^{int/lo}$ progenitors ($T\text{-}bet^{hi}$) and $PD\text{-}1^{hi}$ terminal effectors ($Eomes^{hi}$) established by Paley *et al*.(269, 303, 304) Further studies are necessary to reconcile both models and refine clear phenotypic markers of progenitors and terminal effectors that form during chronic viral infections.

IV- Factors underlying CD8$^+$ T cell exhaustion and potential newcomers

Multiple factors have been shown or are highly suspected to influence CD8$^+$ T cell exhaustion (**Figure. 12**). The most relevant factors are discussed below.



<u>Figure. 12</u>: Factors underlying T cell exhaustion. Several factors directly or indirectly regulate CD8$^+$ T cell exhaustion. These factors can be classified into three classes including i) cell-to-cell contact (e.g. prolonged TCR signals), ii) soluble factors (i.e. cytokines), and iii) tissues and microenvironment.

Wherry Nature Reviews Immunology 2015

4.1- Antigen persistence

$CD8^+$ T cell differentiation to exhaustion only occurs in conditions with elevated and prolonged antigenic stimulation. These parameters represent key prerequisites for exhaustion establishment. However, the question of whether $CD8^+$ T cell exhaustion results from or causes Ag-persistence remains unanswered. Several studies have demonstrated that the strength and duration of Ag-exposure are leading causes of $CD8^+$ T cell exhaustion. In the LCMV model, decreasing the viral load with antiviral treatment (Ribavirin) or abolition of Ag-presentation by stromal cells (targeted MHC class I KO on the non-hematopoietic compartment) restrains the development of severe exhaustion and rescues the functionality of virus-specific $CD8^+$ T cells.(46, 391) In mice and HIV patients, a clear correlation exists between the level of viral loads and the extent of $CD8^+$ T cell dysfunction.(2, 392) Further, Ag-stimulation alone drives some aspects of $CD8^+$ T cell exhaustion.(393) TCR signals also directly drive PD-1 expression on T cells through induction of NFATc1.(120) The strength of TCR signaling is an important parameter dictating the severity of $CD8^+$ T cell exhaustion. The best example of that phenomenon is the collapse of the immunodominance hierarchy that occurs during a chronic viral infection.(2) In the LCMV model, the NP396 epitope binds to its related MHC class 1 molecule with higher affinity compared to either GP33 or GP276 epitopes, and the rate of NP396 presentation by DCs largely outcompetes that of glycoprotein (GP)-derived epitopes.(2, 53) Therefore, the intensity of TCR signals perceived by NP396-specific $CD8^+$ T cells is far greater compared to that of GP33 or GP276-specific cells. Interestingly, NP396 $CD8^+$ T cells lose their effector functions more rapidly and are preferentially deleted whereas GP33 and GP276-specific effectors are maintained.(2) Time of exposure to Ag is also an important parameter. Unlike IL-2 and TNFα secretion, the loss of IFNγ production by Ag-specific $CD8^+$ T cells is more resistant to exhaustion and occurs gradually along chronicity establishment.(2) This suggests that prolonged periods of antigenic stimulation are necessary to commit $CD8^+$ T cells to a fully exhausted state. Consistently, early removal of $CD8^+$ T cells from the chronic environment, or ribavirin treatment during the acute phase of infection, rescues $CD8^+$ T cells from severe exhaustion.(51, 391) These effectors even retain memory development

potential.(51) At later time points, $CD8^+$ T cells are irreversibly committed to exhaustion.(51, 391) Together, the strength and duration of Ag-exposure are essential parameters in the development of $CD8^+$ T cell exhaustion. However, the relative impact of Ag-stimulation versus environmental factors in directing $CD8^+$ T cell exhaustion remains to be defined.

4.2- $CD4^+$ T cell help

Interest over the “helper” functions of $CD4^+$ T cells during chronic viral infections stem from observations in HIV patients. $CD4^+$ T cell numbers decline in these patients during progression to AIDS, and this is correlated with a dramatic loss of $CD8^+$ T cell number and function.(56, 394, 395) Such a correlation has suggested an essential helper function of $CD4^+$ T cells in the maintenance of $CD8^+$ T cell responses during chronic viral infections. Studies using LCMV models in mice demonstrated that $CD4^+$ T cells are dispensable for clearance of an acute viral infection (LCMV Arm or low dose of LCMV WE) but essential to contain more virulent and fast-replicating strains of the virus capable of persisting in the host. Infection of C57Bl/6 mice with the LCMV strains t.1B, 28b, M2.1A, F506b1, DOCILE, Clone 13 or a high dose of WE results in persistent infections that gradually resorbs from most tissues within 1 to 3 months. In the absence of $CD4^+$ T cells, these mice fail to contain the infection and become lifelong carriers.(11, 18, 59) This was correlated with a drastic reduction in the number of functional virus-specific $CD8^+$ T cells capable of secreting antiviral cytokines and mediating CTL functions.(11, 18, 59) Additional studies demonstrated that virus-specific $CD4^+$ T cells provide the help necessary to sustain $CD8^+$ T cell responses to chronic viral infection.(18) Differentiation of these helper cells is skewed from a Th1 to a Tfh phenotype during chronic viral infections, and these effectors rapidly cease to produce IL-2 in favor of IL-21.(345, 396, 397) IL-21 provides direct and essential signals for the maintenance of Ag-specific $CD8^+$ T cell responses to chronic viral infection and sustains their functionality.(345-347) Thus, IL-21 likely represents the “helper” signal provided by Ag-specific $CD4^+$ T cells to support $CD8^+$ T cell responses. A recent study further proposed that IL-21 mediates its impact on $CD8^+$ T cells by upregulating the expression of the TF

BATF in a STAT3-dependent manner.(344) BATF overexpression rescues $CD8^+$ T cell responses in CD4-depleted mice or in the absence of IL-21-signaling.(344) However, how BATF mediates its impact and whether other signals are involved in the process remains enigmatic.

In addition to $CD8^+$ T cells, Tfh cells and IL-21 are essential to promote germinal center (GC) reactions and to produce neutralizing and non-neutralizing antibodies (Ab) by B cells.(398-400) Production of neutralizing and, notably non-neutralizing Ab are important factors involved in the control of a chronic viral infection in mice and humans.(401-403) In the absence of $CD4^+$ T cells, production of LCMV-specific IgG is dramatically reduced.(397) Thus, in addition to providing essential help to $CD8^+$ T cells, $CD4^+$ T cells are also critical for mounting efficient Ab-mediated virus-specific humoral immunity during chronic viral infections.

4.3- Lack of co-stimulation

Co-stimulatory signals play a major role in the initial activation of $CD8^+$ T cells and the absence of co-stimulation at the time of T cell-priming leads to anergy. During the initiation of a chronic viral infection, the priming of $CD8^+$ T cells occurs normally consistent with anergy and exhaustion been distinct developmental programs.(48, 404) However, in the context of a chronic viral infection, dendritic cells (DCs) gradually lose their ability to efficiently prime $CD8^+$ T cells and become more tolerogenic.(2, 404, 405) This was associated with changes in the pattern of expression of co-stimulatory and co-inhibitory molecules expressed by these APCs and, the type of cytokines produced. These factors likely contribute to $CD8^+$ T cell dysfunctions during a chronic viral infection and are discussed below.

4.3.1- Altered DC priming

DCs are professional APCs that are essential for the optimal initiation of T cell responses.(406) Persistent viral infections critically affect the maturation and

functionality of all DC subtypes and substantially impact their capacity to sustain T cell responses. CD8α^- myeloid DCs (now referred to as DCz) and CD8α^+ lymphoid DCs (DC1) isolated from patients with chronic HIV or HBV inefficiently stimulate T cells.(406, 407) This correlates in Cl-13-infected mice which have cells that show lower expression of MHC class I and II molecules at their surface as well as lower co-stimulatory factors including CD80, CD86, and CD40, which is consistent with a defective maturation of the cells.(408) CD8α^- and CD8α^+ DCs also up-regulate suppressive molecules notably PD-L1, IL-10 and IDO.(409, 410) Neutralization of type I IFNs rescues some aspects of DC maturation and decreases PD-L1 levels and IL-10 secretion.(289, 290, 411, 412) This demonstrates an unexpected detrimental impact of type I IFNs in DC maturation during chronic viral infections. IL-10 itself decreases the proportion of CD8α^+ over CD8α^- DCs.(405) During a chronic viral infection, CD8α^- DCs produce more IL-10 and are less efficient at stimulating T cells ex vivo compared to CD8α^+ DCs.(409, 410, 413) Together, inadequate DC priming likely contributes to the ineffective T cell responses that develop during chronic viral infections.

4.3.2- Tumor Necrosis Factor Receptors (TNFRs) superfamily

Co-stimulatory molecules namely members of the TNFR superfamily, greatly impact T cell responses to chronic viral infections. Most of their impact is generally limited to the early phase of infection due to rapid down-regulation of their ligands on APCs or adaptor molecules in T cells. TNFR-signals mostly provide a survival advantage to T cells but also minimally affect exhaustion development. CD4$^+$ T cells are generally more dependent on TNFR-signals, but CD8$^+$ T cells are also directly and/or indirectly affected. Finally, these molecules offer noteworthy therapeutic opportunities to sustain T cell responses during chronic viral infections.

4.3.2.1- CD27 (Tnfrsf7)/CD70 (Tnfsf7)

CD8$^+$ T cells responding to a chronic viral infection express substantial levels of CD27 throughout chronicity (higher to levels found in acutely stimulated effectors) and

sustained expression of its ligand CD70.(48, 414, 415) In HIV patients, CD70 expression on virus-specific $CD8^+$ T cells increases with progression to AIDS and correlates with poor CTL function.(416, 417) This suggests a detrimental and perhaps direct impact of the CD27/CD70 axis on $CD8^+$ T cell responses to chronic viral infections, which is in contrast with the beneficial role seen during acute viral infections.(414) $CD27^{-/-}$ mice clear a chronic LCMV Docile infection more rapidly than WT mice. This improvement in viral control likely results from an impact of CD27-signaling on $CD4^+$ T cells. CD27-deficiency impairs IFNγ and TNFα secretion by $CD4^+$ T cells precluding lymphoid architecture disorganization, and restoring germinal center formation and the production of neutralizing Abs (nAbs) which subsequently improve viral control.(418) However, direct CD27-signaling has little impact on $CD8^+$ T cell dysfunction during chronic viral infection.(418) Blockade of CD70 during LCMV Cl-13 infection improves the number of Ag-specific $CD8^+$ T cells that accumulate during chronicity.(414) Despite this, effectors present similar dysfunctions and do not improve viral elimination.(414) Together, CD70-dependent signaling erodes Ag-specific $CD8^+$ T cell populations but do not impact their functions. Whether this effect acts directly on $CD8^+$ T cells remains to be established. By contrast, the CD27-CD70 axis was shown to increase immune protection in several mouse preclinical tumor models.(419-422) In addition, CD27 was successfully used to boost the efficacy of T-cells genetically engineered to express a chimeric antigen receptor (CAR T cells).(423) This demonstrates that the function of CD27 is context dependent and rather protective during cancer. This observation led to the development of a monoclonal human antibody targeting CD27 (IF5; Varlilumab).(424) This CD27-agonist was first tested in mouse genetically modified to express the human CD27 (hCD27Tg mice). Varlilumab increased $CD8^+$ T cell responses in a vaccination context and provides $CD4^+$ and $CD8^+$ T cells-dependent protection against distinct types of cancers including lymphoma (Bcl1), colon carcinoma CT26) and thymoma (EG.7).(425) Based on these promising results in mouse, Varlilumab in now on phase II clinical for the treatment of several human cancers (colorectal cancer, metastatic melanoma, ovarian cancer, renal cell carcinoma, head and neck squamous carcinoma and glioblastoma).

4.3.2.2- GITR (Tnfrsf18)/GITR-L (Tnfsf18)

T cells responding to a chronic LCMV infection express high levels of GITR at day 8 post-infection (with the highest levels found on Tfh cells), and GITR expression remains upregulated during chronicity.(426-428) The timing of GITR-triggering on T cells is limited to the acute phase of infection due to the rapid down-regulation of its ligand (GITR-L) on DCs and macrophages.(427) Mice deficient for GITR exhibit a critical delay in the control of an LCMV Cl-13 infection, in association with a lower magnitude and greater exhaustion of $CD8^+$ T cell responses.(428) Concomitantly, transgenic expression of GITR-L on B cells largely increases the early accumulation of polyfunctional Ag-specific $CD8^+$ T cells, preventing the establishment of chronicity and subsequent exhaustion.(429) The beneficial effects of GITR on $CD8^+$ T cell responses is indirect and $CD4^+$ T cell dependent. Adoptively transferred $GITR^{-/-}$ Tg P14 $CD8^+$ T cells present similar kinetics, dysfunction and exhaustion (PD-1, TIM-3) compared to WT P14.(428) However, GITR intrinsically sustains early helper functions of Th1 cells (IFNγ, IL-2, CD40L) and Tfh cell development.(428, 429) Therefore during chronic viral infection, GITR is an essential early component for the proper development of $CD4^+$ T cell helper function which subsequently sustains Ag-specific $CD8^+$ T cell responses. The therapeutic potential of GITR is noteworthy. Providing LCMV Cl-13 mice with a single dose of GITR agonist during chronicity doubles the number of Ag-specific $CD8^+$ T cells in a cell-intrinsic manner and largely decreases viral loads without causing pathology.(427) Accumulating effector $CD8^+$ T cells present unaltered exhaustion traits demonstrating that triggering GITR does not reverse nor restrain exhaustion but rather provides proliferative and/or survival boost to these effectors.(427, 430)

4.3.2.3- OX40 (Tnfrsf4)/OX40-L (Tnfsf4)

OX40 is an important co-stimulatory molecule known to enhance T cell survival, function, and memory development.(431-433) During chronic LCMV infection, OX40 is elevated and sustained (following PD-1 kinetic) on Ag-specific $CD4^+$ T cells, outcompeting levels found on their acutely stimulated counterpart.(434) On the other

hand, only a small fraction of KLRG1^{-} CD8^{+} T cells (exhausted cell precursors)(51) express OX40.(434) The ligand for OX40 (OX40-L) is expressed on professional APCs, notably on macrophages, but rapidly vanishes during the acute phase of a LCMV Cl-13 infection.(434) OX40-deficient mice fail to control an LCMV Cl-13 infection. This correlates with a defective accumulation of CD4^{+} and CD8^{+} T cells and ineffective Tfh and humoral responses.(434) Defective accumulation of Ag-specific CD4^{+} and CD8^{+} T cells in the absence of OX40 appears early, is cell-intrinsic, and particularly affects the CD4^{+} T cell compartment that is nearly eliminated by day 10 p.i. The survival, but not the proliferative potential, of CD4^{+} and to a lesser extent CD8^{+} T cells is readily affected by the absence of OX40 which is shown by higher expression of FAS and lower expression of Bcl-2 and Bcl-xL. However, cytokine secretion profiles of WT and OX40$^{-/-}$ CD4^{+} and CD8^{+} T cells are identical, demonstrating that this co-stimulatory molecule does not directly impact exhaustion establishment.(434) Collectively, OX40 critically sustains Ag-specific T cell survival during chronic viral infection, particularly considering CD4^{+} T cells, but has little impact on exhaustion development.

4.3.2.4- 4-1BB (Tnfrsf9)/4-1BBL (Tnfsf9)

During chronic viral infections, 4-1BB expression is maintained on Ag-specific CD8^{+} T cells and participates in the early control of infection while exhibiting little impact thereafter.(435) Mice deficient for 4-1BB-ligand ([4-1BBL] mostly expressed by APCs) and infected with LCMV Cl-13 control the virus less efficiently at day 8 p.i.. This correlates with an early loss of immunodominant NP396-specific CD8^{+} T cells while other less dominant populations are not affected.(435) Late control of infection occurs normally in 4-1BBL$^{-/-}$ mice.(435) Therefore, 4-1BB signals selectively sustain the accumulation of immunodominant populations of CD8^{+} T cells, favoring early control of a chronic viral infection. 4-1BB is known to promote memory CD8^{+} T cell survival through TRAF1-mediated downregulation of the pro-apoptotic molecule Bim.(436) In HIV, the 4-1BB/TRAF1 axis also ameliorates CD8^{+} T cell responses by modulating Bim.(83, 435) Thus, 4-1BB likely sustains early CD8^{+} T cell survival during chronic viral infection through TRAF-1 mediated Bim modulation. TRAF1 levels decline in CD8^{+} T

cells during the course of a chronic viral infection in a TGF-β-dependent manner, desensitizing effectors from 4-1BB-mediated signals.(435) That discovery explains the lack of impact of 4-1BB at later time points post-infection. Interestingly, IL-7 treatment increases TRAF1 levels in $CD8^+$ T cells and synergizes with 4-1BB agonist in the control of a chronic LCMV infection.(435) A low dose of 4-1BB agonist also synergizes with PD-L1 blockade to increase the number and functionality of exhausted $CD8^+$ T cells leading to better viral control.(137) Together, triggering 4-1BB signals represents a salient therapeutic alternative for combinational treatment of chronic viral infection and cancer.

4.4- Regulatory T cells (Tregs)

The immunosuppressive functions of FoxP3-positive regulatory T cells (Tregs), which is provided by their ability to produce IL-10 and TGF-β, downregulate adaptive immune responses to acute viral infections and are required to maintain peripheral tolerance.(437-440) During chronic viral infection, the persistence of Ag coincides with the maintenance of a population of activated Tregs expressing high levels of activation markers (e.g. CD103, GITR, ICOS, PD-1, CD69).(441) Selective depletion of this population restores Ag-driven proliferation of virus-specific $CD8^+$ T cells that massively accumulate in lymphoid and non-lymphoid tissues.(441, 442) The functionality of exhausted $CD8^+$ T cells is also enhanced, namely through their capacity to produce cytokines (e.g. IFNγ, TNFα, and IL-2), to generate cytotoxic molecules (GRZB), and to kill infected targets.(441, 442) This substantial accumulation and regain of functions is dependent on co-stimulatory molecules (B7.1 and B7.2) expression by dendritic cells and requires the presence of CD4 T cells.(441) Therefore, Tregs might inhibit $CD8^+$ T cell responses indirectly by acting on co-stimulatory molecule expression on DCs and limiting $CD4^+$ T cell function. The protective cytokine IL-21, which is produced by Ag-specific $CD4^+$ T cells in such a context, limits Treg cell development.(443) Importantly, Treg cell depletion in the LCMV model enhances viral control in some studies(442) but not others.(441) Failure of Treg cell depletion to reduce viral loads likely results from higher PD-L1 expression by DCs in the absence of Tregs, most likely due to higher

serum levels of IFNγ which is known to increase PD-L1 expression.(441, 444) Consistently, depletion of Tregs supplemented by PD-L1 blockade leads to a significant drop in viral titers.(441) Together, Treg cells largely affect $CD8^+$ T cell responses during chronic viral infections. Direct impact of Tregs on $CD8^+$ T cell exhaustion remains to be established, along with the potential mediating molecules or soluble factors.

4.5- Myeloid-derived suppressor cells (MDSCs)

MDSCs represent a heterogeneous population of innate immune cells of the myeloid lineage that often accumulate during chronic viral infections and cancers.(445, 446) In these contexts, myeloid cells development is skewed towards MDSCs. Increased and prolonged exposure of BM myeloid progenitors to certain growth factors (GM-CSF, G-CSF) and/or cytokines (IL-6, IL-10, VEGF, PGE2 and IL-1) likely favors the development of MDSCs.(446) Interestingly, these cells display strong immunosuppressive properties and possess the ability to inhibit T-cell activation and proliferation.(447) Recently, *Norris et al* demonstrated a role for MDSCs in the alteration of $CD8^+$ T cell responses in a mouse model of chronic viral infection (LCMV Cl-13).(404) Throughout the chronic phase of a LCMV Cl-13 infection, a greater number of $CD11c^-CD11b^+$ myeloid cells (notably $Ly6C^+$ monocytes and GR-1^{hi} neutrophils) accumulate in the blood and lymphoid organs compared to LCMV Arm. The monocytic fraction of these cells displays morphologic, phenotypic (increased PD-L1 and CD115) and transcriptional features of MDSCs and suppresses T cell-proliferation ex vivo.(404) Mice deficient for the C-C chemokine receptor type 2 ($CCR2^{-/-}$; impaired migration of monocytes from the BM to the blood) or antibody-mediated depletion of myeloid cells (anti GR-1), enhances the number of functional Ag-specific $CD8^+$ T cells.(404) This demonstrates that MDSCs alter the $CD8^+$ T cell response during a chronic viral infection. However, targeting myeloid cells only slightly improves viral control and whether these cells impact other aspects of $CD8^+$ T cell exhaustion (inhibitory receptors expression, loss of memory plasticity) remains elusive.

V- Cytokines and persistent viral infections: Implication on T cell exhaustion (see Appendix. 1 for the published review)

5.1- Immunoregulatory cytokines

5.1.1- IL-10

Several hematopoietic cell types produce IL-10 including dendritic cells (DCs), B cells, monocytes, macrophages, $CD4^+$ T cells, $CD8^+$ T cells and regulatory T cells.(448) IL-10 signals via a heterodimeric class II cytokine receptor composed of an inducible IL10R1-chain and an IL10R2-chain constitutively expressed on both hematopoietic and non-hematopoietic cell types that are also shared by the class II cytokines IL-22, IL-26, IL-28, IL-29 and interferon lambdas.(449, 450) Engagement of IL-10 to its cognate receptor triggers activation of JAK1 and TYK2 and phosphorylation of STAT3, STAT1 and also STAT5.(451-455) Subsequent signals allow IL-10 to mediate its immuno-suppressive functions that affect multiple hematopoietic cell types.

In several persistent infections, notably in patients with HIV, HBV, HCV or EBV infections, IL-10 levels were reported to be elevated.(448, 456-460) Concomitantly, polymorphisms within the IL-10 promoter region that reduce the secretion of the cytokine were correlated with enhanced control of these viruses.(461-467) Moreover, numerous viruses including EBV, human cytomegalovirus (HCMV) and some poxviruses produce their own IL-10 homologs to dampen antiviral immunity and favour their persistence in the hosts.(468) Interestingly, IL-10 blockade potently increases the proliferation and cytokine secretion of T cells isolated from chronically infected HIV or HCV patients.(469-473) On the contrary, engagement of PD-1 with its ligand PDL1 can trigger IL-10 expression, thus contributing to functional T-cell exhaustion.(472, 474) Together, these cumulative evidence suggest a potential immuno-suppressive role of IL-10 during chronic viral infections in humans.

Two concomitant studies of Brooks and Ejrnaes modeled chronic viral infection in mice and unraveled an essential role of IL-10 in promoting viral persistence.(405, 475) Similar to what was observed in persistent human infections, both teams reported superior IL-10 levels after infection with the variant LCMV strain clone 13 (Cl-13), which induces a 2-3 month viremia in the host, compared to the LCMV Armstrong strain (Arm), which is rapidly cleared.(2, 3) Remarkably, infection of IL-10-deficient mice by LCMV Cl-13 led to rapid viral control by day 9 post infection (p.i.). Early blockade of IL-10 signals with anti-IL10R specific antibodies similarly resulted in accelerated clearance of Cl-13 infection.(405, 475) Rapid elimination of the Cl-13 virus was associated with enhanced magnitude and functionality of $CD4^+$ and $CD8^+$ T cells. Importantly, IL-10 blockade at later time points also led to enhanced viral control and T-cell responses. However, in both studies, these effects were less impressive than what was observed in IL-$10^{-/-}$ mice or mice treated early with anti-IL10R specific antibodies. Hence, IL-10 acts essentially as an early determinant of viral persistence, while it continuously dampens immune functions throughout chronicity. This latter point was emphasized in a study that demonstrated that IL10R-antibody treatment of mice with a well-established persistent Cl-13 infection still improved T-cell immunity and substantially decreased viral loads.(136) More importantly, this study revealed that IL-10 blockade was even more effective when combined with anti-PDL1 treatment, suggesting that this dual regimen might be effective in combating established persistent infections.

Remaining is the question of how IL-10 favors viral persistence (**Figure. 13**). IL-10 was previously shown to alter DC maturation by reducing the expression of MHC class I and II as well as the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2).(448, 451, 476, 477). Hence, IL-10-primed DCs or DCs isolated from HIV patients are poor inducers of $CD4^+$ T-cell proliferation.(476-478) Further, DCs from chronically-infected mice diverge naïve $CD4^+$ T cells from a Th1 $IFN\gamma^+$ profile to an IL-10 secretion profile (**Figure. 13**).(405) That effect was not observed when DCs were isolated from anti-IL10R treated mice.(405) This is in support of a direct impact of IL-10 on DCs' priming abilities that favor the accumulation of suppressive IL-10^+ $CD4^+$ T cells (**Figure. 13**). Thus, IL-10 indirectly promotes $CD4^+$ T-cell exhaustion by altering DC

maturation leading to dampened priming of the cells and divergence to a suppressive IL-10 producing profile. Conversely, IL-10 can behave as a proliferative factor for $CD8^+$ T cells *in vitro* and as a growth factor *in vivo* during primary response to *Listeria monocytogenes*.(479-481) However, IL-10 restrains HIV-specific $CD8^+$ T-cell proliferation *in vitro*.(469) Hence, the impact of IL-10 on $CD8^+$ T-cell response is likely context-dependent and preferentially inhibiting during chronic viral infections (**Figure. 13**). Those assertions define IL-10 as an overall suppressor of T-cell response during persistent viral infections. However, whether IL-10 favors viral persistence by directly promoting T-cell exhaustion remains to be determined.

5.1.2- Transforming Growth Factor *β* (TGF*β*)

The TGFβ superfamily in mammals includes three isoforms, TGFβ1, TGFβ2 and TGFβ3, that are encoded by distinct genes.(482) The dominant TGFβ1 form can be selectively produced by activated T cells, macrophages, DCs as well as non-immune cell types including stromal cells from lymphoid tissues.(482-486) TGFβ1 is first secreted in an inactive "latent form", trapped by the so-called latency-associated proteins (LAP) that form the small latent complex (SLC). Eventually, other latent TGFβ binding protein (LTBP) can bind the SLC to form a large latent complex (LLC).(487, 488) Once freed from these accessory proteins, TGFβ1 homodimers bind to their cognate receptor composed of two subunits of TGFβRI and two subunits of TGFβRII.(489-491) Engagement of TGFβ1 activates the kinase activity of TGFβRI and II. This allows for recruitment and phosphorylation of SMADs proteins notably SMAD 2 and 3 that complex with SMAD4 to access the nucleus and achieve the transcription of SMAD-responsive genes.(489-496) Thereby, TGFβ1 exerts several regulatory functions in almost all immune cell types and particularly affects the T-cell compartment.(497)

TGF-β has long been associated with a plethora of regulatory functions in multiple hematopoietic cell types. Indeed, mice deficient for TGFβ1 (TGF$\beta 1^{-/-}$) develop a lethal inflammatory syndrome associated with massive infiltration of immune cells

including lymphocytes in several organs.(498, 499) Interestingly, depletion of $CD4^+$ or $CD8^+$ T cells alleviates this overwhelming inflammatory syndrome suggesting that TGFβ directly suppresses T-cell auto-activation.(500, 501) Accordingly, mice with a T-cell-restricted deficiency for TGFβRII develop uncontrolled expansion and activation of peripheral CD4 and mostly $CD8^+$ T cells associated with severe organ lesions and rapid demise.(502, 503) Consistent with these observations, TGFβ was shown to directly suppress several primary T-cell functions that are also lost during chronic viral infection including proliferation, antiviral cytokine production (IFNγ) and cytotoxicity.(2, 497, 504-508) Besides its suppressive functions, TGFβ also greatly influences the differentiation of activated T cells. Hence, TGFβ restrains Th1 and Th2 $CD4^+$ T-cell differentiation while directing regulatory T-cell and Th17 cells development *in vitro*.(497, 509) TGFβ-mediated alteration of Th1 $CD4^+$ T-cell differentiation is at least partially due to its capacity to repress the expression of the T-box transcription factor T-BET.(510) Of note, T-BET directly represses PD-1 expression on $CD8^+$ T cells, restrains terminal CD8 T-cell exhaustion and sustains $CD4^+$ T- cell response during a chronic viral infection.(123, 269, 396) Thus, we may speculate that TGFβ directly aggravates T-cell exhaustion through repression of T-BET, although this has not been demonstrated to date (see below).

In mice, Tinoco et al. examined the impact of cell-intrinsic TGFβ in the silencing of T-cell responses during a chronic viral infection (**Figure. 13**).(84) In accordance with the heightened TGFβ production often observed in humans during chronic viral infections – although no clear correlations have been established between TFGβ1 levels and disease progression in humans (511-513) – TGFβ production and signaling were increased in T cells isolated from chronically infected Cl-13 mice compared to Arm mice. This confirmed that elevated and persistent levels of TGFβ are a hallmark of chronic viral infections. However, attenuation of TGFβ signaling in T cells by the transgenic expression of a dominant negative form of the TGF-β receptor II (dnTGFBRII) mainly limited the BIM-dependent apoptosis of effector T cells (**Figure. 13**).(84, 85, 88) This resulted in a greater accumulation of Ag-specific $CD8^+$ T cells that appeared less

exhausted as they produced more cytokines, expressed lower levels of PD1 and displayed enhanced killing functions.(84) However, these functional enhancements reflected the more rapid viral clearance in mice with attenuated TGFβ signaling rather than a cell-intrinsic effect of TGFβ. Indeed, dnTGFBRII mice rapidly cleared a Cl-13 infection without presenting organ pathologies. This allowed for the development of a competent memory pool that fails to develop normally in that model.(19, 20, 84)

The demonstration by Tinoco and colleagues that a T-cell-restricted attenuation of TGFβ signals led to the rapid clearance of Cl-13 infection suggested that *in vivo* neutralization of TGFβ might represent a relevant therapeutic option for purging a chronic viral infection.(84) Unfortunately, several groups tested the impact of TGFβ blockade *in vivo* with disappointing results. In fact, TGFβ blockade during priming or during the chronic phase of Cl-13 infection only slightly increased the number of $CD4^+$ and $CD8^+$ T cells.(514, 515) Yet, the functionality of these effectors was barely improved and no reduction in inhibitory receptor expression (i.e. PD1, 2B4) was reported. Importantly, the modest effects of TGFβ neutralization had no impact on viral control regardless of the timing of treatment.(514, 515) These results imply that targeting TGFβ alone is neither sufficient to prevent the establishment of chronic viral infection nor to promote the clearance of an established chronic viral infection (**Figure. 13**). However, it remains possible that combining TGFβ blockade with other regimens might efficiently improve the control of chronic viral infections.

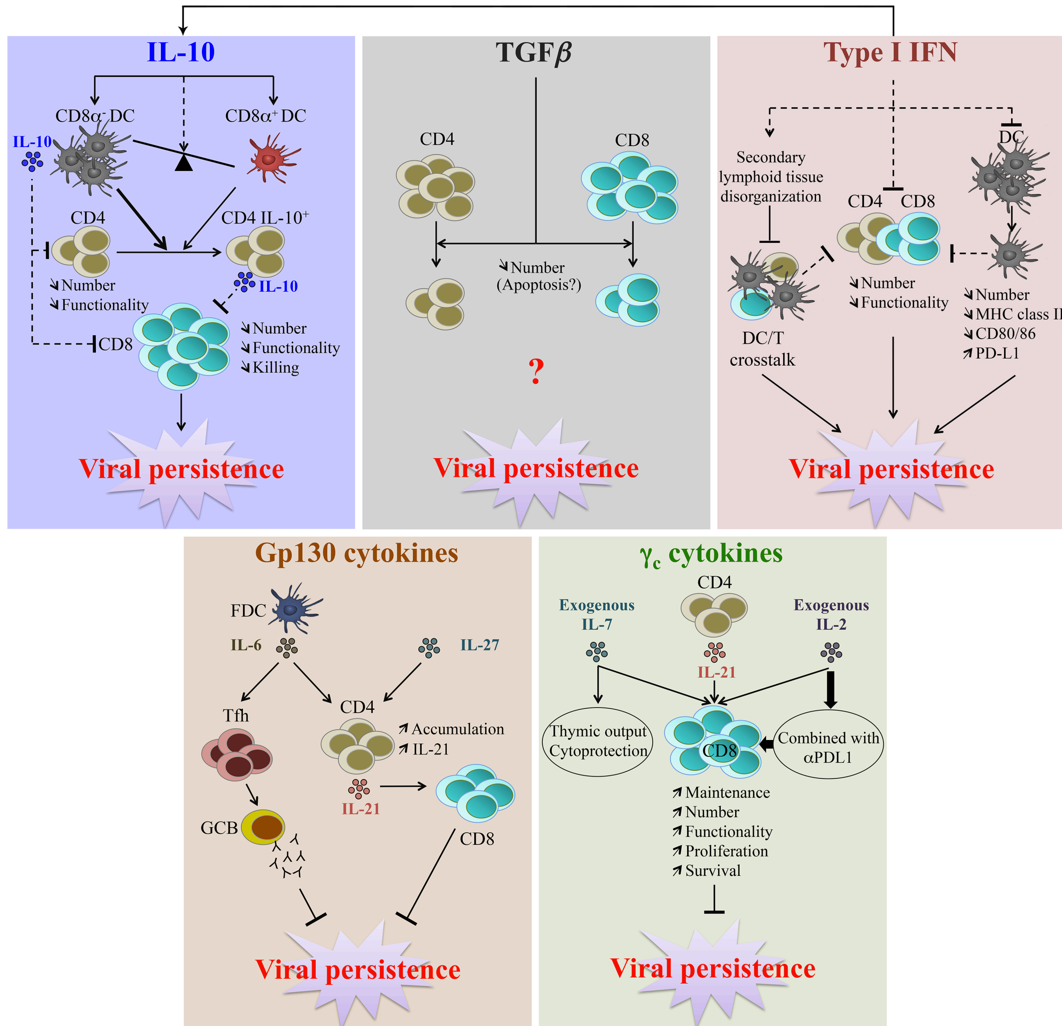


Figure. 13. Cytokines regulate T-cell responses during chronic viral infection

IL-10: Infection of mice with LCMV Cl-13 causes a gradual decline in the number of CD8α^+ DCs, shifting the ratio in favour of the suppressive CD8α- DCs. This imbalance during chronic viral infection is sustained by IL-10, which supports the subsequent differentiation of suppressive IL-10$^+$ CD4$^+$ T cells. IL-10 produced by CD8α- DCs, CD4$^+$ T cells directly and/or indirectly reduces T-cell numbers and hinders their functions leading to viral persistence. ***TGFβ***: TGFβ directly reduces the number of Ag-specific CD4$^+$ and CD8$^+$ T cells likely through increased Bim-mediated apoptosis. However, TGFβ neutralization *in vivo* has minimal to no impact on viral control during a chronic infection. ***Type I IFNs***: Type I IFNs cause a dramatic disorganization of secondary lymphoid tissues that limits efficient cross-talk between APCs and T cells. Type I IFNs further limit DCs number and impair their maturation by decreasing MHC Class II and CD80/86

levels while promoting PDL1 expression. These cytokines also increase circulating levels of IL-10. These combined alterations likely hamper the magnitude of $CD4^+$ and $CD8^+$ T-cell responses, reducing their functionality and favoring viral persistence. ***Gp130-dependent cytokines***: During a chronic viral infection, IL-6 (mainly provided by follicular dendritic cells [FDCs], promotes the development of Tfh cells that in turn support GCB accumulation and anti-LCMV antibody production necessary for viral control. IL-6 and IL-27 redundantly promote IL-21 production by Ag-specific $CD4^+$ T cells. IL-21 maintains the number of Ag-specific $CD8^+$ T cells leading to better viral control, while IL-27 has the unique capacity to support the maintenance Ag-specific $CD4^+$ T cells during chronicity. ***γ_c-dependent cytokines*****:** CD4-derived IL-21 provides cell-intrinsic signals that are essential for the maintenance of a functional Ag-specific $CD8^+$ T-cell population. Prolonged IL-7 therapy augments the number of polyfunctional $CD8^+$ T cells through increased proliferation and survival leading to accelerated viral clearance. IL-7 also displays other beneficial effects including the improvement of thymic output and prevention of organ pathology through IL-22 induction. Low-dose IL-2 regimen in chronically infected mice increases the number of polyfunctional $CD8^+$ T cells, especially when combined with αPDL1 treatment, to sustain viral decline. *Beltra Cytokine Review 2016*

5.2- Inflammatory cytokines

5.2.1- Type I interferons (IFN)

Type I interferons are a large family of soluble mediators comprising the most described IFNα and IFNβ and the less defined classes IFNκ, ω, ε, δ, τ and ζ. Plasmacytoid dendritic cells are a major source of IFNα while several other cell types can produce type I IFNs after viral sensing by pathogen recognition receptors.(516, 517) Once secreted, type I IFNs engage a unique heterodimeric receptor composed of the IFNAR1 and IFNAR2 subunits; activate JAK1 and TYK2 and trigger several downstream molecules (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) and signaling pathways (PI3K and MAPK).(517, 518) Activation of those signaling pathways leads to the transcription of hundreds of interferon-stimulated genes (ISGs) creating a typical type I IFN signature.(519, 520) ISGs encode proteins with direct

antiviral functions and are responsible for type I IFN-mediated enhancement of innate and adaptive immune functions and maturation.

While often being associated with positive antiviral functions, type I IFNs recently received attention regarding their potential deleterious effects during persistent viral infections. Indeed, heightened inflammation and sustained type I IFN signature often correlate with disease progression in chronic viral infections. For example, HIV infection is associated with chronic immune activation and unremitting type I IFN responses, which correlate with disease severity and reduction of $CD4^+$ T-cell counts.(521-524) Concomitantly, comparative transcriptional analysis of $CD4^+$ and $CD8^+$ T cells between HIV progressor patients and viral "elite" controllers revealed an elevated level of ISGs in the former, implying that type I IFN signals may contribute to disease progression.(525, 526) Similarly, the relative resistance of African green monkeys or sooty mangabeys to simian immunodeficiency virus (SIV)-induced immunodeficiency is associated with a restrained type I IFN signature as opposed to SIV-infected rhesus macaques who often progress to AIDS and present persistent high levels of ISGs.(527, 528) Hence, levels and persistence of type I IFN signals have become a relevant biological determinant of viral persistence and disease progression during chronic viral infections. In addition to those correlative observations, type I IFNs exert a set of immunosuppressive functions intimately linked to viral persistence. Indeed, type I IFNs are critical inducers of IL-10 and PDL1 expression, two major components that promote viral persistence.(529-531) In addition, priming of naïve $CD8^+$ T cells with type I IFNs reduced their proliferative potential to cognate Ag-encounter.(532) IFNα also enhanced the FAS/FASL-mediated antigen-induced cell death in human T cells.(533) Further, type I IFNs potently damped IFNγ secretion by murine T and NK cells in a STAT1-dependent manner.(534) Hence, sustained inflammatory responses, induction of immuno-suppressive factors and attenuation of T-cell responses through PD-1 ligation represent three distinct pathways by which type I IFNs can mediate virus persistence.

Two studies by Teijaro et al. and Wilson et al. examined more thoroughly the role of IFNα/β during a chronic viral infection using the LCMV Cl-13 mouse model.(289,

290) As expected, persistent infection with Cl-13 led to superior levels of IFNα and IFNβ compared to mice infected with LCMV Arm. In addition, viral persistence in Cl-13-infected mice was associated with prolonged expression of ISGs, STATs and IFN-I regulatory elements, in accordance with the sustained type I IFN response observed in humans with HIV and HCV.(522-524, 535) Importantly, both studies reported that early IFNAR1 blockade, which prevents ligation of all type I IFNs to their common receptor chain, although initially permissive for viral replication, led to enhanced control of viremia and accelerated viral clearance at later time points. Moreover, blockade of IFNAR1 when chronicity was well established similarly decreased viral titers. This last point was further emphasized in two recent studies showing beneficial effects of type I IFNs blockade in HIV-infected humanized mice during chronicity (particularly in combination with ART).(536, 537) Conversely, Wang and colleagues demonstrated that providing exogenous IFNα/β efficiently boosted T-cell responses and accelerated Cl-13 elimination when given at the time of T-cell priming, while providing IFNα/β at earlier time points or after the peak of the T-cell expansion was ineffective.(538) This timing-dependent impact of prophylactic type I IFNs treatment correlated with recent observations in SIV-infected rhesus macaques. In this model, early treatment with type I IFNs (IFNα2a) prevented systemic infection while continuous therapy enabled infection, increased SIV reservoir and accelerated $CD4^+$ T cell loss.(539) Hence, type I IFNs appear beneficial during the early phase of a chronic viral infection but deleterious during chronicity. This temporality implies that type I IFNs modulation needs to be finely tuned since divergent outcomes may arise depending on the timing since infection. This latter point is of critical importance for the treatment of chronic viral infections in humans. Indeed, IFNα has been successfully used for years as a first-line treatment of HCV in combination with the antiviral drug ribavirin.(540) However a significant number of patients are refractory to IFNα therapy. In fact, treatment outcome appears to correlate with the extent of type I IFN signature in HCV patients, with refractory patients presenting the strongest signature.(540, 541) Hence, defining the optimal time frame for type I IFNs modulation to achieve a positive viral response in patients remains to be studied.

Disrupting type I IFNs signaling with anti-IFNAR1 antibodies revealed how these cytokines suppressed antiviral responses during a chronic viral infection (**Fig. 13**). Early IFNα/β burst sustained the secretion of multiple pro-inflammatory cytokines and caused a dramatic disorganization of secondary lymphoid tissues (**Figure. 13**).(289, 290) IFNAR1 blockade efficiently prevented secondary lymphoid tissue disorganization and increased the number of several innate and adaptive immune cells including DCs, NK, B cells and $CD4^+$ T cells. Levels of suppressive factors such as IL-10 and PDL1 were also largely reduced with IFNAR1 blockade, while DC maturation and $CD4^+$ T-cell functions were readily improved (**Figure. 13**).(289, 290) Notably, the rapid control of viremia with early IFNAR1 blockade strictly required the presence of $CD4^+$ T cells. Interestingly, neutralization of IFNβ-signals alone - although to a lesser extent than IFNAR1 blockade - also enhanced $CD4^+$ T-cell responses, preserved lymphoid tissue architecture and accelerated viral clearance while IFNα blockade did not.(411) Rather, IFNα likely dispensed early anti-viral effects while being dispensable for viral clearance thereafter. However, IFNβ blockade did not alter IL-10 and PDL1 levels. In addition, while IFNAR1 blockade had little impact on CD8 T-cell responses, IFNβ neutralization greatly improved their number and functionality.(289, 290, 411) Together, these results point to a dominant role for IFNβ over IFNα in mediating suppressive functions during a chronic viral infection, but also suggest that type I IFNs use redundant and unique pathways to impair anti-viral immune responses and favor viral persistence.

5.2.2- Glycoprotein (gp130)-dependent cytokines

The gp130 family of cytokines is a subclass of type I cytokines composed of the prototypical IL-6 as well as IL-11, IL-27, leukemia inhibitory factor (LIF), cardiotrophin-1, ciliary neurotrophic factor and oncostatin M (OSM).(542) These soluble mediators bind to and signal through unique multimeric receptors composed of one (IL-27, LIF, OSM) or two (IL-6, IL-11) common gp130 subunits associated with distinct α-chains inherent to each cytokine.(542, 543) The gp130 receptor subunit is essential for signal transduction and predominantly activates JAK/STAT molecules (notably JAK1, JAK2, TYK2 and STAT1, STAT3, STAT5) but also the PI3K/AKT and MAPK pathways.(544-

547) Gp130 is ubiquitously expressed within hematopoietic and non-hematopoietic tissues.(548) Thus, cytokines that signal through the gp130 receptor chain mediate a broad spectrum of actions in the development of innate and adaptive immune responses.

Several aspects of the biology of gp130-dependent cytokines have suggested a role for these cytokines in the control of chronic viral infection. First, numerous studies uncovered the dominant role of gp130-dependent cytokines in the regulation of inflammatory responses. Particularly, IL-6 displays key pro-inflammatory properties essential for the initiation of inflammatory responses and subsequent recruitment of innate and adaptive mediators to inflamed tissues.(542) These functions of IL-6, besides being associated to multiple inflammatory autoimmune diseases, are also essential to sustain immune responses to pathogenic infections.(549) Indeed, several reports have underlined the incapacity of IL-$6^{-/-}$ mice to control fungal, parasitic, bacterial and viral infections as a result of defective innate cells recruitment and suboptimal T-cell responses.(550-556) Conversely, gp130-dependent cytokines, notably IL-6 and IL-27, also display anti-inflammatory properties.(542, 557) For instance, IL-6 prevents uncontrolled accumulation of neutrophils to inflamed tissues and limits severe lung pathology in a mouse model of influenza infection.(558-561) Further, IL-27 tempers overwhelming T-cell activation during infection and prevents the development of pathogenic Th17 cells in multiple autoimmune diseases, in contrast to IL-6.(542, 561, 562) Thus, not only gp130-dependent cytokines have the potency to sustain adaptive responses during infection but also prevent T-cell hyperactivation and limit organ immunopathologies.

These observations suggest that gp130-dependent cytokines sustain T-cell responses during chronic viral infection while limiting exacerbated responses. Of importance, IL-6 is known for its direct impact on the differentiation of T follicular helper (Tfh) cells.(557, 563, 564) Interestingly, a higher proportion of Tfh cells was reported in mice infected with LCMV Cl-13 compared to LCMV Arm, and that CD4 T-cell subset was critical to support B-cell responses and viral control.(396, 397) In line with its capacity to promote Tfh-cell differentiation, IL-6 but also IL-27 induce IL-21

production by $CD4^+$ T cells, an essential cytokine for the maintenance of $CD8^+$ T-cell responses during chronic viral infection (discussed below).(345-347, 565-568) These results suggest that gp130-dependent cytokines are required to sustain both cellular and humoral immune responses during chronic viral infection.

In humans, elevated serum levels of IL-6 were reported in patients with HIV, HCV and HBV infections, and in the former, disease progression was correlated with lower levels of IL-6.(569-571) Additional studies also reported an inverse correlation between serum levels of IL-27 and markers of disease progression in HIV and HCV patients, while others did not.(572-575) Finally, patients suffering from rheumatoid arthritis (or other inflammatory autoimmune diseases) and treated with tocilizumab, a monoclonal antibody against IL6R that induces rapid and sustained clinical responses, may reactivate *Mycobacterium tuberculosis*, HBV or HCV, suggesting a role for IL-6 in the control of latent infections.(549) Taken together, these observations are highly predictive of a role for gp130-dependent cytokines in the control of adaptive responses to chronic viral infection.

Using a mouse model carrying a T-cell restricted deletion of gp130, Harker et al. uncovered an essential role for gp130-dependent signals for the control of LCMV Cl-13.(576) Indeed, while being dispensable during the acute phase of infection (day 9 p.i.), gp130-dependent signals were essential for late accumulation of Ag-specific $CD4^+$ and $CD8^+$ T cells during viral persistence.(576) More in depth analysis revealed that the $CD4^+$ T-cell compartment was particularly affected in the absence of gp130. In fact, T-cell restricted gp130-deficiency impaired the accumulation of Tfh cells during chronicity, restrained Ag-specific $CD4^+$ T-cell survival and production of both IFNγ and IL-21.(576) In contrast, gp130-dependent signals only modestly affected the functionality of CD8 T cells. However, as for $CD4^+$ T cells, the maintenance of Ag-specific $CD8^+$ T cells was defective during viral persistence. This observation likely resulted from the lack of CD4-derived IL-21 rather than a direct impact of gp130-dependent signals on $CD8^+$ T-cell maintenance.(345-347, 576) Of note, defective Tfh cell generation and lack of IL-21 production correlated with a critical reduction in the number of germinal center B cells

(GCB) and anti-LCMV antibody production.(576) Together, these results demonstrate that gp130-dependent signals directly and indirectly sustain T and B cell responses during chronic viral infection and are critically required for the control of viremia.

Additional work by the same group investigated the relevant gp130-dependent cytokines involved in the control of persistent viral infections.(576, 577) Interestingly, the decline in T-cell numbers seen in infected gp130-deficient mice correlated with IL-6 and IL-27 being the only two gp130-dependent cytokines able to trigger the phosphorylation of STAT3 in Ag-specific $CD4^+$ T cells *ex vivo,* while IL-27 also selectively induced the phosphorylation of STAT1.(576) Infection of either IL-$6^{-/-}$ or IL27R$\alpha^{-/-}$ mice with LCMV Cl-13 resulted in impaired viral control similar to what was observed in gp130-deficient mice.(576, 577) This demonstrated that both cytokines were individually essential for the control of chronic viral infection. In IL27Rα-deficient mice, this correlated with a gradual decline in the number of Ag-specific $CD4^+$ T cells during chronicity that was not observed in IL-$6^{-/-}$ mice.(576, 577) However, the proportion of Tfh cells was substantially reduced in the absence of IL-6, but not within IL27R$\alpha^{-/-}$ mice. The lack of Tfh cells in IL-$6^{-/-}$ mice impaired the development of germinal center B cells (GBC) and led to severe reduction in anti-LCMV IgG levels, which led to uncontrolled viremia (**Figure. 13**).(577, 578) Of note, neither IL-6 nor IL27Rα-deficiency impaired Ag-specific $CD4^+$ T-cell capacity to produce IL-21 while this function was critically diminished in gp130-deficient mice. This indicated that IL-6 and IL-27 likely act redundantly to sustain IL-21 production by Ag-specific $CD4^+$ T cells during chronic viral infection (**Figure. 13**).(576, 577) This is in accordance with the fact that both IL-6 and IL-27 induce IL-21 production by Ag-specific $CD4^+$ T cells *ex vivo*.(576) Taken together, these results demonstrate that IL-6 and IL-27 display individual and redundant functions to regulate adaptive immune responses to chronic viral infections. IL-27 further inhibits HIV replication in human monocytes, macrophages, DCs and CD4 T cells *in vitro*.(579-582) These combined abilities would be of great interest in the design of novel therapeutic strategies for chronic viral infections.

5.3- Common gamma (γ_c) chain-dependent cytokines

The γ_c-dependent cytokines that include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are pleiotropic factors, dispensing multiple functions in several immune cell types.(583) This family of cytokines has long been associated with T-cell development. Notably, γ_c-dependent cytokines display essential signals for the generation, differentiation and homeostasis of naïve and memory T cells.(66, 584-586) *De facto*, the potential of these cytokines as immuno-therapeutic agents to boost T-cell responses during persistent viral infections and cancers has been under the scope for numerous years.(587, 588)

5.3.1- IL-21

IL-21 is the most recently discovered member of the γ_c-dependent cytokine family.(589) During viral infections, IL-21 is primarily produced by Th17 $CD4^+$ T cells.(590-592) IL-21 signals through a dimeric receptor composed of the γ_c chain (CD132) and a unique IL-21-receptor (IL21R) chain (CD360). Engagement of IL-21 to its cognate receptor activates JAK1, JAK3 and subsequently STATs notably STAT1, STAT5a, STAT5b and predominantly STAT3.(593) In addition, IL-21 signals though the canonical PI3K and MAPK pathways. As multiple immune cell types express the IL21R, this cytokine exerts pleiotropic modulatory functions in both innate and adaptive cells including NK cells, B cells, CD4 and CD8 T cells.(593)

$CD4^+$ T-cell help is essential for maintaining an effective antiviral $CD8^+$ T-cell response during chronic viral infections.(11, 59, 594) For example, loss of $CD4^+$ T cells during acute HCV infection often leads to viral persistence.(595-597) In rodents, $CD4^+$ T-cell depletion before LCMV Cl-13 inoculation causes lifelong viremia.(11) These observations were correlated with critical loss of $CD8^+$ T-cell immunity. Hence, efforts were made to identify the CD4-derived “helper” factor required to maintain a potent antiviral $CD8^+$ T-cell response during chronic viral infection. Because IL-2 production by T cells rapidly vanishes due to functional exhaustion, IL-21 became the candidate cytokine. Three independent studies used the LCMV Cl-13 model to explore the impact

of IL-21 in maintaining efficient T-cell responses during chronic viral infection.(345-347) IL-21 was readily detected at distinct phases of chronic LCMV infection and $CD4^+$ T cells were uniformly reported to be the main source of the cytokine.(345-347) Interestingly, chronically stimulated $CD4^+$ T cells appeared to rapidly shift from an IL-2 to an IL-21 secretion profile.(345) The importance of IL-21 was further underlined by the fact that $IL21R^{-/-}$ or $IL-21^{-/-}$ mice failed to control chronic LCMV infection despite the maintenance of high numbers of $CD4^+$ T cells. More in depth analysis revealed that CD4-derived IL-21 critically sustained the maintenance and polyfunctionality of CD8 T cells in a cell-intrinsic manner (**Figure. 13**).(345-347) Treatment of chronically infected mice with exogenous IL-21 improved the number and functionality of $CD8^+$ T cells, decreased viral titers, but led to animal demise in 70% of cases.(347) However, in this study, IL-21 treatment was only attempted during the acute phase of the infection and in severe conditions, when $CD4^+$ T cells were depleted. Hence, further work is needed to determine if IL-21 administration in conditions of reduced viral loads can improve $CD8^+$ T-cell responses without lethal adverse effects. Concomitantly, determining how IL-21 intrinsically sustains $CD8^+$ T-cell maintenance and effector functions is an important future research question.

5.3.2- IL-7

IL-7 is a central cytokine for the development of T cells and an essential component for the homeostasis of naïve and memory T cells.(259, 584, 598-602) Primarily produced by stromal cells in primary and secondary lymphoid tissues, IL-7 signals through a dimeric receptor composed of the IL7Rα-chain (CD127) and the γ_c chain (CD132). IL7R principally triggers JAK1 and JAK3 and subsequently STAT5, but can also activate the PI3K and MAPK pathways.(600, 603) Hence IL-7 regulates several key functions of T-cell biology including metabolism, migration, cell cycle progression and survival.(600, 603)

The unique capacity of IL-7 to promote T-cell development, survival and homeostasis raised interest in using this cytokine as an immunotherapeutic agent to

reverse the severe lymphopenia that often comes with chronic viral infections.(45, 604, 605) IL-7 administration to patients with HIV or primates with SIV greatly enhanced the number of circulating $CD4^+$ and $CD8^+$ T cells with a preferential peripheral expansion of naïve and central memory T cells.(606-610) Thus, IL-7 appeared as a powerful supportive agent for maintaining substantial numbers of T cells during chronic viral infection. In addition, when used as antitumor vaccine adjuvant in mice, IL-7 increases the proliferation and cytotoxicity of $CD8^+$ T cells.(611, 612) Further, IL7R expression on effector $CD8^+$ T cells from chronically infected mice was inversely correlated with the severity of exhaustion.(613) Thus, besides its capacity to enhance the number of circulating T cells, IL-7 might also prevents their exhaustion.

Studies by Nanjappa et al. and Pellegrini et al. recently examined more accurately the therapeutic benefits of prophylactic IL-7 in mice that were chronically infected with LCMV Cl-13.(614, 615) Similar to observations in humans, both studies reported that prolonged IL-7 treatment largely expanded the number of polyfunctional $CD4^+$ and $CD8^+$ T cells which led to accelerated viral elimination (**Figure. 13**).(614, 615) This later effect was dependent on T cells as depletion of either $CD4^+$ or $CD8^+$ T cells abrogated the beneficial effects of IL-7.(615) In parallel, IL-7 treatment also increased the number of non-Ag specific CD8 T cells in the periphery, likely by enhancing thymic output (**Figure. 13**).(615) This suggests that IL-7 treatment could be particularly interesting for HIV patients to restore a normal T-cell repertoire and prevent bystander infections associated with AIDS. Importantly, exogenous IL-7 augmented IL-22 levels, a cytoprotective cytokine that prevented liver damage that could arise from the important expansion of T cells after treatment (**Figure. 13**).(615-617) Together, these observations suggest that IL-7 represents a promising immunotherapeutic agent capable of boosting Ag-specific T-cell immunity without causing collateral damage to the host.

More in depth analysis of adaptive responses also revealed that IL-7 treatment critically reduced levels of the suppressor of cytokine signaling 3 (SOCS3), a known inhibitor of IL-6 signaling, within T cells.(615, 618) Interestingly, IL-6 levels were increased in the serum of treated mice and IL-7-mediated viral control was hindered in

IL-6-deficient animals.(615) Further, mice with T-cell restricted Socs3-deficiency recapitulated some aspects of IL-7 treatment and rapidly cleared Cl-13 infection.(615) Taken together, IL-7 therapy augments T-cell immunity directly by restoring T-cell responsiveness to IL-6 through Socs3 modulation and indirectly by augmenting IL-6 serum levels. So far, a direct impact of IL-7 in preventing and/or reversing T-cell exhaustion remains a possibility that requires further investigations.

Despite the beneficial effect of IL-7 therapy in mouse models of chronic viral infections, serious complications limit its use for the treatment of HIV patients. In combination with ART, IL-7 increases the number of circulating $CD4^+$ T cells consistent with the results obtained in LCMV Cl-13 mice.(619) However, this cytokine also increases HIV replication in “virally-active” cells.(619, 620) IL-7 therapy also induces the proliferation of latently infected memory $CD4^+$ T cells without disrupting latency. This results in a dramatic increase of the size of the latent HIV reservoir.(619) Therefore, IL-7 does not appear as a relevant cytokine in the treatment of HIV.

5.3.3- IL-2

$CD4^+$ and $CD8^+$ T cells are the primary source of IL-2 during viral infection.(621-623) IL-2 signals on T cells via a trimeric receptor composed of a unique IL2Rα chain (CD25); an IL2Rβ chain (CD122) that is also shared with IL-15; and the γ_c chain (CD132).(623) As other members of the γ_c-dependent cytokine family, IL-2 binding to its cognate receptor engages JAK/STAT molecules, particularly STAT5a and STAT5b and also triggers the PI3K and MAPK pathways.(624) Since its first description as a powerful growth factor for T cells *in vitro*, IL-2 was shown to mediate a broad spectrum of positive and negative functions on T-cell immunity.(623, 624)

Numerous *in vitro* and *in vivo* studies using IL-$2^{-/-}$ or IL2R$\alpha^{-/-}$ transgenic (Tg) mouse models have elucidated multiple positive functions of IL-2 in the enhancement of $CD8^+$ T-cell responses. During acute viral infection, IL-2 augments the late proliferation of effector $CD8^+$ T cells, especially at non-lymphoid locations, magnifying the overall

antiviral T-cell responses.(313, 625-627) In addition to the quantity, IL-2 also fine-tunes the quality of primary effectors by promoting the secretion of cytolytic and antiviral molecules (i.e. granzyme, perforin, IFNγ).(31, 282) During chronic viral infection, numbers of those functions decrease due to functional exhaustion and T cells rapidly cease to produce IL-2.(2) Hence, providing exogenous IL-2 to compensate for this early loss was considered a relevant therapeutic option.

Recent studies examined both the therapeutic potential of providing low-dose exogenous IL-2 in mice chronically infected with LMCV Cl-13 and the effects of such treatment on Ag-specific $CD8^+$ T-cell responses.(111, 628) Daily low-dose IL-2 regimen for eight days in mice with established Cl-13 infection greatly improved the number of polyfunctional Ag-specific $CD8^+$ T cells at multiple lymphoid and non-lymphoid anatomical locations (**Figure. 13**).(111) This increased number of $CD8^+$ effectors correlated with both a reduction in plasma viral titers assessed directly after treatment, and a subsequent reduction in inhibitory receptor levels, a typical signature of exhaustion.(48, 49, 111, 628) Importantly, combining IL-2 therapy with PDL1 blockade exponentially increased the number of Ag-specific $CD8^+$ T cells and led to viral clearance in a majority of mice (**Figure. 13**).(111) Those exciting results provided a new therapeutic opportunity for treating established chronic viral infections in humans.

There are however serious concerns about the use of IL-2 as a treatment for boosting CD8 T-cell responses in humans. First, a plethora of clinical trials in HIV patients demonstrated the limited impact of IL-2 for increasing the number of functional $CD8^+$ T cells and decreasing HIV titers.(629-633) Second, Second, IL-2 therapy increases the number of suppressive Tregs in HCV and HIV patients.(632, 634) Third, providing IL-2 alone in mice with severe life-long LCMV Cl-13 infection conversely increased viral titers.(111) Also, $CD8^+$ effectors expanded *in vivo* by exogenous IL-2 during an Arm infection were less efficient in containing a subsequent infection by the more virulent Cl-13 strain.(628) *In vitro*, IL-2-expanded CD8 T cells were not maintained after transfer into tumour-bearing mice and displayed modest anti-tumour effects.(295) Hence, care must be taken when designing therapeutic regimens with this cytokine. Finally, while IL-

2 delivers critical signals to shape the differentiation of $CD8^+$ T cells during an acute viral infection,(31, 282, 313, 314, 586) the possible contribution of this cytokine in the deflection toward T-cell exhaustion in chronic settings remains unknown. Hence, a better understanding of the physiological functions of IL-2 in that process should decipher the positive and potential suppressive functions of this cytokine on $CD8^+$ T-cell responses to chronic viral infection.

5.4- Concluding remarks

It has now become clear that cytokines have a major impact in immune responses to persistent viral infections. From the studies reviewed in this section, there are three major points worth keeping in mind. First, although certain cytokines (e.g. TGF-β, IL-10, Type I IFNs) are permissive to viral spread, there are no evidence to date of an intrinsic impact of these soluble mediators in directing $CD8^+$ T cell exhaustion. Second, the impact of a cytokine can be dramatically different depending on the infectious context. For instance, IL-21 is of negligible importance during both primary and secondary T-cell responses to acute LCMV Arm infection,(346, 635) while it is vital during Cl-13 infection.(345-347) Finally, studies regarding the impact of type I IFNs during chronic viral infections emphasized the notion of temporality. One cytokine can be beneficial during the early phase of a chronic viral infection but deleterious during chronicity. This aspect is particularly important to determine optimal windows for therapeutic intervention in patients with chronic viral infections. This notion of temporality also renders difficult the interpretation of KO mouse models in which cytokine or cytokine receptors are completely absent throughout the infection.

VI- Reasoning and hypothesis

6.1- Relevance of research project

T cell exhaustion is now considered as the main mechanism responsible for the silencing of adaptive immune responses during chronic viral infections. Beyond this context, it appears increasingly clear that $CD8^+$ T cell exhaustion is also largely responsible for the failure of immune responses in several types of human cancers. Over the last years, therapeutic approaches aiming to prevent and/or reverse exhaustion notably the use of checkpoint inhibitors (e.g. αPD-L1) demonstrated a great efficacy in reinvigorating exhausted $CD8^+$ T cells in mouse models of chronic viral infections.(49, 60, 104, 106) Such approaches were successfully transposed to human and provided promising results in the treatment of several types of cancers.(135) These studies unequivocally show that preventing and/or reversing $CD8^+$ T cell exhaustion with PD-L1 blockade is the cornerstone of a new generation of extremely promising immunotherapeutic approaches for the treatment of human cancers.(135) Despite, long-term outcome of patients remains a major issue given the high prevalence of relapse after treatment.(135) The main factor underling cancer relapse in these patients is the failure of actual treatment to generate a long-term protective immunity and this for two reasons: (i) reinvigorated $CD8^+$ T cells that massively expand after PD-L1 blockade irremediably convert to a state of terminal exhaustion ($Eomes^{hi}$) after interruption of treatment and (ii) αPD-L1 blockade fails to rescue memory cells development.(104, 636) Such limitations underline the need to combine PD-L1 therapy with approaches targeting the causative factors of $CD8^+$ T cell exhaustion. **However, despite great advances in our understanding of the molecular events governing exhaustion, the factors directly instigating such program in $CD8^+$ T cells and associated loss of memory potential remain unknown.**

6.2- Cytokines: master regulators of T cell differentiation

Among the factors that potently direct $CD8^+$ T cell exhaustion, cytokines are of particular interest. These soluble mediators directly act on activated T cells and orchestrate their differentiation to adapt invading pathogens. To achieve this goal, cytokines directly modify the transcriptional program of activated T cells with particular impact on lineage-specific TFs.(356) Recent studies also evidenced that cytokine-dependent signals can fined-tune the level of transcription of lineage-specific genes by modifying the epigenetic landscape of activated T cells.(356) Such impact of cytokines is particularly evident on $CD4^+$ T cells. Indeed, numerous subtypes of $CD4^+$ effectors with distinct properties can be generated from a single naïve precursor depending on the cocktail of cytokines encountered. To a lesser extent, $CD8^+$ T cell differentiation is also largely governed by cytokines. Several mediators notably IL-2, IL-15, IL-12 and type I IFNs direct the cell-fate decision toward SLECs terminal differentiation while lower amounts of these cytokines rather promote MPECs development.(28, 29, 31, 67, 313, 314) Given the major importance of cytokines in driving T cell differentiation *in vitro* and during acute infections, it is legitimate to speculate that these soluble mediators might also direct $CD8^+$ T cell exhaustion during a chronic viral infection that is considered as an alternative differentiation program.(48) **However, this question remains unanswered to date and determinant cytokines potently involved in the process are yet to be identified.**

6.3- IL-2 and IL-15: instigators of $CD8^+$ T cell exhaustion?

6.3.1- IL-2 and IL-15: biology and impact on $CD8^+$ T cell differentiation

IL-2 and IL-15 belong to the gamma-chain (γ_c)-dependent family of cytokines that share a common γ_c on their respective receptors. That family of cytokines also includes IL-4, -7, -9 and -21 but among them, IL-2 and IL-15 are intimately related because they also share a common IL2Rβ-chain (CD122) in addition to the common γ_c.(583, 637)

Despite such similarities IL-2 and IL-15 possess unique α-chains in their trimeric receptors (IL2Rα [CD25] and IL15Rα [CD215] respectively) and differ in how they are presented to T cells. IL-2 first binds with low affinity to its IL2Rα-chain present on the T-cell surface and then engages the IL2Rβ/γ complex. IL-15 is rather trans-presented to the IL2Rβ/γ complex on T cells by IL-15Rα/IL-15 expressing cells notably macrophages, monocytes, DCs (particularly $CD8^+$ DCs) and also parenchymal cells (**Figure. 14**).(637-642) Once engaged on their respective trimeric receptors, IL-2 and IL-15 trigger similar signaling pathways in $CD8^+$ T cells including JAK3/STAT5, PI3K/AKT and MAPK establishing a similar transcriptional program.(68) De facto, IL-2 and IL-15 display cooperative but also redundant functions in the development of $CD8^+$ T cell responses. During acute infections, IL-2 promotes effector $CD8^+$ T cell proliferation and instigates key changes in their transcriptional program promoting the terminal differentiation of SLECs at the expense of MPECs.(31, 67, 282, 313, 314, 625, 626) This cytokine also enhances CTLs effector functions including granzyme B, perforin and IFNγ secretion (**Figure. 15**) .(31, 282, 311) Albeit IL-15 sustains $CD8^+$ T cell primary expansion (VSV model)(261) and redundantly promotes SLECs development,(67) the impact of this cytokine in the primary phase of infection is extremely limited compared to IL-2. However, IL-15 plays determinant functions at later time-points. During the contraction phase, IL-15-dependent induction of BCL2 in $CD8^+$ T cells sustains MPECs survival and is essential for the maintenance of SLECs.(28, 67, 69, 314, 643, 644) IL-15 is also fundamental for the homeostatic proliferation of memory $CD8^+$ T cells.(107, 109, 645) Importantly, the role of IL-15 in $CD8^+$ T cell responses is not limited to proliferative and survival signals. After a secondary challenge, IL-15 modifies the phenotype of secondary Tem reinforcing traits associated with terminal differentiation including lower CD27 and elevated granzyme B (**Figure. 15**).(646) So, whereas IL-15 has little impact in shaping $CD8^+$ T cell differentiation during primary infections, this cytokine sustains terminal differentiation traits within secondary memory cells. This is in line with the fact that both IL-2 and IL-15 program $CD8^+$ T cell terminal differentiation *in vitro*.(295) **Collectively, IL-2 and IL-15 provide determinant signals that shape $CD8^+$ T cell terminal differentiation during acute viral infections. However, their impact on $CD8^+$ T cell responses to a chronic viral infection remains to be defined.**

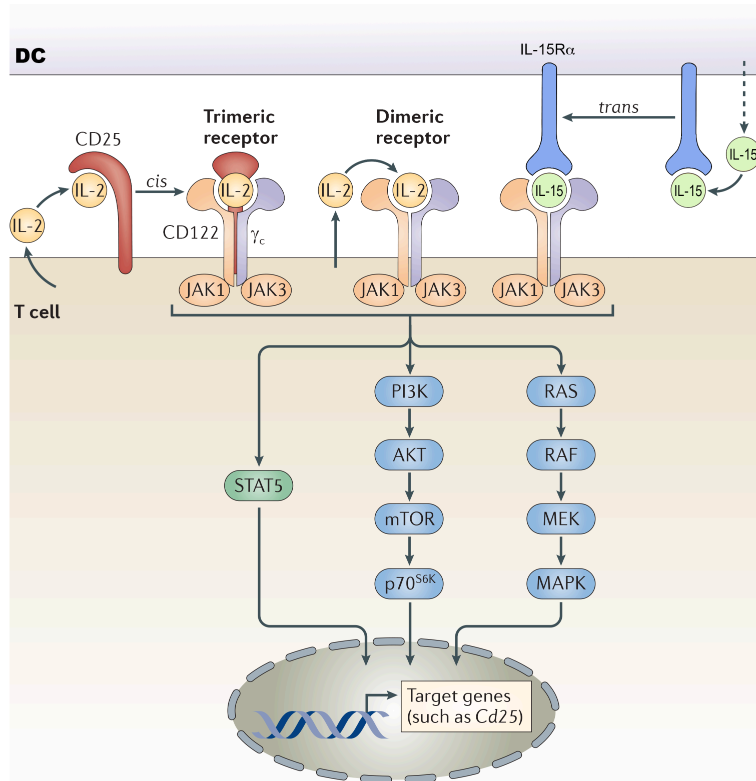


Figure. 14: Biology of IL-2 and IL-15. IL-2 and IL-15 are highly related members of the common γ_c family of cytokines due to shared receptor components (CD122[IL2Rβ], CD132 [γ-chain]) and signaling pathways but differ the ways they are presented to CD8$^+$ T cells. IL-2 (mostly produced by T cells) first bind with low affinity to its unique IL2Rα-chain (CD25) and is subsequently presented in cis to the IL2Rβγ complex on CD8$^+$ T cells. IL-15 produced by DCs, macrophages and parenchymal cells is trans presented complexed to its unique IL15Rα-chain (CD215) to the IL2Rβγ complex on CD8$^+$ T cells.

Adapted from Boyman Nature Reviews Immunology 2012

6.3.2- IL-2 and IL-15: potential implication on CD8$^+$ T cell exhaustion

Studies examining the role of IL-2 and IL-15 in CD8$^+$ T cell responses both *in vitro* and during acute viral infections provided several clues regarding their potential involvement in directing the exhaustion program in chronic settings. As mentioned above, IL-2 and IL-15 are critical for proper differentiation and development of SLECs and these effectors share intriguing similarities with exhausted CD8$^+$ T cells despites being distinct effector subtypes and lineage unrelated.(48, 51) First, both SLECs and

exhausted CD8$^+$ T cells (Tex) have elevated and sustained expression of the TF BLIMP-1.(30, 31, 162) This TF is mainly triggered by IL-2 in CD8$^+$ T cells and is pivotal for both SLECs terminal differentiation and exhaustion establishment.(30, 35, 162, 311) Second, both Tex and SLECs loose potential to differentiate into memory cells and acquire their characteristics including homeostatic proliferation, heightened IL-2 production and strong secondary expansion.(2, 19, 20, 27, 28, 34, 51, 60, 106) This is in line with the fact that both IL-2 and IL-15 restrain memory CD8$^+$ T cell development and/or acquisition of memory features.(67, 295, 647) Third SLECs and Tex present a defective ability to trigger the PI3K/AKT pathway in response to IL-15.(355, 359) Fourth, both IL-2 and IL-15 trigger EOMES and granzyme B expression in CD8$^+$ T cells, molecules that specifically mark the most terminally exhausted population (PD-1^{hi}) during chronic.(269, 282, 295) Finally, while IL-21 protects CD8$^+$ T cells from severe exhaustion,(345-347) IL-2 and IL-15 confers an opposite transcriptional program.(295) **Altogether, these evidences led us to the hypothesis that IL-2 and IL-15 are critical instigators of CD8$^+$ T cell exhaustion during a chronic viral infection.**

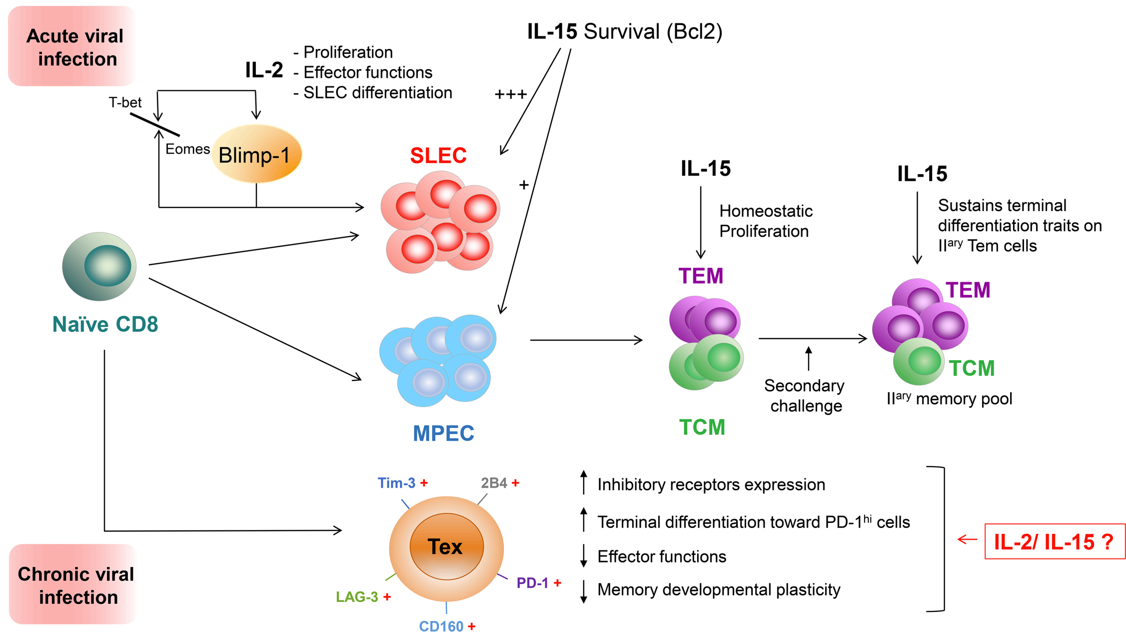


Figure. 15: Impact of IL-2 and IL-15 on CD8$^+$ T cell responses. During the priming phase of an acute viral infection IL-2 sustains effector CD8$^+$ T cells proliferation and promotes

acquisition of effector functions (e.g. GRZB). IL-2 also modifies the transcriptional program of effector $CD8^+$ T cells to promote their terminal differentiation toward SLECs. IL-15 supports the survival of SLECs and MPECs during the contraction phase, direct the homeostatic proliferation of memory cells and imprints terminal effector traits on $CD8^+$ T cells upon several Ag-encounter. During a chronic viral infection however, the impact of IL-2 and IL-15 on multiple parameters associated with $CD8^+$ T cell exhaustion remain unknown.

Results

Article I. IL2Rβ-dependent signals drive terminal exhaustion and suppress memory development during chronic viral infection

Jean-Christophe Beltra[a,b], Sara Bourbonnais[a], Nathalie Bédard[c], Tania Charpentier[d], Moana Boulangé[a,b], Eva Michaud[a,b], Ines Boufaied[e], Julie Bruneau[c,f], Naglaa H. Shoukry[c,g], Alain Lamarre[d], and Hélène Decaluwe[a,b,h]

This article is published in the journal "Proceedings of the National Academy of Sciences" PNAS, vol. 113 no. 37, doi: 10.1073/pnas.1604256113 (2016) PMID 27573835.

This article allowed me to define IL2Rβ as a novel marker of terminal exhaustion in mice and humans and to evidence key functions of IL-2 and IL-15 in directing $CD8^+$ T cell exhaustion. I designed all the experiments (conceptual contribution 90%) with advises from H. Decaluwe. I performed the majority of the experiments and analyzed the results (technical contribution 90%). During busy days I had help from S. Bourbonnais. I formed E. Michaud and M. Boulangé for *in vitro* protocols and they participated in these experiments. I received technical help from T. Charpentier. N. Bédard processed human samples and I analyzed the results. I. Boufaied performed cell-sorting. I wrote and corrected the entire manuscript with corrections from A. Lamarre, N. H. Shoukry and H. Decaluwe.

IL2Rβ-dependent signals drive terminal exhaustion and suppress memory development during chronic viral infection

Jean-Christophe Beltra[a,b], Sara Bourbonnais[a], Nathalie Bédard[c], Tania Charpentier[d], Moana Boulangé[a,b], Eva Michaud[a,b], Ines Boufaied[e], Julie Bruneau[c,f], Naglaa H. Shoukry[c,g], Alain Lamarre[d], and Hélène Decaluwe[a,b,h]

[a]Cytokines and Adaptive Immunity Laboratory, CHU Sainte-Justine Research Center, Montreal, Qc, H3T1C5, Canada

[b]Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Montreal, Qc, H3C3J7, Canada

[c]University of Montreal Hospital Research Center-CRCHUM, Montreal, Qc, H2X0A9, Canada

[d]Immunovirology Laboratory, INRS-Institut Armand-Frappier, Laval, Qc, H7V1B7, Canada

[e]Flow cytometry platform, Sainte-Justine University Hospital Research Center, Montreal, Qc, H3T1C5, Canada

[f]Department of Family Medicine and Emergency Medicine, Faculty of Medicine, University of Montreal, Montreal, Qc, H2X0A9, Canada

[g]Department of Medicine, Faculty of Medicine, University of Montreal, Montreal, Qc, H3T1J4, Canada

[h]Immunology and Rheumatology Division, Department of Pediatrics, Faculty of Medicine, University of Montreal, Montreal, Qc H3T1C5, Canada

***Corresponding author:** Hélène Decaluwe, MD PhD

Mailing address: CHU Sainte-Justine, 3175 Cote-Sainte-Catherine, Montreal, Quebec, H3T 1C5, Canada

Phone number: 1-514-345-4713; Fax: 1-514-345-4897

Email: helene.decaluwe@umontreal.ca

Short title: IL2Rβ-dependent signals direct T cell exhaustion

Classification: Biological Sciences; Immunology and Inflammation

Keywords: $CD8^+$ T cell; IL-2; IL-15; memory T cell; exhaustion

ABSTRACT

Exhaustion of $CD8^+$ T cells severely impedes the adaptive immune response to chronic viral infections. Despite major advances in our understanding of the molecular regulation of exhaustion, the cytokines that directly control this process during chronicity remain unknown. We demonstrate a direct impact of IL-2 and IL-15, two common gamma-chain-dependent cytokines, on $CD8^+$ T cell exhaustion. Common to both cytokine receptors, the IL-2 receptor β (IL2Rβ) chain is selectively maintained on $CD8^+$ T cells during chronic lymphocytic choriomeningitis virus and hepatitis C virus infections. Its expression correlates with exhaustion severity and identifies terminally exhausted $CD8^+$ T cells both in mice and humans. Genetic ablation of the IL2Rβ chain on $CD8^+$ T cells restrains inhibitory receptor induction, in particular 2B4 and Tim-3; precludes terminal differentiation of highly defective $PD\text{-}1^{hi}$ effectors; and rescues memory T-cell development and responsiveness to IL-7-dependent signals. Together, we ascribe a previously unexpected role to IL-2 and IL-15 as instigators of $CD8^+$ T cell exhaustion during chronic viral infection.

SIGNIFICANCE STATEMENT

During chronic viral infection, $CD8^+$ T cells are gradually deprived of their principal effector functions and irreversibly loose their plasticity to develop into memory populations, precluding the establishment of long-lasting protective immunity. Relevant host-derived factors directing this T-cell exhaustion process have remained elusive. Growing evidence suggests that the cytokine milieu dramatically impacts the outcome of chronic viral infection. However, it is unclear if cytokines directly promote $CD8^+$ T-cell exhaustion and, if so, which specific cytokines are involved in this process. Here, we demonstrate a critical role for two highly related cytokines, IL-2 and IL-15, in the terminal differentiation of highly exhausted $CD8^+$ T cells and the lack of immunological memory observed during chronic viral infection.

INTRODUCTION

$CD8^+$ T cells are the cornerstone of antiviral immunity. Throughout their differentiation, $CD8^+$ T cells integrate signals that define their cellular fate and functional capacities (1-3). Persistent infections with viruses such as HIV and hepatitis C (HCV) and B are associated with exhaustion of $CD8^+$ T cells (4, 5). Exhaustion is characterized by progressive loss of effector functions (cytokine secretion, cytolytic potential, antigen-driven proliferation), increased cell death and eventually physical deletion (6-9). This unresponsive state stems from increased expression of multiple inhibitory receptors on $CD8^+$ T cells [including programmed cell death 1 (PD-1), lymphocyte-activation gene 3 (LAG-3), 2B4 (CD244), T-cell Ig and mucin domain-containing molecule 3 (Tim-3), CD160 and cytotoxic T-lymphocyte associated protein 4 (CTLA4)] that use distinct and non-redundant ways to suppress T-cell functions (10-12). Further, $CD8^+$ T cells are gradually and irreversibly deprived of their developmental plasticity as they commit to exhaustion, restricting the cell-fate potential for memory development and precluding the establishment of long-lasting protective immunity (13-15).

Factors underlying T cell exhaustion are not understood (5, 16). Antigen persistence is a culprit, but little is known about the intrinsic factors that program $CD8^+$ T cell differentiation to exhaustion. Certain transcription factors that regulate the differentiation of $KLRG1^{hi}$ $IL7R\alpha^{lo}$ short-lived effector cells (SLECs) during acute infection, are also fundamental for sustaining the differentiation of highly exhausted T cells during chronic infection (1, 5). Despite being distinct effector subtypes (11, 13), both SLECs and exhausted CD8+ T cells have elevated and sustained expression of Blimp-1 (encoded by *prdm1*) (11, 17, 18). Blimp-1 is indispensable for SLEC development and terminal differentiation in acute infection (17, 19), but is also pivotal to the establishment of exhaustion in chronic infection (18). Similarly, the T-box transcription factor Eomes, which matures SLECs with full effector functions (20-22), is indispensable to generate $PD\text{-}1^{hi}$ terminally exhausted cells in chronic settings (23). Thus, some of the cardinal transcriptional requirements for SLEC terminal differentiation during acute infection are also fundamental to directing the exhaustion of $CD8^+$ T cells during chronic infections. Therefore, we hypothesize that determinants shaping the transcriptional program toward

SLEC terminal differentiation during acute infections might also instigate $CD8^+$ T-cell exhaustion in chronic viral infections.

During acute viral infection, SLEC terminal differentiation is partly controlled by the common gamma chain (γ_c)-dependent cytokines IL-2 and IL-15. Indeed, we have previously shown that these closely related cytokines that share common receptor components [i.e., IL-2 receptor β (IL2Rβ; CD122), IL2Rγ (CD132)] and signaling pathways (24), directly promote SLEC development in both primary and secondary responses to acute viral infection (25). Concomitantly, $CD8^+$ T cells deficient for the IL2Rβ chain preferentially turn to $KLRG1^{lo}IL7R\alpha^{hi}$ memory precursor effector cells and convert rapidly to $CD62L^{hi}CD127^{hi}$ central memory T cells (Tcm) (25). These results demonstrate that IL-2 and IL-15-dependent signals direct $CD8^+$ T-cell lineage choices toward SLEC terminal differentiation. Importantly, IL-2 and IL-15 mediate this effect through the modulation of key transcription factors that are also pivotal during the establishment of $CD8^+$ T-cell exhaustion (i.e., Blimp-1 and Eomes). Indeed, IL-2 directly induces Blimp-1 expression (26, 27), and both IL-2 and IL-15 trigger high levels of Eomes in $CD8^+$ T cells in vitro (21, 28). These pieces of evidence strongly predict a role for IL-2 and IL-15 in $CD8^+$ T cell responses during a chronic viral infection. This role remains to be investigated.

Using a mouse model of chronic viral infection, lymphocytic choriomeningitis virus (LCMV) clone 13 (Cl-13), we observed selective maintenance of CD122, common to IL-2 and IL-15 receptors, on $CD8^+$ T cells throughout antigen persistence. CD122 expression levels on $CD8^+$ T cells correlated with severe exhaustion and clearly discriminated $PD\text{-}1^{hi}$ terminal effectors in LCMV-infected mice and humans with persistent HCV infection. Using adoptive transfer experiments of transgenic (Tg) P14 cells deficient for the IL2Rβ chain, we also observed that IL-2 and IL-15 directly regulated several aspects of $CD8^+$ T-cell exhaustion. These cytokines controlled the expression of 2B4 and Tim-3 and augmented the levels of several other inhibitory receptors on $CD8^+$ T cells, leading to higher dysfunction. Further, signaling through IL2Rβ instigated key transcriptional changes in $CD8^+$ T cells that fostered the terminal

differentiation of highly exhausted PD-1^{hi} effectors while precluding memory development and IL-7-dependent signaling. Together, our results show a previously unexpected role for IL-2 and IL-15 in the deviation of $CD8^{+}$ T cells toward terminal exhaustion during chronic viral infection.

RESULTS

Increased and Sustained Expression of CD122 on $CD8^+$ T Cells Marks Severe Exhaustion

To delineate the impact of IL-2 and IL-15 on the $CD8^+$ T-cell response to chronic viral infection, we first analysed the expression kinetics of key IL-2 and IL-15 (IL-2/15) receptor components on H-$2D^b$-restricted LCMV-specific $CD8^+$ T cells throughout infection, focusing on the dominant epitope gp33-41 (D^bgp33). Following infection with LCMV Cl-13, CD25 (IL2Rα) expression was induced early, but vanished after 6 d post infection (p.i.) (Fig. 1*A*, *Left*), whereas CD132, the receptor chain shared by all cytokines of the common γ_c family, returned to baseline levels (Fig. 1*A*, Right and Fig. S1*A*). By contrast, CD122, exclusive to both IL-2 and IL-15, was expressed early and was sustained throughout the infection (Fig. 1*A*, Center), and its expression level declined gradually, mirroring the drop in viral titers (Fig. 1*B*). This association suggests that antigen persistence supports CD122 expression on $CD8^+$ T cells. In line with this notion, the levels of CD122 were substantially higher at day 8 and 15 p.i. on chronically stimulated D^bgp33 $CD8^+$ T cells than on D^bgp33 $CD8^+$ T cells activated by the acute strain of LCMV, LCMV Armstrong (Arm), until day 30 p.i., when they reached comparable levels (Fig. 1*C*). Thereafter, CD122 expression continued to decline in mice previously infected with LCMV Cl-13 consistent with the inability of chronically stimulated cells to sustain its expression after viral elimination (14, 15). Maintenance of the IL2Rβγ complex promoted signal transducer and activator of transcription 5 (STAT5) phosphorylation in response to IL-2 and, to a greater extent, to IL-15 at day 21 p.i., in agreement with previous reports (Fig. 1*D*) (29). In response to IL-2 and IL-15, $CD8^+$ T cells chronically stimulated with Cl-13 expressed levels of phospho-STAT5 similar to those in their counterparts acutely infected with Arm at day 30 p.i. (Fig. S1*B*). These results suggested that CD122-dependent signals supported important physiological functions during antigen persistence. To understand these functions, we performed direct ex vivo comparative analysis between freshly isolated $CD8^+$ T cells expressing high ($CD122^{hi}$) or low ($CD122^{lo}$) levels of IL2Rβ (see Fig. S1*C* for the gating strategy). We noted that a lower frequency of $CD122^{hi}$ D^bgp33-specific $CD8^+$ T cells secreted IFNγ compared with $CD122^{lo}$ cells (22% versus 33%, p=0.0086) (Fig. 1*E* and Fig.

S1*D*). IFNγ/TNFα double producers were also less frequent within CD122^{hi} effectors than in CD122^{lo} cells (5% versus 9%, p=0.0463); however, TNFα production was uniformly low (Fig. 1*E* and Fig. S1*D*). CD122^{hi} effectors were also more apoptotic, as demonstrated by the higher frequency of 7-amino-actinomycin D (7AAD)$^{+}$ and AnnexinV^{+}/7AAD^{+} cells compared with CD122^{lo} effectors (39% versus 13%, ***p<0.0001 and 22% versus 9%, ***p<0.0001 respectively) (Fig. 1*F*). This restricted cytokine profile and limited survival potential were suggestive of T-cell exhaustion. To correlate these findings with the exhaustion status of the cells, we assessed the expression of a panel of inhibitory receptors. On day 21 p.i., CD122^{hi} effectors expressed superior levels of several inhibitory receptors including PD-1, LAG-3, CD160, 2B4 and Tim-3 than did CD122^{lo} D^{b}gp33-specific T cells (Fig. 1*G*). Together, these results imply that high IL-2- and IL-15-dependent signaling may restrict T-cell function and survival through increased expression of inhibitory receptors, committing effector cells to severe exhaustion.

Elevated CD122 Expression on CD8^{+} T Cells Identifies PD-1^{hi} Terminal Effectors in Mice Infected with LCMV Cl-13 and Humans with Persistent HCV Infection

During chronic infection, it was demonstrated that PD-1$^{int/lo}$ progenitor cells serve as a reservoir for continuously replenishing a PD-1^{hi} progeny population (23). Conversion into PD-1^{hi} is terminal and is associated with enhanced exhaustion, increased expression of inhibitory receptors, loss of cytokine production capacity and increased apoptosis (7, 10, 23). As CD122^{hi} cells presented these same characteristics (Fig. 1), we questioned the lineage relationship between CD122^{hi} and PD-1^{hi} cells. At day 21 p.i., we observed a correlation between CD122 and PD-1 expression on Ag-specific CD8^{+} T cells (Fig. 2*A*). PD-1^{hi} cells expressed higher levels of CD122 than PD-1$^{int/lo}$ progenitors (see Fig. 2*B* and Fig. S2*A* for gating strategy). The majority of CD122^{hi} D^{b}gp33 CD8^{+} T cells were also PD-1^{hi} progenies and inversely, CD122^{lo} cells were largely composed of PD-1$^{int/lo}$ progenitors (Fig. 2*C*). Conversion from PD-1$^{int/lo}$ to PD-1^{hi} terminal effectors and subsequent increase in PD-1 levels follows extensive proliferation (23). Similarly, CD122 levels increased with proliferation as most CD122^{hi} D^{b}gp33 CD8^{+} T cells incorporated 5-bromo-2-deoxyuridine (BrDU) between day 15 and 30 p.i. and preferentially expressed

the proliferative marker Ki67 (Fig. 2*D* and *E* and Fig. S2*B*). To delineate the lineage relationship between $CD122^{lo}$ and $CD122^{hi}$ cells, we tested whether $CD122^{lo}$ cells were the progenitors of the $CD122^{hi}$ terminal effector population. To do so, $CD122^{lo}$ and $CD122^{hi}$ $CD45.2^{+}$ cells sorted at day 15 p.i. were transferred into infection-matched $CD45.1^{+}$ mice (Fig. S2*C* and *D*). One week later (day 22 p.i.), 46% ± 4% of $CD122^{lo}$ transferred cells presented a $CD122^{hi}$ PD-1^{hi} phenotype, while 74% ± 2% of $CD122^{hi}$ remained $CD122^{hi}$ PD-1^{hi} (Fig. 2*F*). Conversion of $CD122^{lo}$ transferred cells into $CD122^{hi}$ PD-1^{hi} effectors was accompanied by intense proliferation, as demonstrated by the incorporation of BrdU over one week, while the majority of $CD122^{lo}$ PD-1^{lo} cells were $BrdU^{-}$ (Fig. 2*G*). Differentiation into PD-1^{hi} effectors is associated with specific transcriptional changes including elevated levels of Eomes, Blimp-1, and the Forkhead box protein O1 (FoxO1) (18, 23, 29). Accordingly, $CD122^{hi}$ D^{b}gp33 $CD8^{+}$ T cells isolated at day 21 p.i. expressed higher levels of Eomes and FoxO1 than their $CD122^{lo}$ counterparts (Fig. 2*H* and Fig. S2*E*). *Prdm1* mRNA levels also appeared higher in $CD122^{hi}$ cells, although the difference did not reach statistical significance (Fig. 2*I*). Together, these results define CD122 as an independent marker distinguishing the PD-$1^{int/lo}$ progenitors from the terminally exhausted PD-1^{hi} progeny population and reveal $CD122^{lo}$ cells as the progenitors of the $CD122^{hi}$ PD-1^{hi} population.

To validate and extend our observations to human chronic viral infection, we performed similar analysis on peripheral blood samples obtained at different stages p.i. from individuals who developed persistent HCV infection (Fig. S3*A*). In these patients, we detected a substantial fraction of peripheral HCV-specific $CD8^{+}$ T cells expressing CD122 (54.6% ± 7%, Fig. S3*B*). Interestingly, $CD122^{+}$ HCV-specific $CD8^{+}$ T cells were enriched in cells expressing PD-1, Eomes or both markers (Fig. 3*A* and *B*), indicating that these effectors were more exhausted and terminally differentiated (23). Concomitantly, the frequency of cells expressing these markers was reduced in $CD122^{-}$ HCV-specific $CD8^{+}$ T cells (Fig. 3*A* and *B*). Similar patterns were observed when we gated bulk $CD8^{+}$ T cells with an effector memory $CD45RA^{lo}CCR7^{lo}$ phenotype, which constitutes the majority of effector cells in HCV-infected patients (Fig. 3*C* and *D* and Fig. S3*C*). Taken

together, our results demonstrate that CD122 is a relevant biological marker of $CD8^+$ T cell exhaustion and terminal differentiation in both mice and humans.

Deleting CD122 on $CD8^+$ T Cells Abrogates their Differentiation into PD-1^{hi} Exhausted Cells

To elucidate the cell-intrinsic significance of increased CD122 levels during PD-1^{hi} conversion, we performed adoptive transfer of IL2Rβ-deficient P14 (P14 IL2R$\beta^{-/-}$) cells or IL2Rβ-sufficient controls (P14) into distinct C57BL/6 hosts 20 hours prior infection with LCMV Cl-13 (25). As expected, P14 cells could be identified in the spleen as two distinct populations on day 35 p.i.: PD-$1^{int/lo}$ progenitors (PD-$1^{int/lo}$CD44^{hi}; 2.8×10^4 cells) and PD-1^{hi} progenies (PD-1^{hi}CD$44^{int/lo}$; 1.8×10^4 cells) (Fig. 4*A* and *B* and Fig. S4*A*) (7, 23). Strikingly, PD-1^{hi} effectors were nearly undetectable in the absence of IL2Rβ (8×10^2 cells) while the number of PD-$1^{int/lo}$ cells was unaffected (1.8×10^4 cells) (Fig. 4*A* and *B* and Fig. S4*A*). Absence of PD-1^{hi} cells correlated with a 3.8-fold reduction in the absolute number of P14 IL2R$\beta^{-/-}$ cells compared with P14 controls at day 35 p.i. (Fig. S4*B*). This salient difference in cell numbers resulted mainly from reduced proliferation of IL2R$\beta^{-/-}$ cells at day 8 p.i. rather than from a survival defect, as demonstrated by the lower frequency of Ki67^{hi} cells in the absence of IL2Rβ but equally low expression of active caspase-3 (Fig. S4*C*). The fold difference in absolute numbers did not increase but rather decreased between day 8 and 35 p.i., and both groups presented similar contraction phases (Fig. S4*B* and S4*D*). We and others have previously demonstrated that IL2Rβ-dependent signals improved the survival of terminal effectors during the contraction phase of an acute viral infection in a B-cell lymphoma (Bcl2)-dependent manner (24, 25, 30, 31). Only 16 ± 8% of P14 PD-1^{hi} cells, which present the highest levels of CD122 (Fig. 2*B*), expressed Bcl2, as compared with 30 ± 10% of P14 PD-$1^{int/lo}$ cells (Fig. 4*C*). The absence of IL2Rβ further increased the proportion of cells expressing Bcl2 in PD-$1^{int/lo}$ cells (45% ± 6%, Fig. 4*C*). This increase resulted in a superior ratio of P14 IL2R$\beta^{-/-}$ PD-$1^{int/lo}$ cells expressing Bcl2 over Bim as compared with either P14 PD-1^{hi} or PD-$1^{int/lo}$ cells (Fig. 4*D*). Collectively, these data demonstrate that IL2Rβ-dependent signals are essential for the development of PD-1^{hi} cells rather than for their maintenance, and further

hamper the survival of the PD-$1^{int/lo}$ progenitor pool. Because PD-1^{hi} development is the result of extensive proliferative events (23), we then questioned whether the defective development into PD-1^{hi} cells seen in the absence of IL2Rβ was caused by altered proliferation during viral persistence. To test this hypothesis, mice were given daily BrdU starting at day 15 p.i. At day 30 p.i., P14 and P14 IL2R$\beta^{-/-}$ CD8^+ T cells had incorporated equivalent amounts of BrdU (Fig. 4*E*). However, the immediate proliferation rate of P14 controls was superior to P14 IL2R$\beta^{-/-}$ cells as assessed by direct Ki67 staining at day 30 p.i. (31 ± 2% versus 21 ± 4% respectively, Fig. 4*F)*. Although 21% of P14 IL2R$\beta^{-/-}$ cells underwent proliferation, they never acquired granzyme B expression, a cytotoxic molecule known to increase with PD-1^{hi} terminal differentiation (Fig. 4*G*) (23). These findings indicate that IL2Rβ-dependent signals sustain the immediate proliferation of CD8^+ T cells during progression to chronic infection but also affect intrinsic differentiation pathways critical for the generation of terminally exhausted PD-1^{hi} cells. To understand how IL2Rβ-dependent signals impacted on the differentiation of PD-1^{hi} progenies, we evaluated known transcription factors involved in the differentiation of PD-1^{hi} terminal effectors (23). Unexpectedly, levels of T-bet were nearly equivalent in both groups of cells, while Eomes levels were slightly increased in P14 IL2R$\beta^{-/-}$ cells, although not reaching statistical significance (Fig. 4*H* and Fig. S4*E* and *F)*. However, we detected a marked reduction in *Prdm1* mRNA levels (gene encoding Blimp-1) in P14 IL2R$\beta^{-/-}$ cells at day 35 p.i. compared to P14 controls (Fig. 4*I*). These results point to a critical role for IL-2 and IL-15 in driving the lineage choice to PD-1^{hi} cells by promoting the expression of Blimp-1, a key transcription factor associated with CD8^+ T cell terminal differentiation and exhaustion (17-19).

Lack of IL2Rβ-Dependent Signaling Restrains the Development of Highly Exhausted Effector Cells and Blocks 2B4 and Tim-3 Induction on CD8^+ T cells

To confirm the importance of IL2Rβ-dependent signals on the development of highly exhausted CD8^+ T cells, we analyzed the composition of the CD8^+ T cell pool on day 35 p.i.. First, we measured the frequency of highly dysfunctional P14 and P14 IL2R$\beta^{-/-}$ cells co-expressing multiple inhibitory receptors (10). We observed that IL2Rβ deficiency

enhanced the quality of the effector pool by favouring the accumulation of less exhausted $CD8^+$ T cells, that co-expressed only 1 or 2 inhibitory receptors (Fig. 5*A*) (10). Conversely, most P14 controls were found to express 3 or 4 inhibitory receptors simultaneously, indicative of more pronounced exhaustion (10). In $IL2R\beta^{-/-}$ cells, this correlated not only with a preserved capacity to produce IFNγ and increased IFNγ production on a per-cell basis (MFI) (Fig. 5*B-C* and Fig. S5*A*) but also the ability to proliferate in response to antigenic stimulation (Fig. 5*D* and, Fig. S5*B*), two fundamental characteristics lost by exhausted T cells (6, 8). Together, these results indicated that IL2Rβ deficiency protected effector $CD8^+$ T cells from severe exhaustion. When analyzed individually, we also observed that IL2Rβ deficiency was associated with a major reduction in the expression of all tested inhibitory receptors (PD-1, LAG-3, CD160, 2B4 and Tim-3), with the exception of CD160, which was also reduced on P14 cells from the lymph nodes (Fig. 5*E* and, Fig. S5*C*). Most striking was the near complete abolition of 2B4 and Tim-3 expression on $IL2R\beta^{-/-}$ cells (Figure. 5*E-F*). These observations did not reflect discrepancies between the two groups of mice as endogenous D^bgp33 (CD45.1.2) cells of mice adoptively transferred with either P14 or P14 $IL2R\beta^{-/-}$ cells expressed equivalent levels of PD-1 (Fig. S5*D*). Moreover, both groups had similar viral titers by day 35 p.i. (Fig. S5*E*). Because $PD\text{-}1^{hi}$ cells are known to express higher levels of inhibitory receptors (ref. 10 and Fig. S5*F*), we questioned whether the differences observed between both groups might reflect the defective development of $PD\text{-}1^{hi}$ cells in the absence of IL2Rβ. We compared the expression of LAG-3, CD160, 2B4 and Tim-3 in the $PD\text{-}1^{int/lo}$ compartment of both P14 and P14 $IL2R\beta^{-/-}$ cells. Highly significant differences in 2B4 and Tim-3 expression between both groups were observed while LAG-3 and CD160 expression were comparable (Fig. 5*G*). The restricted expression of 2B4 and Tim-3 in the absence of IL2Rβ was detectable at earlier time points (Fig. S5*G*), excluding a potential selective loss of this population during chronicity. These results imply that LAG-3 and CD160 increase in expression resulted from an efficient conversion in $PD\text{-}1^{hi}$ progenies, while IL-2 and IL-15 directly controlled 2B4 and Tim-3 expression. To confirm this finding, we designed an *in vitro* assay to assess the impact of IL-2 and IL-15 on the induction of inhibitory receptors, while keeping constant the antigenic stimulation throughout the assay. IL-2 or IL-15 was added

on day 2 of the culture, a time when IL-2/15 receptor components were efficiently induced (Fig. S5*H*). Adequate TCR stimulation was sufficient to trigger high levels of PD-1, LAG-3 and CD160 on P14 cells at day 2 of the culture, while IL-2 and IL-15 maintained their expression thereafter (Fig. 5*H*). In contrast, 2B4 and Tim-3 expression were strictly dependent on the addition of IL-2 or IL-15 to the culture media (Fig. 5*I***)**. Our findings provide evidence that IL-2 and IL-15 use both direct and indirect routes to regulate $CD8^+$ T cell exhaustion. First, IL-2 and IL-15 directly and redundantly promote 2B4 and Tim-3 expression. Second, these cytokines control the terminal differentiation of $PD\text{-}1^{hi}$ effectors and subsequent increase in LAG-3 and CD160 levels. These data point to the critical role of IL-2 and IL-15 in the development of severe T cell exhaustion during chronic viral infection.

IL2Rβ deficiency restores $CD8^+$ memory T cell development and IL-7-dependent signaling

Resolution of an acute infection allows for the differentiation of a CD8 memory T cell pool that persists and protects the host from re-infection. During chronic viral infection however, CD8 memory T cell development is completely blocked (13-15). The factors involved in this arrested memory differentiation remain unclear, and very few immunomodulatory methods used to limit exhaustion have been shown to rescue CD8 memory T cells. Strikingly, we noted that abrogation of IL2Rβ-dependent signals led to the rapid re-expression of the cardinal memory marker CD127 from day 8 p.i. onwards (Fig. 6*A*). At that time point, frequency of $CD127^+$ P14 $IL2R\beta^{-/-}$ already exceeded that of P14 controls and rapidly increased thereafter (Fig. S6*A*). This suggested that IL2Rβ-deficiency caused an early differentiation bias allowing a substantial fraction of effector $CD8^+$ T cells to escape exhaustion and conserve memory potential. Indeed, at day 65 p.i., when the virus was eliminated from most tissues except the kidneys (Fig. S6*B*), 74% ± 5% of P14 $IL2R\beta^{-/-}$ cells expressed CD127 in contrast to P14 controls (21% ± 3%), in which CD127 expression remained repressed, in line with previous reports (Fig. 6*A* and, Fig. S6*A*) (14, 15). 27% ± 2% of P14 $IL2R\beta^{-/-}$ memory T cells ($2.6.10^3$ cells) efficiently converted to $CD127^{high}CD62L^{high}$ central memory T cells (Tcm) phenotype (Fig. 6*B*-*C*), whereas this population was numerically inferior ($1.2.10^3$ cells) in P14 controls; 65% ±

4% of the cells remaining $CD127^{neg}CD62L^{neg}$ ($1.6.10^4$ cells), a state we refer to as exhausted T cells (Tex) (Fig. 6*B*-*C*). Rescued Tcm development in P14 $IL2R\beta^{-/-}$ cells correlated with increased expression of the Tcm-associated transcription factor Bcl6 (32, 33), while the levels of Eomes remained equivalent to P14 controls (Fig. 6*D*). Although equivalent numbers of $CD127^{high}CD62L^{neg}$ T effector memory cells (Tem) were generated with or without of IL2Rβ (Fig. 6*C*), these cells expressed higher levels of both CD127 and Bcl6 in the absence of IL2Rβ compared to their P14 counterparts (Fig. 6*E*). Collectively, these results indicate that both the quality and the quantity of the memory T cell pool generated in the absence of IL2Rβ-dependent signals is greatly enhanced. We show that Ag persistence associated with chronic viral infections does not impede the capacity of $CD8^+$ T cells to differentiate into memory T cells as previously thought. Rather, we demonstrate that IL-2 and IL-15 are the main mediators of this arrested developmental process during viral persistence. Conventional memory T cells provide long-lasting protection and persist in the host through their ability to respond to the homeostatic cytokine IL-7 (34, 35). We thus tested if enhanced memory generation in the absence of IL2Rβ was sufficient to restore responsiveness to IL-7-dependent signals. We first assessed STAT5 phosphorylation in response to IL-7 and showed that day 65 P14 $IL2R\beta^{-/-}$ memory T cells efficiently triggered STAT5 phosphorylation compared to P14 controls albeit not to the same levels as conventional memory T cells (Fig. 6*F*). However, the majority of P14 $IL2R\beta^{-/-}$ memory T cells re-expressed the pro-survival molecule Bcl2, a known target of IL-7, and its expression strictly overlapped with that of CD127 (Fig. 6*G*). Conversely, few P14 control cells co-expressed CD127 and Bcl2, and the level of Bcl2 in $CD127^+$ cells remained always inferior to that of their $IL2R\beta^{-/-}$ counterparts (Fig. 6*G*-*H*). These results indicate that lack of IL2Rβ-dependent signaling reinstated responsiveness to IL-7-mediated homeostatic pathways signals (14, 15). This led to the generation of a stable population of $CD127^+$ $IL2R\beta^{-/-}$ memory T cells over time, in contrast to P14 controls (Fig. 6*I*). Together, our results strongly demonstrate that IL-2 and IL-15 are the main repressors of memory $CD8^+$ T cell development during chronic viral infection. Absence of these signals restored Tcm development as well as responsiveness to IL-7-dependent signals and subsequent Bcl2 expression. This indicates that chronically

stimulated $CD8^+$ T cells are able to overcome the typical Ag addiction that comes with exhaustion and restore a functional IL-7-STAT5-Bcl2 survival axis when IL2Rβ-dependent signals are interrupted.

DISCUSSION

The physiological impact of IL-2 and IL-15, two key cytokines involved in the differentiation of $CD8^+$ effector T cells (25, 36), has not been thoroughly evaluated during chronic infections. Here, we demonstrate a critical role for these two cytokines in the development of severe $CD8^+$ T cell exhaustion and the associated abrogation of memory T cell development. We provide evidence for preferential maintenance of CD122, the IL2Rβ chain common to IL-2 and IL-15, on $CD8^+$ T cells during chronic viral infection in both humans and mice. We show that IL-2 and IL-15-dependent signals direct key aspects of $CD8^+$ T cell exhaustion including i) the terminal differentiation of highly exhausted PD-1^{hi} cells, ii) the expression of multiple inhibitory receptors at the surface of the cells, in particular 2B4 and Tim3, and their associated cellular dysfunctions and iii) the arrested CD8 memory T cell differentiation observed during chronic viral infection. These observations demonstrate for the first time a direct role of cytokines, in this case IL-2 and IL-15, in instigating $CD8^+$ T cell exhaustion during chronic viral infection.

Of first importance, we identified distinct subsets of exhausted $CD8^+$ T cells expressing high ($CD122^{hi}$) or low ($CD122^{lo}$) levels of the IL2Rβ chain, the latter being the progenitors of the former. These subsets had distinct patterns of exhaustion and selectively marked PD-1^{hi} and PD-$1^{int/lo}$ effectors respectively (7, 18, 23). Hence, we defined CD122 as a new biological marker of severe exhaustion and terminal differentiation in both humans and mice with chronic viral infections. Moreover, our observations expanded the understanding of the dynamics involved in the conversion from PD-$1^{int/lo}$ progenitors to PD-1^{hi} terminal effectors. Previous studies proposed that the development of PD-1^{hi} effectors was dependent on proliferative events driven by Ag and Eomes (23). Here, IL2Rβ-deficient $CD8^+$ T cells expressed normal or even slightly increased Eomes levels, proliferated during chronicity albeit at a lower rate than control cells but did not terminally differentiate into PD-1^{hi} progenies. This underscored a new level of regulation in which IL-2 and IL-15-dependent signals drove the terminal differentiation of PD-1^{hi} effectors, likely through the induction of Blimp-1. This is in line

with the essential role of this transcriptional repressor in directing $CD8^+$ T cell terminal differentiation in acute infection models and severe exhaustion in chronic settings (17-19). We propose that conversion to PD-1^{hi} effectors is a two-step process where Ag first drives the proliferation of PD-$1^{int/lo}$ progenitors. Then, IL-2 and IL-15 sustain this proliferation and cooperate to perfect PD-1^{hi} terminal maturation and exhaustion. This defines a novel role for IL-2 and IL-15 in directing cell-fate decision to PD-1^{hi} terminal differentiation during chronic viral infection.

We further provided evidence for IL2Rβ-dependent signals in supporting TCR-dependent PD-1, LAG-3 and CD160 expression during chronic antigenic stimulation. In addition, these two cytokines are mandatory for the induction of 2B4 and Tim-3 at the surface of the cells in conjunction with TCR signals. These results present evidence for distinct regulatory pathways for the induction of inhibitory receptor expression. Previous studies support the dichotomy observed in the regulation of inhibitory receptors (18, 37). Indeed, T-bet directly repressed PD-1 and decreased LAG-3 and CD160 expression on $CD8^+$ T cells, while promoting Tim-3 and 2B4 to a lesser extent (37). Blimp-1 deficiency in $CD8^+$ T cells prevented 2B4 induction while PD-1, LAG-3 and CD160 were less affected (18). These results point to specific inherent signals for the induction of distinct inhibitory receptors at the surface of the cells, fine-tuning the level of inhibition that is needed in different contexts. We unveil a novel immuno-regulatory role for IL-2 and IL-15 in governing the induction of a specific pattern of inhibitory receptors (i.e. 2B4 and Tim-3) on $CD8^+$ T cells during chronic viral infection. Future research will entail determining the mechanisms underlying such selective induction.

Our data strongly support that IL-2 and IL-15 cooperate to sustain the exhaustion of $CD8^+$ T cells during chronic infections. Indeed, despite the early loss of CD25 expression on $CD8^+$ effectors during chronic stimulation, exhausted $CD8^+$ T cells integrate both IL-2 and IL-15-dependent signals at day 21 p.i., likely through the IL2Rβγ complex (38). However, in a setting where IL-2 production is severely hindered (6, 39), IL-15 phosphorylated STAT5 at day 21 of infection more efficiently than IL-2, pointing to an

increased sensitivity of exhausted T cells to IL-15-dependent signals. Interestingly, IL-15 triggered Blimp-1 expression in $CD8^+$ T cells *in vitro*, albeit to a lesser extent than IL-2 (unpublished data), in accordance with the notion that both cytokines induce similar transcriptional programs in $CD8^+$ T cells (24). Furthermore, IL-2 and IL-15 redundantly supported 2B4 and Tim-3 expression on $CD8^+$ T cells *in vitro*. These results imply that IL-2 and IL-15 act in concert to direct the differentiation of exhausted cells during chronic viral infection. Determining the exact timeframe during which IL-2 and IL-15 program and regulate this process will be an important question to address in the future.

Finally, we demonstrate that IL-2 and IL-15 are responsible for the progressive and irreversible loss of memory development potential that marks $CD8^+$ T cells during chronic viral infection (13-15). IL2Rβ deficiency on $CD8^+$ T cells allowed for CD127 re-expression and Tcm development despite viral persistence, and reinstated a potent CD127-Stat5-Bcl2 survival axis. Transcriptionally, memory resurgence in IL2Rβ-deficient $CD8^+$ T cells resulted from an imbalance in favour of Bcl6 over Blimp-1 expression, two transcription factors promoting central memory development and terminal differentiation respectively (17, 32, 40). Similarly, in acute infection settings, $CD25^{-/-}$ and $CD122^{-/-}$ $CD8^+$ T cells rapidly turned to memory precursor effector cells and preferentially developed into Tcm cells, through regulation of the same transcription factors (21, 25, 36, 41). Yet, the impact of IL-2 and IL-15 in chronic settings is far more dramatic since it leads to the complete abrogation of memory development. This interesting observation indicates that although chronic antigenic stimulation is a pre-requisite to $CD8^+$ memory T cell defects (14, 15), IL-2 and IL-15-dependent signals direct the arrested memory differentiation of $CD8^+$ T cells during chronic viral infection.

Not only chronic infections preclude memory T cell development, conversion to PD-1^{hi} terminal effectors has dramatic consequences for the long-term outcome of the host. In fact, PD-1^{hi} cells present critical survival defects and shorter lifespan than PD-$1^{int/lo}$ progenitors (7, 10, 23). Importantly, these terminal effectors barely proliferate to antigenic stimulation and mediate a poor immune protection upon secondary challenge

despite having better killing potential (7, 23). More importantly, prolonged and elevated viral loads in mice and humans continuously enforce the transition to PD-1^{hi} cells leading to a gradual erosion of the progenitor pool and ultimately the loss of T cell immunity (23). The demonstration that IL2Rβ-deficiency halts PD-1^{hi} conversion, supports the survival of PD-$1^{int/lo}$ progenitors and promote memory T cell development paves the way to new therapeutic approaches aiming to restore T cell immunity during chronic viral infection. It gives salient therapeutic opportunities to limit poor outcomes associated with the loss of $CD8^+$ memory T cells in patients with chronic viral infections by interfering with IL2Rβ-dependent signals. By sustaining an IL-7-Stat5-Bcl2 survival axis, it would provide considerable survival advantage to anti-viral T cells following treatment-induced viral decline. Moreover, it provides an interesting complementary approach to anti-PDL1 therapies, since limitations of such treatment is not only its ineffectiveness at rescuing terminally differentiated PD-1^{hi} cells that represent the majority of the $CD8^+$ T cell pool (7, 23), but also its inability to generate a stable pool of $CD8^+$ memory T cells (42). Hence, limiting the conversion to PD-1^{hi} cells (i.e. by blocking IL-2 and/or IL-15-dependent signals) might increase the proportion of PD-$1^{int/lo}$ progenitors that are highly responsive to αPDL1 therapy, and improve treatment efficacy. Globally, our findings provide new promising therapeutic perspectives for the treatment of chronic viral infection.

Taken together, we identified previously unknown immunosuppressive functions for IL-2 and IL-15 on $CD8^+$ T cell responses during chronic viral infection. This includes the induction of specific inhibitory receptors, the enforced terminal differentiation of $CD8^+$ effectors and the suppression of memory potential. These findings provide the next crucial steps to innovative therapeutic measures for preventing the dramatic loss of immunity that comes with exhaustion during chronic viral infection.

MATERIALS AND METHODS

Mice, virus and infection

Six-week-old C57BL/6 (B6) mice were obtained from The Jackson laboratory. $CD45.2^{+}$ P14 transgenic (Tg) mice bearing the $H\text{-}2D^{b}$gp33-41-specific TCR were kindly provided by A. Freitas (Institut Pasteur, Paris, France). $RAG2^{-/-}$ IL2Rβ-deficient P14 Tg mice ($CD45.2^{+}$) were obtained by breeding P14 mice with $RAG2^{-/-}$ $IL2R\beta^{-/-}$ mice (both strains from The Jackson laboratory), and were previously shown to generate a monoclonal population of naïve $CD8^{+}$ T cells (25). Adoptive transfer recipients (B6 CD45.1.2) were obtained by breeding Tg^{-} littermate (CD45.2) mice with CD45.1 B6.SJL mice (The Jackson laboratory) to ensure genetic compatibility between donor and recipient mice avoiding rejection of transferred cells. P14 and P14 $IL2R\beta^{-/-}$ chimeric mice were obtained by infusing splenocytes from 6 to 9-weeks-old Tg mice containing 10^{4} P14 $IL2R\beta^{+/-}$ or P14 $IL2R\beta^{-/-}$ cells (CD45.2) into 6 to 10-weeks-old recipient mice (CD45.1/2) 20 hours prior to infection. To confirm the adoptive transfer of naïve cells, the activation status (CD69, CD25, CD62L, Ly6C) of Tg cells was verified before each experiment. LCMV Arm and LCMV Cl-13 were kindly provided by Rolf M.Zinkernagel (Zurich University Hospital, Switzerland), produced on BHK-21 and L929 cell respectively and titrated by plaque assays on MC57G cells as previously described (43, 44). Mice were infected i.p with 2.10^{5} PFU of LCMV Arm or i.v with 2.10^{6} PFU of LCMV Cl-13 to generate chronic infection. All donor and virus-free recipient mice were housed in specific pathogen-free facilities at the CHU Sainte-Justine Research Center. Infectious experiments were conducted at the Experimental Biological Centre of the Institut Armand Frappier and at the CHU Sainte-Justine Research Center in accordance with the Canadian Council on Animal Care guidelines.

Study subjects

HCV acutely-infected subjects were recruited among high-risk HCV-seronegative injection drug users participating in the Montreal Hepatitis C Cohort (HEPCO) at the Saint-Luc Hospital of the University of Montreal Health Center as previously described (45). Blood samples were processed and analysed as previously described (46). The

institutional ethics committee at the University of Montreal Hospital Research Center approved the human study (SL05.014). All participants signed informed consent forms upon enrolment and experiments were performed in accordance with the Declaration of Helsinki.

Flow cytometry, intracellular staining and cell sorting

Spleen and lymph nodes (axillary and inguinal) harvested from infected mice were filtered on a 100μM nylon mesh and treated with NH4Cl for erythrocyte removal. For all experiments, dead cells were stained with fixable LIVE/DEAD Aqua (ThermoFisher) and excluded from the analysis. Cells were Fc-blocked (BD) and extra-cellular staining was performed in 50-100μl of PBS with 2% FBS during 20 min on ice, before fixation. For cytokine release assays, splenocytes were re-stimulated 4 h with cognate gp33 peptide (0.1mM) in the presence of GolgiStop (BD). Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD) and stained for IFNγ, TNFα and IL-2. Intracellular Granzyme B and active caspase 3 were assessed directly *ex vivo* using the same BD Cytofix/Cytoperm kit (BD). Transcription factors and intracellular proteins were detected using the Foxp3 Fixation/Permeabilization kit (eBioscience). To allow for Bim detection an additional 1 h staining was performed with a PE-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch). Annexin V/7AAD stainings were realized using the Apoptosis detection Kit I (BD) in accordance with manufacturer's protocols. Phosphorylated STAT5 was detected as follows. Briefly, splenocytes from P14 and P14 IL2R$\beta^{-/-}$ transferred mice were rested for 2–4 h in nude medium at 37°C as previously described (29). Cells were then stained with LIVE/DEAD Aqua prior to 30 min of re-stimulation with IL-2, IL-15 (R&D; 20ng/ml) or IL-7 (PeproTech; 5ng/ml) in complete RPMI medium. Splenocytes were then fixed with methanol-free 4% formaldehyde, permeabilized with 90% ice-cold methanol and stained for 1 h at room temperature with surface and p-STAT5 antibodies in staining buffer (BD). Anti-mouse antibodies were purchased from eBioscience (CD8, TCRβ, CD45.1, CD45.2, CD127, CD62L, CD25, CD122, LAG-3, CD160, 2B4, Tim-3 [RMT3-23 clone], CD69, Ly6C, Bcl2, Ki67, Eomes and Bcl6), BD Bioscience (active-Caspase 3, CD44, CD132, IFNγ, TNFα, IL-2), BioLegend (PD-1 [RMP1-30 clone] and Tim-3 [RMT3-23 clone]), Santa

Cruz Biotech (T-bet), ThermoFisher (Granzyme B), Cell Signalling Technology (p-STAT5, FoxO1, Bim). H-2D^{b}gp33 tetramers were kindly provided by F. Lemaître (Institut Pasteur, Paris, France) and coupled to ultra-avidin-R-phycoerythrin (Leinco). H-2D^{b}np396 tetramers were homemade and coupled to streptavidin R phycoerythrin (ThermoFisher). Human PBMC samples were stained with MHC class I tetramers carrying the HLA-A2 restricted NS3-1073 epitope (A2/NS3-1073) (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA). Anti-human antibodies were purchased from BD Bioscience (CD3, CD8, CCR7, CD45RA, CD122, PD-1) and eBioscience (Eomes). All data were acquired on a LSR FORTESSA II (BD) and analysed with FlowJo v9.7.6 (Tree Star). For quantitative PCR analysis, cells were sorted on an FACS Aria II (BD) based on Aqua^{-}CD8^{+}CD44^{+}Tet^{+} for endogenous responses and an additional gating on CD45.2^{+}CD45.1^{-} congenic markers for transferred cells. Purity was routinely >95%.

Adoptive transfers of CD122^{hi} and CD122^{lo} subsets

CD45.2^{+} mice were infected with LCMV Cl-13 and spleens were harvested at day 15 p.i.. Activated CD11a^{+} PD-1^{+} CD8^{+} T cells were sorted based on their differential expression of CD122. CD122^{hi} and CD122^{lo} cells (1.10^{6} each) were infused i.v. into infection-matched CD45.1^{+} recipients and analysed one week later.

BrdU treatment

Mice were injected daily with 1 mg of BrdU (Sigma-Aldrich) intraperitoneally between day 15 and day 30 p.i.. Harvested splenocytes were stained with extracellular markers and BrdU incorporation was measured with the BrdU flow Kit (BD) in accordance with manufacturer's protocol.

***In vitro* stimulations**

For proliferative assay, splenocytes from day 35-infected mice were labeled with CFSE (2µM) and cultured with H-2D^{b}-restricted gp33 peptide (0.1µM) in complete RPMI medium. Proliferation of CD45.2^{+} P14 and P14 IL2Rβ$^{-/-}$ was assessed 72 h later by CFSE dilution. Inhibitory receptor induction was performed by co-culturing (ratio 1:1) naive

P14 cells with gp33-pulsed DCs (0.1μM) isolated by magnetic separation with CD11c MicroBeads (Miltenyi). At days 2, 4 and 6 of co-culture, P14 cells were numbered, normalized and cultured back in triplicates in RPMI medium complemented with gp33 peptide (1nM) alone or in combination with IL-2 (R&D) or IL-15 (R&D) at 10 U/ml.

Quantitative PCR

Endogenous H-2D^{b}gp33 and adoptively transferred P14 and P14 IL2Rβ$^{-/-}$ cells were sorted directly in Trizol (Invitrogen). Total RNA was precipitated by adding chloroform (Sigma-Aldrich), washed with 70% ethanol and purifyied using the RNeasy Micro Kit (Qiagen) according to manufacturer's protocol. cDNA was generated with the SuperScript® Vilo cDNA Synthesis Kit (ThermoFisher) and quantitative PCR was realized in two steps using the Brilliant II SYBR Green qRT-PCR low ROX master mix (Agilent Technologies) on a Mx 3000p qRT-PCR system (Stratagene). PCR were performed in triplicates for mouse Blimp-1 (*prdm1*) (forward, 5′-ACA CAC AGG AGA GAA GCC ACA TGA-3′ and reverse, 5′-TCG AAG GTG GGT CTT GAG ATT GCT-3′) and hypoxanthine guanine phosphoribosyl transferase (HPRT) as internal control (forward, 5′-CTC CTC AGA CCG CTT TTT GC-3′ and reverse, 5′-TAA CCT GGT TCA TCA TCG CTA ATC-3′). For all experiments, mRNA levels are expressed as a fold increase compared to naive P14.

Statistical analyses

Statistical significance was determined by a standard Student's *t* test or One-way ANOVA non-parametric tests (Dunnett's *t*) using ABI Prism 6. Significance was set as any *P* value *<0.05, **<0,005 and ***<0,0005.

ACKNOWLEDGMENTS

We thank A. Freitas for P14 mice; Rolf M. Zinkernagel for LCMV virus; F. Lemaître for gp33 tetramers; F. Le Deist for flow cytometry and cell sorting; V. Abadie for insightful comments; V. Abadie, L. Barreiro, E. Kritikou and J. Kashul for critical reading of the manuscript; C. Mathieu, E. Tihic, N. Cotta-Grand, E. Tarrab, S. Condotta and M. Richer for technical assistance and all the members of the animal facilities at CHU Ste Justine Research Center and Experimental Biological Centre at Institut Armand Frappier for mice care. H.D. is supported by grants from the Canadian Institutes of Health Research (CIHR) (MOP-130469), the Fonds de Recherche du Québec – Santé (FRQS), the Leukemia and Lymphoma Society of Canada, the Cole Foundation and Clinician Scientists salary awards from the FRQS and the Canadian Child Health Clinician Scientist Program. A.L. acknowledges support from the CHIR (MOP-89797) and from the Jeanne and J.-Louis Lévesque Research Chair in Immunovirology from the J.-Louis Lévesque Foundation. N.H.S. is supported by grants from the CIHR (MOP-133680), the FRQS AIDS and Infectious Diseases Network (Réseau FRQS SIDA-MI) and a FRQS Chercheurs Boursier-Senior award. J-C.B. has research scholarships from the University of Montreal, the CHU Sainte-Justine Foundation and The American Association of Immunologists (AAI).

REFERENCES

1. Gray SM, Kaech SM, & Staron MM (2014) The interface between transcriptional and epigenetic control of effector and memory CD8(+) T-cell differentiation. *Immunol Rev* 261(1):157-168.
2. Zhang N & Bevan MJ (2011) CD8(+) T cells: foot soldiers of the immune system. *Immunity* 35(2):161-168.
3. Decaluwe H*, et al.* (2010) Gamma(c) deficiency precludes CD8+ T cell memory despite formation of potent T cell effectors. *Proc Natl Acad Sci U S A* 107(20):9311-9316.
4. Zehn D & Wherry EJ (2015) Immune Memory and Exhaustion: Clinically Relevant Lessons from the LCMV Model. *Adv Exp Med Biol* 850:137-152.
5. Wherry EJ & Kurachi M (2015) Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15(8):486-499.
6. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, & Ahmed R (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77(8):4911-4927.
7. Blackburn SD, Shin H, Freeman GJ, & Wherry EJ (2008) Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc Natl Acad Sci U S A* 105(39):15016-15021.
8. Barber DL*, et al.* (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439(7077):682-687.
9. Jin HT*, et al.* (2010) Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 107(33):14733-14738.
10. Blackburn SD*, et al.* (2009) Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10(1):29-37.
11. Wherry EJ*, et al.* (2007) Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27(4):670-684.
12. Odorizzi PM & Wherry EJ (2012) Inhibitory receptors on lymphocytes: insights from infections. *J Immunol* 188(7):2957-2965.
13. Angelosanto JM, Blackburn SD, Crawford A, & Wherry EJ (2012) Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. *J Virol* 86(15):8161-8170.
14. Wherry EJ, Barber DL, Kaech SM, Blattman JN, & Ahmed R (2004) Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A* 101(45):16004-16009.
15. Shin H, Blackburn SD, Blattman JN, & Wherry EJ (2007) Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J Exp Med* 204(4):941-949.
16. Beltra JC & Decaluwe H (2016) Cytokines and persistent viral infections. *Cytokine*.
17. Rutishauser RL*, et al.* (2009) Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31(2):296-308.

18. Shin H, *et al.* (2009) A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31(2):309-320.
19. Kallies A, Xin A, Belz GT, & Nutt SL (2009) Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity* 31(2):283-295.
20. Banerjee A, *et al.* (2010) Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. *J Immunol* 185(9):4988-4992.
21. Pipkin ME, *et al.* (2010) Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32(1):79-90.
22. Belz GT & Masson F (2010) Interleukin-2 tickles T cell memory. *Immunity* 32(1):7-9.
23. Paley MA, *et al.* (2012) Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science* 338(6111):1220-1225.
24. Ring AM, *et al.* (2012) Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat Immunol* 13(12):1187-1195.
25. Mathieu C, *et al.* (2015) IL-2 and IL-15 regulate CD8(+) memory T-cell differentiation but are dispensable for protective recall responses. *Eur J Immunol* 45(12):3324-3338.
26. Gong D & Malek TR (2007) Cytokine-dependent Blimp-1 expression in activated T cells inhibits IL-2 production. *J Immunol* 178(1):242-252.
27. Xin A, *et al.* (2016) A molecular threshold for effector CD8(+) T cell differentiation controlled by transcription factors Blimp-1 and T-bet. *Nat Immunol* 17(4):422-432.
28. Hinrichs CS, *et al.* (2008) IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood* 111(11):5326-5333.
29. Staron MM, *et al.* (2014) The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8(+) T cells during chronic infection. *Immunity* 41(5):802-814.
30. Yajima T, *et al.* (2006) IL-15 regulates CD8+ T cell contraction during primary infection. *J Immunol* 176(1):507-515.
31. Sanjabi S, Mosaheb MM, & Flavell RA (2009) Opposing effects of TGF-beta and IL-15 cytokines control the number of short-lived effector CD8+ T cells. *Immunity* 31(1):131-144.
32. Ichii H, Sakamoto A, Kuroda Y, & Tokuhisa T (2004) Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells. *J Immunol* 173(2):883-891.
33. Ichii H, *et al.* (2002) Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells. *Nat Immunol* 3(6):558-563.
34. Schluns KS, Kieper WC, Jameson SC, & Lefrancois L (2000) Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* 1(5):426-432.

35. Kaech SM, *et al.* (2003) Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4(12):1191-1198.
36. Mitchell DM, Ravkov EV, & Williams MA (2010) Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells. *J Immunol* 184(12):6719-6730.
37. Kao C, *et al.* (2011) Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat Immunol* 12(7):663-671.
38. Nakamura Y, *et al.* (1994) Heterodimerization of the IL-2 receptor beta- and gamma-chain cytoplasmic domains is required for signalling. *Nature* 369(6478):330-333.
39. Brooks DG, Teyton L, Oldstone MB, & McGavern DB (2005) Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. *J Virol* 79(16):10514-10527.
40. Cui W & Kaech SM (2010) Generation of effector CD8+ T cells and their conversion to memory T cells. *Immunol Rev* 236:151-166.
41. Obar JJ, *et al.* (2010) CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc Natl Acad Sci U S A* 107(1):193-198.
42. Wherry EJ (2016) Molecular Basis of T cell Exhaustion: Insights for Immunotherapy. in *Keystone symposia 2016 Cell Biology and Immunology of Persistent Infection (A8)*.
43. Dutko FJ & Oldstone MB (1983) Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *The Journal of general virology* 64 (Pt 8):1689-1698.
44. Battegay M, *et al.* (1991) Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. *Journal of virological methods* 33(1-2):191-198.
45. Grebely J, *et al.* (2013) Cohort profile: the international collaboration of incident HIV and hepatitis C in injecting cohorts (InC3) study. *International journal of epidemiology* 42(6):1649-1659.
46. Kared H, Fabre T, Bedard N, Bruneau J, & Shoukry NH (2013) Galectin-9 and IL-21 mediate cross-regulation between Th17 and Treg cells during acute hepatitis C. *PLoS Pathog* 9(6):e1003422.

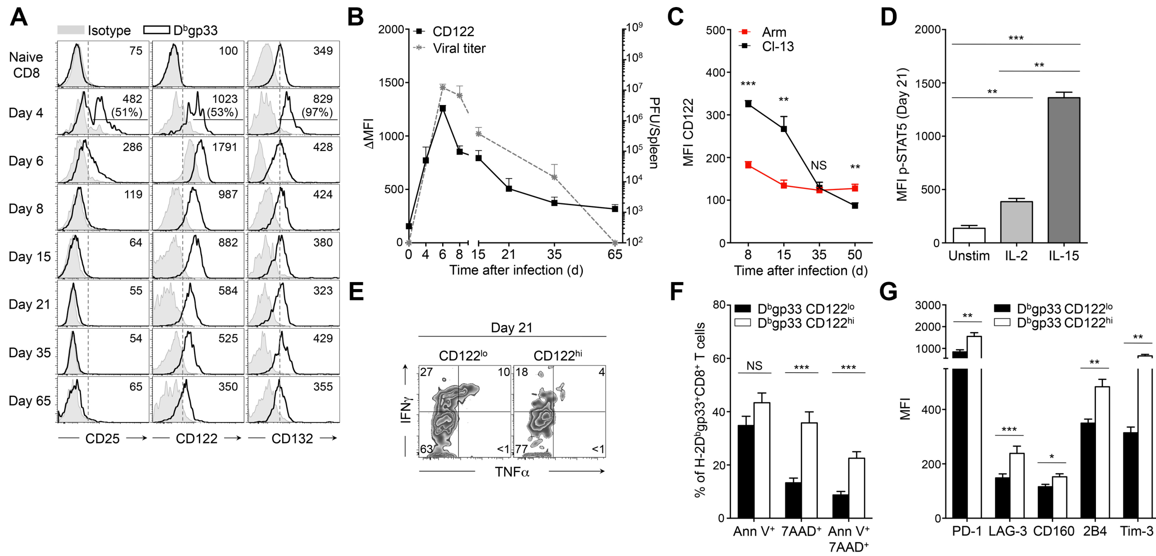


Fig. 1. CD122 expression is maintained on CD8$^+$ T cells and identifies severely exhausted cells. C57BL/6 (B6) mice were infected with LCMV Cl-13; analyses were performed in spleens on LCMV-specific H-2D^{b}gp33$^+$CD8$^+$ T cells at day 21 p.i. (unless otherwise indicated). (*A*) Cell-surface expression of CD25, CD122 and CD132 at distinct time-points after infection. Values in quadrants indicate MFI (mean fluorescence intensity) and/or frequency of highly positive cells (day 4, below); grey filled histograms are isotype controls. (*B*) Cell-surface expression of CD122 (left Y axis; filled squatters representing Δ mean fluorescent intensity (Δ MFI) [MFI minus isotype control MFI]) and viral titers (right Y axis; dotted grey line) at indicated time-points. (*C*) Cell-surface expression of CD122 on H-2D^{b}gp33$^+$CD8$^+$ T cells isolated from either LCMV Arm (acute infection; red squatters) or LCMV Cl-13 (chronic infection; black squatters) infected mice at indicated time-points. (*D*) Intracellular expression of p-STAT5 in activated (CD11a$^+$) CD8$^+$ T cells following stimulation with the indicated cytokines; NS for unstimulated control. (*E*) Intracellular production of IFNγ and TNFα in CD122^{lo} (left quadrant) and CD122^{hi} (right quadrant) H-2D^{b}gp33$^+$CD8$^+$ T cells. Values indicate frequency of cells in each quadrant. (*F*) Frequency of CD122^{lo} (black bars) and CD122^{hi} (white bars) H-2D^{b}gp33$^+$CD8$^+$ T cells positive for Annexin V and/or 7AAD. (*G*) Expression of the indicated inhibitory receptors on CD122^{lo} (black bars) and CD122^{hi} (white bars) H-2D^{b}gp33$^+$CD8$^+$ T cells. Bars representing the MFI. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, NS $p\geq 0.05$ (two-tailed unpaired Student's *t*-test). Data are pooled from

two (*C, G*) or three (*B*, *F*) independent experiments with at least five mice per group or representative of three independent experiments (*A*, *D*, *E*) with similar results (two to three mice per group in each). (error bars (*B, C*, *D*, *F, G*), mean±s.e.m.).

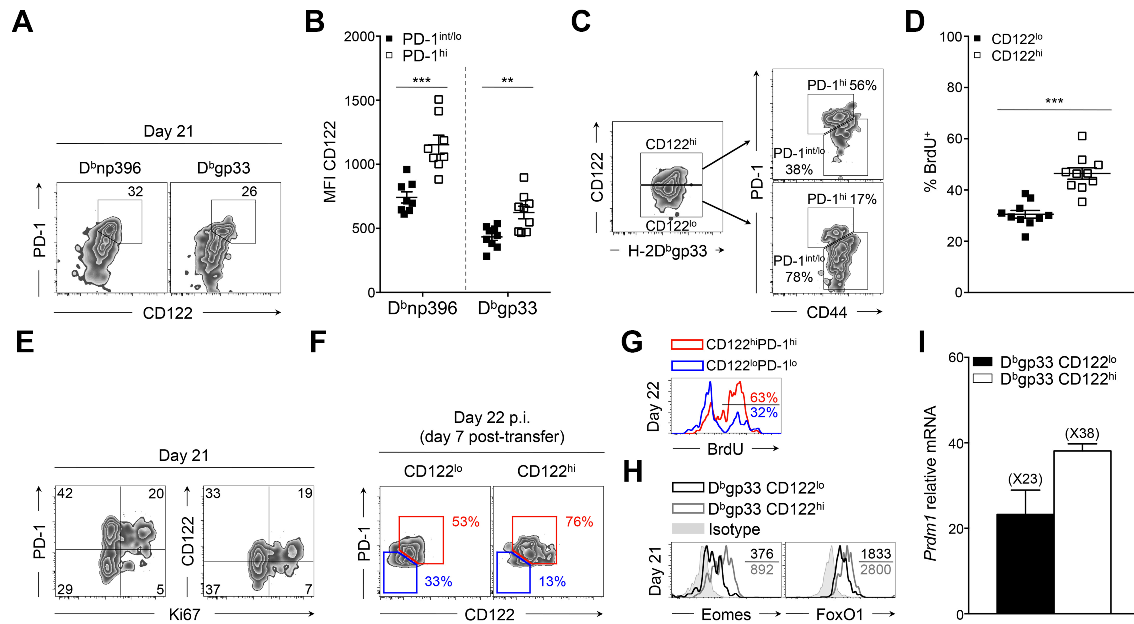


Fig. 2. Lineage relationship between CD122^{hi} cells and PD-1^{hi} cells. Mice were infected and analysed as in Fig. 1. (*A*) Cell-surface expression of CD122 and PD-1 on H-2D^{b}np396^{+}CD8^{+} T cells (left panel) and H-2D^{b}gp33^{+}CD8^{+} T cells (right panel). Values indicate the frequency of CD122^{hi}PD-1^{hi} cells. (*B*) Expression of CD122 on PD-1$^{int/lo}$ (filled squatters) and PD-1^{hi} (opened squatters) H-2D^{b}np396^{+} (left) and H-2D^{b}gp33^{+} (right) CD8^{+} T cells. (*C*) Expression of PD-1 and CD44 on CD122^{hi} (upper panel) and CD122^{lo} (lower panel) H-2D^{b}gp33^{+}CD8^{+} T cells. Values indicate the frequency of PD-1^{hi}CD44^{int} and PD-1$^{int/lo}$CD44^{hi} cells in each panel. (*D*) Frequency of CD122^{lo} (filled squatters) and CD122^{hi} (opened squatters) H-2D^{b}gp33^{+}CD8^{+} T cells that incorporated BrdU between day 15 and 30 p.i. (*E*) Cell-surface expression of CD122, PD-1 and intracellular Ki67 in H-2D^{b}gp33^{+}CD8^{+} T cells. Values indicate frequency of cells in each gate. (*F*) Cell-surface expression of CD122 and PD-1 on CD122^{lo} (left panel) and CD122^{hi} (right panel) transferred cells (CD45.2^{+}) at day 22 p.i. (day 7 post-transfer). Values indicate the frequency of CD122^{hi}PD-1^{hi} (red) and CD122^{lo}PD-1^{lo} (blue) cells. (G) BrdU incorporation (day 15 to 22 p.i.) by CD122^{hi}PD-1^{hi} (red histogram) and CD122^{lo}PD-1^{lo} (blue histogram) CD45.2^{+} cells recovered from mice adoptively transferred with CD122^{lo} (CD45.2^{+}) cells. Values indicate the frequency of positive cells. (*H*) Intracellular Eomes and FoxO1 expression in CD122^{lo} (black histograms) and

CD122^{hi} (grey histograms) H-2D^{b}gp33^{+}CD8^{+} T cells. Values indicate the MFI; grey filled histograms are isotype controls. (*I*) Expression of *Prdm1* assessed by quantitative RT-QPCR analysis in sorted CD122^{lo} (black bar) and CD122^{hi} (white bar) H-2D^{b}gp33^{+}CD8^{+} T cells pooled from at least four mice. Values indicate the fold increase relative to naive P14 CD8^{+} T cells. **$p<0.005$, ***$p<0.0005$; two-tailed unpaired Student's *t*-test. Data are pooled from two (*I*) or three (*B*) independent experiments with at least eight mice per group or representative of one (*G*), two (*F, H*) or three (*A*, *C*) independent experiments with similar results (two to three mice per group in each). (*D*, *E*) Data are representative of one experiment with ten mice per group. (error bars (*B*, *D*, *E*, *I*), mean±s.e.m.).

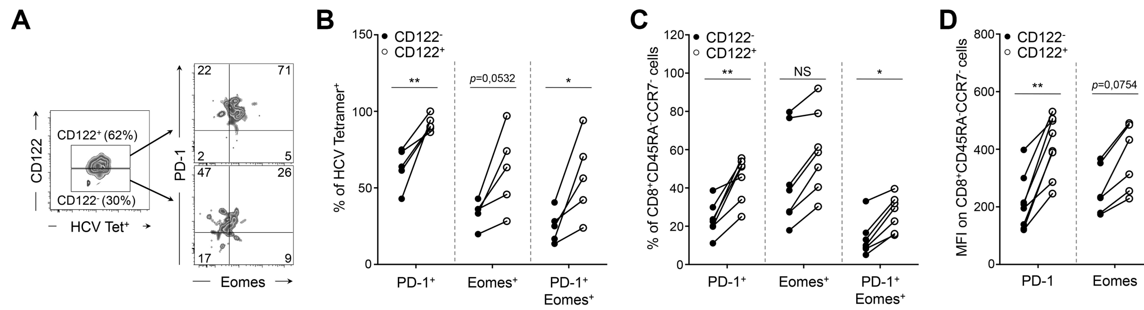


Fig. 3. CD122 identifies exhausted cells in individuals infected with HCV. PBMCs were collected during the first year of HCV infection from individuals with high viral titers and developing persistent viremia as described in Fig. S3*A*. (*A*) Expression of extracellular PD-1 and intracellular Eomes in $CD122^+$ (upper panel) and $CD122^-$ (lower panel) HCV-specific $CD8^+$ T cells. . Gates for $CD122^+$ and $CD122^-$ cells are set based on FMO controls. Values indicate the frequency of cells in each quadrant. (*B*) Frequency of $CD122^-$ (filled circles) and $CD122^+$ (opened circles) HCV-specific $CD8^+$ T cells positive for the indicated molecules. (*C*) Frequency of $CD122^-$ (filled circles) and $CD122^+$ (opened circles) $CD45RA^-CCR7^-$ effector memory $CD8^+$ T cells positive for the indicated molecules. (*D*) Expression of PD-1 and Eomes in $CD122^-$ (filled circles) and $CD122^+$ (opened circles) $CD45RA^-CCR7^-$ effector memory $CD8^+$ T cells represented by their MFI. *$p<0.05$, **$p<0.005$, NS $p\geq 0.05$; two-tailed unpaired Student's *t*-test. Data are pooled from five to eight distinct time-points from two to three infected patients processed independently.

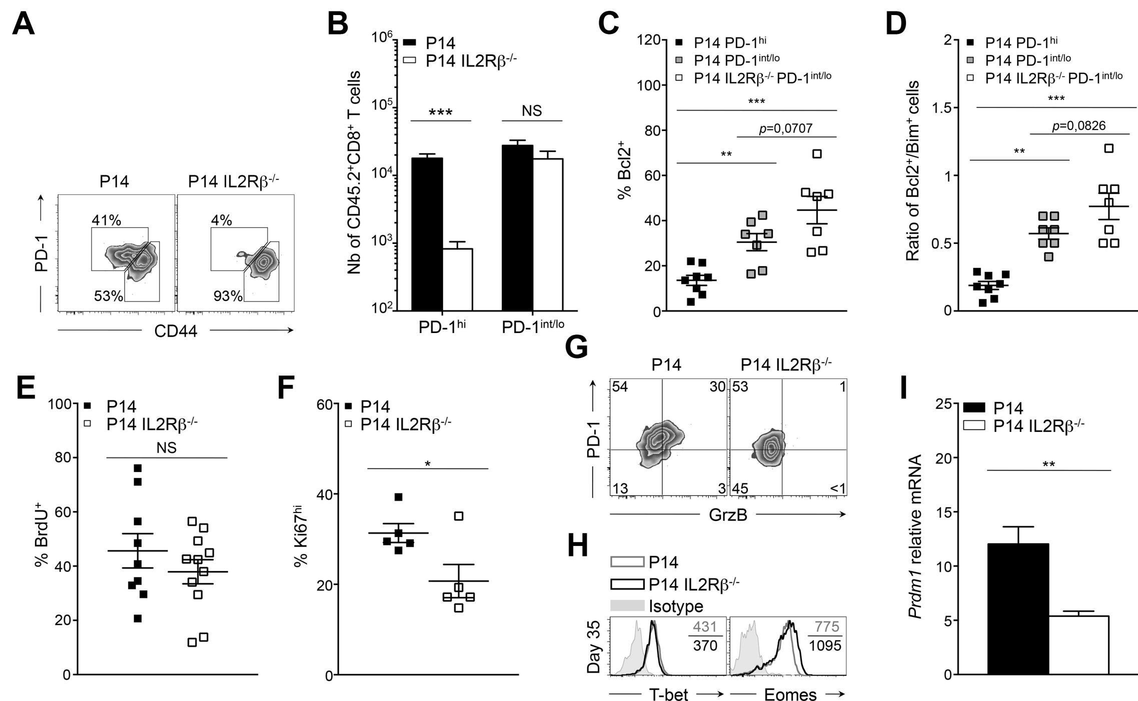


Fig. 4. IL2Rβ deficiency abrogates PD-1^{hi} terminal differentiation. Tg P14 or P14 IL2Rβ$^{-/-}$ (CD45.2) cells were adoptively transferred into recipient mice (CD45.1.2) prior to LCMV Cl-13 infection. Tg cells (CD45.2) were compared in the spleen at day 35 p.i. (*A*) Representative dot plots of PD-1^{hi} and PD-1$^{int/lo}$ P14 (left) and P14 IL2Rβ$^{-/-}$ (right) cells. Values indicate frequency of cells in each gate. (*B*) Absolute numbers of PD-1^{hi} (left panel) and PD-1$^{int/lo}$ (right panel) splenic cells from P14 (black bars) and P14 IL2Rβ$^{-/-}$ (white bars) chimeric mice. (C) Frequency of cells expressing Bcl2 in P14 PD-1^{hi} (filled squatters), P14 PD-1$^{int/lo}$ (grey squatters) and P14 IL2Rβ$^{-/-}$ PD-1$^{int/lo}$ cells (opened squatters). (D) Frequency of Bcl2^{+} over Bim^{+} P14 PD-1^{hi} (filled squatters), P14 PD-1$^{int/lo}$ (grey squatters) and P14 IL2Rβ$^{-/-}$ PD-1$^{int/lo}$ cells (opened squatters). (E) Frequency of P14 (filled squatters) and P14 IL2Rβ$^{-/-}$ cells (opened squatters) that incorporated BrdU between day15 and 30 p.i.. (*F*) Frequency of Ki67^{hi} P14 (filled squatters) and P14 IL2Rβ$^{-/-}$ (opened squatters) cells. Data are representative of one experiment with five mice per group. (*G*) Expression of PD-1 and intracellular granzyme B in P14 (left dot plot) and P14 IL2Rβ$^{-/-}$ (right dot plot) cells. Values indicate the frequency of cells in each gate. (*H*) Intracellular T-bet and Eomes expression in P14 (grey histograms) and P14 IL2Rβ$^{-/-}$

(black histograms) cells. Values indicate the MFI; grey filled curves are isotype controls. (*I*) *Prdm1* levels assessed by quantitative RT-QPCR analysis in sorted P14 (black bar) and P14 IL2R$\beta^{-/-}$ (white bar) cells relative to naïve P14 cells. *p<0,05, **p<0.005, ***p<0.0005, NS p≥ 0.05; two-tailed unpaired Student's *t*-test. Data are pooled from two (*C, D, I*) or three (*B*, *E*,) independent experiments with at least seven mice per group or representative of two (*G*, *H*) or three (*A*) independent experiments with similar results (two to four mice per group in each). (error bars (*B*, *C*, *D*, *E, F, I*), mean± s.e.m.).

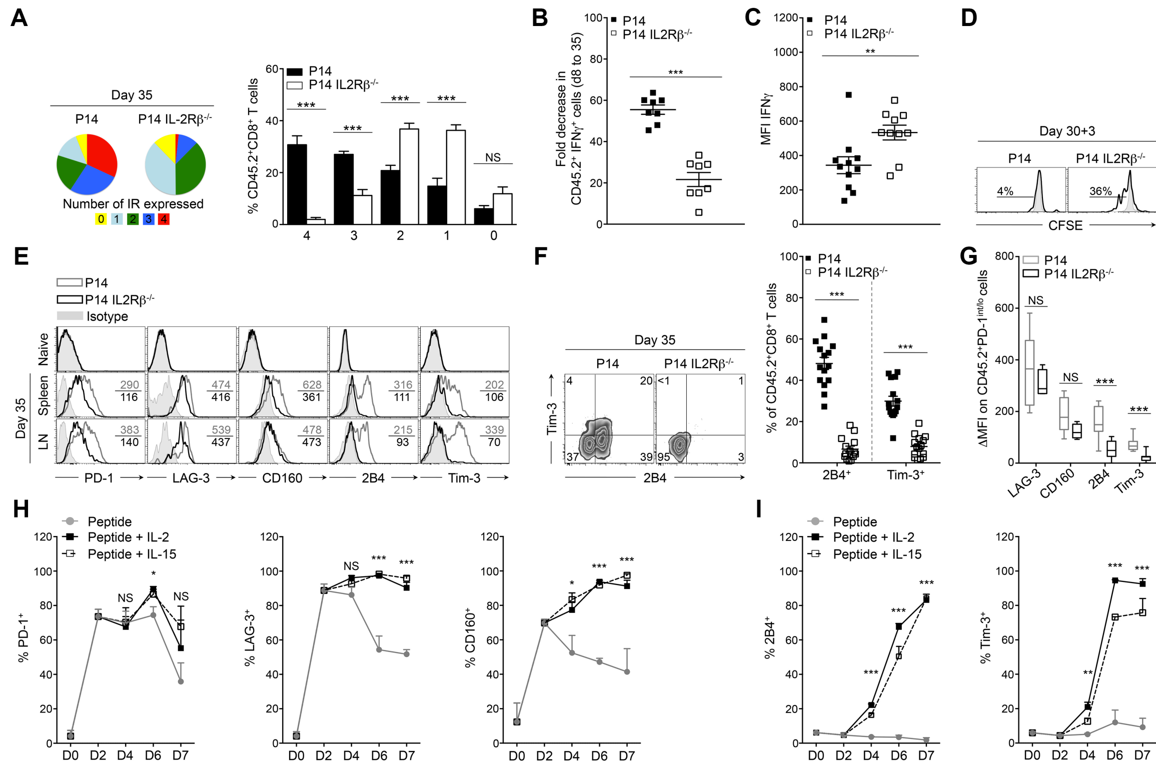


Fig. 5. IL2Rβ-dependent signals regulate the levels of inhibitory receptor expression. (*A*-*G*) P14 or P14 IL2Rβ$^{-/-}$ chimeric mice were generated and analysed as described in Fig. 4. (*A*) Frequency of P14 (left pie; black bars) and P14 IL2Rβ$^{-/-}$ (right pie; white bars) cells co-expressing PD-1, LAG-3, 2B4 and CD160. (*B*) Fold decrease in the frequency of P14 (filled squatters) and P14 IL2Rβ$^{-/-}$ (opened squatters) cells producing IFNγ between days 8 and 35 p.i. (*C*) Production of IFNγ in CD8^{+}CD45.2^{+} P14 (filled squatters) and P14 IL2Rβ$^{-/-}$ (opened squatters) cells; squatters representing the MFI. (*D*) Proliferation of P14 (left histogram) and P14 IL2Rβ$^{-/-}$ cells (right histogram) in response to gp33 stimulation. Proliferation is represented as CFSE dilution over 3 days with values indicating the frequency of CFSE^{int} cells. Grey filled histograms are unstimulated controls. (*E*) Inhibitory receptors expression on P14 (grey histograms) and P14 IL2Rβ$^{-/-}$ (black histograms) cells. Values indicate the MFI; grey filled histograms are isotype controls. (*F*) Cell-surface expression of 2B4 and Tim-3 on P14 (left plot) and P14 IL2Rβ$^{-/-}$ (right plot) cells (left panel) and cumulative frequencies of 2B4^{+} and Tim-3^{+} P14 (filled squatters) and P14 IL2Rβ$^{-/-}$ (opened squatters) cells (right panel). Values in the left panel

indicate frequency of cells in each quadrant. (*G*) Inhibitory receptors expression on PD-$1^{int/lo}$ P14 (grey boxes) and P14 IL2R$\beta^{-/-}$ (black boxes) cells represented by their Δ MFI. (*H–I*) Frequency of P14 cells expressing the indicated inhibitory receptors during co-culture with gp33 peptide alone (grey lines) or with additional IL-2 (black lines) or IL-15 (dotted lines). Data pooled from five independent experiments with triplicates in each. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, NS $p\geq 0.05$; two-tailed unpaired Student's *t*-test or (*G*, *H*) one-way ANOVA non parametric tests; Dunnett's *t*). Data are pooled from two (*A*), three (*B*, *C, G*) or four (*F*) independent experiments with five to sixteen mice per group or representative of two (*D*) or five (*E*) independent experiments (*D*) with similar results (two to five mice per group in each). (error bars (*A*, *B*, *C*, *F, G*, *H, I*), mean± s.e.m.).

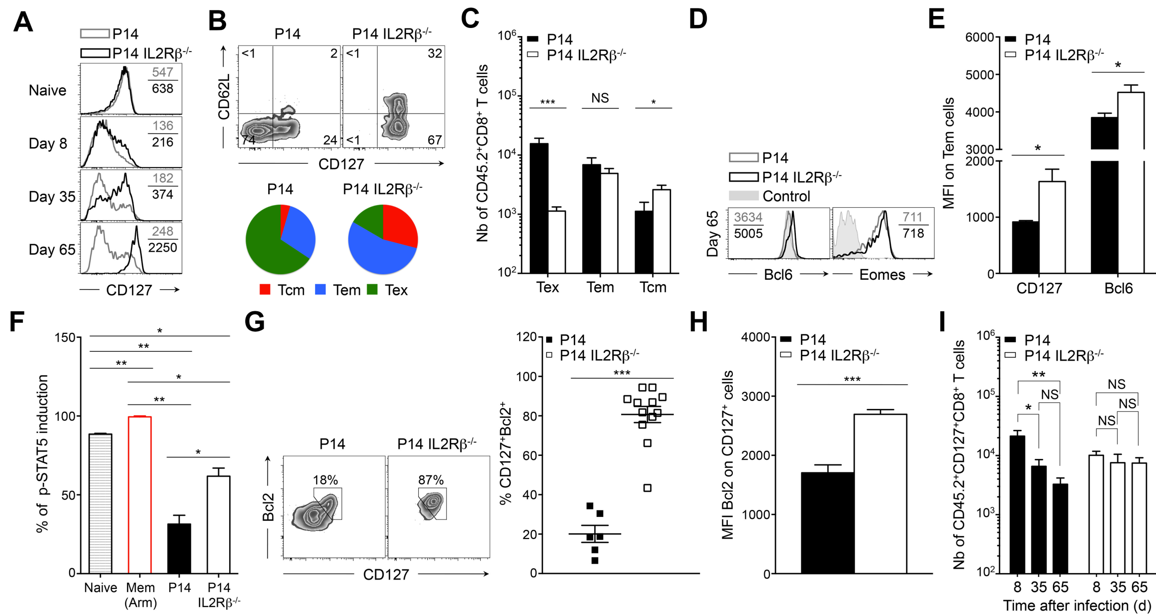


Fig. 6. IL2Rβ deficiency restores CD8^{+} memory T cell development. P14 or P14 IL2Rβ$^{-/-}$ chimeric mice were generated as described in Fig. 4. Cells were analysed in the spleen at day 65 p.i., unless otherwise indicated. (*A*) CD127 expression on P14 (grey histograms) and P14 IL2Rβ$^{-/-}$ (black histograms) cells at the indicated time points. Values indicate the MFI. (*B*) Expression of CD62L and CD127 (contour plots; upper panel) and cumulative frequency of Tcm (CD62L^{hi}CD127^{hi}; red), Tem (CD62L^{lo}CD127^{hi}; blue) or Tex (CD62L^{lo}CD127^{lo}; green) cells (pie charts; lower panel) on P14 (left) and P14 IL2Rβ$^{-/-}$ (right) cells. Values indicated the frequency in each quadrant. (*C*) Absolute numbers of Tex (CD62L^{lo}CD127^{lo}; left), Tem (CD62L^{lo}CD127^{hi}; middle) or Tcm (CD62L^{hi}CD127^{hi}; right) cells in P14 (filled bars) and P14 IL2Rβ$^{-/-}$ (opened bars) cells. (*D*) Intracellular expression of Bcl6 and Eomes in P14 (grey histograms) and P14 IL2Rβ$^{-/-}$ (black histograms) cells. Values indicate the MFI; grey filled histograms are isotype controls (Eomes) and TCRβ-negative cells (Bcl6). (*E*) Expression of CD127 (left) and Bcl6 (right) on P14 (black bars) and P14 IL2Rβ$^{-/-}$ (white bars) Tem cells. (*F*) Induction of p-STAT5 in P14 (black bar) and P14 IL2Rβ$^{-/-}$ (white bar) cells following stimulation with IL-7. Naive P14 cells are represented by stripped bar; Ag-specific memory cells are represented by red bar. (*G*) Expression of CD127 and Bcl2 (left panel) and cumulative

frequency of $CD127^{+}Bcl2^{+}$ cells (right panel) in P14 (left plot; filled squatters) and P14 $IL2R\beta^{-/-}$ (right plot; opened squatters) cells. Values in contour plots indicate frequency of double positive cells. (*H*) Expression of Bcl2 in $CD127^{+}$ P14 (black bars) and P14 $IL2R\beta^{-/-}$ (white bars) cells. (*I*) Absolute numbers of $CD127^{+}$ P14 (left) and $CD127^{+}$ P14 $IL2R\beta^{-/-}$ cells (right) at the indicated time points. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, NS $p\geq 0.05$; two-tailed unpaired Student's *t*-test. Data are pooled from two (*G, H*) or three (*C, I*) independent experiments with six to seventeen mice per group or representative of two (*D*, *F*) or three (*A*, *B*, *E*) independent experiments with similar results (two to seven mice per group in each).(error bars (C, *E*, *F, G, H, I*), mean± s.e.m.).

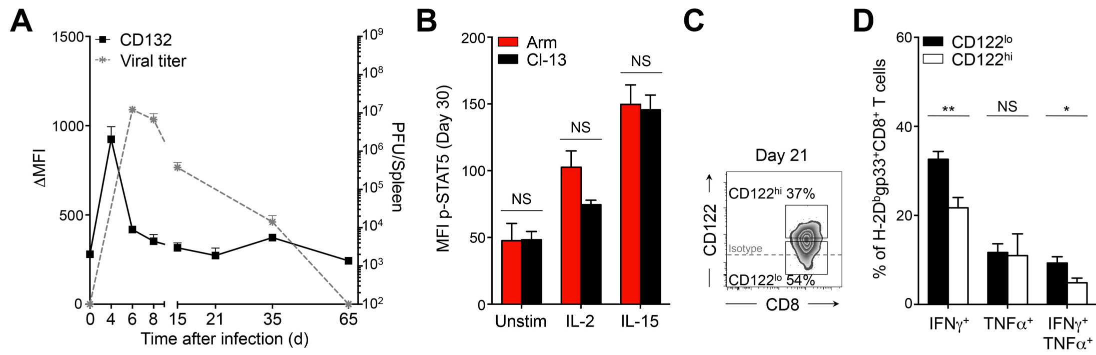


Fig. S1. CD122 expression on CD8$^+$ T cells identifies severely exhausted cells. C57BL/6 (B6) mice were infected with LCMV Cl-13; analyses were performed in spleens on day 21 p.i. (unless otherwise indicated). (*A*) Cell-surface expression of CD132 (left Y axis; filled squatters representing Δ mean fluorescent intensity (Δ MFI) [MFI minus isotype control MFI]) and viral titers (right Y axis; dotted grey line) at indicated time-points. (*B*) Intracellular expression of p-STAT5 in H-2D^{b}gp33$^+$CD8$^+$ T cells following stimulation with the indicated cytokines from animals infected with LCMV Arm (acute infection; red boxes) or LCMV Cl-13 (chronic infection; black boxes) at day 30 p.i.; NS; unstimulated control. (*C*) Gating strategy for CD122^{hi} (upper gate) and CD122^{lo} (lower gate) H-2D^{b}gp33$^+$CD8$^+$ T cells. Gates are set above and below the center of the population. CD122$^{int/lo}$ cells include cells negative for CD122. Values indicate frequency in each gate. (*D*) Frequency of CD122^{lo} (black bars) and CD122^{hi} (white bars) H-2D^{b}gp33$^+$CD8$^+$ T cells producing IFNγ, TNFα or both IFNγ and TNFα simultaneously. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, NS $p\geq 0.05$; two-tailed unpaired Student's *t*-test. Data are pooled from two (*A*) or three (*D*) independent experiments with four to eight mice per group or representative of three (*C*) or four (*B*) independent experiments with similar results (two to three mice per group in each). (error bars (*A*, *B, D*)**,** mean±s.e.m.).

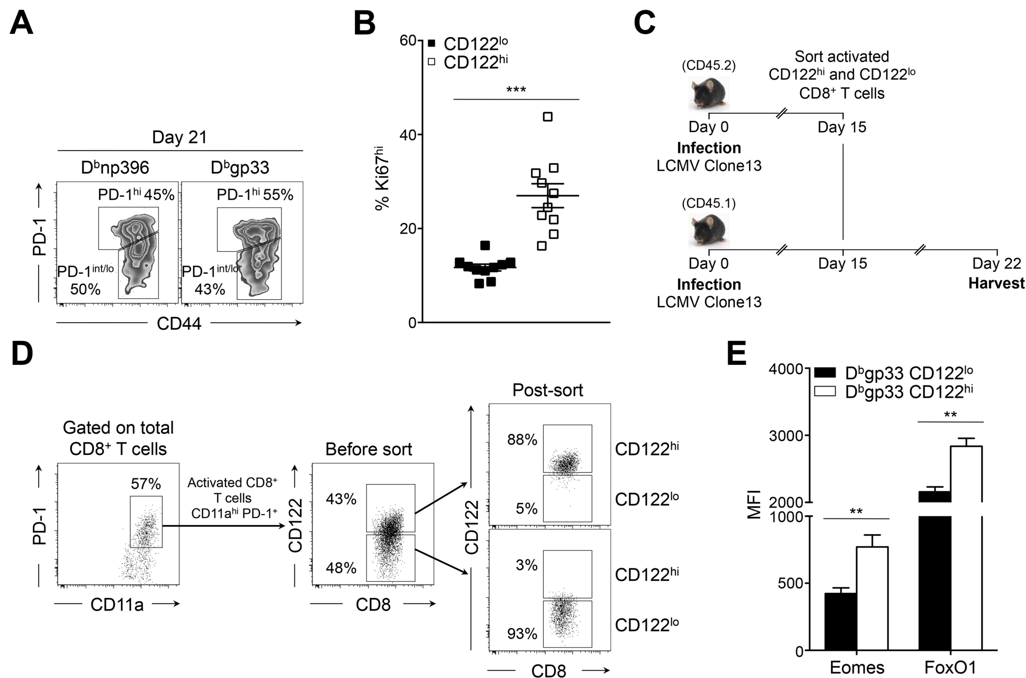
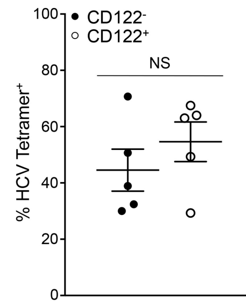


Fig. S2. CD122^{hi} cells share functional and transcriptional features with PD-1^{hi} cells. Mice were infected and analysed as in Fig. S1. (*A*) Gating strategy for PD-1^{hi} (upper gates) and PD-1$^{int/lo}$ (bottom gates) H-2D^{b}np396^{+} (left panel) and H-2D^{b}gp33^{+} (right panel) CD8^{+} T cells. Values indicate the frequency of cells in each gate. (*B*) Cumulative frequency of CD122^{lo} (filled squatters) and CD122^{hi} (opened squatters) H-2D^{b}gp33^{+}CD8^{+} T cells expressing high levels of Ki67. Data are representative of one experiment with ten mice per group. (*C-D*) Experimental approach (*C*) and sorting strategy (*D*) for adoptive transfer experiments of CD122^{lo} and CD122^{hi} cells. Splenocytes were harvested from CD45.2^{+} donor mice at day 15 p.i.. Activated CD11a^{+} PD-1^{+} CD8^{+} T cells were sorted based on high (CD122^{hi}) or intermediate/low (CD122^{lo}) expression of CD122 and infused i.v. in distinct CD45.1^{+} matched-infected recipient mice. Recipient mice were given daily BrdU from day 15 to 22 p.i. and CD45.2^{+} transferred cells were harvested at day 22 and analysed for surface markers and BrdU incorporation. (*E*) Intracellular Eomes and FoxO1 expression in CD122^{lo} (black bars) and CD122^{hi} (white bars) H-2D^{b}gp33^{+}CD8^{+} T cells. $^{**}p<0.005$, $^{***}p<0.0005$; two-tailed unpaired Student's *t*-test. Data are pooled from two (*E*) independent experiments with at least six mice per group or representative of three (*A*) independent experiments with similar results (two to three mice per group in each). (error bars (*B*, *E*), mean±s.e.m.).

A

Patients	Gender /Age (yr)	HLA Class I	HCV Genotype	Reactive tetramers	Estimate time post-infection (d)	Viral loads (IU/ml of plasma)	Infection outcome
C1	M (28)	A2, B49/B51	1a	A2/NS3-1073	250	2.047.920	Chronic HCV
C2	M (33)	A2/A68, B18/B40	1a	A2/NS3-1073	66 83	60.130.386 19.174.736	Chronic HCV
C3	M (48)	A2/A3, B14/B35	1a	A2/NS3-1073	46 89 137 168 195	10.639.243 11.487.369 8.721.915 812.189 3.854.523	Chronic HCV

B



C

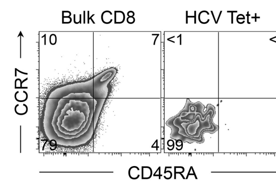


Fig S3. Chronically-infected patients with HCV. PBMCs were collected during the first year of HCV infection from individuals with high viral titers and developing persistent viremia. (*A*) Patients characteristics. (*B*) Frequency of CD122$^-$ (filled circles) and CD122$^+$ (opened circles) HCV-specific CD8$^+$ T cells. (*C*) Expression of CCR7 and CD45RA on total (left plot) and HCV-specific (right plot) CD8$^+$ T cells. Values indicate frequency of cells in each gate. Data is representative of five to eight samples from three infected patients processed independently.

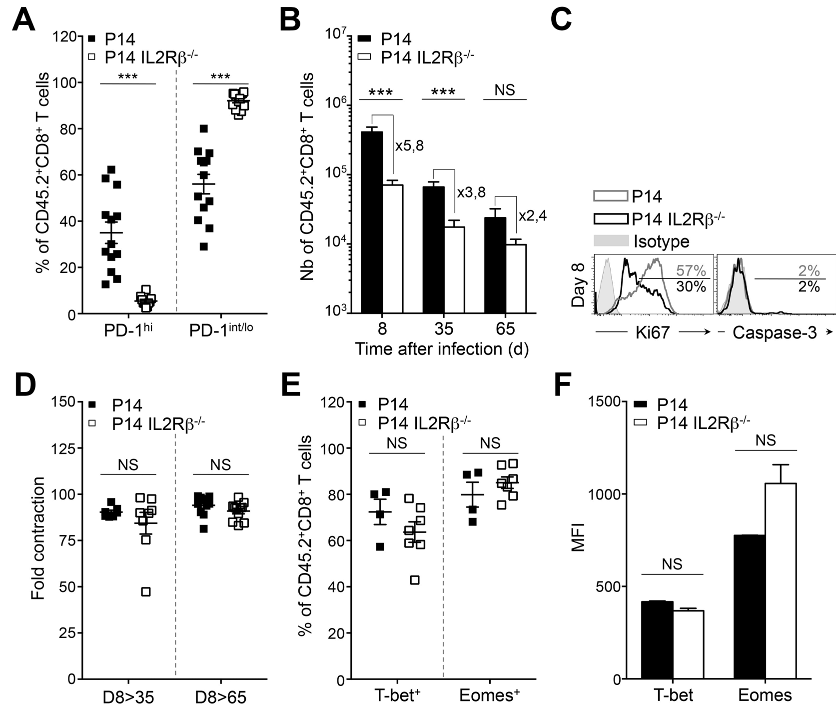


Fig. S4. IL2Rβ deficiency abrogates PD-1^{hi} terminal differentiation. Tg P14 or P14 IL2Rβ$^{-/-}$ (CD45.2) CD8^{+} T cells, specific for the H-2D^{b}gp33 epitope of LCMV, were adoptively transferred into recipient mice (CD45.1.2) prior to LCMV Cl-13 infection. Development of Tg CD8^{+}CD45.2^{+} PD-1^{hi} and PD-1$^{int/lo}$ cells was compared between the two groups of chimeric mice at day 35 p.i. (*A*) Cumulative frequencies of PD-1^{hi} and PD-1$^{int/lo}$ P14 and P14 IL2Rβ$^{-/-}$ cells. (B) Absolute numbers of CD8^{+}CD45.2^{+} P14 (black bars) and P14 IL2Rβ$^{-/-}$ (white bars) splenic cells calculated based on H-2D^{b}gp33 tetramer and CD45.2 congenic marker staining at the indicated time points. Values indicate the fold difference in the absolute numbers of P14 cells over P14 IL2Rβ$^{-/-}$ at that precise time point. (C) Intracellular expression of Ki-67 (left) and active-caspase 3 (right) in CD8^{+}CD45.2^{+} P14 (grey histograms) and P14 IL2Rβ$^{-/-}$ cells (black histograms) at day 8 p.i.. Values indicate the frequency of Ki-67^{hi} or active caspase-3^{+} cells. (*D*) Fold contraction of P14 (filled squatters) and P14 IL2Rβ$^{-/-}$ (opened squatters) cells in the indicated time frames and calculated by dividing the average number of CD45.2^{+}CD8^{+} T cells in the spleen at day 8 by the number of CD45.2^{+}CD8^{+} T cells recovered from individual mice in the spleen at day 35 and 65 p.i.. (*E-F*) Frequency (*E*) and mean fluorescent intensity (*F*) of intracellular T-bet and Eomes in P14 (filled squatters) and

P14 IL2R$\beta^{-/-}$ (opened squatters) cells at day 35 p.i. *p<0,05, ***p<0,0005, NS p≥ 0,05; two-tailed unpaired Student's *t*-test. Data are pooled from two (*E*, *F*), three (*A, D)* or four (*B*) independent experiments with three to ten mice per group or representative of three (*C*) independent experiments with similar results (three mice per group in each). (error bars (*A*, *B*, *D, E, F*), mean±s.e.m.).

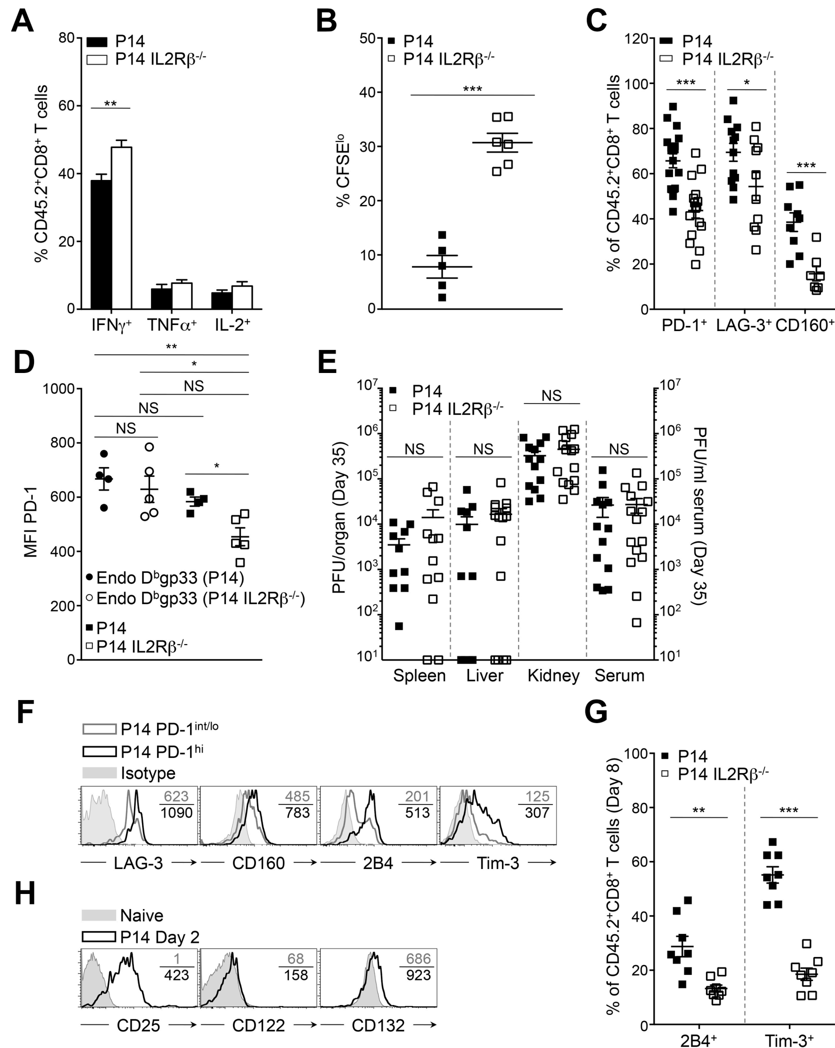


Fig. S5. IL2Rβ-dependent signals regulate the level of inhibitory receptor expression and the associated cellular dysfunctions. P14 or P14 IL2R$\beta^{-/-}$ chimeric mice were generated as described in Fig. S4. and cells were analysed at day 35 p.i. (*A*) Intracellular production of the indicated cytokines by $CD8^{+}CD45.2^{+}$ P14 (black bars) and P14 IL2R$\beta^{-/-}$ (white bars) cells. (B) Cumulative frequency of P14 (filled squatters) and P14 IL2R$\beta^{-/-}$ (opened squatters) cells proliferating to gp33 peptide stimulation, as assessed by a CFSE dilution assay. (C) Cumulative frequencies of PD-1^{+} (left), LAG-3^{+} (middle) and $CD160^{+}$ (right) P14 (filled squatters) and P14 IL2R$\beta^{-/-}$ (opened squatters) cells at day 35 p.i.. (*D*) Cumulative cell-surface expression of PD-1 on endogenous $CD45.1.2^{+}$H-$2D^{b}gp33^{+}CD8^{+}$ T cells from chimeric mice transferred with P14 (filled circles) and P14 IL2R$\beta^{-/-}$ (opened

circles) cells. Tg P14 CD45.2$^+$H-2D^{b}gp33$^+$CD8$^+$ T (filled squatters) and Tg P14 IL2R$\beta^{-/-}$ CD45.2$^+$H-2D^{b}gp33$^+$CD8$^+$ T (opened squatters) cells are also represented. Grey filled histograms are isotype controls. (*E*) Viral titers in indicated organs (left axis) and serum (right axis) at day 35 p.i. from P14 (filled squatters) and P14 IL2R$\beta^{-/-}$ (opened squatters) chimeric mice. (*F*) Expression of the indicated inhibitory receptors on P14 PD-1$^{int/lo}$ (grey histograms) and P14 PD-1^{hi} (black histograms) cells. Values indicate the MFI; filled histograms are isotype controls. (G) Cumulative frequencies of 2B4$^+$ (left) and Tim-3$^+$ (right) P14 (filled squatters) and P14 IL2R$\beta^{-/-}$ (opened squatters) cells at day 8 p.i.. (*H*) Expression of CD25, CD122 and CD132 on P14 cells after two days of *in vitro* stimulation with dendritic cells loaded with gp33-41. Values indicate the MFI; grey filled histograms are isotype controls. *$p<0.05$, **$p<0.005$, NS $p\geq 0.05$; two-tailed unpaired Student's *t*-test. Data are pooled from one (*D*), two (*B*), three (*A*, *E*, *G*) or five (*C*) or three (*A*, *B*, *D*) independent experiments with four to eighteen mice per group or representative of one (*H*) or three (*F*) independent experiments with similar results (two to four mice per group in each). (error bars (*A*, *B*, *C*, *D, E, G*), mean±s.e.m.).

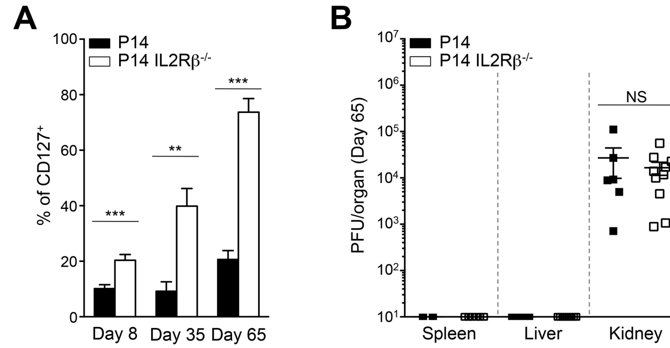


Fig. S6. IL2Rβ deficiency restores CD127 expression. P14 or P14 IL2Rβ$^{-/-}$ chimeric mice were generated as described in Fig. S4. (*A*) Frequency of CD127^{+} P14 (black bars) and P14 IL2Rβ$^{-/-}$ (white bars) cells at indicated time points. (*B*) Viral titers in indicated organs at day 65 p.i. from P14 (filled squatters) and P14 IL2Rβ$^{-/-}$ (opened squatters) chimeric mice. **$p<0.005$, ***$p<0.0005$, NS $p\geq 0.05$; two-tailed unpaired Student's *t*-test. Data are pooled from two (*B*) or three (*A*) independent experiments with five to ten mice per group. (error bars (*A*, *B*), mean±s.e.m.).

Article II. Cutting Edge: IL-2 and IL-15 cooperate to program CD8 T cell exhaustion through STAT5

Jean-Christophe Beltra[*,†], Chloé Berthe[*], and Hélène Decaluwe[*,†,‡]

This manuscript is in preparation for submission to “The Journal of Immunology” JI.

This article allowed me to complete several questions arising from our first article including the relative contribution of IL-2 and IL-15 on $CD8^+$ T cell exhaustion, the determinant time-frame(s) of both cytokines and the dominant signaling pathway by which these cytokines mediate their impact. I designed all the experiments (conceptual contribution 95%) with advises from H. Decaluwe. I performed all the experiments with help from C. Berthe and analyzed the results (technical contribution 95%). I wrote and corrected the entire manuscript with corrections from H. Decaluwe.

Cutting Edge: IL-2 and IL-15 cooperate to program CD8 T cell exhaustion through STAT5

Jean-Christophe Beltra[*,†], Chloé Berthe[*], and Hélène Decaluwe[*,†,‡]

[*]Cytokines and Adaptive Immunity Laboratory, CHU Sainte-Justine Research Center, Montreal, Qc, H3T1C5, Canada

[†]Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Montreal, Qc, H3C3J7, Canada

[‡]Immunology and Rheumatology Division, Department of Pediatrics, Faculty of Medicine, University of Montreal, Montreal, Qc H3T1C5, Canada

***Corresponding author:** Hélène Decaluwe, MD PhD

Mailing address: CHU Sainte-Justine, 3175 Cote-Sainte-Catherine, Montreal, Quebec, H3T 1C5, Canada

Phone number: 1-514-345-4713; Fax: 1-514-345-4897

Email: helene.decaluwe@umontreal.ca

Short title: IL-15-dependent signals direct T cell exhaustion

Classification: Biological Sciences; Immunology and Inflammation

Keywords: $CD8^+$ T cell; IL-2; IL-15; memory T cell; exhaustion; STAT5

ABSTRACT

IL2Rβ (the β receptor chain common to IL-2 and IL-15)-dependent signals direct key aspects of $CD8^+$ T cell exhaustion during chronic viral infections. However, the relative contribution of IL-2 and IL-15 in the process, the time-frame(s) during which each cytokine delivers critical signals to $CD8^+$ T cells and the determinant intracellular mechanisms involved remain unclear. Using a mouse model of chronic viral infection (LCMV clone-13), we show that IL-2 and IL-15 redundantly control the primary induction of 2B4 and Tim-3 on $CD8^+$ T cells, cooperatively promote their terminal differentiation but direct distinct aspects of memory developmental arrest. IL-2-dependent signals during priming abrogate subsequent Tcm development while continuous IL-15 production throughout infection precludes Tem differentiation. Both cytokines selectively trigger STAT5 in exhausted $CD8^+$ T cells. STAT5 inhibition restrains IL-2 and IL-15-induced 2B4 and Tim-3 expression and facilitates memory development. Together, we ascribe unique and redundant functions for IL-2 and IL-15 in directing $CD8^+$ T cell exhaustion and identify JAK3/STAT5 as a determinant pathway in the process.

INTRODUCTION

T cell exhaustion is as a major immunosuppressive mechanism hampering adaptive immune responses to severe pathologies including chronic viral infections and cancers (1). This unique and context specific differentiation program causes severe $CD8^+$ T cell dysfunctions (loss of cytokines secretion, decreased proliferation potential and killing abilities) as a result of increased expression of several inhibitory molecules at the cell surface (PD-1, LAG-3, CD160, 2B4, Tim-3, CTLA4, TIGIT) (2-8). As antigen (Ag) persists, $CD8^+$ T cells converge into a terminal exhaustion state and irreversible epigenetic marks are set up to perpetuate cell dysfunctions.(9-12) Fully exhausted $CD8^+$ T cells irremediably lose developmental plasticity towards protective memory precluding establishment of long-term immune protection (6, 13, 14). Disruption of the PD-1/PD-L1 axes and/or other immune checkpoints reinvigorates exhausted $CD8^+$ T cells functions (cytokines secretion [IFNγ, TNFα], proliferation and survival) and shows impressive results in the treatment of several human cancers.(3-5, 15) However, such approaches failed to overcome the exhaustion program or restore memory development and reinvigorated $CD8^+$ T cells eventually fallback into terminal exhaustion after treatment interruption.(16, 17) To prevent such outcome, immunotherapeutic regimens are now converging into combinational therapies reinforcing our need to identify the determinant factors directing T cell exhaustion program and the relevant time-frame for therapeutic interventions.(18)

Among the potential factors, cytokines are of particular interest since these soluble mediators greatly influence $CD8^+$ T cell differentiation and also have a critical impact on the outcome of chronic viral infections.(19-23) We previously demonstrated the critical role of the common gamma-chain (γ_c)-dependent cytokines IL-2 and IL-15 on $CD8^+$ T cell exhaustion.(24) These cytokines directly control the induction of a specific pattern of immune checkpoint molecules (i.e., 2B4 and Tim3) on $CD8^+$ T cells, instigate terminal exhaustion onto PD-1^{hi} effectors and adversely preclude memory development.(24) However, the relative contribution of IL-2 and IL-15 in these processes as well as the critical time-frame and intrinsic mechanisms involved remain to be determined.

Using a mouse model of chronic viral infection (lymphocytic choriomeningitis virus [LCMV] strain clone-13 [Cl-13]), we demonstrate that IL-2 and IL-15 redundantly direct the primary induction of 2B4 and Tim-3 on $CD8^+$ T cells, cooperatively promote terminal differentiation but regulate distinct aspects of memory developmental arrest. Early IL-2-dependent signals suppress the developmental plasticity towards central memory T cells (Tcm) while prolonged exposure to IL-15 throughout infection arrests effector memory T cells (Tem) differentiation. Both cytokines selectively trigger signal transducer and activator of transcription 5 (STAT5) in exhausted $CD8^+$ T cells. STAT5 inhibition restrains IL-2 and IL-15-induced 2B4 and Tim-3 expression and facilitates memory development. Together, we ascribe unique and redundant functions for IL-2 and IL-15 in directing $CD8^+$ T cell exhaustion and identify JAK3/STAT5 as a determinant pathway in this process.

MATERIAL AND METHODS

Mice, virus and infection

Six-week-old C57BL/6 (B6) mice were obtained from The Jackson laboratory. *Il15*$^{-/-}$ mice were kindly provided by V. Abadie, CHU Sainte-Justine Research Center, Montreal. CD45.2^{+} P14 transgenic (Tg) mice were bred in-house and crossed with IL2R$\alpha^{-/-}$ mice (The Jackson laboratory) to generate P14 IL2R$\alpha^{-/-}$ animals. Adoptive transfer experiments of P14 and P14 IL2R$\alpha^{-/-}$ were performed as previously described.(24) LCMV Arm and LCMV Cl-13 were produced on BHK-21 cells and titrated by plaque assays on VERO cells. Mice were infected i.p with 2.10^{5} PFU of LCMV Arm or i.v with 2.10^{6} PFU of LCMV Cl-13. For cytokine dosages both viral strains were given i.v. and standardized at 2.10^{6} PFU. All donor and virus-free recipient mice were housed in specific pathogen-free facilities at the CHU Sainte-Justine Research Center. Infectious experiments were conducted at the CHU Sainte-Justine Research Center in accordance with the Canadian Council on Animal Care guidelines.

Flow cytometry

For each experiment, splenocytes were stained with LIVE/DEAD Aqua (Thermo Fisher). Endogenous gp33 were identified as follows: Aqua^{-}TCRβ^{+}CD8^{+}CD44^{+}tetramer^{+} and adoptively transferred P14 and P14 IL2R$\alpha^{-/-}$ as Aqua^{-}TCRβ^{+}CD8^{+}CD45.1^{-}CD45.2^{+}. Antibodies were purchased from Affymetrix eBioscience (CD8, TCRβ, CD45.1, CD45.2, CD127, CD62L, LAG-3, CD160, 2B4, Tim-3 [clone RMT3-23], Bcl2 and Ki67), BD Bioscience (CD44, IFNγ, TNFα, IL-2), BioLegend (Tim-3 [clone RMT3-23], PD-1 [clone RMP1-30]) and Cell Signalling Technology (p-STAT5, p-S6). H-2D^{b} gp33 tetramers were from NIH Tetramer Core Facility. Intracellular staining for IFNγ, TNFα, IL-2, Ki67, Bcl2 and p-STAT5 were performed as described previously.(24) For p-S6 detection, splenocytes were rested 2-4h in nude medium at 37°C and re-stimulated with IL-15 (20 ng/ml) for 1h. Cells were then fixed with paraformaldehyde, permeabilized with methanol and stained for surface antibodies and p-S6 according to manufacturer's protocol. All data were acquired on a LSR FORTESSA II (BD) and analyzed with FlowJo v9.9.3 (Tree Star).

Blocking antibody experiments

Groups of B6 mice were given blocking antibodies against CD25 (clone 7D4, 100μg, i.p., BioXCell), CD122 (clone TM-β1, 200μg, i.p., BioXCell) or control antibodies (clone LTF2, 200μg, i.p., BioXCell) every two days starting at day -1 to day 7 post LCMV Cl-13 infection.

ELISA

Serum and spleen sections were harvested at indicated time points. Spleen frozen sections were lysed in RIPA buffer and proteins were collected from lysate supernatants after centrifugation. Serum and splenic proteins were dosed using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) for normalization. Cytokine levels were detected using Mouse IL-2/IL-15 ELISA Ready-SET-go!® (affymetrix) according to manufacturer's protocol and plates were read on a Multiskan Go instrument (Thermo Fisher Scientific).

***In vitro* culture**

In vitro stimulation of P14 cells was performed as previously described.(24) STAT5 inhibitor (N'-[(4-oxo-4H-chromen-3-yl)methylene]nicotinohydrazide, 100μM, Calbiochem) was added at day 2 and 4 of the culture.

Quantitative PCR

Total RNA was extracted from *in vitro* stimulated P14 cells (day 6) using the RNeasy Micro Kit (Qiagen) and cDNA was generated with the SuperScript® Vilo cDNA Synthesis Kit (ThermoFisher) as previously described.(24) Quantitative PCR were realized in two steps using the Brilliant II SYBR Green qRT-PCR low ROX master mix (Agilent Technologies) on a Mx 3000p qRT-PCR system (Stratagene). PCR were performed in triplicates for mouse KLF2 (*klf2*) (forward, 5'-TGT GAG AAA TGC CTT TGA GTT TAC TG-3' and reverse, 5'CCC TTA TAG AAA TAC AAT CGG TCA TAG TC-3'), Bcl6 (*bcl6*) (forward, 5'-GGG ACA TCT TGA CGG ACG TT-3' and reverse 5'-TCA CGG GAG GTT TAA GTG C-3'), Blimp-1 (*prdm1*) (forward, 5′-ACA CAC AGG AGA GAA GCC ACA TGA-3′ and reverse, 5′-TCG AAG GTG GGT CTT GAG ATT

GCT-3′) and hypoxanthine guanine phosphoribosyl transferase (HPRT) as internal control (forward, 5′-CTC CTC AGA CCG CTT TTT GC-3′ and reverse, 5′-TAA CCT GGT TCA TCA TCG CTA ATC-3′).

Statistical analyses

Statistical significance was determined by a standard Student's *t* test or One-way ANOVA non-parametric tests using ABI Prism 6. Significance was set as any *P* value *<0.05, **<0,005 and ***<0,0005.

RESULTS AND DISCUSSION

IL-2 and IL-15 redundantly initiate 2B4 and Tim-3 expression on CD8 T cells

We previously demonstrated in a mice model of chronic viral infection that IL2Rβ-dependent signals (triggered by both IL-2 and IL-15) on $CD8^+$ T cells increase inhibitory receptor expression and are mandatory for the induction of 2B4 and Tim-3 during a chronic viral infection.(24) Given the dominant role of IL-2 over IL-15 in the differentiation of short-lived effector cells (SLECs) during primary responses to acute viral infections, we first addressed the individual and direct contribution of that cytokine during a chronic viral infection. Therefore, we performed adoptive transfer of gp33-restricted P14 TCR transgenic $CD8^+$ T cells ($CD45.2^+$) deficient for the IL2Rα-chain (unique to IL-2) or their sufficient controls (P14) into separate groups of recipient mice ($CD45.1.2^+$). Chimeric mice were infected 20h later with LCMV Cl-13 and $CD45.2^+$ P14 and P14 $IL2R\alpha^{-/-}$ cells were analysed in the spleen at relevant time points as previously described.(24) We detected similar frequencies and numbers of P14 and P14 $IL2R\alpha^{-/-}$ cells in the spleen of infected mice throughout infection (Fig. 1*A* and Supplemental Fig. S1*A*). Accordingly, both groups of cells proliferated equivalently at days 8 and 21 p.i. (Supplemental Fig. S1*B*). This demonstrates that IL-2-dependent signals alone are dispensable to mount and maintain $CD8^+$ T cell responses during a chronic viral infection. We then assessed the expression of inhibitory receptors and found equivalent levels of PD-1, LAG-3, CD160 but also 2B4 and Tim-3 between splenic P14 and P14 $IL2R\alpha^{-/-}$ cells at days 8 and 21 p.i. (Fig. 1*B*). This result contrasted with the critical reduction and silencing of 2B4 and Tim-3 expression we previously reported at days 8 and 35 p.i. respectively in the absence of IL2Rβ-dependent signals.(24) Accordingly, P14 and P14 $IL2R\alpha^{-/-}$ cells presented similar patterns of inhibitory receptors co-expression, cytokine profiles and secreted similar amounts of IFNγ on a per cell basis testifying equivalent exhaustion severity (Fig. 1*C* and *D* and Supplemental Fig. S1*C*). These results raised two possibilities (i) IL-2 does not play any role in the establishment of $CD8^+$ T cell exhaustion and IL-15 is prominent in the process or (ii) both IL-2 and IL-15 participate in the development of $CD8^+$ T cell exhaustion but the absence of one cytokine is compensated by the presence of the other. To explore these possibilities, we infected

C57BL/6 (B6) and IL-15$^{-/-}$ (KO) mice with LCMV Cl-13 and examined inhibitory receptors induction on gp33-specific CD8^{+} T cells. The use of IL-15KO mice ensured a complete abrogation of IL-15-dependent signals compared to the cognate IL15RαKO model that does not entirely hamper these signals.(25) We found no differences in the induction of PD-1, CD160, 2B4 or Tim-3 in the blood at day 8 p.i. demonstrating that similar to IL-2, IL-15-deficiency alone barely affected inhibitory receptors levels at this time-point (Fig. 1*E*). These data suggested that IL-2 and IL-15 redundantly promote CD8^{+} T cell exhaustion and that each cytokine compensate for the lack of the other. In order to confirm this point, B6 mice were infected with LCMV Cl-13 and treated with blocking antibodies against CD25 (7D4), CD122 (TM-β1) or control antibody (LTF2) between day-1 and 7 p.i.. One-day post-treatment (day 8 p.i.), splenic gp33-specific CD8^{+} T cells from αCD122-treated mice expressed lower levels of 2B4 and Tim-3 (and CD160 to lesser extent) in accordance with our previous observations (Fig. 1*F*-*G*), while others did not.(24) Collectively, these data suggest that IL-2 and IL-15 redundantly direct exhaustion traits on CD8^{+} T cells and that each cytokine alone is sufficient for optimal induction of 2B4 and Tim-3.

IL2Rα-deficiency does not prevent terminal exhaustion but partially rescues memory differentiation during a chronic viral infection

We next assessed the impact of IL2Rα-deficiency on the commitment of CD8^{+} T cells to terminal exhaustion and *de novo* acquisition of memory markers. At day 21 p.i., we detected similar frequencies and numbers of PD-1^{hi} and PD-1$^{int/lo}$ cells between P14 and P14 IL2Rα$^{-/-}$ cells (Fig. 2*A* and *B* and Supplemental Fig. S1*D*). These results were in contrast with the complete loss of PD-1^{hi} terminal differentiation observed in the combined absence of IL-2 and IL-15-dependent signals on CD8^{+} T cells at day 35 p.i..(24) Furthermore, IL2Rα-deficiency did not allow for the rapid re-expression of the memory marker CD127 (IL7Rα) at time points when virus titers were still elevated (days 8 and 21 p.i.) (Fig. 2*C*), in contrast to what we previously observed in the absence of the IL2Rβ-chain.(24) However, by day 60p.i. when LCMV Cl-13 is generally cleared from most tissues except kidneys ,(2, 24) we detected a superior fraction of P14 IL2Rα$^{-/-}$ cells

(47,5% ± 3,1) expressing CD127 in the spleen compared to P14 cells (33% ± 4,8) (Fig. 2*C*). In similar settings, around 50% of IL2Rβ-deficient P14 cells had already re-expressed CD127 at day 35p.i. despite high viral titers and a vast majority of these effectors were CD127-positive at day 65p.i.(24) This demonstrated that a fraction of IL2Rα-deficient effectors retained memory developmental plasticity after viral decline without reaching the levels of recovery observed with combined abrogation of IL-2 and IL-15-dependent signals.(24) It suggests that IL-2 and IL-15 likely cooperate to achieve complete loss of memory $CD8^+$ T cell development during a chronic viral infection. More in depth analysis revealed that 20% ± 2 (8.5 x 10^3 cells) of P14 IL2Rα$^{-/-}$ cells had regained a central memory phenotype ($CD62L^+CD127^+$) by day 60 p.i. while this population was nearly absent amongst P14 cells (4% ± 1; 1.1 x 10^3 cells), as previously described (Fig. 2*D-E,* and Supplemental Fig. S1*E*).(24) The frequency of Tcm generated without IL2Rα-dependent signals nearly reached what we previously reported in the absence of IL2Rβ-dependent signals at day 65 p.i. (20% ± 2 and 27% ± 2 respectively).(24) This demonstrated that IL-2 mainly governs the arrested development of Tcm cells during a chronic viral infection. In contrast to our previous observations with the IL2Rβ$^{-/-}$ model, IL2Rα-deficiency neither rescued Tem cells development nor provided any developmental advantage to this population (Fig. 2*D-E* and Supplemental Fig. S1*E-F*).(24) Instead, a large proportion of P14 IL2Rα$^{-/-}$ cells remained deprived of their developmental plasticity toward Tem and retained a $CD62L^-CD127^-$ Tex phenotype (Fig. 2*D-E* and Supplemental Fig. S1*E*). Thus, IL2Rα-deficiency rescued Tcm cells development but unlike IL2Rβ-deficiency, failed to convert remaining Tex into Tem cells.(24) This implied a dominant role of IL-15 in the suppression of Tem cells development during a chronic viral infection. Superior central memory conversion of P14 IL2Rα$^{-/-}$ cells resulted in higher proportion of cells co-expressing CD127 and the pro-survival molecule B-cell lymphoma 2 (Bcl2) *in vivo* compared to P14 cells (Fig. 2*F*). Most $Bcl2^+$ P14 IL2Rα$^{-/-}$ cells were actually Tcm cells (Supplemental Fig. S1*G*). This suggested that Tcm conversion in the absence IL-2-dependent signals was accompanied by Ag-independent, IL-7-Bcl2-dependent survival signals. Further, albeit IL2Rα-deficiency did not restore Tem cells development, P14 IL2Rα$^{-/-}$ Tem cells gained survival

potential compared to P14 Tem (Supplemental Fig. S1*G*). Altogether, these results evidenced a critical cooperation between IL-2 and IL-15 in the arrested memory development of exhausted $CD8^+$ T cells during a chronic viral infection and also suggested selective functions of the cytokines in the suppression of Tcm and Tem cells differentiation respectively.

Early IL-2 and prolonged IL-15-dependent signals arrest memory $CD8^+$ T cell development.

To elucidate the relevant time-frame during which IL-2 and IL-15 instigate the loss of memory development, serum and splenic levels of the cytokines were quantified in C57BL/6 mice chronically infected with LCMV Cl-13. Substantial IL-2 levels were detected in the spleen (but not the serum) of infected mice between days 1 and 8 p.i., but the cytokine was undetectable thereafter (Fig. 3*A* and Supplemental Fig. S2*A*). This was in accordance with the rapid impairment of IL-2 production by $CD4^+$ and $CD8^+$ T cells during a chronic viral infection.(2, 26, 27) Further, we and others previously reported that the IL2Rα-chain expression vanished from effector $CD8^+$ T cells from day 6 p.i..(7, 24) Thus, IL-2-dependent signals on $CD8^+$ T cells that are able to suppress Tcm development are likely provided during the priming phase of a chronic viral infection.

IL-15 production was also elevated during the first few days p.i. but as opposed to IL-2, substantial levels of the cytokine remained detectable during chronicity (Fig. 3*A* and Supplemental Fig. S2*A*). By day 6 and onwards, the amounts of IL-15 protein in LCMV Cl-13 infected mice even outcompeted levels found within animals acutely infected with LCMV Arm (Fig. 3*B* and *C*). These data suggest that Ag-persistence selectively sustains IL-15 production *in vivo*. This is in accordance with the heightened serum levels of IL-15 found in HIV patients and the prolonged IFNα responses demonstrated in LCMV Cl-13 mice, IFNα being a known enhancer of IL-15 production.(28-32) In addition, populations of monocytes/macrophages ($CD11c^-CD11b^+$) at day 8 and $CD8^+$ and $CD8^-$ DCs ($CD11c^+CD11b^-$) at days 8 and 15 post LCMV Cl-13 infection expressed superior levels of the IL15Rα-chain compared to their equivalent in LCMV Arm mice (Fig. 3*D* and Supplemental Fig. S2*B for gating strategy*). This implied superior trans-presentation of IL-15 by professional APCs during Ag-persistence. Taken together, these results pointed

to important physiologic functions of IL-15 during chronicity, a time-frame during which exhausted $CD8^+$ T cells are gradually deprived of their capacity to re-express CD127.(14) Collectively, our data and others suggest that IL-2 abolishes Tcm cells plasticity during priming while IL-15-signals gradually suppress CD127 re-expression potential during chronicity depriving Tex cells of their developmental potential into Tem cells.

IL-2 and IL-15 direct $CD8^+$ T cell exhaustion through STAT5

We finally investigated the determinant signalling pathways by which IL-2 and IL-15 direct $CD8^+$ T cell exhaustion and preclude memory development. These cytokines activate multiple pathways in $CD8^+$ T cells including Pi3K/AKT, MAPK and JAK/STATs (predominantly JAK3/STAT5) pathways.(33) During a chronic viral infection, responding $CD8^+$ T rapidly lose their ability to trigger Pi3K/AKT and MAPK pathways in response to TCR and cytokine signals as a result of proximal interference dispensed by inhibitory receptors.(8, 34) Accordingly, we also detected insubstantial phosphorylation of the Pi3K/AKT downstream ribosomal S6 kinase (S6) in chronically stimulated $CD8^+$ T cells at days 8 and 30 p.i. compared to their acute counterpart in response to IL-15 (Fig. 4*A*). In contrast, IL-2 and IL-15 triggered optimal STAT5 phosphorylation in chronically-stimulated $CD8^+$ T cells throughout infection (Fig. 4*B* and *C*).(24) This points to JAK3/STAT5 as being the prominent signalling pathway operating downstream of IL-2 and IL-15 in $CD8^+$ T cells during a chronic viral infection. We previously demonstrated that IL-2 and IL-15 induces high expression of 2B4 and Tim-3 on $CD8^+$ T cells *in vitro*.(24) Using 2B4 and Tim-3 expression as a surrogate and IL-2 as a trigger, we found that specific inhibition of STAT5 with small molecules *in vitro* largely restrained 2B4, Tim-3 and to a lesser extent CD160 induction on $CD8^+$ T cells while PD-1 and LAG-3 were unaffected (Fig. 4*D*). Similar results were obtained with IL-15 (data not shown). STAT5 inhibition also accelerated re-expression of the L-selectine CD62L and favored establishment of a pro-memory transcriptional program in $CD8^+$ T cells (i.e., higher KLF2 and Bcl6 levels) (Fig. 4*E* and *F*).(35, 36) Further, STAT5 inhibition lowered Blimp-1 expression, a transcriptional repressor associated with $CD8^+$ T cells terminal differentiation and exhaustion (Fig. 4*F*).(37-39) Together, these data

provide evidences that during a chronic viral infection, IL-2 and IL-15 instigate $CD8^+$ T cell exhaustion and memory developmental arrest through STAT5.

Together, we refine our understanding of the combined and individual functions of IL-2 and IL-15 on CD8 T cell responses during a chronic viral infection. Impact of IL-2 is limited to the priming phase of infection due to arrested production of the cytokine and rapid downregulation of the IL2Rα-chain on CD8 T cells.(24) During this time-frame IL-2 and IL-15 act in concert to promote the initial induction of 2B4 and Tim-3 on $CD8^+$ T cells. Our data also indicate that IL-2-dependent signals provided during priming mainly direct the irreversible loss of Tcm cells development marking exhausted $CD8^+$ T cells.(6, 13) Previous work in acute infection settings also reported that early effectors (day 3.5 post LCMV Arm infection) expressing higher levels of the IL2Rα-chain are fated to terminal differentiation and lose their developmental plasticity toward Tcm cells.(40) This unique function of IL-2 is far more dramatic during a chronic viral infection as it leads to a complete loss of Tcm cell development. Conversely, IL-2-deficiency failed to rescue Tem cells. Interestingly, exhausted $CD8^+$ T cells retain the ability to re-express CD127 beyond the priming phase of a chronic viral infection (when removed onto an Ag-free environment).(14) IL-15 but not IL-2 is still produced during this time-frame and this cytokine is known to restrains CD127 expression on $CD8^+$ T cells.(41, 42) Thus, it is tempting to speculate that prolonged IL-15-dependent signalling during chronicity arrest Tem development during a chronic viral infection. Finally, we identify STAT5 as the dominant signalling molecule by which IL-2 and IL-15 signal in exhausted $CD8^+$ T cells. STAT5 inhibition *in vitro* specifically restrains IL-2-mediated induction of 2B4 and Tim-3 expression on $CD8^+$ T cells, reinstates a pro-memory transcriptional program and dampens Blimp-1 expression. Previous works also suggested that STAT5 sustains a terminal differentiation program in $CD8^+$ T cells at that expense of memory.(43-45) De facto, modulating STAT5 activity in $CD8^+$ T cells might represent a relevant therapeutic approach to restrain exhaustion and rescue memory cell development in patients with chronic viral infections.

ACKNOWLEDGMENTS

We thank V. Abadie for insightful comments; V. Abadie and S. Bourbonnais for critical reading of the manuscript and all the members of the animal facility at CHU Ste Justine Research Center for mice care.

REFERENCES

1. Wherry EJ (2016) Molecular Basis of T cell Exhaustion: Insights for Immunotherapy. in *Keystone symposia 2016 Cell Biology and Immunology of Persistent Infection (A8)*.
2. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, & Ahmed R (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77(8):4911-4927.
3. Barber DL*, et al.* (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439(7077):682-687.
4. Blackburn SD*, et al.* (2009) Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10(1):29-37.
5. Blackburn SD, Shin H, Freeman GJ, & Wherry EJ (2008) Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc Natl Acad Sci U S A* 105(39):15016-15021.
6. Wherry EJ, Barber DL, Kaech SM, Blattman JN, & Ahmed R (2004) Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A* 101(45):16004-16009.
7. Wherry EJ*, et al.* (2007) Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27(4):670-684.
8. Odorizzi PM & Wherry EJ (2012) Inhibitory receptors on lymphocytes: insights from infections. *J Immunol* 188(7):2957-2965.
9. Paley MA*, et al.* (2012) Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science* 338(6111):1220-1225.
10. Youngblood B*, et al.* (2011) Chronic virus infection enforces demethylation of the locus that encodes PD-1 in antigen-specific CD8(+) T cells. *Immunity* 35(3):400-412.
11. Youngblood B*, et al.* (2013) Cutting edge: Prolonged exposure to HIV reinforces a poised epigenetic program for PD-1 expression in virus-specific CD8 T cells. *J Immunol* 191(2):540-544.
12. Sen DR*, et al.* (2016) The epigenetic landscape of T cell exhaustion. *Science*.
13. Shin H, Blackburn SD, Blattman JN, & Wherry EJ (2007) Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J Exp Med* 204(4):941-949.
14. Angelosanto JM, Blackburn SD, Crawford A, & Wherry EJ (2012) Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. *J Virol* 86(15):8161-8170.
15. Topalian SL, Drake CG, & Pardoll DM (2015) Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* 27(4):450-461.
16. Pauken KE & Wherry EJ (2015) Overcoming T cell exhaustion in infection and cancer. *Trends Immunol* 36(4):265-276.
17. Pauken KE*, et al.* (2016) Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. *Science*.
18. Minn AJ & Wherry EJ (2016) Combination Cancer Therapies with Immune Checkpoint Blockade: Convergence on Interferon Signaling. *Cell* 165(2):272-275.

19. Beltra JC & Decaluwe H (2016) Cytokines and persistent viral infections. *Cytokine* 82:4-15.
20. Decaluwe H*, et al.* (2010) Gamma(c) deficiency precludes CD8+ T cell memory despite formation of potent T cell effectors. *Proc Natl Acad Sci U S A* 107(20):9311-9316.
21. Mathieu C*, et al.* (2015) IL-2 and IL-15 regulate CD8+ memory T-cell differentiation but are dispensable for protective recall responses. *Eur J Immunol* 45(12):3324-3338.
22. Joshi NS*, et al.* (2007) Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27(2):281-295.
23. Wiesel M*, et al.* (2012) Type-I IFN drives the differentiation of short-lived effector CD8+ T cells in vivo. *Eur J Immunol* 42(2):320-329.
24. Beltra JC*, et al.* (2016) IL2Rbeta-dependent signals drive terminal exhaustion and suppress memory development during chronic viral infection. *Proc Natl Acad Sci U S A* 113(37):E5444-5453.
25. Schluns KS, Williams K, Ma A, Zheng XX, & Lefrancois L (2002) Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J Immunol* 168(10):4827-4831.
26. Brooks DG, Teyton L, Oldstone MB, & McGavern DB (2005) Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. *J Virol* 79(16):10514-10527.
27. Elsaesser H, Sauer K, & Brooks DG (2009) IL-21 is required to control chronic viral infection. *Science* 324(5934):1569-1572.
28. Swaminathan S*, et al.* (2016) Interleukin-15 (IL-15) Strongly Correlates with Increasing HIV-1 Viremia and Markers of Inflammation. *PLoS One* 11(11):e0167091.
29. Wilson EB*, et al.* (2013) Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science* 340(6129):202-207.
30. Teijaro JR*, et al.* (2013) Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* 340(6129):207-211.
31. Colpitts SL*, et al.* (2012) Cutting edge: the role of IFN-alpha receptor and MyD88 signaling in induction of IL-15 expression in vivo. *J Immunol* 188(6):2483-2487.
32. Richer MJ*, et al.* (2015) Inflammatory IL-15 is required for optimal memory T cell responses. *J Clin Invest* 125(9):3477-3490.
33. Ring AM*, et al.* (2012) Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat Immunol* 13(12):1187-1195.
34. Staron MM*, et al.* (2014) The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8(+) T cells during chronic infection. *Immunity* 41(5):802-814.
35. Rao RR, Li Q, Gubbels Bupp MR, & Shrikant PA (2012) Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8(+) T cell differentiation. *Immunity* 36(3):374-387.
36. Ichii H*, et al.* (2002) Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells. *Nat Immunol* 3(6):558-563.

37. Shin H, *et al.* (2009) A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31(2):309-320.
38. Rutishauser RL, *et al.* (2009) Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31(2):296-308.
39. Kallies A, Xin A, Belz GT, & Nutt SL (2009) Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity* 31(2):283-295.
40. Kalia V, *et al.* (2010) Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity* 32(1):91-103.
41. Quinci AC, *et al.* (2012) IL-15 inhibits IL-7Ralpha expression by memory-phenotype CD8(+) T cells in the bone marrow. *Eur J Immunol* 42(5):1129-1139.
42. Cassese G, *et al.* (2007) Bone marrow CD8 cells down-modulate membrane IL-7Ralpha expression and exhibit increased STAT-5 and p38 MAPK phosphorylation in the organ environment. *Blood* 110(6):1960-1969.
43. Hand TW, *et al.* (2010) Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. *Proc Natl Acad Sci U S A* 107(38):16601-16606.
44. Grange M, *et al.* (2013) Active STAT5 regulates T-bet and eomesodermin expression in CD8 T cells and imprints a T-bet-dependent Tc1 program with repressed IL-6/TGF-beta1 signaling. *J Immunol* 191(7):3712-3724.
45. Mitchell DM & Williams MA (2013) Disparate roles for STAT5 in primary and secondary CTL responses. *J Immunol* 190(7):3390-3398.

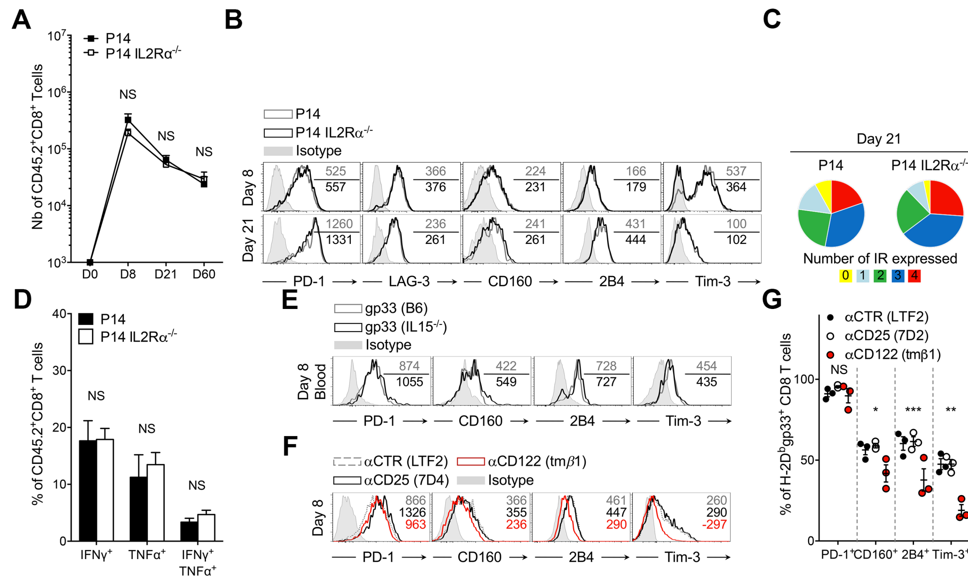


Fig. 1. IL-2 and IL-15 conjointly promote inhibitory receptors expression. Tg P14 or P14 IL2Rα$^{-/-}$ (CD45.2^{+}) cells were adoptively transferred into distinct groups of recipient mice (CD45.1.2^{+}) prior to LCMV Cl-13 infection. Tg P14 and P14 IL2Rα$^{-/-}$ cells (CD45.2^{+}) were compared in the spleen at indicated time points. (*A*) Absolute numbers of CD8^{+}CD45.2^{+} P14 (black squatters) and P14 IL2Rα$^{-/-}$ (white squatters) splenic cells calculated based on H-2D^{b}gp33 tetramer and CD45.2 congenic marker staining at the indicated time points. (*B*) Inhibitory receptors expression on P14 (grey histograms) and P14 IL2Rα$^{-/-}$ (black histograms) cells. Values indicate the MFI; grey filled histograms are isotype controls. (*C*) Frequency of P14 (left pie) and P14 IL2Rα$^{-/-}$ (right pie) cells co-expressing PD-1, LAG-3, 2B4 and CD160. (*D*) Intracellular production of the indicated cytokines by CD8^{+}CD45.2^{+} P14 (black bars) and P14 IL2Rα$^{-/-}$ (white bars) cells at day 21 p.i.. (*E*) Inhibitory receptors expression on gp33-specific CD8^{+} T cells at day 8 p.i. in the blood of B6 (grey histograms) and IL-15$^{-/-}$ (black histograms) mice at day 8 p.i. Values indicate the MFI; grey filled histograms are isotype controls. (*F*) Inhibitory receptors expression on gp33-specific CD8^{+} T cells isolated at day 8 p.i. from the spleen of infected mice treated with αCD25 (black histograms), αCD122 (red histograms) or αCTR (grey dashed histograms). Values indicate the MFI; grey filled histograms are isotype controls. (*G*) Frequency of splenic gp33-specific CD8^{+} T cells expressing the indicated inhibitory receptors at day 8p.i.. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, NS $p \geq 0.05$ (two-tailed

unpaired Student's *t*-test). Data are pooled from three (*A*) or one (*C*, *D*, *G*) independent experiments with three to five mice per group or representative of one independent experiments (*B*, *E*, *F*) (three to five mice per group). (error bars (*A, D*, *G*), mean±s.e.m.).

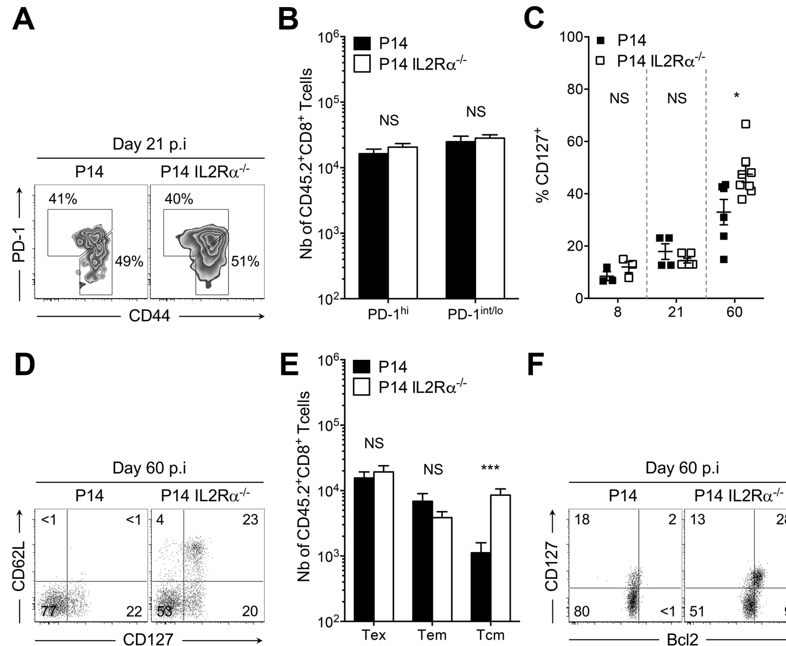


Fig. 2. IL2Rα-deficiency rescues Tcm development. P14 and P14 IL2Rα$^{-/-}$ chimeric mice were generated as described in Fig. 1. (*A-B*) Representative dot plots (*A*) and absolute numbers (*B*) of PD-1^{hi} and PD-1$^{int/lo}$ P14 (left; black bars) and P14 IL2Rα$^{-/-}$ (right; white bars) cells. Values indicate frequency of cells in each gate. (*C*) Frequency of CD127-positive P14 (black squatters) and P14 IL2Rα$^{-/-}$ (white squatters) at indicated time points p.i.. (*D-E*) Representative dot plots (*D*) and absolute numbers (*E*) of splenic P14 (left; black bars) and P14 IL2Rα$^{-/-}$ (right; white bars) Tex (CD62L$^-$CD127$^-$), Tem (CD62L$^-$ CD127$^+$) and Tcm (CD62L$^+$CD127$^+$) at day 60 p.i.. Values indicate frequency of cells in each gate. (*F*) Expression of CD127 and Bcl2 in P14 (left plot) and P14 IL2Rα$^{-/-}$ (right plot) cells. Values indicate frequency of double positive cells. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, NS $p\geq 0.05$ (two-tailed unpaired Student's *t*-test). Data are pooled from two (*B*, *C*, *E*) independent experiments with three to eight mice per group or representative of two independent experiments (*A*, *D*, *F*) with similar results (three to nine mice per group in each). (error bars (*B, C*, *E*), mean±s.e.m.).

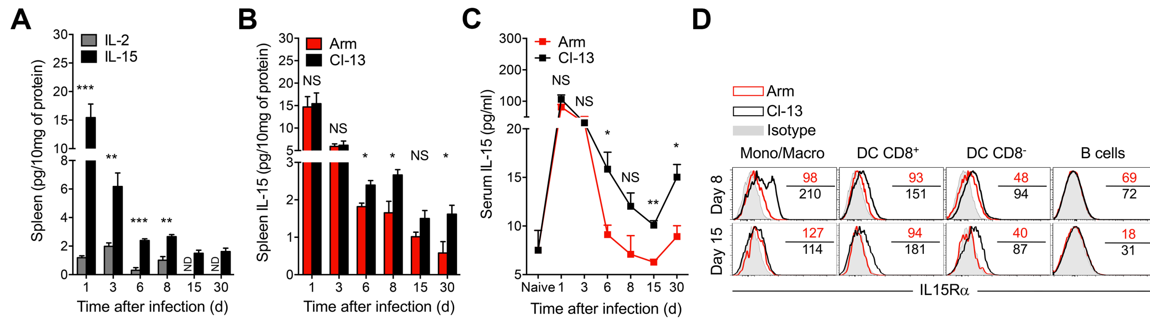


Fig. 3. Distinct kinetics of IL-2 and IL-15 production during a chronic viral infection. (*A*) Splenic protein levels of IL-2 (grey bars) and IL-15 (black bars) in the spleen of infected mice at indicated time points assessed by ELISA dosage. (*B*-*C*) Splenic (*B*) and serum (*C*) levels of IL-15 proteins in LCMV Arm (red bars; red line) and LCMV Cl-13 (black bars; black lines) infected mice at indicated time points p.i. assessed by ELISA dosage (*D*) Cell surface expression of IL15Rα on monocytes/macrophages (TCRβ$^-$NK1.1$^-$CD19$^-$CD11c$^-$CD11b$^+$, CD8$^+$ and CD8$^-$ DCs (TCRβ$^-$NK1.1$^-$CD19$^-$CD11c$^+$CD11b$^-$CD8$^+$ and CD8$^-$ respectively) and B cells (TCRβ$^-$NK1.1$^-$CD19$^+$) isolated from the spleen of LCMV Arm (red histograms) and LCMV Cl-13 (black histograms) at indicated time points p.i.. Values indicate the MFI; grey filled histograms are isotype controls. Data are pooled from two (*A*, *B*, *C*) independent experiments with four to six mice per group or representative of two independent experiments (*D*) with similar results (two to eight mice per group in each). (error bars (*A*, *B, C*), mean±s.e.m.).

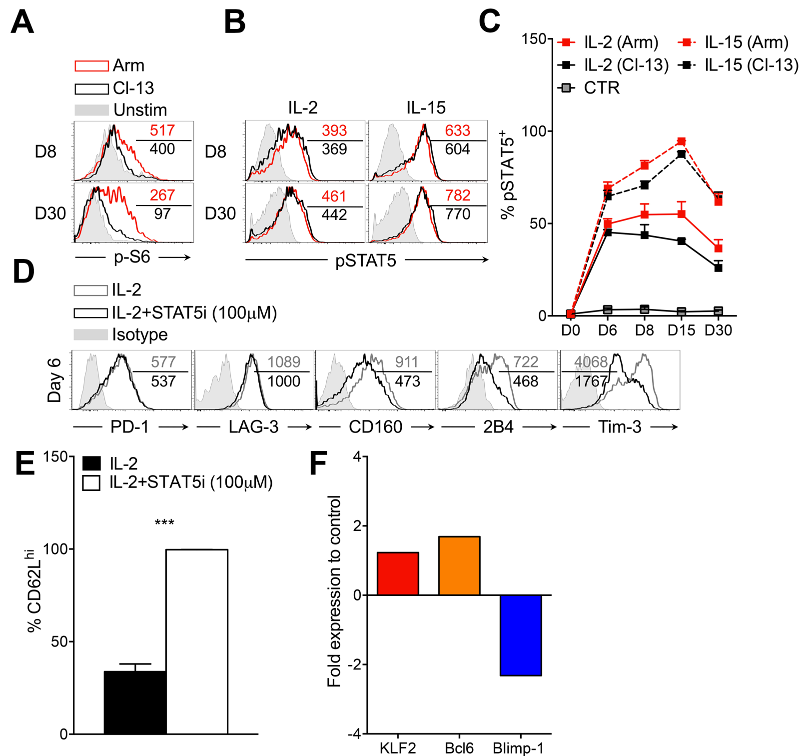


Fig. 4. Prominent role of STAT5 in IL-2 and IL-15-induced $CD8^+$ T cell exhaustion. (*A*) Intracellular expression of p-S6 in activated ($CD11a^{hi}$) $CD8^+$ T cells from LCMV arm (red histograms) and LCMV Cl-13 (black histograms) at indicated time points following 1h of stimulation with IL-15 (20ng/ml). Unstim stands for unstimulated control; Values indicate the MFI. (*B*) Intracellular expression of p-STAT5 in activated ($CD11a^{hi}$) $CD8^+$ T cells from LCMV arm (red histograms) and LCMV Cl-13 (black histograms) infected mice at indicated time points following 30 min of stimulation with IL-2 (left panels) or IL-15 (right panels) (20ng/ml). Unstim stands for unstimulated control; Values indicate the MFI. (*C*) Kinetic of p-STAT5 induction in activated ($CD11a^{hi}$) $CD8^+$ T cells from LCMV arm (red lines) and LCMV Cl-13 (black lines) infected mice at indicated time points following 30 min of stimulation with IL-2 (solid lines) or IL-15 (dotted lines) (20ng/ml) or without stimulation (grey lines). (*D*) Inhibitory receptors expression on P14 cells after 6 days of co-culture with gp33 peptide complemented with IL-2 (10U/ml) in the presence (black histograms) or absence (grey histograms) of a STAT5 inhibitor (100μM). Values indicate the MFI; grey filled histograms are isotype controls (N=5 with each condition realized in triplicates) (*E*) Frequency of $CD62L^{hi}$ P14 cells recovered after

6 days of co-culture with gp33 peptide complemented with IL-2 (10U/ml) in the presence (white bars) or absence (black bars) of STAT5 inhibitor (100μM) (N=4 with each condition realized in triplicates). (*F*) Relative expression of indicated genes in P14 cells co-culture for 6 days with gp33 peptide complemented with IL-2 in the presence of STAT5 inhibitor (100μM) referred to P14 cells co-culture with gp33 peptide and IL-2 alone (data pooled from one experiment). Data are pooled from five (*C*) independent experiments with four to nine mice per group or representative of five (*B*) or two (*A*) independent experiments (two to three mice per group in each). (error bars *(C*, *E*), mean±s.e.m.).

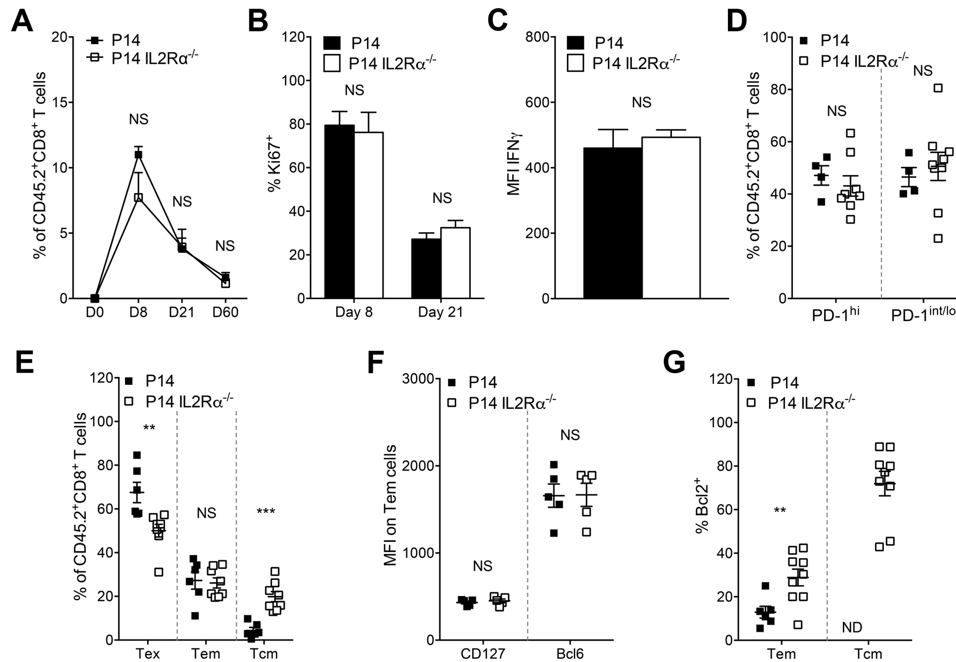


Fig. S1. IL2Rα-deficiency prevents complete loss of memory $CD8^+$ T cell development. (*A*) Cumulative frequencies of $CD8^+CD45.2^+$ P14 (black squatters) and P14 IL2Rα$^{-/-}$ (white squatters) splenic cells calculated based on H-2D^{b}gp33 tetramer and CD45.2 congenic marker staining at the indicated time points. (*B*) Cumulative frequencies of Ki67-positive P14 (black bars) and P14 IL2Rα$^{-/-}$ (white bars) at indicated time points. (C) Production of IFNγ in P14 (black bars) and P14 IL2Rα$^{-/-}$ (white bars) cells; bars representing the MFI. (*D*) Cumulative frequencies of splenic P14 (black squatters) and P14 IL2Rα$^{-/-}$ (white squatters) PD-1^{hi} (left panel) and PD-1$^{int/lo}$ (right panel) cells at day 21 p.i.. (*E*) Cumulative frequencies of splenic P14 (black squatters) and P14 IL2Rα$^{-/-}$ (white squatters) Tex ($CD62L^-CD127^-$), Tem ($CD62L^-CD127^+$) and Tcm ($CD62L^+CD127^+$) cells at day 60 p.i.. (*F*) Expression of CD127 (left panel) and Bcl6 (right panel) in P14 (black squatters) and P14 IL2Rα$^{-/-}$ (white squatters) Tem at day 60 p.i. (*G*) Frequency of P14 (black squatters) and P14 IL2Rα$^{-/-}$ (white squatters) Tem (left panel) and Tcm (right panel) cells expressing Bcl2 at day 60 p.i. Data are pooled from three (*A*), two (*D*, *E*, *F*, *G*) or one (*B*, *C*) independent experiments with five to nine mice per group. (error bars (*A*, *B, C*, *D*, *E*, *F, G*), mean±s.e.m.).

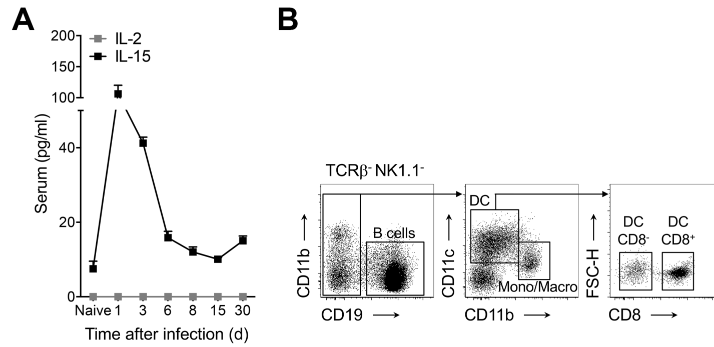


Fig. S2. Chronic environment favors IL-15 production and trans-presentation by myeloid cells. (*A*) Serum protein levels of IL-2 (grey bars) and IL-15 (black bars) at indicated time points assessed by ELISA dosage. (*B*) Gating strategy for identification of monocytes/macrophages, $CD8^+$ and $CD8^-$ DCs and B cells. Dot plots display a representative splenic sample harvested at day 8 post LCMV Cl-13 infection. Data are pooled from three (*A*) independent experiments with four to six mice per group or representative of two independent experiments (*B*) with similar results (two to eight mice per group in each). (error bars (*A*), mean±s.e.m.).

Discussion

CD8$^+$ T cell exhaustion has been the focus of intense investigations over the last decade. This unique differentiation program is characterized by key phenotypic, functional, developmental, transcriptional and epigenetic features which dramatically hinder CD8$^+$ T cell responses. Despite our growing knowledge of the molecular signature of T cell exhaustion, a precise understanding of the factors that directly control the establishment of such program in CD8$^+$ T cells during a chronic viral infection is still lacking. Particularly, the involvement of cytokines in the process remains unknown. In the first article of the present thesis, we uncover for the first time one major impact of cytokines (i.e., IL-2 and IL-15) in directing key aspects CD8$^+$ T cell exhaustion during a chronic viral infection. IL-2 and IL-15-dependent signals control the induction of a specific pattern of inhibitory receptors (i.e., 2B4 and TIM-3), instigate the terminal differentiation of highly exhausted PD-1^{hi} effectors and preclude memory development. In the second article, we tackle several questions arising from the first article including i) the relative contribution of IL-2 versus IL-15 in these processes ii) the determinant time-frame(s) during which IL-2 and/or IL-15 mediate their functions on CD8$^+$ T cells and iii) the intracellular mechanisms involved. Our main results are discussed in this last section.

I- Regulation of inhibitory receptors expression

Elevated and sustained expression of several inhibitory receptors (e.g., PD-1, LAG-3, CD160, 2B4 and TIM-3) are a hallmark of CD8$^+$ T cell exhaustion.(48, 49) These receptors use distinct and non-redundant ways to suppress CD8$^+$ T cell functions and are prime targets of current immunotherapeutic regimens in several human cancers.(50, 135, 648) However, little is known about factors controlling their expression on CD8$^+$ T cells *in vivo*.

1.1- IL-2 and IL-15 direct 2B4 and Tim-3 expression on CD8 T cells

Our first article provides clues that IL-2 and IL-15-dependent signals chiefly direct the expression of 2B4 and TIM-3 on CD8 T cells during a chronic viral infection. Using adoptive transfer experiments of P14 and P14 IL2R$\beta^{-/-}$ cells, we demonstrated that the combined absence of IL-2 and IL-15-dependent signals critically restrained the initial induction of 2B4 and TIM-3 on CD8^{+} T cells and abrogated their expression during chronicity (**Article 1**, Fig. 5*E* and *F* and S5*G*).(649) *In vitro*, TCR signals alone were unable to trigger 2B4 and TIM-3 but expression of these receptors strictly required additional stimulation with IL-2 or IL-15 (**Article 1**, Fig. 5*I*).(649) These data definitively prove that IL-2 and IL-15 directly control 2B4 and TIM-3 expression on CD8^{+} T cells and are the main actors of their induction during a chronic viral infection.

Our second article further explore the relative contribution of IL-2 versus IL-15 in the induction of 2B4 and TIM-3 on CD8^{+} T cells. Selective abrogation of IL-2-dependent signals alone using P14 cells deficient for the IL2Rα-chain (P14 IL2R$\alpha^{-/-}$) or blocking antibodies against CD25 (7D4) had virtually no impact in the initial induction of 2B4 and TIM-3 on Ag-specific CD8^{+} T cells (**Article 2**, Fig. 1*B* and *F*).(649) Infection of IL15KO mice did not either influence 2B4 and TIM-3 expression on Ag-specific CD8 T cells at day 8p.i. (**Article 2**, Fig. 1*E*).(649) These results suggest that IL-2 and IL-15 redundantly instigate 2B4 and TIM-3 expression on CD8^{+} T cells during the priming phase of a chronic viral infection and each cytokine is able to compensate for the lack of the other. According to that assumption, our *in vitro* data showed that IL-2 and IL-15 similarly promote 2B4 and TIM-3 expression on CD8^{+} T cells (**Article 1**. Fig. 5*I*).(649) One way to definitively prove the point would be to perform adoptive transfer of P14 and P14 IL2R$\alpha^{-/-}$ into WT and IL15KO recipients and to compare 2B4 and TIM-3 expression at day 8 p.i.. This approach would allow the direct comparison of CD8^{+} T cell responses in the absence of either IL-2, IL-15 and both signals. We would expect to observe a decrease in the induction of 2B4 and TIM-3 only in the condition in which IL-2 and IL-15-dependent signals are neutralized (P14 IL2R$\alpha^{-/-}$ transferred into IL15KO mice).

The relative contribution of IL-2 and IL-15 in the maintenance of 2B4 and TIM-3 expression during chronicity remains unclear. IL-2-dependent signals were limited to the priming phase of infection (due to arrested production of the cytokine (**Article 2**, Fig. 3*A*) and rapid downregulation of CD25 by $CD8^+$ T cells (**Article 1**, Fig. 1*A*)(2, 48, 649) while IL-15 was still produced and likely trans-presented throughout chronicity (**Article 2**, Fig. 3*B-D*). P14 $IL2R\alpha^{-/-}$ cells expressed optimal levels of 2B4 and TIM-3 during chronicity demonstrating that IL-15-dependent signals alone were sufficient to maintain their expression (**Article 2**, Fig. 1*B*). So far, IL-15 seems to play a unique role in maintaining 2B4 and Tim-3 expression on $CD8^+$ T cells during chronicity. However, we cannot exclude the contribution of IL-2. As proposed in the above paragraph, comparing 2B4 and TIM-3 expression (this time during chronicity [day 30p.i.]) on P14 and P14 $IL2R\alpha^{-/-}$ adoptively transferred into either WT and IL-15KO mice would allow to depict the individual and combined contribution of IL-2 and IL-15 in the maintenance of these receptors throughout infection. If IL-15 has a unique function in sustaining 2B4 and TIM-3 during chronicity, we would expect to observe a similar decrease in the expression of these receptors in the condition in which only IL-15 signals are selectively extinguished (P14 transferred into IL-15KO mice) compared to the condition in which IL-2 and IL-15-dependent signals are neutralized (P14 $IL2R\alpha^{-/-}$ transferred into IL-15KO mice).

1.2- Regulation of 2B4

Several evidences point out a critical role of the TF BLIMP-1 in the expression of 2B4 on $CD8^+$ T cells. *In vivo*, 2B4 expression kinetic opposes that of other inhibitory receptors and increases from day 8 p.i. (until day 30p.i.) on $CD8^+$ T cells following the pattern of BLIMP-1 expression.(48, 162) During a chronic viral infection, conditional deletion of BLIMP-1 in CD8 T cells abrogates 2B4 expression during chronicity.(162) Accordingly, we observed a critical reduction of 2B4 levels on BLIMP-1-deficient CD8 T cells *in vitro* after stimulation with either IL-2 or IL-15 (**Appendix. 2**). We demonstrated that $IL2R\beta$-deficiency reduced *Prdm1* (BLIMP-1) mRNA levels in $CD8^+$ T cells and abrogated 2B4 expression (**Article 1**, Fig. 4*I* and Fig. 5*E* and *F*).(649) In our second article, we showed that IL-2-mediated induction of 2B4 *in vitro* was partially

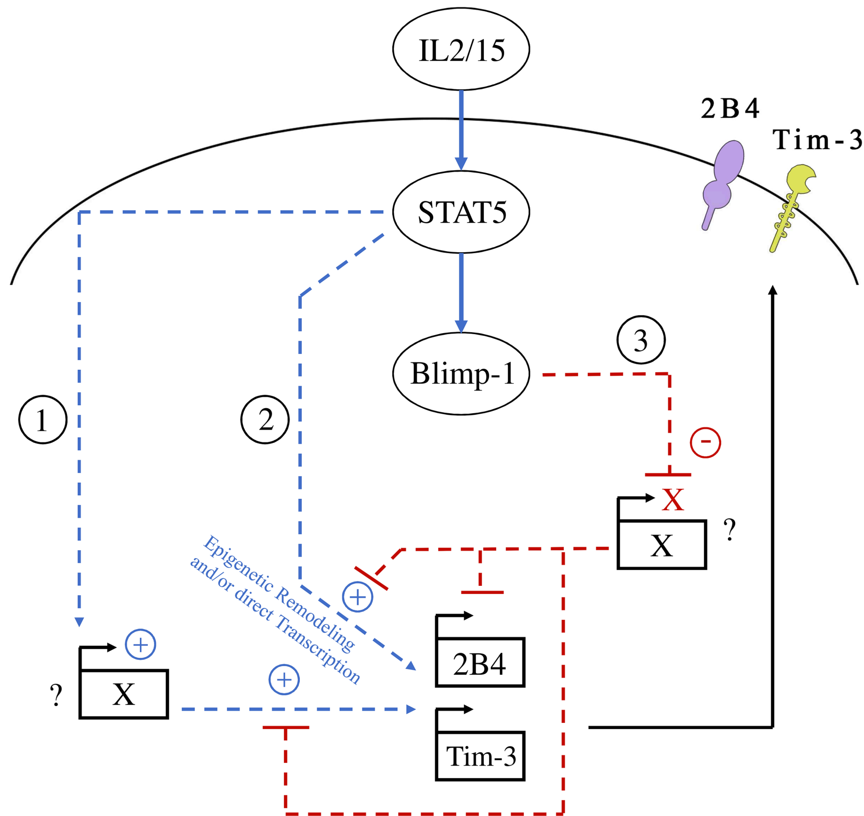
blocked in the presence of a STAT5 inhibitor (**Article 2**, Fig. 4*D*). STAT5 directly promotes BLIMP-1 expression in $CD8^+$ T cells and inhibition of STAT5 *in vitro* reduced BLIMP-1 levels (**Article 2**, Fig. 4*F*).(312, 650, 651) Finally, we demonstrated that IL-2 and IL-15 selectively trigger activation of STAT5 in $CD8^+$ T cells *in vivo* during a chronic viral infection in accordance with previous observations (**Article 2**, Fig. 4*A-C*).(359) Together, we show that 2B4 expression on $CD8^+$ T cells is controlled by an IL-2/15>>STAT5>>BLIMP-1 axis during a chronic viral infection (**Figure. 16**).

1.3- Regulation of Tim-3

During a chronic viral infection in mice, TIM-3 expression peaks at day 8 p.i. on Ag-specific $CD8^+$ T cells and gradually declines thereafter. The TF T-bet plays an important role in the initial induction of TIM-3 on $CD8^+$ T cells. Indeed, Tim-3 is barely detectable on $CD8^+$ T cells at day 8p.i. in the absence of T-bet.(123) Similar control of Tim-3 expression by T-BET was also described on Th1 $CD4^+$ T cells.(291) However, neither T-bet haploinsufficiency nor retrovirally overexpressed T-BET modifies TIM-3 expression on $CD8^+$ T cells during the chronic phase of infection.(123) Ag-specific $CD8^+$ T cells from CD4-depleted LCMV Cl-13 infected mice (severe model of chronic infection) express lower levels of T-BET but persistent high expression of TIM-3 during the chronic phase.(110, 123) Still during chronicity, TIM-3 expression levels are higher on $PD\text{-}1^{hi}$ effectors that express the lowest levels of T-BET (**Article 1**, Fig. S5*F*).(269, 649) Thus, T-BET is critical for initial induction of TIM-3 on $CD8^+$ T cells but dispensable for its maintenance during chronicity. Similar to T-BET-deficient cells, we showed a critical reduction of TIM-3 at day 8 p.i. in the absence of IL2Rβ-dependent signals (**Article 1**, Fig. S5*G*).(649) At this time-point, we often noticed a modest but reproducible reduction of T-BET expression within P14 $IL2R\beta^{-/-}$ compared to P14 cells (**Appendix. 3**). IL-2 and IL-15 are poor inducers of T-BET in $CD8^+$ T cells.(282) However, substantial decrease of that TF is often reported (including by our group) in effector $CD8^+$ T cells deficient for the IL2Rα-chain or the IL2Rβ-chain during the priming phase of an acute viral infection.(67, 282, 314) Thus, it is legitimate to speculate that during the priming phase of a chronic viral infection, IL2Rβ-dependent signals favor

optimal levels of T-BET on effector $CD8^+$ T cells and subsequent Tim-3 induction. Our data also indicate that STAT5 is critical for TIM-3 expression on CD8 T cells (**Article 2**, Fig. 4*D*). Interestingly STAT5 is a direct inducer of T-BET.(651) Collectively, primary induction of TIM-3 on $CD8^+$ T cells during a chronic viral infection might rely on a IL-2/15>>STAT5>>T-BET axis. However, as mentioned above, levels of T-BET at the peak of the response are only slightly lower without $IL2R\beta$-signals (**Appendix. 3**). This suggests that TIM-3 induction at this time point requires optimal levels of T-BET or that additional mechanisms set up by IL-2/15 and STAT5 facilitate its expression (e.g. epigenetic remodeling facilitating the accessibility of TIM-3 promoter region for T-BET).

Similar to previous observations with 2B4, BLIMP-1-deficiency severely compromised TIM-3 expression on IL-2 and IL-15-stimulated $CD8^+$ T cells *in vitro* (**Appendix. 2**). A recent report demonstrated that T-BET and BLIMP-1 display some overlapping functions in developing effectors $CD8^+$ T cells during an acute viral infection.(312) RNAseq analysis evidenced a lower expression of TIM-3 (*HAVCR2*) in the absence of either T-BET or BLIMP-1 but the more striking decrease was observed in the combined absence of both TFs.(312) Hence, T-BET and BLIMP-1 operate redundantly to promote TIM-3 expression on $CD8^+$ T cells. BLIMP-1 levels increase during chronicity while T-BET expression falls.(123, 162) On the other hand, $IL2R\beta$-signals sustained BLIMP-1 expression in exhausted $CD8^+$ T cells *in vivo* likely through activation of STAT5 (**Article 1**, Fig. 4*I* and **Article 2**, Fig 4*F*).(312, 651) These observations strongly indicate that IL-2/15-STAT5-mediated BLIMP-1 expression supplants T-BET to sustain TIM-3 expression on $CD8^+$ T cells during the chronic phase of infection (**Figure. 16**).



<u>Figure. 16</u>: Regulation of 2B4 and Tim-3 expression by IL-2 and IL-15. Unique and/or overlapping pathways might contribute to the IL-2/15-mediated induction of 2B4 and Tim-3 expression on CD8^{+} T cells. 1 IL-2/15-induced STAT5 might direct the transcription of yet unidentified TF(s) that subsequently trigger 2B4 and TIM-3 expression. 2 STAT5 could also directly promote 2B4 and TIM-3 expression and/or establish permissive epigenetic marks on their respective genomic sequences. 3 Blimp-1 functions as a transcriptional repressor. De facto, its impact might involve repression of regulatory genes responsible for the silencing 2B4 and TIM-3 expression.

1.4- 2B4 and Tim-3 expression on secondary effectors

The direct control of 2B4 and TIM-3 expression by IL-2 and IL-15 is interesting in many ways. A previous study in mice has demonstrated that conventional memory CD8^{+} T cells challenged with a chronic viral infection exhaust more rapidly than naïve cells and are quickly eliminated.(214) This dramatic effect likely explains some therapeutic issues in the treatment of chronic diseases notably the poor efficacy of cell-

mediated immunity vaccines against chronic viral infections (HIV).(652) In their study, *West et al* demonstrate that levels of 2B4 and TIM-3 but not PD-1 and LAG-3 are selectively magnified on secondary effectors (derived from memory $CD8^+$ T cells) compared to primary effectors (derived from naïve $CD8^+$ T cells) and genetic ablation of 2B4 partially restores their maintenance.(214) Hence, 2B4 (and likely also TIM-3) possess the unique ability to rapidly terminate potent secondary T cell responses after challenge with a chronic viral strain. By challenging P14 $IL2R\beta^{-/-}$ memory cells (generated after resolution of an acute LCMV Arm infection), we showed that IL-2 and IL-15 largely contributed to the massive induction of 2B4 and TIM-3 on secondary effectors (**Appendix. 4**). Hence, control of 2B4 and TIM-3 expression by IL-2 and IL-15 not only exhausts primary effectors more severely but causes the rapid eradication of secondary effectors arising from fully competent memory cells.(649) Controlling IL-2 and IL-15-dependent signals might help to design memory $CD8^+$ T cells capable of protecting the host during chronic viral infections. This strategy should definitively be tested in mice but also in experimental models closer to the human pathology. For example, SIV-infected non-human primates (NHP) are a reliable predictive model of vaccine efficacy against HIV. Several constructions of SIV T-cell based vaccine have been developed. Combining these vaccination protocols with IL-2 and/or IL-15 blockade could result in the development of competent memory cells capable of performing robust secondary responses and overcoming 2B4-mediated deletion. Subsequent challenge of vaccinated animals and monitoring of SIV-specific responses in groups treated or not with IL-2/15 blocking antibodies should be a good approach to evaluate vaccine efficacy.

1.5- Three classes of inhibitory receptors

While IL-2 and IL-15 are critical for the induction of 2B4 and TIM-3, the impact of these cytokines on other inhibitory receptors is more modest.(649) *In vivo*, IL2Rβ-deficiency reduced PD-1, LAG-3 and CD160 expression on $CD8^+$ T cells at day 35 p.i. (**Article 1**, Fig. 5*E* and S5*C*).(649) However, this difference in expression resulted from the blockade of $PD\text{-}1^{hi}$ cell development in the absence of IL2Rβ-signals. Indeed, $PD\text{-}1^{hi}$ cells expressed higher levels of PD-1, LAG-3 and CD160 compared to $PD\text{-}1^{int/lo}$ cells

(**Article 1**, Fig. 4*A*, S4*A* and S5*F*).(49, 649) LAG-3 and CD160 levels were equivalent between P14 and P14 IL2Rβ$^{-/-}$ PD-1$^{int/lo}$ effectors demonstrating that IL-2 and IL-15 had little impact on their expression *in vivo* (**Article 1**, Fig. 5*G*). *In vitro*, TCR signals alone were sufficient to promote maximal levels of PD-1 and LAG-3 on CD8^{+} T cells (day 2 of culture) while IL-2 and IL-15 acted as supportive signals to maintain their expression thereafter (**Article 1**, Fig. 5*H*). This is reminiscent of the fact that PD-1 mainly relies on TCR-signals (and downstream NFATc1) for induction but that its expression is supported by cytokines in vitro (e.g., IL-6 and IL-12).(120, 122) Requirements for CD160 expression on CD8^{+} T cells are two-sided. TCR signals promoted substantial levels of CD160 *in vitro* (day 2 of culture) but IL-2 and IL-15 were required to magnify its expression thereafter (**Article 1**, Fig. 5*H*). Conversely, 2B4 and TIM-3 expression were mainly controlled by IL-2 and IL-15 *in vivo* and *in vitro (***Article 1**, Fig. 5*E-F* and *I*) (649) Previous studies also support this dichotomy in the regulation of inhibitory receptors expression. Indeed, T-BET represses PD-1, CD160 and LAG-3 but not 2B4 expression on CD8^{+} T cells and conversely promotes TIM-3 induction.(123) In addition, Blimp-1 deficiency on CD8^{+} T cells precludes 2B4 induction, strikingly reduces TIM-3 and CD160 while PD-1 and LAG-3 are less affected (**Appendix. 2**).(162) Hence, inhibitory receptors require specific and inherent signals for optimal induction on CD8^{+} T cells. Based on these requirements, we define three classes of inhibitory receptors with graded dependency over TCR and/or cytokines signals for their expression (**Figure. 17**).

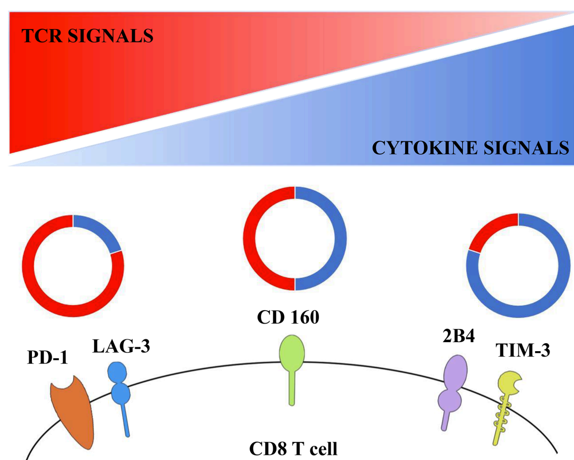


Figure. 17: Classification of inhibitory receptors based on their induction requirements. PD-1 and LAG-3 expression is mainly driven by TCR signals. CD160 expression is efficiently triggered by TCR signals but cytokines magnify its expression *in vitro*. 2B4 and TIM-3 expression are mainly induced by cytokines (e.g. IL-2 and IL-15).

1.6- Perspectives

From the assertions presented on this section, some points remain unclear. First, the relative importance of IL-2 versus IL-15 in the maintenance of 2B4 and TIM-3 during chronicity is uncertain. Our data strongly point to a selective role of IL-15 during that time frame but IL-2-signals during priming might also facilitate their expression during chronicity. Second, BLIMP-1 appears to have a central role in both 2B4 and TIM-3 expression on $CD8^+$ T cells, particularly during chronicity when it reaches maximal level.(162) However, BLIMP-1 behaves as a transcriptional repressor. Then it is unlikely that BLIMP-1 directly promotes 2B4 and TIM-3 expression in $CD8^+$ T cells. One possibility is that BLIMP-1 represses key regulators of 2B4 and TIM-3 expression allowing STAT5-induced TF(s) and/or STAT5 itself to bind to their promoter regions and activate transcription. STATs family of proteins has recently been shown to modify the epigenetic landscape of lineage specific genes in T cells.(356) Thus, STAT5 might be required to fully unlock 2B4 and TIM-3 promoter regions while BLIMP-1 might repress the expression of transcriptional regulators (**Figure. 16**).

II- Acquisition and maintenance of effector functions

During a chronic viral infection, exhausted $CD8^+$ T cell effector functions are severely compromised including production of cytokines (IFNγ, TNFα, IL-2), Ag-driven proliferation, killing ability and survival potential (see Introduction section 1.2.3).(2, 60, 106, 110) Our data show that the impact of IL-2 and IL-15 on $CD8^+$ T cell effector functions during a chronic viral infection is dichotomous and biphasic. During the priming phase of infection, IL2Rβ-dependent signals sustained the acquisition of full effector functions (IFNγ production, GRZB) by primary effectors (**Appendix. 5**, Fig. 3).(67) However, this effect was reversed during chronicity and some functions that are dramatically lost during exhaustion (IFNγ production, Ag-driven proliferation)(2, 106) were better preserved without IL-2 and IL-15-signals (**Article 1**, Fig. 5*B-D* and S5*A* and *B*).(67, 649) However, the absence of GRZB without IL2Rβ-dependent signals during

chronicity suggested decreased cytolytic potential. These functions of IL-2 and IL-15 are discussed below.

2.1- Priming phase

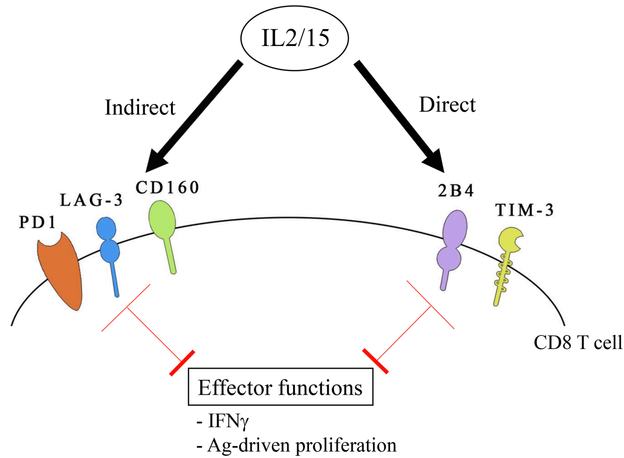
During the priming phase of a chronic viral infection, IL2Rβ-dependent signals on CD8$^+$ T cells supported the production of IFNγ and the expression of the cytotoxic molecule GRZB while being dispensable for degranulation (**Appendix. 5**, Fig. 3).(67) Reduced IFNγ production in the absence of IL2Rβ-dependent signals contrasted with previous observations (including by our group) demonstrating that IL-2 and IL-15 are dispensable to initiate IFNγ production on effector CD8$^+$ T cells during an acute viral infection (**Appendix. 5**, Fig. 2*A*).(67, 314, 627) However, IL-2 induces IFNγ production in developing Th1 CD4$^+$ T cells in a JAK3/STAT5 dependent-manner.(653) *In vitro*, IL2Rβ-signals on CD8$^+$ T cells increase IFNγ protein and mRNA levels.(311, 654) The TF Eomes, a direct target of IL-2 and IL-15-dependent signals also directly promotes IFNγ expression in CD8$^+$ T cells.(282) Hence, IL2Rβ-dependent signals are potent inducers of IFNγ production in CD8$^+$ T cells but at physiological level their impact on this precise function is limited and/or compensated during an acute viral infection. Thus, we evidence a context-specific role for IL2Rβ-dependent signals in supporting IFNγ production by CD8$^+$ T cells during the priming phase of a chronic viral infection (**Appendix. 5**, Fig. 3*A* and *B*).(67)

A number of IL2Rβ-induced TFs (BLIMP-1, STAT5, EOMES) directly or indirectly promote GRZB expression in CD8$^+$ T cells.(30, 35, 286, 288, 355, 651) In acute infection models, IL2Rβ-deficiency largely decreases GRZB induction in developing effectors.(67, 654, 655) We found that similar to acute infection settings, IL2Rβ-dependent signals chiefly drive the initial GRZB expression in effector CD8$^+$ T cells during a chronic viral infection. (**Appendix. 5**, Fig. 3*D*).(67) However, suboptimal induction of IFNγ and GRZB on IL2Rβ-deficient effectors had little impact on their ability to eliminate target cells *in vivo* (**Appendix. 5**, Fig 3*E*). Together, IL2Rβ-

dependent signals sustain IFNγ and GRZB expression during the priming phase of a chronic viral infection but absence of these signals minimally affects killing potential.

2.2- Chronic phase

We show that IL2Rβ-dependent signals accentuate $CD8^+$ T cell dysfunctions during chronicity.(649) Ag-specific $CD8^+$ T cells retained better IFNγ production capacity (but not TNFα and IL-2) and higher proliferative potential to antigenic stimulation at day 35p.i. in the absence of IL2Rβ-signals (**Article 1**, Fig. 5*B*-*D* and S5*A* and *B*).(649) These observations contrasted with the stimulatory functions which are usually assigned to IL-2 and IL-15. One logical explanation is that IL-2 and IL-15 indirectly cause these dysfunctions through up-regulation of several inhibitory receptors on $CD8^+$ T cells. IL2Rβ-dependent signals magnify PD-1, LAG-3, CD160, 2B4 and TIM-3 levels on $CD8^+$ T cells during chronicity (**Article 1**, Fig. 5*E* and *F* and S5*C*). Previous studies demonstrated that PD-L1 (PD-1 ligand) blockade *in vivo* restores IFNγ production and $CD8^+$ T cell proliferation. These effects are amplified by co-blockade of PD-L1 and LAG-3.(49, 106) 2B4 and TIM-3 are virtually absent on IL2Rβ-deficient $CD8^+$ T cells. Both receptors have been implicated in the loss of IFNγ production and proliferative potential of exhausted $CD8^+$ T cells in mice and patients with HIV, HBV or HCV.(49, 110, 210, 213, 226, 227) Inhibitory receptors cooperate to silence T cell functions during a chronic viral infection and the co-expression of several of these receptors at the cell surface causes higher dysfunctions.(49, 50) We showed that IL2Rβ-dependent signals fostered the co-expression of multiple inhibitory receptors on $CD8^+$ T cells during chronicity (**Article 1**, Fig. 5*A*). This phenomenon likely participates in the aggravation of T cell dysfunctions.(49) Together, we ascribe new regulatory functions of IL-2 and IL-15 during a chronic viral infection. By promoting the expression of several inhibitory receptors, IL-2 and IL-15 instigate a regulatory loop that severely compromises cardinal functions of effector $CD8^+$ T cells during Ag-persistence (**Figure. 18**).



<u>Figure. 18</u>: Regulation of effector functions by IL-2 and IL-15 during a chronic viral infection. By directly (2B4, TIM-3) and indirectly (PD-1, LAG-3 and CD160) promoting inhibitory receptors expression, IL-2 and IL-15 aggravate the silencing of effector $CD8^+$ T cell functions during a chronic viral infection.

Another point to consider is the heterogeneity of the $CD8^+$ T cell pool that accumulated at day 35 p.i. in the presence or absence of IL2Rβ-dependent signals. IL2Rβ-deficiency abrogated the development of highly exhausted $PD\text{-}1^{hi}$ cells (**Article 1**, Fig. 4*A* and *B* and S4*A*).(649) De facto, IL2Rβ-deficient effectors were mainly (92±1%) $PD\text{-}1^{int/lo}$ progenitors whereas IL2Rβ-proficient cells were composed of both $PD\text{-}1^{int/lo}$ and $PD\text{-}1^{hi}$ effectors (56±4% versus 35±5% respectively) (**Article 1**, Fig S4*A*). $PD\text{-}1^{hi}$ terminal effectors produce limited amounts of cytokines and barely proliferate to Ag compared to $PD\text{-}1^{int/lo}$ progenitors.(269, 649) Thus, superior functionality of IL2Rβ-deficient cells might have resulted from the absence of $PD\text{-}1^{hi}$ cells development. However, several points indicate otherwise. First of all, when examined thoroughly, $PD\text{-}1^{hi}$ and $PD\text{-}1^{int/lo}$ effectors secrete similar amount of IFNγ and only differ in their capacity to make TNFα.(269) Second, even if the frequency of $PD\text{-}1^{int/lo}$ cells was largely decreased within P14 controls compared to P14 $IL2R\beta^{-/-}$ cells (56±4% versus 92±1% respectively) their absolute number remained equivalent at day 35p.i. (**Article 1**, Fig. 4*B* and S4*A*). Yet, P14 controls were still unable to proliferate to Ag in accordance with previous study(106) whereas P14 $IL2R\beta^{-/-}$ cells performed several rounds of division (**Article 1**, Fig. 5*D* and S5*B*). <u>Together, these observations indicate that IL2Rβ-dependent signals intrinsically worsen $CD8^+$ T cell effector functions independently of their role in promoting terminal differentiation onto $PD\text{-}1^{hi}$ effectors.</u>

Similar to our observations during the priming phase of infection, we showed a critical defect in the expression of the cytolytic molecule GRZB on $CD8^+$ T cells lacking the IL2Rβ-chain during chronicity (**Article 1**, Fig. 4*G*). Such defect likely resulted from the lowered induction of BLIMP-1 observed in the absence of IL2Rβ-dependent signals (**Article 1**, Fig. 4*G*). Indeed, BLIMP-1 expression is critical to support GRZB production by $CD8^+$ T cells during the chronic phase of infection.(162) We also propose that reduced BLIMP-1 levels in IL2Rβ-deficient $CD8^+$ T cells precluded the development of PD-1^{hi} cells (discussed below) that despite been more severely exhausted retain higher GRZB expression compared to PD-$1^{int/lo}$ cells.(269) Thereby, lower BLIMP-1 expression and subsequent impaired terminal differentiation likely precluded optimal production of GRZB in the absence of IL2Rβ-dependent signals. However, BLIMP-1-deficiency does not completely abrogate GRZB expression by exhausted $CD8^+$ T cells while this cytolytic molecule remained nearly undetectable in IL2Rβ-deficient cells (**Article 1**, Fig 4*G*).(162) This suggests that IL-2 and/or IL-15 use BLIMP-1-dependent and -independent routes (e.g. STAT5)(355, 651) to support GRZB expression in $CD8^+$ T cells during the chronic phase of infection. Together, we demonstrate that IL2Rβ-dependent signals mediate dichotomous functions in the maintenance of $CD8^+$ T cell effector functions during chronicity. IL2Rβ-dependent signals first aggravate $CD8^+$ T cells dysfunctions through up-regulation of inhibitory receptors but these signals also sustain GRZB expression. These findings might represent a regulatory mechanism elaborated to prevent overwhelming T cell responses in conditions of prolonged exposure to Ag.

III- Development of PD-1^{hi} terminal effectors

As discussed in section 3.2.2 of the introduction, exhausted $CD8^+$ T cells are not a homogenous population. Rather two subtypes and lineage related effectors so called PD-$1^{int/lo}$ (T-bet^{hi}) and PD-1^{hi} ($Eomes^{hi}$) cells cooperate to contain the infection, the former being the progenitor of the later.(269) The discovery of these populations is relatively recent and the factors involved in the dynamic transition of PD-$1^{int/lo}$ progenitors into PD-1^{hi} terminal effectors remain to be defined. During this thesis, we unraveled a central role

for IL2Rβ-dependent signals in the development of PD-1^{hi} cells. Our results are discussed below.

3.1- IL2Rβ-dependent signals are dispensable for CD8^{+} T cells proliferation and survival during chronicity

Our results showed that IL2Rβ-deficiency on CD8^{+} T cells selectively abrogated PD-1^{hi} cells development during chronicity without altering the number of PD-1$^{int/lo}$ cells (**Article 1**, Fig. 4*A* and *B* and S4*A*).(649) Based on these findings we first considered that IL2Rβ-dependent signals might be mandatory for the survival of PD-1^{hi} terminal effectors during a chronic viral infection. However, several points excluded this possibility. Firstly, we barely detected any PD-1^{hi} cells in the absence of IL2Rβ-dependent signals during chronicity (day 35p.i.) whereas P14 IL2Rβ$^{-/-}$ cells and P14 controls contracted equivalently between day 8 and 35p.i. (**Article 1**, Fig. 4*A* and *B* and S4*A* and *D*). Moreover, lower numbers of P14 IL2Rβ$^{-/-}$ cells observed at day 35p.i. resulted from an altered proliferation at the peak of the response (day 8p.i.) rather than a selective loss of PD-1^{hi} cells during chronicity (**Article 1**, Fig S4*B* and *C*). These results strongly argued toward a defective development rather than a selective loss of PD-1^{hi} cells in the absence of IL-2 and IL-15-dependent signals. Secondly, CD122 levels were particularly elevated on PD-1^{hi} cells compared to their PD-1$^{int/lo}$ counterpart (**Article 1**, Fig. 2*B*). Conversely PD-1^{hi} cells expressed the lowest amounts of the IL-15-inducible survival factor BCL2 compared to PD-1$^{int/lo}$ cells, that molecule even being nearly absent in the former. (**Article 1**, Fig. 4*C*).(649) Bcl2 expression even increased in CD8^{+} T cells lacking the IL2Rβ-chain (**Article 1**, Fig. 4*C*).(649) These results excluded the possibility of an IL-15-Bcl2 axis governing PD-1^{hi} cells survival during a chronic viral infection in contrast to the major role of IL-15-induced BCL2 expression in the survival of SLECs during an acute viral infection.(67, 69, 70) Previous studies also reported extremely low levels of BCL2 in exhausted CD8^{+} T cells that mainly rely on Ag for survival.(19, 20) Together, our data indicate that IL2Rβ-deficiency hampers the development rather than the survival of PD-1^{hi} terminal effectors during a chronic viral infection.

The development of PD-1^{hi} cells relies on extensive proliferative events initiated at the PD-1$^{int/lo}$ state.(269) During an acute viral infection, IL-2 and IL-15 sustain the late expansion of effector CD8^{+} T cells and IL-15 is mandatory for the proliferative renewal of memory cells (**Appendix. 5**, Fig. 1*B*).(67, 107, 261, 313, 626, 645, 655) Then we explored the possibility that IL2Rβ-deficiency might arrest the proliferation of exhausted CD8^{+} T cells during chronicity resulting in a defective development of PD-1^{hi} cells. The absence of IL2Rβ-dependent signals on CD8^{+} T cells did not affect the overall proliferation of CD8^{+} T cells during the chronic phase of infection (**Article 1**, Fig. 4*E*).(649) Nevertheless, we showed a slight decrease in the proliferation rate of P14 IL2Rβ$^{-/-}$ cells compared to P14 controls at day 35p.i. (**Article 1**, Fig. 4*F*).(649) Previous studies demonstrated that exhausted CD8^{+} T cells proliferate *in vivo* in response to exogenous IL-2 provided during chronicity.(111) Accordingly, we showed that exhausted CD8^{+} T cells were responsive to IL-2 and IL-15-dependent signals during the chronic phase as assessed by their capacity to trigger STAT5 phosphorylation *ex vivo* (**Article 1**, Fig. 1*D* and S1*B* and **Article 2**. Fig. 4*B* and *C*).(649) Hence, exhausted CD8^{+} T cells retain the capacity to expand *in vivo* in response to IL2Rβ-dependent signals. However, at physiological level, IL-2 and IL-15 only played a modest role in driving CD8^{+} T cells proliferation during chronicity (**Article 1**, Fig. 4*E* and *F*).(649) Interestingly, *Paley et al* demonstrated that gp33-specific PD-1^{hi} and PD-1$^{int/lo}$ cells stop proliferating after transfer in mice chronically infected with the LCMV Cl-13 variant V35A (this variant generates a chronic viral infection in mice similar to the parental Cl-13 strain but specifically lacks the gp33 epitope).(269) This demonstrates that the inflammatory milieu alone is not sufficient to sustain CD8^{+} T cell proliferation but Ag-dependent signals have a dominant role in this process.(269) Collectively, IL2Rβ-dependent signals modestly supported CD8^{+} T cell proliferation during chronicity but were not mandatory in the process. In the absence of IL2Rβ-dependent signals, a substantial fraction of CD8^{+} T cells still proliferated but these effectors did not convert into PD-1^{hi} cells (**Article 1**, Fig. 4*A*, *B*, *E* and *F* and S4*A*). We then conclude that IL2Rβ-dependent signals arrest PD-1^{hi} cell development independently of their slight impact on CD8^{+} T cell proliferation. Of note, abrogation of IL-2-dependent signals alone did not impact CD8^{+} T cell proliferation during a chronic viral infection (**Article 2**, Fig. S1*B*). This suggests that the lack of IL-2

is compensated by IL-15 *in vivo* or that IL-15 selectively sustains $CD8^+$ T cell proliferation during chronicity.

3.2- IL2Rβ-dependent signals instigate $CD8^+$ T cell terminal differentiation during chronicity

In the previous section, we show that the absence of PD-1^{hi} cells without IL2Rβ-dependent signals is not caused by an altered proliferation or survival defect. Our data rather indicate a new role for IL-2 and/or IL-15 in directing the differentiation of PD-1^{hi} terminal effectors during a chronic viral infection.(649) This conclusion challenges previous work which showed that Ag-dependent signals and EOMES are the main actors in PD-1^{hi} cells development.(269) As mentioned, Ag-dependent signals but also EOMES promote $CD8^+$ T cell proliferation during chronicity that subsequently leads to the generation of PD-1^{hi} cells.(269) But it remains undefined whether these factors instigate a terminal differentiation program in proliferating effectors.(269) In acute infection settings, the "decreasing potential" model supports the notion that prolonged exposure to antigenic signals enforces $CD8^+$ T cells terminal differentiation (albeit that model does not rule out the involvement of other signals).(43) It remains a possibility that prolonged antigenic signals also sustain PD-1^{hi} terminal differentiation in synergy with IL2Rβ-dependent signals during a chronic viral infection. However, PD-1^{hi} cells failed to develop without IL2Rβ-signals despite high viral loads (**Article 1**, Fig 4*A*, *B*, S4*A* and S5*E*).(649) In the absence of IL2Rβ-dependent signals, EOMES levels in exhausted $CD8^+$ T cells were conserved and even slightly superior compared to control (**Article 1**, Fig. 4*H*).(649) Hence, combination of Ag-dependent signals and EOMES are not sufficient to trigger PD-1^{hi} cells development in the absence of IL2Rβ-dependent signals.(649) These observations further emphasize the central role of IL2 and IL-15 in directing $CD8^+$ T cells terminal differentiation during a chronic viral infection.

3.3- A new model of PD-1^{hi} terminal effector development

Similar to PD-1, we demonstrated that IL2Rβ expression on $CD8^+$ T cells increased during the transition from PD-1$^{int/lo}$ to PD-1^{hi} cells (**Article 1**, Fig. 2*D* and *E*).(649) Both TCR-signals and EOMES (that directly binds to the IL2Rβ promoter region) promote IL2Rβ expression on $CD8^+$ T cells.(286) Further, PD-1^{hi} cells development is characterized by a coordinated increase in EOMES and IL2Rβ expression (**Article 1**, Fig. 2*B*).(269) Thus, beside their role in promoting $CD8^+$ T cell proliferation, Ag-dependent signals and/or EOMES likely magnify IL2Rβ-expression on proliferating effectors during chronicity. Expression of the transcriptional repressor BLIMP-1 also increases during the conversion onto PD-1^{hi} cells.(162) IL-2 is a key inducer of BLIMP-1 and IL2Rβ-deficiency reduced BLIMP-1 levels in exhausted $CD8^+$ T cells during chronicity (**Article 1**, Fig. 4*I*).(311) A previous study demonstrated that IL-15 is a poor inducer of BLIMP-1 in $CD8^+$ T cells *in vitro*.(311) However, IL-2 and IL-15 trigger a similar transcriptional program in $CD8^+$ T cells and the impact of IL-15 on BLIMP-1 expression *in vivo* during a chronic viral infection is unknown.(68) The IL-2-BLIMP-1 axis is critical to drive SLECs terminal differentiation in acute infection settings and BLIMP-1 is a central TF involved in $CD8^+$ T cells terminal differentiation and exhaustion.(30, 33, 35, 162) As mentioned earlier, STAT5 is a direct inducer of BLIMP-1 and we showed that STAT5 inhibition *in vitro* restrained BLIMP-1 expression in $CD8^+$ T cells (**Article 2** , Fig. 4*F*). Together, we propose a new model for the generation of PD-1^{hi} cells during a chronic viral infection. Firstly, Ag-dependent signals and EOMES trigger PD-1$^{int/lo}$ cell proliferation and likely increase IL2Rβ expression at the cell surface. Finally, IL2Rβ-dependent signals direct the terminal differentiation of proliferating effectors into PD-1^{hi} cells through a STAT5-BLIMP-1 axis (**Figure. 19**).

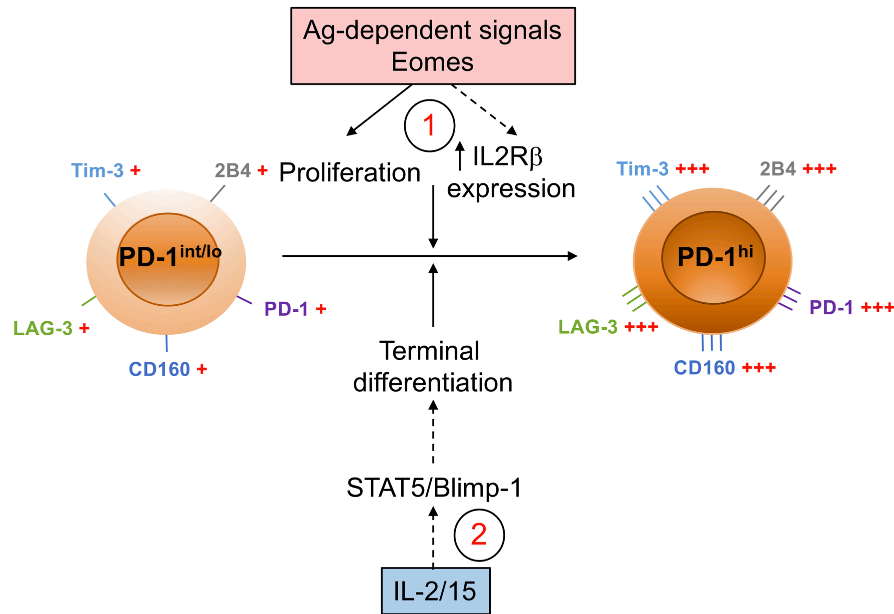


Figure. 19: IL-2 and IL-15 direct PD-1^{hi} cells terminal differentiation. Our data suggest that the development of PD-1^{hi} cells is multifactorial and biphasic. 1 Ag-dependent signals and Eomes promote PD-1$^{int/lo}$ cells proliferation and probably increase CD122 (IL2Rβ) expression on these effectors. 2 IL2Rβ-dependent signals fulfill the terminal differentiation of proliferating effectors into PD-1^{hi} cells likely through a STAT5/BLIMP-1 axis.

3.4- Therapeutic potential

Our findings demonstrate that IL2Rβ-dependent signals on CD8^{+} T cells direct the terminal differentiation of PD-1^{hi} cells opens new perspectives for the treatment of chronic viral infections and cancers. Indeed, the conversion of PD-1$^{int/lo}$ progenitors into PD-1^{hi} cells has dramatic consequences for the establishment of long-term immune protection. Firstly, this transition is terminal and associated with a global increase in exhaustion severity notably a higher level of several inhibitory receptors, a loss of proliferative potential and most particularly a shorter lifespan that precludes long-term persistence of these effectors *in vivo*.(49, 60, 269, 649) Secondly, prolonged periods of Ag exposure gradually erode the PD-1$^{int/lo}$ subset leading to a preferential accumulation of PD-1^{hi} cells and ultimately a complete loss of CD8^{+} T cell responses. This was notably

evidenced in patients with chronic HCV.(269) In cancer patients, relapse after cessation of αPD-L1 therapy coincides with a massive conversion of $CD8^+$ T cells onto PD-1^{hi} ($Eomes^{hi}$) terminal effectors.(636) In contrast, PD-1^{hi} cells conserve higher cytolytic potential compared to PD-$1^{int/lo}$ cells despite been more severely exhausted.(649) Hence, tempering (but not abrogating) IL2Rβ-dependent signals might be a relevant approach to slow down the conversion into PD-1^{hi} cells and prolonged immune protection while preserving cytolytic potential. Treating patients at relevant time-points with the newly described janus kinase-3 inhibitor Tofacitinib (safely used for the treatment of rheumatoid arthritis)(656) might be an interesting therapeutic avenue to explore. For example, providing Tofacitinib to cancer patients during and/or after PD-1 blockade might prevent the massive terminal differentiation of responding $CD8^+$ T cells (often associated with cancer relapse) and provide memory development advantage. Our results suggest that BLIMP-1 is central for PD-1^{hi} cells development.(649) BLIMP-1-deficiency restrains $CD8^+$ T cells cytotoxicity consistent with a lack of PD-1^{hi} cells development and delays viral clearance.(162) However, BLIMP-1 haploinsufficiency conserves $CD8^+$ T cells cytotoxic potential, reduces their exhaustion and accelerates viral clearance. This result further emphasizes our conviction that tempering IL2Rβ-dependent signals on $CD8^+$ T cells and subsequently BLIMP-1 induction might be beneficial for the control of chronic viral infections.

3.5- Perspectives

On this subject, several points need to be fully explored. Firstly, we must define if the differentiation into PD-1^{hi} cells results from a combined impact of IL-2 and IL-15 or if IL-15 alone mainly controls this conversion. The fact that PD-1^{hi} cells developed normally in the absence of IL-2-dependent signals demonstrates that IL-15 alone is sufficient to direct their differentiation (**Article 2**, Fig. 2*A*, *B* and S1*D*). Examining PD-1^{hi} cells development in IL-15KO mice will provide a definitive answer to this question. Given the central role of BLIMP-1 on $CD8^+$ T cell terminal differentiation and exhaustion,(30, 35, 162) we propose that this TF mainly controls PD-1^{hi} cells development.(649) However, this must be proven using a mouse model carrying a $CD8^+$

T cell-restricted deletion of BLIMP-1. We would expect to observe a defective differentiation of PD-1^{hi} cells in these mice mirroring the results we observed with the IL2Rβ-deficient model. Finally, since STAT5 directly modulates BLIMP-1 expression in CD8^{+} T cells, we postulate that IL2Rβ-dependent signals instigate CD8^{+} T cell terminal differentiation through a STAT5-BLIMP-1 axis. This point remains to be fully demonstrated. A first step would be to observe PD-1^{hi} cells development in P14 cells retrovirally transduced with a constitutively active form of STAT5. If our assumption is correct, we would expect to observe an increase expression of BLIMP-1 accompanied by an accelerated development of PD-1^{hi} cells. In a second time, we could perform similar experiments in BLIMP-1-deficient P14 cells. The aim would be to demonstrate that constitutive STAT5 activation requires BLIMP-1 to accelerate PD-1^{hi} cells development. These experiments will definitively prove that the differentiation of PD-1^{hi} cells during a chronic viral infection rely on an IL-2/15>STAT5>BLIMP-1 axis.

IV- Development of memory CD8^{+} T cells

The complete loss of developmental plasticity towards competent memory cells is another dramatic consequence of CD8^{+} T cell exhaustion during a chronic viral infection.(19, 20) Reversing and/or preventing such outcome is of first importance to ensure long-term immune protection in patients with chronic viral infections. Recent advances in the field underline that this process is progressive and might involve repressive epigenetic modifications.(51, 104, 105) However, the causative factors of such memory developmental arrest that could represent relevant therapeutic targets needs to be identified. During this thesis, we demonstrate for the first time that IL-2 and IL-15 coordinately suppress memory CD8^{+} T cell development during a chronic viral infection and that these cytokines are the main actors in the process. Our main results are discussed below.

4.1- IL2Rβ-dependent signals preclude memory CD8+ T cell differentiation during a chronic viral infection

4.1.1- Persistent antigenic stimulation versus IL2Rβ-dependent signals

Previous studies demonstrated that cardinal markers of memory CD8+ T cell (e.g. CD127 and CD62L) are critically repressed during a chronic viral infection.(19, 20, 48) Our first striking observation was that in the absence of IL-2 and IL-15-dependent signals, a substantial fraction of CD8+ T cells re-express CD127 in the midst of the chronic phase (day35p.i.) despite heightened viral loads (**Article 1**, Fig. 6*A*, S5*E* and S6*A*).(649) At day 65p.i., when LCMV Cl-13 virus was cleared from most tissues except kidneys (**Article 1**, Fig. S6*B*), a large majority of IL2Rβ-deficient CD8+ T cells had re-expressed CD127 and a fraction even converted into a Tcm phenotype (**Article 1**, Fig. 6*A*-*C* and S6*A*).(649) These data demonstrate that persistent antigenic stimulation is not the primary cause of memory developmental arrest that often occurs during a chronic viral infection. Rather, IL2Rβ-dependent signals are the main instigators of such defect.

Importantly, we provide evidence that the restoration of memory CD8+ T cell development in the absence of IL2Rβ-dependent signals results from a bias of differentiation rather than a selective loss of CD127-negative cells over time. First, we demonstrated that at day 65p.i., IL2Rβ-deficiency resulted in a superior number of Tcm cells in spite of a 2.4 fold reduction in the total number of P14 IL2Rβ$^{-/-}$ cells compared to P14 controls at this time point (**Article 1**, Fig. 6*C* and S4*B*).(649) Similarly, IL2Rα-deficiency also generated more Tcm cells compared to P14 controls despite equivalent numbers of cells at day 60 p.i. (**Article 2**, Fig. 1*A* and 2*E*). Globally, P14 controls failed to generate Tcm cells in accordance with previous observations (19, 20, 48) and this population only differentiated in the absence of either the IL2Rα or the IL2Rβ-chain (**Article 1**, Fig. 6*B* and *C* and **Article 2**, Fig. 2*D* and *E*).(649) Secondly, at day 65p.i., we demonstrated that Tem cells generated within P14 and P14 IL2Rβ$^{-/-}$ cell populations were not similar.(649) IL2Rβ-deficient Tem expressed higher levels of CD127 and the pro-

memory TF BCL6 (**Article 1**, Fig. 6*E*). This demonstrates that even if a small fraction of Tem are generated within P14 controls, these cells do not reach a state of memory differentiation as advanced as that of P14 IL2Rβ-deficient Tem. Finally, previous studies clearly define that exhausted $CD8^+$ T cells mainly rely on Ag rather than homeostatic signals (provided by IL-7 and IL-15) for survival.(19, 20) Together, we demonstrate that IL2Rβ-deficiency causes a bias of differentiation that reinstates a memory developmental program in exhausted $CD8^+$ T cells during a chronic viral infection.

4.1.2- Tcm development

In our first article, we demonstrate that the combined absence of IL-2 and IL-15-dependent signals preserved Tcm developmental potential in a substantial fraction of Ag-specific $CD8^+$ T cells (**Article 1**, Fig. 6*B* and *C*). In similar settings, specific abrogation of IL-2-dependent signals alone also restored Tcm differentiation in nearly similar (albeit slightly reduced) proportions compared to the IL2Rβ-deficient model (Compare **Article 1**, Fig. 6*B* and **Article 2**, Fig 2*D*). These results clearly demonstrate that i) IL2Rβ-dependent signals mainly instigate the arrested development of Tcm cells during a chronic viral infection and ii) IL-2 has a dominant role over IL-15 on this particular defect. Despite the apparent limited role of IL-15 in Tcm developmental arrest, it remains to be defined whether Tcm generated in the absence of either the IL2Rα-chain or the IL2Rβ-chain are the exact same cells. In addition, examining the development of Tcm in IL15KO mice will be necessary to definitively rule out a contribution of IL-15 in Tcm developmental defect during a chronic viral infection.

As mentioned before, IL-2-dependent signals were limited to the priming phase of infection (**Article 1**, Fig. 1*A* and **Article 2**, Fig. 2*A* and *B*).(649) This suggests that IL-2-dependent signals instigate an “anti-Tcm” developmental program in $CD8^+$ T cells during the priming phase of infection (**Figure. 20**). This assertion contrasts with previous work which demonstrated that loss of memory potential during a chronic viral infection is progressive and fully acquired only from day 30 p.i. onward.(51) Work by *Angelosanto et al* also demonstrates that signals beyond day 8 p.i. also alter memory $CD8^+$ T cell

development albeit the authors did not thoroughly examine Tcm differentiation and limited their observations to CD127 expression.(51) From these evidences, two possibilities subsist i) by day 8 p.i., chronically stimulated CD8 T cells have already lost their developmental plasticity towards Tcm because of IL-2-dependent signals (but are still able to re-express CD127) or ii) IL-2 instigate the "anti-Tcm" program on $CD8^+$ T cells during priming but that program requires a prolonged chronic environment to persist and to become irreversible. In support of the first hypothesis, early after initiation of an acute LCMV infection, effector $CD8^+$ T cells with distinct cell fate can be identified based on their expression level of the IL2Rα-chain.(31) Adoptive transfer experiments into infection matched recipients revealed that $IL2R\alpha^{hi}$ effectors (sorted early by day 3.5 p.i.) present a defective differentiation into Tcm cells after viral elimination compared to their $IL2R\alpha^{lo}$ counterpart.(31) This demonstrates that even in a "pro-memory" acute infection context, IL-2-dependent signals provided early after infection restrain $CD8^+$ T cells memory developmental plasticity after viral clearance. Further experiments will be necessary to determine whether a similar "programming" occurs during a chronic viral infection. A relevant approach would be to block IL-2-dependent signals during the priming phase of infection (using blocking antibodies against CD25) and determine if such treatment is sufficient to rescue Tcm cells development.

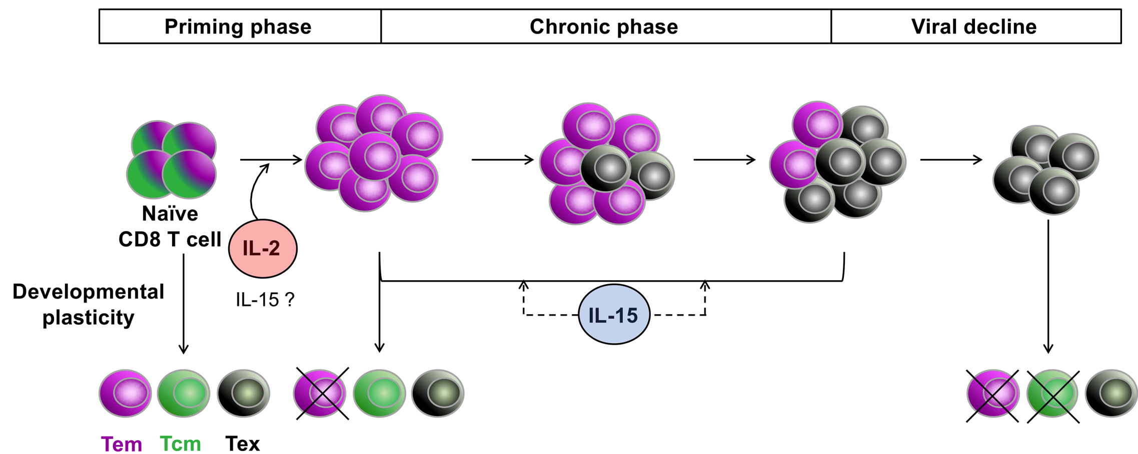


Figure. 20: Gradual loss of memory $CD8^+$ T cell potential. During the priming phase of a chronic viral infection, IL-2 suppresses $CD8^+$ T cells developmental plasticity toward Tcm.

During the chronic phase, prolonged exposure to IL-15 further preclude Tem cell developmental potential. Such developmental failures are irreversible and fully exhausted (Tex) $CD8^+$ T cells remain incapable to differentiate into either Tcm or Tem even after viral decline.

4.1.3- Tem development

We demonstrated that in the absence of IL2Rβ-dependent signals, nearly all $CD8^+$ T cells that failed to convert into Tcm cells presented a $CD62L^-CD127^+$ Tem phenotype at day 65 p.i. whereas the vast majority of P14 controls remained $CD62L^-CD127^-$ (Tex) (**Article 1**, Fig. 6*B*).(649) IL2Rβ-deficient Tem expressed higher CD127 and Bcl6 compared to the few Tem cells generated within P14 controls (**Article 1**, Fig. 6*E*).(649) This demonstrates that IL2Rβ-deficiency confers a developmental advantage to that population during a chronic viral infection. In contrast, IL2Rα-deficiency did not rescue Tem cell development (**Article 2**, Fig. 2*D-E* and S1*E*). This shows that IL-15-dependent signals alone are sufficient to impede Tem cell development during a chronic viral infection. As mentioned above chronically stimulated $CD8^+$ T cells gradually lose their ability to re-express CD127 during chronicity a time-period during which only IL-15 was produced (no IL-2) (**Article 2**, Fig. 3).(51) Together, our results very much indicate that prolonged exposure to IL-15 during chronicity is necessary to abrogate Tem cells development during a chronic viral infection (**Figure. 20**). On the other hand, we cannot at this point exclude the possibility that IL-2 and IL-15 act redundantly during priming to imprint an "anti-Tem" program on $CD8^+$ T cells. But even in the case, our data clearly demonstrate that IL-15 substantiate the lack of IL-2 to silence Tem development. Hence, IL-15 has clearly a dominant role over IL-2 in the repression of Tem differentiation during a chronic viral infection. Further studies using IL-15KO mice will definitively enlighten this point. A relevant experiment would be to transfer P14 $CD8^+$ T cells at day 8p.i. into match-infected WT and IL-15KO recipients and examine Tem development at day 60p.i.. If our assumption is correct, we would expect to observe a rescued Tem development in IL-15KO but not WT recipients. This result would definitively demonstrate that IL-15-dependent signals during chronicity selectively arrest Tem cell development.

4.2- Transcriptional control of memory differentiation arrest during a chronic viral infection

Our data showed that IL2Rβ-dependent signals played a critical role in controlling the relative expression of BLIMP-1 and BCL6 during a chronic viral infection tipping the balance towards terminal differentiation while precluding memory development.(649) BLIMP-1 supports $CD8^+$ T cell terminal differentiation while BCL6 sustains Tcm development and both TFs directly antagonize each other's expression.(30, 35, 657, 658) BLIMP-1-deficient $CD8^+$ T cells are able to re-express CD127 and even CD62L during a chronic viral infection in mice.(162) In our IL2Rβ-deficient model, BLIMP-1 expression was reduced in $CD8^+$ T cells at day 35 p.i. while BCL6 levels increased by day 65 p.i. (**Article 1**, Fig, 4*I* and 6*D*).(649) Growing amounts of literature also evidenced that STAT5 negatively regulates memory $CD8^+$ T cell differentiation.(355, 650, 651, 659, 660) STAT5 directly promotes BLIMP-1 expression in $CD8^+$ T cells and conversely decreases BCL6.(312, 650, 651) In $CD4^+$ T cells, STAT5 inhibits Tfh cells differentiation by increasing BLIMP-1 levels at the expense of BCL6.(310) Finally, we demonstrated that STAT5 was the dominant pathways by which IL-2 and IL-15 signaled in exhausted $CD8^+$ T cells and STAT5 inhibition *in vitro* decreased BLIMP-1 levels but enhanced BCL6 expression (**Article 2**, Fig. 4*A-C* and *F*). Together we propose that IL2Rβ-dependent signals and downstream STAT5 activation arrest memory $CD8^+$ T cells development during a chronic viral infection by tipping the balance in favor of BLIMP-1 at the expense of BCL6.

4.3- Antigen addiction versus IL-7-mediated survival

CD8 T cells committing to severe exhaustion irreversibly loose cell-surface expression of homeostatic cytokine receptor-chains including the IL2Rβ-chain (CD122) and the IL7Rα-chain (CD127) required for IL-15 and IL-7-dependent signaling respectively (**Article 1**, Fig. 1*C* and 6*A*).(649) De facto, exhausted $CD8^+$ T cells become unable to sense homeostatic cytokines and develop an Ag-addiction for survival.(20)

IL2Rβ-deficient memory cells not only re-expressed CD127 but responded to IL-7 *ex vivo* (**Article 1**, Fig. 6*F*).(649) Rescued IL-7 sensitivity coincided *in vivo* with increased levels of the pro-survival molecule BCL2 required for memory $CD8^+$ T cell survival (**Article 1**, Fig. 6*G*).(259, 649) STAT5 is well-known at inducing BCL2 in $CD8^+$ T cells, we conclude that IL2Rβ-deficient memory cells bypass the typical Ag-addiction of exhausted cells and restore an IL-7/STAT5/BCL2 survival axis. However, that point must still be formally addressed by monitoring the maintenance of IL2Rβ-deficient memory cells in an Ag-free environment.

4.4- Therapeutic interests

Current therapeutic trials using αPD-L1 blocking Abs in mice with chronic viral infections encounter two major problematics. First, $CD8^+$ T cells that expand after PD-L1 blockade tend to become more terminally exhausted after cessation of treatment.(104) Secondly, αPD-L1 treatment fails to reinstate memory $CD8^+$ T cells development even when they are combined with repetitive IL-7 infusions.(104) Hence, combining αPD-L1 therapy with well-timed injections of Tofacitinib (JAK3 inhibitor) could represent a powerful approach to i) prevent uncontrolled terminal differentiation and ii) preserve a pool of competent memory cells in patients with chronic viral infections. Infusion of CAR T cells in cancer patients has become a major approach for the treatment of cancers notably B-cell malignancies. Several groups are now trying to apply that technology for the treatment of chronic viral infections. However, several parameters restrain CAR T cell therapy effectiveness including i) the early exhaustion of these cells and ii) their limited capacity to maintain in the host and develop long-term immunological memory. Tempering IL2Rβ-dependent signals in CAR T cells could slow-down their exhaustion, restrain their conversion toward short-lived terminal effectors without altering cytotoxic potential and might preserve memory plasticity. Together, our results answer real clinical issues and provide salient opportunities to improve actual immunotherapeutic approaches to cure human cancers and chronic viral infections.

4.5- Perspectives

An important point to assess is whether memory CD8$^+$ T cells generated in the absence of IL2Rβ-dependent signals are fully functional and efficiently contain a secondary viral burst. A second key question is if these memory cells are maintained in the host in the absence of Ag. This is extremely important regarding actual issues in HIV patients treated with highly active antiretroviral therapy (HAART). In these patients, HAART-induced viral decline coincides with a decrease number of Ag-specific CD8$^+$ T cells that rely on Ag for survival. Another unexplored question is whether IL2Rβ-dependent signals also affect the development of memory CD8$^+$ T cells in peripheral tissue (notably the mucosa). Recently, a population of resident memory T cells (Trm; CD69^{hi}CD103^{hi}) localized in peripheral tissues and mucosa was described in mice.(661, 662) This non-recirculating population of memory CD8$^+$ T cells provides a first-line on-site protection against invading pathogens (e.g. HSV).(663) The development of Trm cells was shown to be partly dependent on IL-15 and TGF-β in acute infection systems.(664, 665) However, how this population evolves during a chronic viral infection and how IL-15 affects its development remains to be defined. These perspectives will be important questions to answer in the future.

V- Conclusion, significance and future directions

T cell exhaustion represents the most recently evidenced differentiation program of CD8$^+$ T cells and tremendous work is still needed to fully elucidate the causative factors and molecular events governing such process. Reversing and/or preventing CD8$^+$ T cell exhaustion has become the primary aim of numerous recent immunotherapeutic regimens against human cancers and chronic viral infections. Our lack of knowledge regarding the causative factors of CD8$^+$ T cell exhaustion impedes the improvement of novel immunotherapeutic approaches. Given that exhaustion establishment in CD8$^+$ T cells requires persistent antigenic stimulation, Ag-dependent signals have been considered the primary cause of CD8$^+$ T cell exhaustion. Hence, involvement of other

factors and particularly cytokines has been neglected. For the first time our study provides evidence that cytokines (i.e. IL-2 and IL-15) control distinct aspects of $CD8^+$ T cell exhaustion.

During this thesis, we define IL2Rβ (CD122) as a new reliable marker of severe exhaustion and terminal differentiation in LCMV-infected mice and patients with chronic HCV. Using IL2Rβ-deficient $CD8^+$ T cells, we also demonstrate that IL-2 and IL-15-dependent signals control key aspects of CD8 T cell exhaustion including i) the induction of a specific pattern of inhibitory receptors (i.e. 2B4 and TIM-3), ii) the terminal differentiation of PD-1^{hi} terminal effectors and iii) the arrested development of memory $CD8^+$ T cells (**Figure. 21**). We also define that IL-2 and IL-15 display overlapping and individual functions in these processes. And, we provide evidences that IL-2 and IL-15 mediate their functions mainly through activation of the TF STAT5. Together our study adds to our understanding on the development of $CD8^+$ T cell exhaustion during a chronic viral infection and reinstates cytokines as a primary cause of such process.

As discussed in this last section, our results and previous work greatly indicate that IL-2 and IL-15 mediate their action through a STAT5-BLIMP-1 axis.(162, 649) Defining whether STAT5 only functions to promote BLIMP-1 expression or displays additional BLIMP-1-independent functions will be an interesting point to decipher. The pro-exhaustion functions of IL-2 and IL-15 contrast with the protective role of IL-21, another member of the γ_c-family of cytokines.(345-347) During a chronic viral infection, cell-intrinsic IL-21-dependent signals are essential for the maintenance of $CD8^+$ T cell responses.(345) In vitro, IL-21 confers an opposing transcriptional program to that of IL-2 and IL-15 in $CD8^+$ T cells.(295) This cytokine restrains terminal differentiation but sustains memory development and our data clearly show that during a chronic viral infection, IL-2 and IL-15 promote $CD8^+$ T cell terminal differentiation at the expense of memory development.(295, 649) IL-21 primarily signals through STAT3 while IL-2 and IL-15 preferentially trigger STAT5. As opposed to STAT5, STAT3 sustains memory $CD8^+$ T cell development.(355, 650, 651, 666) Finally, IL-21 restrains IL2Rβ-expression in $CD8^+$ T cells.(295, 667) Hence, IL-21 might not only counteract IL-2/15-dependent

signals but could also temper IL2Rβ expression on $CD8^+$ T cells. Therefore, the rate of $CD8^+$ T cell terminal exhaustion during a chronic viral infection could be dictated by a balance between IL-2/15-STAT5 and IL-21-STAT3-dependent signals. Examining the reciprocal action of these cytokines in the development of $CD8^+$ T cell exhaustion will be an interesting point to uncover in the future. Particularly, the impact of IL-21 in the development of PD-$1^{int/lo}$ progenitors and PD-1^{hi} terminal effectors remains unanswered.(345-347) Comparing the role of IL-21 in the development of these populations might reveal opposing functions compared to IL-2 and IL-15. This should highlight novel information regarding the involvement of cytokines in the dynamic of PD-$1^{int/lo}$ progenitors and PD-1^{hi} terminal effectors.

Altogether the data presented during this thesis suggest that tempering IL-2 and/or IL-15-dependent signals during a chronic viral infection can be beneficial in several ways. A next step would be to refine the window of opportunity for such therapeutic intervention in patients. One major issue to achieve this goal in mouse is that the viral course of LCMV differs drastically compared to human chronic viral infections. For example, while LCMV and HIV trigger very similar immunological processes, the viral kinetic of these viruses is very different. LCMV Cl-13 causes a persistent infection with heightened viral titers for months before being kept under control by the immune system. HIV primary infection is also controlled in humans but the virus persists in a latent mode in reservoir cells and gradually re-emerged over the years [of note, other determinants including the genetic composition, the replicative strategy and the mechanism of infection vary between HIV and LCMV]. A similar issue is to consider for HCV infections that are characterized by several episodes of viral flare-ups. Therefore, additional experimental models closer to the human pathology and more predictive should be used to determine accurate windows of opportunity to target IL-2 and/or IL-15. The use of rhesus macaques infected with SIV would be a relevant option to consider.

Finally, the work realized during that thesis enlightens new functions of IL-2 and IL-15 in directing several key aspects of $CD8^+$ T cell exhaustion during a chronic viral infection. Our results also represent a great advancement in the field as we

demonstrate for the first time that cytokines play a major role in the development of $CD8^+$ T cell exhaustion challenging the previous dogma that persistent Ag-dependent signals were the main culprits. That result is quite important as it might explain why IL-2 treatment (provided to HIV patients during the late 90's) did nor produced the expected results. Restraining $CD8^+$ T cell terminal differentiation and restoring memory development represent the main challenge of actual immunotherapeutic strategies including immune checkpoint blockade and engineered T cells. Until now such goal could not be achieved as we ignored the causative factors of such developmental issues. Our results identified IL-2/IL-15 and downstream STAT5 as the main regulators of $CD8^+$ T cell terminal exhaustion and memory developmental arrest during a chronic viral infection. We are convinced that this discovery will open novel avenues to improve actual immunotherapeutic regimens against chronic viral infections and cancers. We also hope that this work will encourage future research regarding the role of cytokines on the T cell exhaustion process.

Finally, I hope you have appreciated reading this thesis.

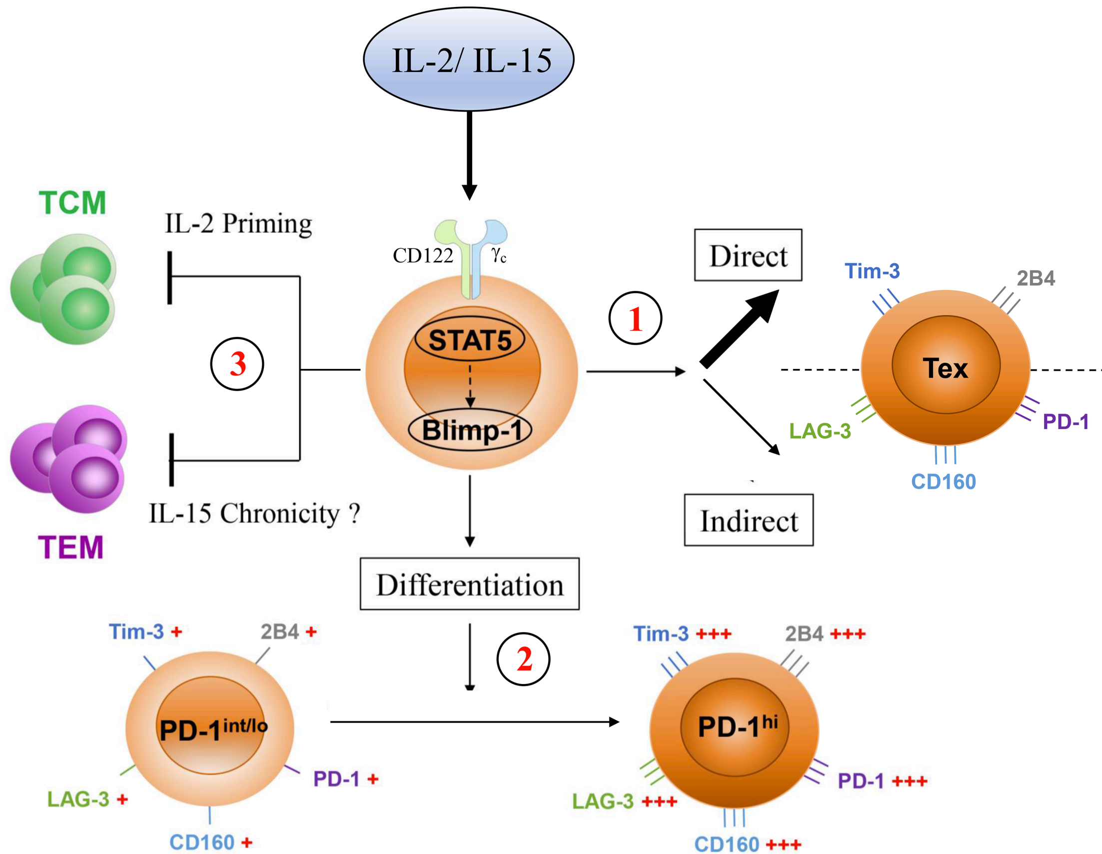


Figure. 21: Conclusion scheme: Impact of IL-2 and IL-15 on CD8$^+$ T cell responses during a chronic viral infection. During a chronic viral infection, selective maintenance of the IL2Rβ-chain (in addition to basal levels of IL2Rγ-chain) allows IL-2 and IL-15 to signal on responding CD8$^+$ T cells. These cytokines selectively trigger STAT5 activation and likely the transcriptional repressor BLIMP-1. Through that axis, IL-2 and IL-15 control several aspects of CD8$^+$ T cell exhaustion. 1 IL-2 and IL-15 directly and redundantly control the expression of 2B4 and TIM-3 on CD8$^+$ T cells. These cytokines also indirectly increase the expression of PD-1, LAG-3 and CD160. 2 IL-2 and IL-15-dependent signals also instigate the terminal differentiation of PD-1^{hi} terminal effectors likely in a BLIMP-1 manner. 3 Finally, IL-2 and IL-15 cooperate to suppress the developmental plasticity of CD8$^+$ T cells to memory. IL-2-dependent signals during priming preclude Tcm differentiation while continuous IL-15-dependent signals are likely required to extinguish Tem development.

References

1. Gilbert SC (2012) T-cell-inducing vaccines - what's the future. *Immunology* 135(1):19-26.
2. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, & Ahmed R (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77(8):4911-4927.
3. Ahmed R, Salmi A, Butler LD, Chiller JM, & Oldstone MB (1984) Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J Exp Med* 160(2):521-540.
4. Zhou X, Ramachandran S, Mann M, & Popkin DL (2012) Role of lymphocytic choriomeningitis virus (LCMV) in understanding viral immunology: past, present and future. *Viruses* 4(11):2650-2669.
5. Rivers TM & Scott TF (1936) Meningitis in Man Caused by a Filterable Virus : Ii. Identification of the Etiological Agent. *J Exp Med* 63(3):415-432.
6. Traub E (1936) The Epidemiology of Lymphocytic Choriomeningitis in White Mice. *J Exp Med* 64(2):183-200.
7. Traub E (1936) Persistence of Lymphocytic Choriomeningitis Virus in Immune Animals and Its Relation to Immunity. *J Exp Med* 63(6):847-861.
8. Traub E (1936) An Epidemic in a Mouse Colony Due to the Virus of Acute Lymphocytic Choriomeningitis. *J Exp Med* 63(4):533-546.
9. Muckenfuss RSA, C; Webster, L (1934) Etiology of the 1933 epidemic of encephalitis. *J. Am. Med. Assoc* (
).
10. Matloubian M, Somasundaram T, Kolhekar SR, Selvakumar R, & Ahmed R (1990) Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. *J Exp Med* 172(4):1043-1048.
11. Matloubian M, Concepcion RJ, & Ahmed R (1994) CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 68(12):8056-8063.
12. Wherry EJ*, et al.* (2003) Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4(3):225-234.
13. Buchmeier MJ, Welsh RM, Dutko FJ, & Oldstone MB (1980) The virology and immunobiology of lymphocytic choriomeningitis virus infection. *Adv Immunol* 30:275-331.
14. Oldstone MB & Dixon FJ (1967) Lymphocytic choriomeningitis: production of antibody by "tolerant" infected mice. *Science* 158(3805):1193-1195.
15. Benson L & Hotchin J (1969) Antibody formation in persistent tolerant infection with lymphocytic choriomeningitis virus. *Nature* 222(5198):1045-1047.
16. Marker O & Volkert M (1973) Studies on cell-mediated immunity to lymphocytic choriomeningitis virus in mice. *J Exp Med* 137(6):1511-1525.
17. Matloubian M, Kolhekar SR, Somasundaram T, & Ahmed R (1993) Molecular determinants of macrophage tropism and viral persistence: importance of single

amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *J Virol* 67(12):7340-7349.
18. Zajac AJ*, et al.* (1998) Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188(12):2205-2213.
19. Wherry EJ, Barber DL, Kaech SM, Blattman JN, & Ahmed R (2004) Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A* 101(45):16004-16009.
20. Shin H, Blackburn SD, Blattman JN, & Wherry EJ (2007) Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J Exp Med* 204(4):941-949.
21. Villarete L, Somasundaram T, & Ahmed R (1994) Tissue-mediated selection of viral variants: correlation between glycoprotein mutation and growth in neuronal cells. *J Virol* 68(11):7490-7496.
22. Dockter J, Evans CF, Tishon A, & Oldstone MB (1996) Competitive selection in vivo by a cell for one variant over another: implications for RNA virus quasispecies in vivo. *J Virol* 70(3):1799-1803.
23. Klepper A & Branch AD (2015) Macrophages and the Viral Dissemination Super Highway. *EC Microbiol* 2(3):328-336.
24. Bonhomme CJ, Knopp KA, Bederka LH, Angelini MM, & Buchmeier MJ (2013) LCMV glycosylation modulates viral fitness and cell tropism. *PLoS One* 8(1):e53273.
25. Joshi NS & Kaech SM (2008) Effector CD8 T cell development: a balancing act between memory cell potential and terminal differentiation. *J Immunol* 180(3):1309-1315.
26. Butz EA & Bevan MJ (1998) Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* 8(2):167-175.
27. Kaech SM*, et al.* (2003) Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4(12):1191-1198.
28. Joshi NS*, et al.* (2007) Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27(2):281-295.
29. Wiesel M*, et al.* (2012) Type-I IFN drives the differentiation of short-lived effector CD8+ T cells in vivo. *Eur J Immunol* 42(2):320-329.
30. Rutishauser RL*, et al.* (2009) Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31(2):296-308.
31. Kalia V*, et al.* (2010) Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity* 32(1):91-103.
32. Mathieu C*, et al.* (2015) IL-2 and IL-15 regulate CD8(+) memory T-cell differentiation but are dispensable for protective recall responses. *Eur J Immunol* 45(12):3324-3338.
33. Boulet S, Daudelin JF, & Labrecque N (2014) IL-2 Induction of Blimp-1 Is a Key In Vivo Signal for CD8+ Short-Lived Effector T Cell Differentiation. *J Immunol*.

34. Sarkar S, *et al.* (2008) Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med* 205(3):625-640.
35. Kallies A, Xin A, Belz GT, & Nutt SL (2009) Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity* 31(2):283-295.
36. Yang CY, *et al.* (2011) The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets. *Nat Immunol* 12(12):1221-1229.
37. Badovinac VP, Porter BB, & Harty JT (2002) Programmed contraction of CD8(+) T cells after infection. *Nat Immunol* 3(7):619-626.
38. Bouillet P & O'Reilly LA (2009) CD95, BIM and T cell homeostasis. *Nat Rev Immunol* 9(7):514-519.
39. Kaech SM & Cui W (2012) Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol* 12(11):749-761.
40. Masopust D, Vezys V, Marzo AL, & Lefrancois L (2001) Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291(5512):2413-2417.
41. Sallusto F, Lenig D, Forster R, Lipp M, & Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401(6754):708-712.
42. Mazo IB, *et al.* (2005) Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. *Immunity* 22(2):259-270.
43. Kaech SM & Wherry EJ (2007) Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* 27(3):393-405.
44. Homann D, Teyton L, & Oldstone MB (2001) Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat Med* 7(8):913-919.
45. Sprent J, Cho JH, Boyman O, & Surh CD (2008) T cell homeostasis. *Immunology and cell biology* 86(4):312-319.
46. Mueller SN & Ahmed R (2009) High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 106(21):8623-8628.
47. Beltra JC & Decaluwe H (2016) Cytokines and persistent viral infections. *Cytokine* 82:4-15.
48. Wherry EJ, *et al.* (2007) Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27(4):670-684.
49. Blackburn SD, *et al.* (2009) Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10(1):29-37.
50. Odorizzi PM & Wherry EJ (2012) Inhibitory receptors on lymphocytes: insights from infections. *J Immunol* 188(7):2957-2965.
51. Angelosanto JM, Blackburn SD, Crawford A, & Wherry EJ (2012) Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. *J Virol* 86(15):8161-8170.
52. van der Most RG, *et al.* (1997) Uncovering subdominant cytotoxic T-lymphocyte responses in lymphocytic choriomeningitis virus-infected BALB/c mice. *J Virol* 71(7):5110-5114.

53. van der Most RG, *et al.* (1998) Identification of Db- and Kb-restricted subdominant cytotoxic T-cell responses in lymphocytic choriomeningitis virus-infected mice. *Virology* 240(1):158-167.
54. Zhou S, Ou R, Huang L, & Moskophidis D (2002) Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. *J Virol* 76(2):829-840.
55. Ou R, Zhou S, Huang L, & Moskophidis D (2001) Critical role for alpha/beta and gamma interferons in persistence of lymphocytic choriomeningitis virus by clonal exhaustion of cytotoxic T cells. *J Virol* 75(18):8407-8423.
56. Rosenberg ES, *et al.* (1997) Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 278(5342):1447-1450.
57. Bowen DG & Walker CM (2005) Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 436(7053):946-952.
58. Lopes AR, *et al.* (2008) Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection. *J Clin Invest* 118(5):1835-1845.
59. Battegay M, *et al.* (1994) Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. *J Virol* 68(7):4700-4704.
60. Blackburn SD, Shin H, Freeman GJ, & Wherry EJ (2008) Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc Natl Acad Sci U S A* 105(39):15016-15021.
61. Sprent J & Tough DF (2001) T cell death and memory. *Science* 293(5528):245-248.
62. Krammer PH (2000) CD95's deadly mission in the immune system. *Nature* 407(6805):789-795.
63. Strasser A, Harris AW, Huang DC, Krammer PH, & Cory S (1995) Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *The EMBO journal* 14(24):6136-6147.
64. Strasser A (2005) The role of BH3-only proteins in the immune system. *Nat Rev Immunol* 5(3):189-200.
65. Kulinski JM, Tarakanova VL, & Verbsky J (2013) Regulation of antiviral CD8 T-cell responses. *Crit Rev Immunol* 33(6):477-488.
66. Decaluwe H, *et al.* (2010) Gamma(c) deficiency precludes CD8+ T cell memory despite formation of potent T cell effectors. *Proc Natl Acad Sci U S A* 107(20):9311-9316.
67. Mathieu C, *et al.* (2015) IL-2 and IL-15 regulate CD8+ memory T-cell differentiation but are dispensable for protective recall responses. *Eur J Immunol* 45(12):3324-3338.
68. Ring AM, *et al.* (2012) Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat Immunol* 13(12):1187-1195.
69. Yajima T, *et al.* (2006) IL-15 regulates CD8+ T cell contraction during primary infection. *J Immunol* 176(1):507-515.
70. Sanjabi S, Mosaheb MM, & Flavell RA (2009) Opposing effects of TGF-beta and IL-15 cytokines control the number of short-lived effector CD8+ T cells. *Immunity* 31(1):131-144.
71. Wang X (2001) The expanding role of mitochondria in apoptosis. *Genes & development* 15(22):2922-2933.

72. Green DR & Kroemer G (2004) The pathophysiology of mitochondrial cell death. *Science* 305(5684):626-629.
73. Newmeyer DD & Ferguson-Miller S (2003) Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* 112(4):481-490.
74. Green DR (2005) Apoptotic pathways: ten minutes to dead. *Cell* 121(5):671-674.
75. Kurtulus S*, et al.* (2011) Bcl-2 allows effector and memory CD8+ T cells to tolerate higher expression of Bim. *J Immunol* 186(10):5729-5737.
76. Hildeman DA*, et al.* (2002) Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity* 16(6):759-767.
77. Ashkenazi A & Dixit VM (1998) Death receptors: signaling and modulation. *Science* 281(5381):1305-1308.
78. Wallach D, Kovalenko AV, Varfolomeev EE, & Boldin MP (1998) Death-inducing functions of ligands of the tumor necrosis factor family: a Sanhedrin verdict. *Curr Opin Immunol* 10(3):279-288.
79. Pellegrini M, Belz G, Bouillet P, & Strasser A (2003) Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. *Proc Natl Acad Sci U S A* 100(24):14175-14180.
80. Fischer SF, Belz GT, & Strasser A (2008) BH3-only protein Puma contributes to death of antigen-specific T cells during shutdown of an immune response to acute viral infection. *Proc Natl Acad Sci U S A* 105(8):3035-3040.
81. Strasser A & Pellegrini M (2004) T-lymphocyte death during shutdown of an immune response. *Trends Immunol* 25(11):610-615.
82. Arnoult D*, et al.* (2003) Caspase-dependent and -independent T-cell death pathways in pathogenic simian immunodeficiency virus infection: relationship to disease progression. *Cell Death Differ* 10(11):1240-1252.
83. Wang C*, et al.* (2007) 4-1BBL induces TNF receptor-associated factor 1-dependent Bim modulation in human T cells and is a critical component in the costimulation-dependent rescue of functionally impaired HIV-specific CD8 T cells. *J Immunol* 179(12):8252-8263.
84. Tinoco R, Alcalde V, Yang Y, Sauer K, & Zuniga EI (2009) Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo. *Immunity* 31(1):145-157.
85. Grayson JM, Weant AE, Holbrook BC, & Hildeman D (2006) Role of Bim in regulating CD8+ T-cell responses during chronic viral infection. *J Virol* 80(17):8627-8638.
86. Badley AD, Pilon AA, Landay A, & Lynch DH (2000) Mechanisms of HIV-associated lymphocyte apoptosis. *Blood* 96(9):2951-2964.
87. de Oliveira Pinto LM, Garcia S, Lecoeur H, Rapp C, & Gougeon ML (2002) Increased sensitivity of T lymphocytes to tumor necrosis factor receptor 1 (TNFR1)- and TNFR2-mediated apoptosis in HIV infection: relation to expression of Bcl-2 and active caspase-8 and caspase-3. *Blood* 99(5):1666-1675.
88. Hughes PD*, et al.* (2008) Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity. *Immunity* 28(2):197-205.
89. Oldstone MB & Buchmeier MJ (1982) Restricted expression of viral glycoprotein in cells of persistently infected mice. *Nature* 300(5890):360-362.

90. Gallimore A, Dumrese T, Hengartner H, Zinkernagel RM, & Rammensee HG (1998) Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. *J Exp Med* 187(10):1647-1657.
91. Green DR (2008) Fas Bim boom! *Immunity* 28(2):141-143.
92. Strasser A, Jost PJ, & Nagata S (2009) The many roles of FAS receptor signaling in the immune system. *Immunity* 30(2):180-192.
93. Lenardo MJ (1991) Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 353(6347):858-861.
94. Zheng L, Trageser CL, Willerford DM, & Lenardo MJ (1998) T cell growth cytokines cause the superinduction of molecules mediating antigen-induced T lymphocyte death. *J Immunol* 160(2):763-769.
95. Lenardo M*, et al.* (1999) Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. *Annu Rev Immunol* 17:221-253.
96. Boehme SA & Lenardo MJ (1993) Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. *Eur J Immunol* 23(7):1552-1560.
97. Esser MT*, et al.* (1997) IL-2 induces Fas ligand/Fas (CD95L/CD95) cytotoxicity in CD8+ and CD4+ T lymphocyte clones. *J Immunol* 158(12):5612-5618.
98. Owen-Schaub LB, Yonehara S, Crump WL, 3rd, & Grimm EA (1992) DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. *Cellular immunology* 140(1):197-205.
99. Dai Z, Arakelov A, Wagener M, Konieczny BT, & Lakkis FG (1999) The role of the common cytokine receptor gamma-chain in regulating IL-2-dependent, activation-induced CD8+ T cell death. *J Immunol* 163(6):3131-3137.
100. Chinai JM*, et al.* (2015) New immunotherapies targeting the PD-1 pathway. *Trends Pharmacol Sci* 36(9):587-595.
101. Wherry EJ (2011) T cell exhaustion. *Nat Immunol* 12(6):492-499.
102. Wherry EJ & Kurachi M (2015) Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15(8):486-499.
103. Fuller MJ & Zajac AJ (2003) Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol* 170(1):477-486.
104. Pauken KE*, et al.* (2016) Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. *Science*.
105. Sen DR*, et al.* (2016) The epigenetic landscape of T cell exhaustion. *Science*.
106. Barber DL*, et al.* (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439(7077):682-687.
107. Becker TC*, et al.* (2002) Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med* 195(12):1541-1548.
108. Goldrath AW*, et al.* (2002) Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. *J Exp Med* 195(12):1515-1522.
109. Prlic M (2002) Multiple Choices: Regulation of Memory CD8 T Cell Generation and Homeostasis by Interleukin (IL)-7 and IL-15. *Journal of Experimental Medicine* 195(12):49F-52.

110. Jin HT, *et al.* (2010) Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 107(33):14733-14738.
111. West EE, *et al.* (2013) PD-L1 blockade synergizes with IL-2 therapy in reinvigorating exhausted T cells. *J Clin Invest* 123(6):2604-2615.
112. Day CL, *et al.* (2006) PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443(7109):350-354.
113. Boni C, *et al.* (2007) Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 81(8):4215-4225.
114. Urbani S, *et al.* (2006) PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 80(22):11398-11403.
115. Jurado JO, *et al.* (2008) Programmed death (PD)-1:PD-ligand 1/PD-ligand 2 pathway inhibits T cell effector functions during human tuberculosis. *J Immunol* 181(1):116-125.
116. Bhadra R, Gigley JP, Weiss LM, & Khan IA (2011) Control of Toxoplasma reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1-PDL-1 blockade. *Proc Natl Acad Sci U S A* 108(22):9196-9201.
117. Joshi T, Rodriguez S, Perovic V, Cockburn IA, & Stager S (2009) B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against Leishmania donovani infections. *PLoS Pathog* 5(5):e1000431.
118. Velu V, *et al.* (2009) Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 458(7235):206-210.
119. Ishida Y, Agata Y, Shibahara K, & Honjo T (1992) Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *The EMBO journal* 11(11):3887-3895.
120. Oestreich KJ, Yoon H, Ahmed R, & Boss JM (2008) NFATc1 regulates PD-1 expression upon T cell activation. *J Immunol* 181(7):4832-4839.
121. Kinter AL, *et al.* (2008) The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. *J Immunol* 181(10):6738-6746.
122. Austin JW, Lu P, Majumder P, Ahmed R, & Boss JM (2014) STAT3, STAT4, NFATc1, and CTCF regulate PD-1 through multiple novel regulatory regions in murine T cells. *J Immunol* 192(10):4876-4886.
123. Kao C, *et al.* (2011) Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat Immunol* 12(7):663-671.
124. Lu P, *et al.* (2014) Blimp-1 represses CD8 T cell expression of PD-1 using a feed-forward transcriptional circuit during acute viral infection. *J Exp Med* 211(3):515-527.
125. Riley JL (2009) PD-1 signaling in primary T cells. *Immunol Rev* 229(1):114-125.
126. Blackburn SD, *et al.* (2010) Tissue-specific differences in PD-1 and PD-L1 expression during chronic viral infection: implications for CD8 T-cell exhaustion. *J Virol* 84(4):2078-2089.
127. Yokosuka T, *et al.* (2012) Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med* 209(6):1201-1217.

128. Chemnitz JM, Parry RV, Nichols KE, June CH, & Riley JL (2004) SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 173(2):945-954.
129. Okazaki T, Maeda A, Nishimura H, Kurosaki T, & Honjo T (2001) PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci U S A* 98(24):13866-13871.
130. Parry RV*, et al.* (2005) CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Molecular and cellular biology* 25(21):9543-9553.
131. Kamphorst AO*, et al.* (2017) Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. *Science* 355(6332):1423-1427.
132. Quigley M*, et al.* (2010) Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. *Nat Med* 16(10):1147-1151.
133. Blank C*, et al.* (2004) PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. *Cancer Res* 64(3):1140-1145.
134. Iwai Y, Terawaki S, & Honjo T (2005) PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol* 17(2):133-144.
135. Pauken KE & Wherry EJ (2015) Overcoming T cell exhaustion in infection and cancer. *Trends Immunol* 36(4):265-276.
136. Brooks DG*, et al.* (2008) IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. *Proc Natl Acad Sci U S A* 105(51):20428-20433.
137. Vezys V*, et al.* (2011) 4-1BB signaling synergizes with programmed death ligand 1 blockade to augment CD8 T cell responses during chronic viral infection. *J Immunol* 187(4):1634-1642.
138. Petrovas C*, et al.* (2006) PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* 203(10):2281-2292.
139. Brahmer JR*, et al.* (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 366(26):2455-2465.
140. Topalian SL*, et al.* (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 366(26):2443-2454.
141. Odorizzi PM, Pauken KE, Paley MA, Sharpe A, & Wherry EJ (2015) Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted CD8+ T cells. *J Exp Med* 212(7):1125-1137.
142. Triebel F*, et al.* (1990) LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med* 171(5):1393-1405.
143. Bruniquel D, Borie N, & Triebel F (1997) Genomic organization of the human LAG-3/CD4 locus. *Immunogenetics* 47(1):96-98.
144. Huard B*, et al.* (1997) Characterization of the major histocompatibility complex class II binding site on LAG-3 protein. *Proc Natl Acad Sci U S A* 94(11):5744-5749.
145. Sierro S, Romero P, & Speiser DE (2011) The CD4-like molecule LAG-3, biology and therapeutic applications. *Expert Opin Ther Targets* 15(1):91-101.

146. Tian X*, et al.* (2015) The upregulation of LAG-3 on T cells defines a subpopulation with functional exhaustion and correlates with disease progression in HIV-infected subjects. *J Immunol* 194(8):3873-3882.
147. Chen N*, et al.* (2015) Lymphocyte activation gene 3 negatively regulates the function of intrahepatic hepatitis C virus-specific CD8+ T cells. *Journal of gastroenterology and hepatology* 30(12):1788-1795.
148. Woo SR*, et al.* (2012) Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res* 72(4):917-927.
149. Workman CJ, Dugger KJ, & Vignali DA (2002) Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. *J Immunol* 169(10):5392-5395.
150. Workman CJ & Vignali DA (2005) Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J Immunol* 174(2):688-695.
151. Hannier S, Tournier M, Bismuth G, & Triebel F (1998) CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. *J Immunol* 161(8):4058-4065.
152. Triebel F (2003) LAG-3: a regulator of T-cell and DC responses and its use in therapeutic vaccination. *Trends Immunol* 24(12):619-622.
153. Li N*, et al.* (2007) Metalloproteases regulate T-cell proliferation and effector function via LAG-3. *The EMBO journal* 26(2):494-504.
154. Workman CJ & Vignali DA (2003) The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *Eur J Immunol* 33(4):970-979.
155. Workman CJ*, et al.* (2004) Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. *J Immunol* 172(9):5450-5455.
156. Richter K, Agnellini P, & Oxenius A (2010) On the role of the inhibitory receptor LAG-3 in acute and chronic LCMV infection. *Int Immunol* 22(1):13-23.
157. Grosso JF*, et al.* (2007) LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest* 117(11):3383-3392.
158. He Y*, et al.* (2016) Lymphocyte-activation gene-3, an important immune checkpoint in cancer. *Cancer science* 107(9):1193-1197.
159. Cai G*, et al.* (2008) CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat Immunol* 9(2):176-185.
160. Anumanthan A*, et al.* (1998) Cloning of BY55, a novel Ig superfamily member expressed on NK cells, CTL, and intestinal intraepithelial lymphocytes. *J Immunol* 161(6):2780-2790.
161. Maiza H*, et al.* (1993) A novel 80-kD cell surface structure identifies human circulating lymphocytes with natural killer activity. *J Exp Med* 178(3):1121-1126.
162. Shin H*, et al.* (2009) A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31(2):309-320.
163. Tsujimura K*, et al.* (2006) Characterization of murine CD160+ CD8+ T lymphocytes. *Immunology letters* 106(1):48-56.

164. Le Bouteiller P, *et al.* (2002) Engagement of CD160 receptor by HLA-C is a triggering mechanism used by circulating natural killer (NK) cells to mediate cytotoxicity. *Proc Natl Acad Sci U S A* 99(26):16963-16968.
165. Maeda M, *et al.* (2005) Murine CD160, Ig-like receptor on NK cells and NKT cells, recognizes classical and nonclassical MHC class I and regulates NK cell activation. *J Immunol* 175(7):4426-4432.
166. Agrawal S, *et al.* (1999) Cutting edge: MHC class I triggering by a novel cell surface ligand costimulates proliferation of activated human T cells. *J Immunol* 162(3):1223-1226.
167. Barakonyi A, *et al.* (2004) Cutting edge: engagement of CD160 by its HLA-C physiological ligand triggers a unique cytokine profile secretion in the cytotoxic peripheral blood NK cell subset. *J Immunol* 173(9):5349-5354.
168. Nikolova MH, *et al.* (2005) The CD160+ CD8high cytotoxic T cell subset correlates with response to HAART in HIV-1+ patients. *Cellular immunology* 237(2):96-105.
169. Pombo C, Wherry EJ, Gostick E, Price DA, & Betts MR (2015) Elevated Expression of CD160 and 2B4 Defines a Cytolytic HIV-Specific CD8+ T-Cell Population in Elite Controllers. *The Journal of infectious diseases* 212(9):1376-1386.
170. Peretz Y, *et al.* (2012) CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. *PLoS Pathog* 8(8):e1002840.
171. Bengsch B, *et al.* (2010) Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog* 6(6):e1000947.
172. El-Far M, *et al.* (2014) CD160 isoforms and regulation of CD4 and CD8 T-cell responses. *J Transl Med* 12:217.
173. Tangye SG, Phillips JH, & Lanier LL (2000) The CD2-subset of the Ig superfamily of cell surface molecules: receptor-ligand pairs expressed by NK cells and other immune cells. *Seminars in immunology* 12(2):149-157.
174. Boles KS, Stepp SE, Bennett M, Kumar V, & Mathew PA (2001) 2B4 (CD244) and CS1: novel members of the CD2 subset of the immunoglobulin superfamily molecules expressed on natural killer cells and other leukocytes. *Immunol Rev* 181:234-249.
175. Davis SJ, Ikemizu S, Wild MK, & van der Merwe PA (1998) CD2 and the nature of protein interactions mediating cell-cell recognition. *Immunol Rev* 163:217-236.
176. Sidorenko SP & Clark EA (2003) The dual-function CD150 receptor subfamily: the viral attraction. *Nat Immunol* 4(1):19-24.
177. Mathew PA, *et al.* (1993) Cloning and characterization of the 2B4 gene encoding a molecule associated with non-MHC-restricted killing mediated by activated natural killer cells and T cells. *J Immunol* 151(10):5328-5337.
178. Boles KS, *et al.* (1999) Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens* 54(1):27-34.
179. Nakajima H & Colonna M (2000) 2B4: an NK cell activating receptor with unique specificity and signal transduction mechanism. *Hum Immunol* 61(1):39-43.
180. Brown MH, *et al.* (1998) 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med* 188(11):2083-2090.

181. Brown MH, Preston S, & Barclay AN (1995) A sensitive assay for detecting low-affinity interactions at the cell surface reveals no additional ligands for the adhesion pair rat CD2 and CD48. *Eur J Immunol* 25(12):3222-3228.
182. Latchman Y, McKay PF, & Reiser H (1998) Identification of the 2B4 molecule as a counter-receptor for CD48. *J Immunol* 161(11):5809-5812.
183. Nakajima H, Cella M, Langen H, Friedlein A, & Colonna M (1999) Activating interactions in human NK cell recognition: the role of 2B4-CD48. *Eur J Immunol* 29(5):1676-1683.
184. Colonna M, Nakajima H, & Cella M (1999) Inhibitory and activating receptors involved in immune surveillance by human NK and myeloid cells. *J Leukoc Biol* 66(5):718-722.
185. Valiante NM & Trinchieri G (1993) Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes. *J Exp Med* 178(4):1397-1406.
186. Schuhmachers G*, et al.* (1995) 2B4, a new member of the immunoglobulin gene superfamily, is expressed on murine dendritic epidermal T cells and plays a functional role in their killing of skin tumors. *J Invest Dermatol* 105(4):592-596.
187. Dhanji S & Teh HS (2003) IL-2-activated CD8+CD44high cells express both adaptive and innate immune system receptors and demonstrate specificity for syngeneic tumor cells. *J Immunol* 171(7):3442-3450.
188. Speiser DE*, et al.* (2001) The activatory receptor 2B4 is expressed in vivo by human CD8+ effector alpha beta T cells. *J Immunol* 167(11):6165-6170.
189. Garni-Wagner BA, Purohit A, Mathew PA, Bennett M, & Kumar V (1993) A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J Immunol* 151(1):60-70.
190. Chuang SS*, et al.* (2000) 2B4 stimulation of YT cells induces natural killer cell cytolytic function and invasiveness. *Immunology* 100(3):378-383.
191. Chuang SS, Kumaresan PR, & Mathew PA (2001) 2B4 (CD244)-mediated activation of cytotoxicity and IFN-gamma release in human NK cells involves distinct pathways. *J Immunol* 167(11):6210-6216.
192. Coffey AJ*, et al.* (1998) Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nature genetics* 20(2):129-135.
193. Tangye SG*, et al.* (1999) Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J Immunol* 162(12):6981-6985.
194. Sayos J*, et al.* (2000) Potential pathways for regulation of NK and T cell responses: differential X-linked lymphoproliferative syndrome gene product SAP interactions with SLAM and 2B4. *Int Immunol* 12(12):1749-1757.
195. Benoit L, Wang X, Pabst HF, Dutz J, & Tan R (2000) Defective NK cell activation in X-linked lymphoproliferative disease. *J Immunol* 165(7):3549-3553.
196. Nakajima H*, et al.* (2000) Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur J Immunol* 30(11):3309-3318.
197. Parolini S*, et al.* (2000) X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the

inability of natural killer cells to kill Epstein-Barr virus-infected cells. *J Exp Med* 192(3):337-346.
198. Tangye SG, Phillips JH, Lanier LL, & Nichols KE (2000) Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. *J Immunol* 165(6):2932-2936.
199. Stepp SE, Schatzle JD, Bennett M, Kumar V, & Mathew PA (1999) Gene structure of the murine NK cell receptor 2B4: presence of two alternatively spliced isoforms with distinct cytoplasmic domains. *Eur J Immunol* 29(8):2392-2399.
200. Schatzle JD*, et al.* (1999) Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4. *Proc Natl Acad Sci U S A* 96(7):3870-3875.
201. Lee KM*, et al.* (2004) 2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells. *J Exp Med* 199(9):1245-1254.
202. Mooney JM*, et al.* (2004) The murine NK receptor 2B4 (CD244) exhibits inhibitory function independent of signaling lymphocytic activation molecule-associated protein expression. *J Immunol* 173(6):3953-3961.
203. Chlewicki LK, Velikovsky CA, Balakrishnan V, Mariuzza RA, & Kumar V (2008) Molecular basis of the dual functions of 2B4 (CD244). *J Immunol* 180(12):8159-8167.
204. Waggoner SN & Kumar V (2012) Evolving role of 2B4/CD244 in T and NK cell responses during virus infection. *Frontiers in immunology* 3:377.
205. Castro AG*, et al.* (1999) Molecular and functional characterization of mouse signaling lymphocytic activation molecule (SLAM): differential expression and responsiveness in Th1 and Th2 cells. *J Immunol* 163(11):5860-5870.
206. Morra M*, et al.* (2001) Structural basis for the interaction of the free SH2 domain EAT-2 with SLAM receptors in hematopoietic cells. *The EMBO journal* 20(21):5840-5852.
207. Roncagalli R*, et al.* (2005) Negative regulation of natural killer cell function by EAT-2, a SAP-related adaptor. *Nat Immunol* 6(10):1002-1010.
208. Kambayashi T, Assarsson E, Chambers BJ, & Ljunggren HG (2001) Cutting edge: Regulation of CD8(+) T cell proliferation by 2B4/CD48 interactions. *J Immunol* 167(12):6706-6710.
209. Peritt D*, et al.* (1999) C1.7 antigen expression on CD8+ T cells is activation dependent: increased proportion of C1.7+CD8+ T cells in HIV-1-infected patients with progressing disease. *J Immunol* 162(12):7563-7568.
210. Raziorrouh B*, et al.* (2010) The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. *Hepatology* 52(6):1934-1947.
211. Ezinne CC, Yoshimitsu M, White Y, & Arima N (2014) HTLV-1 specific CD8+ T cell function augmented by blockade of 2B4/CD48 interaction in HTLV-1 infection. *PLoS One* 9(2):e87631.
212. Kroy DC*, et al.* (2014) Liver environment and HCV replication affect human T-cell phenotype and expression of inhibitory receptors. *Gastroenterology* 146(2):550-561.

213. Schlaphoff V, *et al.* (2011) Dual function of the NK cell receptor 2B4 (CD244) in the regulation of HCV-specific CD8+ T cells. *PLoS Pathog* 7(5):e1002045.
214. West EE, *et al.* (2011) Tight regulation of memory CD8(+) T cells limits their effectiveness during sustained high viral load. *Immunity* 35(2):285-298.
215. Kuchroo VK, Umetsu DT, DeKruyff RH, & Freeman GJ (2003) The TIM gene family: emerging roles in immunity and disease. *Nat Rev Immunol* 3(6):454-462.
216. van de Weyer PS, *et al.* (2006) A highly conserved tyrosine of Tim-3 is phosphorylated upon stimulation by its ligand galectin-9. *Biochemical and biophysical research communications* 351(2):571-576.
217. Anderson AC, *et al.* (2007) Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. *Science* 318(5853):1141-1143.
218. Zhu C, *et al.* (2005) The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 6(12):1245-1252.
219. Leitner J, *et al.* (2013) TIM-3 does not act as a receptor for galectin-9. *PLoS Pathog* 9(3):e1003253.
220. Monney L, *et al.* (2002) Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* 415(6871):536-541.
221. Sanchez-Fueyo A, *et al.* (2003) Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nat Immunol* 4(11):1093-1101.
222. Sabatos CA, *et al.* (2003) Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat Immunol* 4(11):1102-1110.
223. Chae SC, Park YR, Lee YC, Lee JH, & Chung HT (2004) The association of TIM-3 gene polymorphism with atopic disease in Korean population. *Hum Immunol* 65(12):1427-1431.
224. Chae SC, Park YR, Shim SC, Yoon KS, & Chung HT (2004) The polymorphisms of Th1 cell surface gene Tim-3 are associated in a Korean population with rheumatoid arthritis. *Immunology letters* 95(1):91-95.
225. Graves PE, Siroux V, Guerra S, Klimecki WT, & Martinez FD (2005) Association of atopy and eczema with polymorphisms in T-cell immunoglobulin domain and mucin domain-IL-2-inducible T-cell kinase gene cluster in chromosome 5 q 33. *J Allergy Clin Immunol* 116(3):650-656.
226. Jones RB, *et al.* (2008) Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 205(12):2763-2779.
227. Golden-Mason L, *et al.* (2009) Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol* 83(18):9122-9130.
228. Wu W, *et al.* (2012) Blockade of Tim-3 signaling restores the virus-specific CD8(+) T-cell response in patients with chronic hepatitis B. *Eur J Immunol* 42(5):1180-1191.
229. McMahan RH, *et al.* (2010) Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest* 120(12):4546-4557.

230. Sakhdari A, *et al.* (2012) Tim-3 negatively regulates cytotoxicity in exhausted CD8+ T cells in HIV infection. *PLoS One* 7(7):e40146.
231. Fourcade J, *et al.* (2010) Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* 207(10):2175-2186.
232. Sakuishi K, *et al.* (2010) Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 207(10):2187-2194.
233. Perkins D, *et al.* (1996) Regulation of CTLA-4 expression during T cell activation. *J Immunol* 156(11):4154-4159.
234. Takahashi T, *et al.* (2000) Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192(2):303-310.
235. Rudd CE, Taylor A, & Schneider H (2009) CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev* 229(1):12-26.
236. Egen JG, Kuhns MS, & Allison JP (2002) CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol* 3(7):611-618.
237. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, & Freeman GJ (2007) Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 27(1):111-122.
238. Kristiansen OP, Larsen ZM, & Pociot F (2000) CTLA-4 in autoimmune diseases--a general susceptibility gene to autoimmunity? *Genes and immunity* 1(3):170-184.
239. Ueda H, *et al.* (2003) Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423(6939):506-511.
240. Barrat FJ, *et al.* (1999) Defective CTLA-4 cycling pathway in Chediak-Higashi syndrome: a possible mechanism for deregulation of T lymphocyte activation. *Proc Natl Acad Sci U S A* 96(15):8645-8650.
241. Tivol EA, *et al.* (1995) Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3(5):541-547.
242. Waterhouse P, *et al.* (1995) Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270(5238):985-988.
243. Pentcheva-Hoang T, Egen JG, Wojnoonski K, & Allison JP (2004) B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. *Immunity* 21(3):401-413.
244. Somasundaram R & Herlyn M (2015) Nivolumab in combination with ipilimumab for the treatment of melanoma. *Expert Rev Anticancer Ther* 15(10):1135-1141.
245. Hodi FS, *et al.* (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363(8):711-723.
246. Yu X, *et al.* (2009) The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* 10(1):48-57.
247. Kamran N, *et al.* (2013) Toll-like receptor ligands induce expression of the costimulatory molecule CD155 on antigen-presenting cells. *PLoS One* 8(1):e54406.

248. Reymond N, *et al.* (2004) DNAM-1 and PVR regulate monocyte migration through endothelial junctions. *J Exp Med* 199(10):1331-1341.
249. Stengel KF, *et al.* (2012) Structure of TIGIT immunoreceptor bound to poliovirus receptor reveals a cell-cell adhesion and signaling mechanism that requires cis-trans receptor clustering. *Proc Natl Acad Sci U S A* 109(14):5399-5404.
250. Shibuya K, *et al.* (2003) CD226 (DNAM-1) is involved in lymphocyte function-associated antigen 1 costimulatory signal for naive T cell differentiation and proliferation. *J Exp Med* 198(12):1829-1839.
251. Liu XG, Hou M, & Liu Y (2017) TIGIT, A Novel Therapeutic Target for Tumor Immunotherapy. *Immunological investigations* 46(2):172-182.
252. Liu S, *et al.* (2013) Recruitment of Grb2 and SHIP1 by the ITT-like motif of TIGIT suppresses granule polarization and cytotoxicity of NK cells. *Cell Death Differ* 20(3):456-464.
253. Chauvin JM, *et al.* (2015) TIGIT and PD-1 impair tumor antigen-specific CD8(+) T cells in melanoma patients. *J Clin Invest* 125(5):2046-2058.
254. Kurtulus S, *et al.* (2015) TIGIT predominantly regulates the immune response via regulatory T cells. *J Clin Invest* 125(11):4053-4062.
255. Lozano E, Dominguez-Villar M, Kuchroo V, & Hafler DA (2012) The TIGIT/CD226 axis regulates human T cell function. *J Immunol* 188(8):3869-3875.
256. Georgiev H, *et al.* (2014) To the editor: TIGIT versus CD226: hegemony or coexistence? *Eur J Immunol* 44(1):307-308.
257. Johnston RJ, *et al.* (2014) The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* 26(6):923-937.
258. Joller N, *et al.* (2011) Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* 186(3):1338-1342.
259. Schluns KS, Kieper WC, Jameson SC, & Lefrancois L (2000) Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* 1(5):426-432.
260. Schluns KS & Lefrancois L (2003) Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* 3(4):269-279.
261. Schluns KS, Williams K, Ma A, Zheng XX, & Lefrancois L (2002) Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J Immunol* 168(10):4827-4831.
262. Alter G, *et al.* (2003) Longitudinal assessment of changes in HIV-specific effector activity in HIV-infected patients starting highly active antiretroviral therapy in primary infection. *J Immunol* 171(1):477-488.
263. Jamieson BD, *et al.* (2003) Epitope escape mutation and decay of human immunodeficiency virus type 1-specific CTL responses. *J Immunol* 171(10):5372-5379.
264. Casazza JP, Betts MR, Picker LJ, & Koup RA (2001) Decay kinetics of human immunodeficiency virus-specific CD8+ T cells in peripheral blood after initiation of highly active antiretroviral therapy. *J Virol* 75(14):6508-6516.
265. Jung YW, Rutishauser RL, Joshi NS, Haberman AM, & Kaech SM (2010) Differential localization of effector and memory CD8 T cell subsets in lymphoid organs during acute viral infection. *J Immunol* 185(9):5315-5325.

266. Jung YW, Kim HG, Perry CJ, & Kaech SM (2016) CCR7 expression alters memory CD8 T-cell homeostasis by regulating occupancy in IL-7- and IL-15-dependent niches. *Proc Natl Acad Sci U S A* 113(29):8278-8283.
267. He XS*, et al.* (1999) Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci U S A* 96(10):5692-5697.
268. Youngblood B*, et al.* (2011) Chronic virus infection enforces demethylation of the locus that encodes PD-1 in antigen-specific CD8(+) T cells. *Immunity* 35(3):400-412.
269. Paley MA*, et al.* (2012) Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science* 338(6111):1220-1225.
270. Schwartz RH (2003) T cell anergy. *Annu Rev Immunol* 21:305-334.
271. Macian F*, et al.* (2002) Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* 109(6):719-731.
272. Martinez GJ*, et al.* (2015) The transcription factor NFAT promotes exhaustion of activated CD8(+) T cells. *Immunity* 42(2):265-278.
273. Akbar AN & Henson SM (2011) Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat Rev Immunol* 11(4):289-295.
274. Hoare M*, et al.* (2010) CD4+ T-lymphocyte telomere length is related to fibrosis stage, clinical outcome and treatment response in chronic hepatitis C virus infection. *Journal of hepatology* 53(2):252-260.
275. Klenerman P & Hill A (2005) T cells and viral persistence: lessons from diverse infections. *Nat Immunol* 6(9):873-879.
276. Lichterfeld M*, et al.* (2008) Telomerase activity of HIV-1-specific CD8+ T cells: constitutive up-regulation in controllers and selective increase by blockade of PD ligand 1 in progressors. *Blood* 112(9):3679-3687.
277. Brenchley JM*, et al.* (2003) Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* 101(7):2711-2720.
278. Wirth TC*, et al.* (2010) Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation. *Immunity* 33(1):128-140.
279. Hertoghs KM*, et al.* (2010) Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. *J Clin Invest* 120(11):4077-4090.
280. Dagarag M, Evazyan T, Rao N, & Effros RB (2004) Genetic manipulation of telomerase in HIV-specific CD8+ T cells: enhanced antiviral functions accompany the increased proliferative potential and telomere length stabilization. *J Immunol* 173(10):6303-6311.
281. Hibbert L, Pflanz S, De Waal Malefyt R, & Kastelein RA (2003) IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 23(9):513-522.
282. Pipkin ME*, et al.* (2010) Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32(1):79-90.
283. Szabo SJ*, et al.* (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100(6):655-669.

284. Szabo SJ*, et al.* (2002) Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 295(5553):338-342.
285. Intlekofer AM*, et al.* (2007) Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J Exp Med* 204(9):2015-2021.
286. Intlekofer AM*, et al.* (2005) Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* 6(12):1236-1244.
287. Sullivan BM, Juedes A, Szabo SJ, von Herrath M, & Glimcher LH (2003) Antigen-driven effector CD8 T cell function regulated by T-bet. *Proc Natl Acad Sci U S A* 100(26):15818-15823.
288. Pearce EL*, et al.* (2003) Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science* 302(5647):1041-1043.
289. Wilson EB*, et al.* (2013) Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science* 340(6129):202-207.
290. Teijaro JR*, et al.* (2013) Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* 340(6129):207-211.
291. Anderson AC*, et al.* (2010) T-bet, a Th1 transcription factor regulates the expression of Tim-3. *Eur J Immunol* 40(3):859-866.
292. Cox MA, Harrington LE, & Zajac AJ (2011) Cytokines and the inception of CD8 T cell responses. *Trends Immunol* 32(4):180-186.
293. Takemoto N, Intlekofer AM, Northrup JT, Wherry EJ, & Reiner SL (2006) Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. *J Immunol* 177(11):7515-7519.
294. Banerjee A*, et al.* (2010) Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. *J Immunol* 185(9):4988-4992.
295. Hinrichs CS*, et al.* (2008) IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood* 111(11):5326-5333.
296. Staal FJ, Luis TC, & Tiemessen MM (2008) WNT signalling in the immune system: WNT is spreading its wings. *Nat Rev Immunol* 8(8):581-593.
297. Gattinoni L*, et al.* (2009) Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nat Med* 15(7):808-813.
298. Verbeek S*, et al.* (1995) An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 374(6517):70-74.
299. Zhou X & Xue HH (2012) Cutting edge: generation of memory precursors and functional memory CD8+ T cells depends on T cell factor-1 and lymphoid enhancer-binding factor-1. *J Immunol* 189(6):2722-2726.
300. Zhou X*, et al.* (2010) Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1. *Immunity* 33(2):229-240.
301. Zhao DM*, et al.* (2010) Constitutive activation of Wnt signaling favors generation of memory CD8 T cells. *J Immunol* 184(3):1191-1199.
302. Jeannet G*, et al.* (2010) Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory. *Proc Natl Acad Sci U S A* 107(21):9777-9782.

303. Im SJ, *et al.* (2016) Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* 537(7620):417-421.
304. Utzschneider DT, *et al.* (2016) T Cell Factor 1-Expressing Memory-like CD8(+) T Cells Sustain the Immune Response to Chronic Viral Infections. *Immunity* 45(2):415-427.
305. Martins GA, *et al.* (2006) Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol* 7(5):457-465.
306. Kallies A, *et al.* (2006) Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* 7(5):466-474.
307. Shaffer AL, *et al.* (2002) Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 17(1):51-62.
308. Turner CA, Jr., Mack DH, & Davis MM (1994) Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* 77(2):297-306.
309. Calame KL, Lin KI, & Tunyaplin C (2003) Regulatory mechanisms that determine the development and function of plasma cells. *Annu Rev Immunol* 21:205-230.
310. Nurieva RI, *et al.* (2012) STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem* 287(14):11234-11239.
311. Gong D & Malek TR (2007) Cytokine-dependent Blimp-1 expression in activated T cells inhibits IL-2 production. *J Immunol* 178(1):242-252.
312. Xin A, *et al.* (2016) A molecular threshold for effector CD8(+) T cell differentiation controlled by transcription factors Blimp-1 and T-bet. *Nat Immunol* 17(4):422-432.
313. Obar JJ, *et al.* (2010) CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc Natl Acad Sci U S A* 107(1):193-198.
314. Mitchell DM, Ravkov EV, & Williams MA (2010) Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells. *J Immunol* 184(12):6719-6730.
315. Martins GA, Cimmino L, Liao J, Magnusdottir E, & Calame K (2008) Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival. *J Exp Med* 205(9):1959-1965.
316. Shin HM, *et al.* (2013) Epigenetic modifications induced by Blimp-1 Regulate CD8(+) T cell memory progression during acute virus infection. *Immunity* 39(4):661-675.
317. Rao A, Luo C, & Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15:707-747.
318. Oukka M, *et al.* (1998) The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity* 9(3):295-304.
319. Crabtree GR & Olson EN (2002) NFAT signaling: choreographing the social lives of cells. *Cell* 109 Suppl:S67-79.
320. Hogan PG, Chen L, Nardone J, & Rao A (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes & development* 17(18):2205-2232.
321. Macian F (2005) NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 5(6):472-484.

322. Okamura H, *et al.* (2004) A conserved docking motif for CK1 binding controls the nuclear localization of NFAT1. *Molecular and cellular biology* 24(10):4184-4195.
323. Okamura H, *et al.* (2000) Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell* 6(3):539-550.
324. Gwack Y, *et al.* (2006) A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. *Nature* 441(7093):646-650.
325. Arron JR, *et al.* (2006) NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. *Nature* 441(7093):595-600.
326. Luo C, *et al.* (1996) Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import. *Proc Natl Acad Sci U S A* 93(17):8907-8912.
327. Teixeira LK, *et al.* (2005) IFN-gamma production by CD8+ T cells depends on NFAT1 transcription factor and regulates Th differentiation. *J Immunol* 175(9):5931-5939.
328. Kiani A, Viola JP, Lichtman AH, & Rao A (1997) Down-regulation of IL-4 gene transcription and control of Th2 cell differentiation by a mechanism involving NFAT1. *Immunity* 7(6):849-860.
329. Chen L, Glover JN, Hogan PG, Rao A, & Harrison SC (1998) Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. *Nature* 392(6671):42-48.
330. Macian F, Garcia-Rodriguez C, & Rao A (2000) Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. *The EMBO journal* 19(17):4783-4795.
331. Fehr T, *et al.* (2010) A CD8 T cell-intrinsic role for the calcineurin-NFAT pathway for tolerance induction in vivo. *Blood* 115(6):1280-1287.
332. Heissmeyer V, *et al.* (2004) Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat Immunol* 5(3):255-265.
333. Agnellini P, *et al.* (2007) Impaired NFAT nuclear translocation results in split exhaustion of virus-specific CD8+ T cell functions during chronic viral infection. *Proc Natl Acad Sci U S A* 104(11):4565-4570.
334. Glasmacher E, *et al.* (2012) A genomic regulatory element that directs assembly and function of immune-specific AP-1-IRF complexes. *Science* 338(6109):975-980.
335. Li P, *et al.* (2012) BATF-JUN is critical for IRF4-mediated transcription in T cells. *Nature* 490(7421):543-546.
336. Williams KL, *et al.* (2001) Characterization of murine BATF: a negative regulator of activator protein-1 activity in the thymus. *Eur J Immunol* 31(5):1620-1627.
337. Ciofani M, *et al.* (2012) A validated regulatory network for Th17 cell specification. *Cell* 151(2):289-303.
338. Schraml BU, *et al.* (2009) The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* 460(7253):405-409.
339. Ise W, *et al.* (2011) The transcription factor BATF controls the global regulators of class-switch recombination in both B cells and T cells. *Nat Immunol* 12(6):536-543.

340. Betz BC, *et al.* (2010) Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. *J Exp Med* 207(5):933-942.
341. Kurachi M, *et al.* (2014) The transcription factor BATF operates as an essential differentiation checkpoint in early effector CD8+ T cells. *Nat Immunol* 15(4):373-383.
342. Kuroda S, *et al.* (2011) Basic leucine zipper transcription factor, ATF-like (BATF) regulates epigenetically and energetically effector CD8 T-cell differentiation via Sirt1 expression. *Proc Natl Acad Sci U S A* 108(36):14885-14889.
343. Grusdat M, *et al.* (2014) IRF4 and BATF are critical for CD8(+) T-cell function following infection with LCMV. *Cell Death Differ* 21(7):1050-1060.
344. Xin G, *et al.* (2015) A Critical Role of IL-21-Induced BATF in Sustaining CD8-T-Cell-Mediated Chronic Viral Control. *Cell Rep* 13(6):1118-1124.
345. Elsaesser H, Sauer K, & Brooks DG (2009) IL-21 is required to control chronic viral infection. *Science* 324(5934):1569-1572.
346. Frohlich A, *et al.* (2009) IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* 324(5934):1576-1580.
347. Yi JS, Du M, & Zajac AJ (2009) A vital role for interleukin-21 in the control of a chronic viral infection. *Science* 324(5934):1572-1576.
348. Burgering BM (2008) A brief introduction to FOXOlogy. *Oncogene* 27(16):2258-2262.
349. Salih DA & Brunet A (2008) FoxO transcription factors in the maintenance of cellular homeostasis during aging. *Curr Opin Cell Biol* 20(2):126-136.
350. Gubbels Bupp MR, *et al.* (2009) T cells require Foxo1 to populate the peripheral lymphoid organs. *Eur J Immunol* 39(11):2991-2999.
351. Kerdiles YM, *et al.* (2009) Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat Immunol* 10(2):176-184.
352. Ouyang W, Beckett O, Flavell RA, & Li MO (2009) An essential role of the Forkhead-box transcription factor Foxo1 in control of T cell homeostasis and tolerance. *Immunity* 30(3):358-371.
353. Rao RR, Li Q, Gubbels Bupp MR, & Shrikant PA (2012) Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8(+) T cell differentiation. *Immunity* 36(3):374-387.
354. Kim MV, Ouyang W, Liao W, Zhang MQ, & Li MO (2013) The transcription factor Foxo1 controls central-memory CD8+ T cell responses to infection. *Immunity* 39(2):286-297.
355. Hand TW, *et al.* (2010) Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. *Proc Natl Acad Sci U S A* 107(38):16601-16606.
356. Gray SM, Kaech SM, & Staron MM (2014) The interface between transcriptional and epigenetic control of effector and memory CD8(+) T-cell differentiation. *Immunol Rev* 261(1):157-168.
357. Hess Michelini R, Doedens AL, Goldrath AW, & Hedrick SM (2013) Differentiation of CD8 memory T cells depends on Foxo1. *J Exp Med* 210(6):1189-1200.

358. Tejera MM, Kim EH, Sullivan JA, Plisch EH, & Suresh M (2013) FoxO1 controls effector-to-memory transition and maintenance of functional CD8 T cell memory. *J Immunol* 191(1):187-199.
359. Staron MM*, et al.* (2014) The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8(+) T cells during chronic infection. *Immunity* 41(5):802-814.
360. Weng NP, Araki Y, & Subedi K (2012) The molecular basis of the memory T cell response: differential gene expression and its epigenetic regulation. *Nat Rev Immunol* 12(4):306-315.
361. Wilson CB, Rowell E, & Sekimata M (2009) Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol* 9(2):91-105.
362. Scharer CD, Barwick BG, Youngblood BA, Ahmed R, & Boss JM (2013) Global DNA methylation remodeling accompanies CD8 T cell effector function. *J Immunol* 191(6):3419-3429.
363. Lee PP*, et al.* (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15(5):763-774.
364. Araki Y*, et al.* (2009) Genome-wide analysis of histone methylation reveals chromatin state-based regulation of gene transcription and function of memory CD8+ T cells. *Immunity* 30(6):912-925.
365. Fields PE, Kim ST, & Flavell RA (2002) Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *J Immunol* 169(2):647-650.
366. Zhou W, Chang S, & Aune TM (2004) Long-range histone acetylation of the Ifng gene is an essential feature of T cell differentiation. *Proc Natl Acad Sci U S A* 101(8):2440-2445.
367. Bird JJ*, et al.* (1998) Helper T cell differentiation is controlled by the cell cycle. *Immunity* 9(2):229-237.
368. Agarwal P*, et al.* (2009) Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory. *J Immunol* 183(3):1695-1704.
369. Young HA*, et al.* (1994) Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. *J Immunol* 153(8):3603-3610.
370. Vahedi G*, et al.* (2012) STATs shape the active enhancer landscape of T cell populations. *Cell* 151(5):981-993.
371. O'Shea JJ, Lahesmaa R, Vahedi G, Laurence A, & Kanno Y (2011) Genomic views of STAT function in CD4+ T helper cell differentiation. *Nat Rev Immunol* 11(4):239-250.
372. Chang S, Collins PL, & Aune TM (2008) T-bet dependent removal of Sin3A-histone deacetylase complexes at the Ifng locus drives Th1 differentiation. *J Immunol* 181(12):8372-8381.
373. Tumes DJ*, et al.* (2013) The polycomb protein Ezh2 regulates differentiation and plasticity of CD4(+) T helper type 1 and type 2 cells. *Immunity* 39(5):819-832.
374. Jones PA (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 13(7):484-492.

375. Okano M, Bell DW, Haber DA, & Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99(3):247-257.
376. Chappell C, Beard C, Altman J, Jaenisch R, & Jacob J (2006) DNA methylation by DNA methyltransferase 1 is critical for effector CD8 T cell expansion. *J Immunol* 176(8):4562-4572.
377. Kersh EN (2006) Impaired memory CD8 T cell development in the absence of methyl-CpG-binding domain protein 2. *J Immunol* 177(6):3821-3826.
378. Kersh EN*, et al.* (2006) Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. *J Immunol* 176(7):4083-4093.
379. Northrop JK, Thomas RM, Wells AD, & Shen H (2006) Epigenetic remodeling of the IL-2 and IFN-gamma loci in memory CD8 T cells is influenced by CD4 T cells. *J Immunol* 177(2):1062-1069.
380. Zediak VP, Johnnidis JB, Wherry EJ, & Berger SL (2011) Cutting edge: persistently open chromatin at effector gene loci in resting memory CD8+ T cells independent of transcriptional status. *J Immunol* 186(5):2705-2709.
381. Araki Y, Fann M, Wersto R, & Weng NP (2008) Histone acetylation facilitates rapid and robust memory CD8 T cell response through differential expression of effector molecules (eomesodermin and its targets: perforin and granzyme B). *J Immunol* 180(12):8102-8108.
382. Fann M*, et al.* (2006) Histone acetylation is associated with differential gene expression in the rapid and robust memory CD8(+) T-cell response. *Blood* 108(10):3363-3370.
383. Nolz JC & Harty JT (2014) IL-15 regulates memory CD8+ T cell O-glycan synthesis and affects trafficking. *J Clin Invest* 124(3):1013-1026.
384. Chandele A*, et al.* (2008) Formation of IL-7Ralphahigh and IL-7Ralphalow CD8 T cells during infection is regulated by the opposing functions of GABPalpha and Gfi-1. *J Immunol* 180(8):5309-5319.
385. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, & Greenleaf WJ (2013) Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* 10(12):1213-1218.
386. Youngblood B*, et al.* (2013) Cutting edge: Prolonged exposure to HIV reinforces a poised epigenetic program for PD-1 expression in virus-specific CD8 T cells. *J Immunol* 191(2):540-544.
387. Cui W & Kaech SM (2010) Generation of effector CD8+ T cells and their conversion to memory T cells. *Immunol Rev* 236:151-166.
388. Gerlach C*, et al.* (2016) The Chemokine Receptor CX3CR1 Defines Three Antigen-Experienced CD8 T Cell Subsets with Distinct Roles in Immune Surveillance and Homeostasis. *Immunity* 45(6):1270-1284.
389. Buggert M*, et al.* (2014) T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection. *PLoS Pathog* 10(7):e1004251.
390. Gupta PK*, et al.* (2015) CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. *PLoS Pathog* 11(10):e1005177.

391. Brooks DG, McGavern DB, & Oldstone MB (2006) Reprogramming of antiviral T cells prevents inactivation and restores T cell activity during persistent viral infection. *J Clin Invest* 116(6):1675-1685.
392. Streeck H*, et al.* (2008) Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells. *PLoS Med* 5(5):e100.
393. Bucks CM, Norton JA, Boesteanu AC, Mueller YM, & Katsikis PD (2009) Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion. *J Immunol* 182(11):6697-6708.
394. Porichis F & Kaufmann DE (2011) HIV-specific CD4 T cells and immune control of viral replication. *Current opinion in HIV and AIDS* 6(3):174-180.
395. Virgin HW & Walker BD (2010) Immunology and the elusive AIDS vaccine. *Nature* 464(7286):224-231.
396. Crawford A*, et al.* (2014) Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* 40(2):289-302.
397. Fahey LM*, et al.* (2011) Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* 208(5):987-999.
398. Ozaki K*, et al.* (2002) A critical role for IL-21 in regulating immunoglobulin production. *Science* 298(5598):1630-1634.
399. Linterman MA*, et al.* (2010) IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* 207(2):353-363.
400. Zotos D*, et al.* (2010) IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* 207(2):365-378.
401. Ackerman ME*, et al.* (2013) Natural variation in Fc glycosylation of HIV-specific antibodies impacts antiviral activity. *J Clin Invest* 123(5):2183-2192.
402. Straub T*, et al.* (2013) Nucleoprotein-specific nonneutralizing antibodies speed up LCMV elimination independently of complement and FcgammaR. *Eur J Immunol* 43(9):2338-2348.
403. Richter K & Oxenius A (2013) Non-neutralizing antibodies protect from chronic LCMV infection independently of activating FcgammaR or complement. *Eur J Immunol* 43(9):2349-2360.
404. Norris BA*, et al.* (2013) Chronic but not acute virus infection induces sustained expansion of myeloid suppressor cell numbers that inhibit viral-specific T cell immunity. *Immunity* 38(2):309-321.
405. Ejrnaes M*, et al.* (2006) Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J Exp Med* 203(11):2461-2472.
406. Pulendran B (2004) Modulating vaccine responses with dendritic cells and Toll-like receptors. *Immunol Rev* 199:227-250.
407. Ladell K*, et al.* (2013) A molecular basis for the control of preimmune escape variants by HIV-specific CD8+ T cells. *Immunity* 38(3):425-436.
408. Sevilla N, McGavern DB, Teng C, Kunz S, & Oldstone MB (2004) Viral targeting of hematopoietic progenitors and inhibition of DC maturation as a dual strategy for immune subversion. *J Clin Invest* 113(5):737-745.
409. Ng CT & Oldstone MB (2012) Infected CD8alpha- dendritic cells are the predominant source of IL-10 during establishment of persistent viral infection. *Proc Natl Acad Sci U S A* 109(35):14116-14121.

410. Wilson EB, *et al.* (2012) Emergence of distinct multiarmed immunoregulatory antigen-presenting cells during persistent viral infection. *Cell host & microbe* 11(5):481-491.
411. Ng CT, *et al.* (2015) Blockade of interferon Beta, but not interferon alpha, signaling controls persistent viral infection. *Cell host & microbe* 17(5):653-661.
412. Cunningham CR, *et al.* (2016) Type I and Type II Interferon Coordinately Regulate Suppressive Dendritic Cell Fate and Function during Viral Persistence. *PLoS Pathog* 12(1):e1005356.
413. Ng CT, Snell LM, Brooks DG, & Oldstone MB (2013) Networking at the level of host immunity: immune cell interactions during persistent viral infections. *Cell host & microbe* 13(6):652-664.
414. Penaloza-MacMaster P, *et al.* (2011) Opposing effects of CD70 costimulation during acute and chronic lymphocytic choriomeningitis virus infection of mice. *J Virol* 85(13):6168-6174.
415. Clouthier DL & Watts TH (2015) TNFRs and Control of Chronic LCMV Infection: Implications for Therapy. *Trends Immunol* 36(11):697-708.
416. Brugnoni D, *et al.* (1997) CD70 expression on T-cell subpopulations: study of normal individuals and patients with chronic immune activation. *Immunology letters* 55(2):99-104.
417. Wolthers KC, *et al.* (1996) Increased expression of CD80, CD86 and CD70 on T cells from HIV-infected individuals upon activation in vitro: regulation by CD4+ T cells. *Eur J Immunol* 26(8):1700-1706.
418. Matter M, Odermatt B, Yagita H, Nuoffer JM, & Ochsenbein AF (2006) Elimination of chronic viral infection by blocking CD27 signaling. *J Exp Med* 203(9):2145-2155.
419. Arens R, *et al.* (2004) Tumor rejection induced by CD70-mediated quantitative and qualitative effects on effector CD8+ T cell formation. *J Exp Med* 199(11):1595-1605.
420. French RR, *et al.* (2007) Eradication of lymphoma by CD8 T cells following anti-CD40 monoclonal antibody therapy is critically dependent on CD27 costimulation. *Blood* 109(11):4810-4815.
421. Sakanishi T & Yagita H (2010) Anti-tumor effects of depleting and non-depleting anti-CD27 monoclonal antibodies in immune-competent mice. *Biochemical and biophysical research communications* 393(4):829-835.
422. Roberts DJ, *et al.* (2010) Control of established melanoma by CD27 stimulation is associated with enhanced effector function and persistence, and reduced PD-1 expression of tumor infiltrating CD8(+) T cells. *J Immunother* 33(8):769-779.
423. Song DG, *et al.* (2012) CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo. *Blood* 119(3):696-706.
424. Vitale LA, *et al.* (2012) Development of a human monoclonal antibody for potential therapy of CD27-expressing lymphoma and leukemia. *Clin Cancer Res* 18(14):3812-3821.
425. He LZ, *et al.* (2013) Agonist anti-human CD27 monoclonal antibody induces T cell activation and tumor immunity in human CD27-transgenic mice. *J Immunol* 191(8):4174-4183.

426. Clouthier DL & Watts TH (2014) Cell-specific and context-dependent effects of GITR in cancer, autoimmunity, and infection. *Cytokine & growth factor reviews* 25(2):91-106.
427. Clouthier DL, Zhou AC, & Watts TH (2014) Anti-GITR agonist therapy intrinsically enhances CD8 T cell responses to chronic lymphocytic choriomeningitis virus (LCMV), thereby circumventing LCMV-induced downregulation of costimulatory GITR ligand on APC. *J Immunol* 193(10):5033-5043.
428. Clouthier DL*, et al.* (2015) GITR intrinsically sustains early type 1 and late follicular helper CD4 T cell accumulation to control a chronic viral infection. *PLoS Pathog* 11(1):e1004517.
429. Pascutti MF*, et al.* (2015) Enhanced CD8 T cell responses through GITR-mediated costimulation resolve chronic viral infection. *PLoS Pathog* 11(3):e1004675.
430. Snell LM*, et al.* (2010) CD8 T cell-intrinsic GITR is required for T cell clonal expansion and mouse survival following severe influenza infection. *J Immunol* 185(12):7223-7234.
431. Croft M (2010) Control of immunity by the TNFR-related molecule OX40 (CD134). *Annu Rev Immunol* 28:57-78.
432. Rogers PR, Song J, Gramaglia I, Killeen N, & Croft M (2001) OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity* 15(3):445-455.
433. Salek-Ardakani S*, et al.* (2011) The TNFR family members OX40 and CD27 link viral virulence to protective T cell vaccines in mice. *J Clin Invest* 121(1):296-307.
434. Boettler T*, et al.* (2012) OX40 facilitates control of a persistent virus infection. *PLoS Pathog* 8(9):e1002913.
435. Wang C*, et al.* (2012) Loss of the signaling adaptor TRAF1 causes CD8+ T cell dysregulation during human and murine chronic infection. *J Exp Med* 209(1):77-91.
436. Sabbagh L*, et al.* (2006) A critical role for TNF receptor-associated factor 1 and Bim down-regulation in CD8 memory T cell survival. *Proc Natl Acad Sci U S A* 103(49):18703-18708.
437. Belkaid Y & Tarbell K (2009) Regulatory T cells in the control of host-microorganism interactions (*). *Annu Rev Immunol* 27:551-589.
438. Kim JM, Rasmussen JP, & Rudensky AY (2007) Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* 8(2):191-197.
439. Sakaguchi S, Yamaguchi T, Nomura T, & Ono M (2008) Regulatory T cells and immune tolerance. *Cell* 133(5):775-787.
440. Veiga-Parga T, Sehrawat S, & Rouse BT (2013) Role of regulatory T cells during virus infection. *Immunol Rev* 255(1):182-196.
441. Penaloza-MacMaster P*, et al.* (2014) Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. *J Exp Med* 211(9):1905-1918.

442. Dietze KK*, et al.* (2011) Transient depletion of regulatory T cells in transgenic mice reactivates virus-specific CD8+ T cells and reduces chronic retroviral set points. *Proc Natl Acad Sci U S A* 108(6):2420-2425.
443. Schmitz I*, et al.* (2013) IL-21 restricts virus-driven Treg cell expansion in chronic LCMV infection. *PLoS Pathog* 9(5):e1003362.
444. Dong H*, et al.* (2002) Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 8(8):793-800.
445. Goh C, Narayanan S, & Hahn YS (2013) Myeloid-derived suppressor cells: the dark knight or the joker in viral infections? *Immunol Rev* 255(1):210-221.
446. Gabrilovich DI (2017) Myeloid-Derived Suppressor Cells. *Cancer Immunol Res* 5(1):3-8.
447. Kusmartsev S, Nefedova Y, Yoder D, & Gabrilovich DI (2004) Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J Immunol* 172(2):989-999.
448. Wilson EB & Brooks DG (2011) The role of IL-10 in regulating immunity to persistent viral infections. *Current topics in microbiology and immunology* 350:39-65.
449. Donnelly RP, Sheikh F, Kotenko SV, & Dickensheets H (2004) The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *J Leukoc Biol* 76(2):314-321.
450. Kotenko SV*, et al.* (1997) Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *The EMBO journal* 16(19):5894-5903.
451. Moore KW, de Waal Malefyt R, Coffman RL, & O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.
452. Finbloom DS & Winestock KD (1995) IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes. *J Immunol* 155(3):1079-1090.
453. Lai CF*, et al.* (1996) Receptors for interleukin (IL)-10 and IL-6-type cytokines use similar signaling mechanisms for inducing transcription through IL-6 response elements. *J Biol Chem* 271(24):13968-13975.
454. Wehinger J*, et al.* (1996) IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes. *FEBS letters* 394(3):365-370.
455. Weber-Nordt RM*, et al.* (1996) Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain. *J Biol Chem* 271(44):27954-27961.
456. Woitas RP*, et al.* (2002) HCV-specific cytokine induction in monocytes of patients with different outcomes of hepatitis C. *World journal of gastroenterology : WJG* 8(3):562-566.
457. Hofer H*, et al.* (2005) Bi-allelic presence of the interleukin-10 receptor 1 G330R allele is associated with cirrhosis in chronic HCV-1 infection. *Genes and immunity* 6(3):242-247.
458. Graziosi C*, et al.* (1994) Lack of evidence for the dichotomy of TH1 and TH2 predominance in HIV-infected individuals. *Science* 265(5169):248-252.

459. Clerici M, *et al.* (1997) Type 1 and type 2 cytokines in HIV infection -- a possible role in apoptosis and disease progression. *Annals of medicine* 29(3):185-188.
460. Swaminathan S (2003) Molecular biology of Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus. *Seminars in hematology* 40(2):107-115.
461. Cheong JY, *et al.* (2006) Association between chronic hepatitis B virus infection and interleukin-10, tumor necrosis factor-alpha gene promoter polymorphisms. *Journal of gastroenterology and hepatology* 21(7):1163-1169.
462. Helminen M, Lahdenpohja N, & Hurme M (1999) Polymorphism of the interleukin-10 gene is associated with susceptibility to Epstein-Barr virus infection. *The Journal of infectious diseases* 180(2):496-499.
463. Paladino N, *et al.* (2006) Gender susceptibility to chronic hepatitis C virus infection associated with interleukin 10 promoter polymorphism. *J Virol* 80(18):9144-9150.
464. Shin HD, *et al.* (2000) Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. *Proc Natl Acad Sci U S A* 97(26):14467-14472.
465. Miyazoe S, *et al.* (2002) Influence of interleukin-10 gene promoter polymorphisms on disease progression in patients chronically infected with hepatitis B virus. *The American journal of gastroenterology* 97(8):2086-2092.
466. Persico M, *et al.* (2006) Interleukin-10 - 1082 GG polymorphism influences the occurrence and the clinical characteristics of hepatitis C virus infection. *Journal of hepatology* 45(6):779-785.
467. Knapp S, *et al.* (2003) Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection. *Immunogenetics* 55(6):362-369.
468. Slobedman B, Barry PA, Spencer JV, Avdic S, & Abendroth A (2009) Virus-encoded homologs of cellular interleukin-10 and their control of host immune function. *J Virol* 83(19):9618-9629.
469. Brockman MA, *et al.* (2009) IL-10 is up-regulated in multiple cell types during viremic HIV infection and reversibly inhibits virus-specific T cells. *Blood* 114(2):346-356.
470. Clerici M, *et al.* (1994) Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus. *J Clin Invest* 93(2):768-775.
471. Landay AL, *et al.* (1996) In vitro restoration of T cell immune function in human immunodeficiency virus-positive persons: effects of interleukin (IL)-12 and anti-IL-10. *The Journal of infectious diseases* 173(5):1085-1091.
472. Said EA, *et al.* (2010) Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. *Nat Med* 16(4):452-459.
473. Rigopoulou EI, Abbott WG, Haigh P, & Naoumov NV (2005) Blocking of interleukin-10 receptor--a novel approach to stimulate T-helper cell type 1 responses to hepatitis C virus. *Clin Immunol* 117(1):57-64.
474. Dong H, Zhu G, Tamada K, & Chen L (1999) B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 5(12):1365-1369.
475. Brooks DG, *et al.* (2006) Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 12(11):1301-1309.

476. Steinbrink K, Wolfl M, Jonuleit H, Knop J, & Enk AH (1997) Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 159(10):4772-4780.
477. Kajino K, Nakamura I, Bamba H, Sawai T, & Ogasawara K (2007) Involvement of IL-10 in exhaustion of myeloid dendritic cells and rescue by CD40 stimulation. *Immunology* 120(1):28-37.
478. Carbonneil C, Donkova-Petrini V, Aouba A, & Weiss L (2004) Defective dendritic cell function in HIV-infected patients receiving effective highly active antiretroviral therapy: neutralization of IL-10 production and depletion of CD4+CD25+ T cells restore high levels of HIV-specific CD4+ T cell responses induced by dendritic cells generated in the presence of IFN-alpha. *J Immunol* 172(12):7832-7840.
479. Foulds KE, Rotte MJ, & Seder RA (2006) IL-10 is required for optimal CD8 T cell memory following Listeria monocytogenes infection. *J Immunol* 177(4):2565-2574.
480. Groux H, Bigler M, de Vries JE, & Roncarolo MG (1998) Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J Immunol* 160(7):3188-3193.
481. Kang SS & Allen PM (2005) Priming in the presence of IL-10 results in direct enhancement of CD8+ T cell primary responses and inhibition of secondary responses. *J Immunol* 174(9):5382-5389.
482. Govinden R & Bhoola KD (2003) Genealogy, expression, and cellular function of transforming growth factor-beta. *Pharmacology & therapeutics* 98(2):257-265.
483. Kehrl JH*, et al.* (1986) Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* 163(5):1037-1050.
484. Letterio JJ & Roberts AB (1998) Regulation of immune responses by TGF-beta. *Annu Rev Immunol* 16:137-161.
485. Lagneaux L, Delforge A, Dorval C, Bron D, & Stryckmans P (1993) Excessive production of transforming growth factor-beta by bone marrow stromal cells in B-cell chronic lymphocytic leukemia inhibits growth of hematopoietic precursors and interleukin-6 production. *Blood* 82(8):2379-2385.
486. Filer A, Pitzalis C, & Buckley CD (2006) Targeting the stromal microenvironment in chronic inflammation. *Current opinion in pharmacology* 6(4):393-400.
487. Annes JP, Munger JS, & Rifkin DB (2003) Making sense of latent TGFbeta activation. *Journal of cell science* 116(Pt 2):217-224.
488. Nunes I, Shapiro RL, & Rifkin DB (1995) Characterization of latent TGF-beta activation by murine peritoneal macrophages. *J Immunol* 155(3):1450-1459.
489. Shi Y & Massague J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113(6):685-700.
490. Massague J (1998) TGF-beta signal transduction. *Annual review of biochemistry* 67:753-791.
491. Massague J (2000) How cells read TGF-beta signals. *Nature reviews. Molecular cell biology* 1(3):169-178.
492. Inman GJ, Nicolas FJ, & Hill CS (2002) Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. *Mol Cell* 10(2):283-294.

493. Shi Y, *et al.* (1998) Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* 94(5):585-594.
494. Zawel L, *et al.* (1998) Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* 1(4):611-617.
495. Xu L, Chen YG, & Massague J (2000) The nuclear import function of Smad2 is masked by SARA and unmasked by TGFbeta-dependent phosphorylation. *Nat Cell Biol* 2(8):559-562.
496. Xu L, Kang Y, Col S, & Massague J (2002) Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. *Mol Cell* 10(2):271-282.
497. Li MO, Wan YY, Sanjabi S, Robertson AK, & Flavell RA (2006) Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* 24:99-146.
498. Kulkarni AB, *et al.* (1993) Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* 90(2):770-774.
499. Shull MM, *et al.* (1992) Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359(6397):693-699.
500. Kobayashi S, *et al.* (1999) Beta 2-microglobulin-deficient background ameliorates lethal phenotype of the TGF-beta 1 null mouse. *J Immunol* 163(7):4013-4019.
501. Letterio JJ, *et al.* (1996) Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest* 98(9):2109-2119.
502. Marie JC, Liggitt D, & Rudensky AY (2006) Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity* 25(3):441-454.
503. Lucas PJ, Kim SJ, Melby SJ, & Gress RE (2000) Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 191(7):1187-1196.
504. Lin JT, Martin SL, Xia L, & Gorham JD (2005) TGF-beta 1 uses distinct mechanisms to inhibit IFN-gamma expression in CD4+ T cells at priming and at recall: differential involvement of Stat4 and T-bet. *J Immunol* 174(10):5950-5958.
505. Smyth MJ, Strobl SL, Young HA, Ortaldo JR, & Ochoa AC (1991) Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood CD8+ T lymphocytes. Inhibition by transforming growth factor-beta. *J Immunol* 146(10):3289-3297.
506. Ahmadzadeh M, *et al.* (2009) Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 114(8):1537-1544.
507. Chen ML, *et al.* (2005) Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A* 102(2):419-424.
508. Mempel TR, *et al.* (2006) Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. *Immunity* 25(1):129-141.

509. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, & Stockinger B (2006) TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24(2):179-189.
510. Gorelik L, Constant S, & Flavell RA (2002) Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. *J Exp Med* 195(11):1499-1505.
511. Malherbe G*, et al.* (2014) Circulating biomarkers of immune activation distinguish viral suppression from nonsuppression in HAART-treated patients with advanced HIV-1 subtype C infection. *Mediators of inflammation* 2014:198413.
512. Kekow J*, et al.* (1991) Transforming growth factor-beta and suppression of humoral immune responses in HIV infection. *J Clin Invest* 87(3):1010-1016.
513. Nelson DR*, et al.* (1997) Transforming growth factor-beta 1 in chronic hepatitis C. *Journal of viral hepatitis* 4(1):29-35.
514. Boettler T, Cheng Y, Ehrhardt K, & von Herrath M (2012) TGF-beta blockade does not improve control of an established persistent viral infection. *Viral immunology* 25(3):232-238.
515. Garidou L, Heydari S, Gossa S, & McGavern DB (2012) Therapeutic blockade of transforming growth factor beta fails to promote clearance of a persistent viral infection. *J Virol* 86(13):7060-7071.
516. Colonna M, Trinchieri G, & Liu YJ (2004) Plasmacytoid dendritic cells in immunity. *Nat Immunol* 5(12):1219-1226.
517. McNab F, Mayer-Barber K, Sher A, Wack A, & O'Garra A (2015) Type I interferons in infectious disease. *Nat Rev Immunol* 15(2):87-103.
518. Ivashkiv LB & Donlin LT (2014) Regulation of type I interferon responses. *Nat Rev Immunol* 14(1):36-49.
519. Schoggins JW*, et al.* (2011) A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* 472(7344):481-485.
520. Yan N & Chen ZJ (2012) Intrinsic antiviral immunity. *Nat Immunol* 13(3):214-222.
521. Boasso A & Shearer GM (2008) Chronic innate immune activation as a cause of HIV-1 immunopathogenesis. *Clin Immunol* 126(3):235-242.
522. Hardy GA*, et al.* (2013) Interferon-alpha is the primary plasma type-I IFN in HIV-1 infection and correlates with immune activation and disease markers. *PLoS One* 8(2):e56527.
523. Sedaghat AR*, et al.* (2008) Chronic CD4+ T-cell activation and depletion in human immunodeficiency virus type 1 infection: type I interferon-mediated disruption of T-cell dynamics. *J Virol* 82(4):1870-1883.
524. Stylianou E, Aukrust P, Bendtzen K, Muller F, & Froland SS (2000) Interferons and interferon (IFN)-inducible protein 10 during highly active anti-retroviral therapy (HAART)-possible immunosuppressive role of IFN-alpha in HIV infection. *Clin Exp Immunol* 119(3):479-485.
525. Hyrcza MD*, et al.* (2007) Distinct transcriptional profiles in ex vivo CD4+ and CD8+ T cells are established early in human immunodeficiency virus type 1 infection and are characterized by a chronic interferon response as well as extensive transcriptional changes in CD8+ T cells. *J Virol* 81(7):3477-3486.

526. Rotger M, *et al.* (2011) Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque. *J Clin Invest* 121(6):2391-2400.
527. Jacquelin B, *et al.* (2009) Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response. *J Clin Invest* 119(12):3544-3555.
528. Estes JD, *et al.* (2008) Early resolution of acute immune activation and induction of PD-1 in SIV-infected sooty mangabeys distinguishes nonpathogenic from pathogenic infection in rhesus macaques. *J Immunol* 180(10):6798-6807.
529. Arimori Y, *et al.* (2013) Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. *Antiviral research* 99(3):230-237.
530. Schreiner B, *et al.* (2004) Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. *Journal of neuroimmunology* 155(1-2):172-182.
531. Rudick RA, *et al.* (2001) Impact of interferon beta-1a on neurologic disability in relapsing multiple sclerosis. 1997. *Neurology* 57(12 Suppl 5):S25-30.
532. Marshall HD, Urban SL, & Welsh RM (2011) Virus-induced transient immune suppression and the inhibition of T cell proliferation by type I interferon. *J Virol* 85(12):5929-5939.
533. Kaser A, Nagata S, & Tilg H (1999) Interferon alpha augments activation-induced T cell death by upregulation of Fas (CD95/APO-1) and Fas ligand expression. *Cytokine* 11(10):736-743.
534. Nguyen KB, *et al.* (2000) Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox. *Nat Immunol* 1(1):70-76.
535. Bolen CR, *et al.* (2013) The blood transcriptional signature of chronic hepatitis C virus is consistent with an ongoing interferon-mediated antiviral response. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 33(1):15-23.
536. Zhen A, *et al.* (2017) Targeting type I interferon-mediated activation restores immune function in chronic HIV infection. *J Clin Invest* 127(1):260-268.
537. Cheng L, *et al.* (2017) Blocking type I interferon signaling enhances T cell recovery and reduces HIV-1 reservoirs. *J Clin Invest* 127(1):269-279.
538. Wang Y, *et al.* (2012) Timing and magnitude of type I interferon responses by distinct sensors impact CD8 T cell exhaustion and chronic viral infection. *Cell host & microbe* 11(6):631-642.
539. Sandler NG, *et al.* (2014) Type I interferon responses in rhesus macaques prevent SIV infection and slow disease progression. *Nature* 511(7511):601-605.
540. Heim MH (2013) 25 years of interferon-based treatment of chronic hepatitis C: an epoch coming to an end. *Nat Rev Immunol* 13(7):535-542.
541. Wilson EB & Brooks DG (2013) Decoding the complexity of type I interferon to treat persistent viral infections. *Trends in microbiology* 21(12):634-640.
542. Silver JS & Hunter CA (2010) gp130 at the nexus of inflammation, autoimmunity, and cancer. *J Leukoc Biol* 88(6):1145-1156.

543. Taga T & Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol* 15:797-819.
544. Stahl N*, et al.* (1994) Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* 263(5143):92-95.
545. Lutticken C*, et al.* (1994) Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science* 263(5143):89-92.
546. Takahashi-Tezuka M*, et al.* (1998) Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase. *Molecular and cellular biology* 18(7):4109-4117.
547. Heinrich PC*, et al.* (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *The Biochemical journal* 374(Pt 1):1-20.
548. Saito M, Yoshida K, Hibi M, Taga T, & Kishimoto T (1992) Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. *J Immunol* 148(12):4066-4071.
549. Yao X*, et al.* (2014) Targeting interleukin-6 in inflammatory autoimmune diseases and cancers. *Pharmacology & therapeutics* 141(2):125-139.
550. Romani L*, et al.* (1996) Impaired neutrophil response and CD4+ T helper cell 1 development in interleukin 6-deficient mice infected with Candida albicans. *J Exp Med* 183(4):1345-1355.
551. Dalrymple SA*, et al.* (1996) Interleukin-6 is required for a protective immune response to systemic Escherichia coli infection. *Infect Immun* 64(8):3231-3235.
552. Suzuki Y*, et al.* (1997) Impaired resistance to the development of toxoplasmic encephalitis in interleukin-6-deficient mice. *Infect Immun* 65(6):2339-2345.
553. Kopf M*, et al.* (1994) Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 368(6469):339-342.
554. Jebbari H, Roberts CW, Ferguson DJ, Bluethmann H, & Alexander J (1998) A protective role for IL-6 during early infection with Toxoplasma gondii. *Parasite immunology* 20(5):231-239.
555. Ladel CH*, et al.* (1997) Lethal tuberculosis in interleukin-6-deficient mutant mice. *Infect Immun* 65(11):4843-4849.
556. van der Poll T*, et al.* (1997) Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *The Journal of infectious diseases* 176(2):439-444.
557. Hunter CA & Jones SA (2015) IL-6 as a keystone cytokine in health and disease. *Nat Immunol* 16(5):448-457.
558. Modur V, Li Y, Zimmerman GA, Prescott SM, & McIntyre TM (1997) Retrograde inflammatory signaling from neutrophils to endothelial cells by soluble interleukin-6 receptor alpha. *J Clin Invest* 100(11):2752-2756.
559. McLoughlin RM*, et al.* (2004) Differential regulation of neutrophil-activating chemokines by IL-6 and its soluble receptor isoforms. *J Immunol* 172(9):5676-5683.
560. Hurst SM*, et al.* (2001) Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. *Immunity* 14(6):705-714.

561. Lauder SN, *et al.* (2013) Interleukin-6 limits influenza-induced inflammation and protects against fatal lung pathology. *Eur J Immunol* 43(10):2613-2625.
562. Villarino A, *et al.* (2003) The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. *Immunity* 19(5):645-655.
563. Choi YS, Eto D, Yang JA, Lao C, & Crotty S (2013) Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol* 190(7):3049-3053.
564. Eto D, *et al.* (2011) IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS One* 6(3):e17739.
565. Suto A, *et al.* (2008) Development and characterization of IL-21-producing CD4+ T cells. *J Exp Med* 205(6):1369-1379.
566. Dienz O, *et al.* (2009) The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells. *J Exp Med* 206(1):69-78.
567. Eddahri F, *et al.* (2009) Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities. *Blood* 113(11):2426-2433.
568. Wojno ED & Hunter CA (2012) New directions in the basic and translational biology of interleukin-27. *Trends Immunol* 33(2):91-97.
569. Birx DL, *et al.* (1990) Induction of interleukin-6 during human immunodeficiency virus infection. *Blood* 76(11):2303-2310.
570. Spanakis NE, *et al.* (2002) Cytokine serum levels in patients with chronic HCV infection. *Journal of clinical laboratory analysis* 16(1):40-46.
571. Torre D, *et al.* (1994) Serum levels of interleukin-1 alpha, interleukin-1 beta, interleukin-6, and tumor necrosis factor in patients with acute viral hepatitis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 18(2):194-198.
572. Ashrafi Hafez A, *et al.* (2014) Analytical assessment of interleukin - 23 and -27 cytokines in healthy people and patients with hepatitis C virus infection (genotypes 1 and 3a). *Hepatitis monthly* 14(9):e21000.
573. He L, *et al.* (2014) Interleukin-27 is differentially associated with HIV viral load and CD4+ T cell counts in therapy-naive HIV-mono-infected and HIV/HCV-co-infected Chinese. *PLoS One* 9(5):e96792.
574. Guzzo C, Hopman WM, Che Mat NF, Wobeser W, & Gee K (2010) Impact of HIV infection, highly active antiretroviral therapy, and hepatitis C coinfection on serum interleukin-27. *Aids* 24(9):1371-1374.
575. Swaminathan S, *et al.* (2014) Plasma interleukin-27 (IL-27) levels are not modulated in patients with chronic HIV-1 infection. *PLoS One* 9(6):e98989.
576. Harker JA, Dolgoter A, & Zuniga EI (2013) Cell-intrinsic IL-27 and gp130 cytokine receptor signaling regulates virus-specific CD4(+) T cell responses and viral control during chronic infection. *Immunity* 39(3):548-559.
577. Harker JA, Lewis GM, Mack L, & Zuniga EI (2011) Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* 334(6057):825-829.
578. Bergthaler A, *et al.* (2009) Impaired antibody response causes persistence of prototypic T cell-contained virus. *PLoS biology* 7(4):e1000080.

579. Imamichi T, *et al.* (2008) IL-27, a novel anti-HIV cytokine, activates multiple interferon-inducible genes in macrophages. *Aids* 22(1):39-45.
580. Greenwell-Wild T, *et al.* (2009) Interleukin-27 inhibition of HIV-1 involves an intermediate induction of type I interferon. *Blood* 114(9):1864-1874.
581. Dai L, *et al.* (2013) IL-27 inhibits HIV-1 infection in human macrophages by down-regulating host factor SPTBN1 during monocyte to macrophage differentiation. *J Exp Med* 210(3):517-534.
582. Chen Q, *et al.* (2013) Interleukin-27 is a potent inhibitor of cis HIV-1 replication in monocyte-derived dendritic cells via a type I interferon-independent pathway. *PLoS One* 8(3):e59194.
583. Rochman Y, Spolski R, & Leonard WJ (2009) New insights into the regulation of T cells by gamma(c) family cytokines. *Nat Rev Immunol* 9(7):480-490.
584. Surh CD & Sprent J (2008) Homeostasis of naive and memory T cells. *Immunity* 29(6):848-862.
585. Masse GX, *et al.* (2007) gamma(c) cytokines provide multiple homeostatic signals to naive CD4(+) T cells. *Eur J Immunol* 37(9):2606-2616.
586. Mathieu C, *et al.* (2015) IL-2 and IL-15 regulate CD8 memory T-cell differentiation but are dispensable for protective recall responses. *Eur J Immunol*.
587. Fewkes NM & Mackall CL (2010) Novel gamma-chain cytokines as candidate immune modulators in immune therapies for cancer. *Cancer J* 16(4):392-398.
588. Diallo M, *et al.* (2011) Prospect of IL-2, IL-7, IL-15 and IL-21 for HIV immune-based therapy. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 36(11):1037-1045.
589. Asao H, *et al.* (2001) Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol* 167(1):1-5.
590. Holm C, Nyvold CG, Paludan SR, Thomsen AR, & Hokland M (2006) Interleukin-21 mRNA expression during virus infections. *Cytokine* 33(1):41-45.
591. Korn T, *et al.* (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448(7152):484-487.
592. Nurieva R, *et al.* (2007) Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448(7152):480-483.
593. Spolski R & Leonard WJ (2008) Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol* 26:57-79.
594. Aubert RD, *et al.* (2011) Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection. *Proc Natl Acad Sci U S A* 108(52):21182-21187.
595. Gerlach JT, *et al.* (1999) Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology* 117(4):933-941.
596. Grakoui A, *et al.* (2003) HCV persistence and immune evasion in the absence of memory T cell help. *Science* 302(5645):659-662.
597. Smyk-Pearson S, *et al.* (2008) Spontaneous recovery in acute human hepatitis C virus infection: functional T-cell thresholds and relative importance of CD4 help. *J Virol* 82(4):1827-1837.
598. Puel A, Ziegler SF, Buckley RH, & Leonard WJ (1998) Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nature genetics* 20(4):394-397.

599. Tan JT, *et al.* (2001) IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A* 98(15):8732-8737.
600. Takada K & Jameson SC (2009) Naive T cell homeostasis: from awareness of space to a sense of place. *Nat Rev Immunol* 9(12):823-832.
601. Hand TW, Morre M, & Kaech SM (2007) Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc Natl Acad Sci U S A* 104(28):11730-11735.
602. Bradley LM, Haynes L, & Swain SL (2005) IL-7: maintaining T-cell memory and achieving homeostasis. *Trends Immunol* 26(3):172-176.
603. Kittipatarin C & Khaled AR (2007) Interlinking interleukin-7. *Cytokine* 39(1):75-83.
604. Jiang Q, *et al.* (2005) Cell biology of IL-7, a key lymphotrophin. *Cytokine & growth factor reviews* 16(4-5):513-533.
605. Wojciechowski S, *et al.* (2007) Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. *J Exp Med* 204(7):1665-1675.
606. Levy Y, *et al.* (2009) Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. *J Clin Invest* 119(4):997-1007.
607. Sereti I, *et al.* (2009) IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. *Blood* 113(25):6304-6314.
608. Fry TJ, *et al.* (2003) IL-7 therapy dramatically alters peripheral T-cell homeostasis in normal and SIV-infected nonhuman primates. *Blood* 101(6):2294-2299.
609. Nugeyre MT, *et al.* (2003) IL-7 stimulates T cell renewal without increasing viral replication in simian immunodeficiency virus-infected macaques. *J Immunol* 171(8):4447-4453.
610. Beq S, *et al.* (2006) IL-7 induces immunological improvement in SIV-infected rhesus macaques under antiviral therapy. *J Immunol* 176(2):914-922.
611. Chu YW, *et al.* (2004) Exogenous IL-7 increases recent thymic emigrants in peripheral lymphoid tissue without enhanced thymic function. *Blood* 104(4):1110-1119.
612. Pellegrini M, *et al.* (2009) Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies. *Nat Med* 15(5):528-536.
613. Lang KS, *et al.* (2005) Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation. *Eur J Immunol* 35(3):738-745.
614. Nanjappa SG, Kim EH, & Suresh M (2011) Immunotherapeutic effects of IL-7 during a chronic viral infection in mice. *Blood* 117(19):5123-5132.
615. Pellegrini M, *et al.* (2011) IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology. *Cell* 144(4):601-613.
616. Radaeva S, Sun R, Pan HN, Hong F, & Gao B (2004) Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. *Hepatology* 39(5):1332-1342.
617. Zenewicz LA, *et al.* (2007) Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity* 27(4):647-659.

618. Croker BA*, et al.* (2003) SOCS3 negatively regulates IL-6 signaling in vivo. *Nat Immunol* 4(6):540-545.
619. Vandergeeten C*, et al.* (2013) Interleukin-7 promotes HIV persistence during antiretroviral therapy. *Blood* 121(21):4321-4329.
620. Imamichi H*, et al.* (2011) HIV-1 viruses detected during episodic blips following interleukin-7 administration are similar to the viruses present before and after interleukin-7 therapy. *Aids* 25(2):159-164.
621. Pfizenmaier K*, et al.* (1984) Quantitative representation of all T cells committed to develop into cytotoxic effector cells and/or interleukin 2 activity-producing helper cells within murine T lymphocyte subsets. *Eur J Immunol* 14(1):33-39.
622. D'Souza WN & Lefrancois L (2004) Frontline: An in-depth evaluation of the production of IL-2 by antigen-specific CD8 T cells in vivo. *Eur J Immunol* 34(11):2977-2985.
623. Liao W, Lin JX, & Leonard WJ (2013) Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. *Immunity* 38(1):13-25.
624. Boyman O & Sprent J (2012) The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol* 12(3):180-190.
625. D'Souza WN, Schluns KS, Masopust D, & Lefrancois L (2002) Essential role for IL-2 in the regulation of antiviral extralymphoid CD8 T cell responses. *J Immunol* 168(11):5566-5572.
626. D'Souza WN & Lefrancois L (2003) IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. *J Immunol* 171(11):5727-5735.
627. Williams MA, Tyznik AJ, & Bevan MJ (2006) Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441(7095):890-893.
628. Blattman JN*, et al.* (2003) Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo. *Nat Med* 9(5):540-547.
629. Levy Y*, et al.* (1999) Comparison of subcutaneous and intravenous interleukin-2 in asymptomatic HIV-1 infection: a randomised controlled trial. ANRS 048 study group. *Lancet* 353(9168):1923-1929.
630. Hardy GA*, et al.* (2007) A phase I, randomized study of combined IL-2 and therapeutic immunisation with antiretroviral therapy. *Journal of immune based therapies and vaccines* 5:6.
631. Kovacs JA*, et al.* (1996) Controlled trial of interleukin-2 infusions in patients infected with the human immunodeficiency virus. *N Engl J Med* 335(18):1350-1356.
632. Group I-ES*, et al.* (2009) Interleukin-2 therapy in patients with HIV infection. *N Engl J Med* 361(16):1548-1559.
633. Pett SL, Kelleher AD, & Emery S (2010) Role of interleukin-2 in patients with HIV infection. *Drugs* 70(9):1115-1130.
634. Saadoun D*, et al.* (2011) Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* 365(22):2067-2077.
635. Yi JS, Ingram JT, & Zajac AJ (2010) IL-21 deficiency influences CD8 T cell quality and recall responses following an acute viral infection. *J Immunol* 185(8):4835-4845.

636. Wherry EJ (2016) Molecular Basis of T cell Exhaustion: Insights for Immunotherapy. in *Keystone symposia 2016 Cell Biology and Immunology of Persistent Infection (A8)*.
637. Waldmann TA (2006) The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 6(8):595-601.
638. Schluns KS, Stoklasek T, & Lefrancois L (2005) The roles of interleukin-15 receptor alpha: trans-presentation, receptor component, or both? *Int J Biochem Cell Biol* 37(8):1567-1571.
639. Jabri B & Abadie V (2015) IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction. *Nat Rev Immunol* 15(12):771-783.
640. Mortier E*, et al.* (2009) Macrophage- and dendritic-cell-derived interleukin-15 receptor alpha supports homeostasis of distinct CD8+ T cell subsets. *Immunity* 31(5):811-822.
641. Schluns KS, Klonowski KD, & Lefrancois L (2004) Transregulation of memory CD8 T-cell proliferation by IL-15Ralpha+ bone marrow-derived cells. *Blood* 103(3):988-994.
642. Colpitts SL*, et al.* (2012) Cutting edge: the role of IFN-alpha receptor and MyD88 signaling in induction of IL-15 expression in vivo. *J Immunol* 188(6):2483-2487.
643. Rubinstein MP*, et al.* (2008) IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response. *Blood* 112(9):3704-3712.
644. Tripathi P*, et al.* (2010) STAT5 is critical to maintain effector CD8+ T cell responses. *J Immunol* 185(4):2116-2124.
645. Tan JT*, et al.* (2002) Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J Exp Med* 195(12):1523-1532.
646. Sandau MM, Kohlmeier JE, Woodland DL, & Jameson SC (2010) IL-15 regulates both quantitative and qualitative features of the memory CD8 T cell pool. *J Immunol* 184(1):35-44.
647. Quinci AC*, et al.* (2012) IL-15 inhibits IL-7Ralpha expression by memory-phenotype CD8(+) T cells in the bone marrow. *Eur J Immunol* 42(5):1129-1139.
648. Minn AJ & Wherry EJ (2016) Combination Cancer Therapies with Immune Checkpoint Blockade: Convergence on Interferon Signaling. *Cell* 165(2):272-275.
649. Beltra JC*, et al.* (2016) IL2Rbeta-dependent signals drive terminal exhaustion and suppress memory development during chronic viral infection. *Proc Natl Acad Sci U S A* 113(37):E5444-5453.
650. Mitchell DM & Williams MA (2013) Disparate roles for STAT5 in primary and secondary CTL responses. *J Immunol* 190(7):3390-3398.
651. Grange M*, et al.* (2013) Active STAT5 regulates T-bet and eomesodermin expression in CD8 T cells and imprints a T-bet-dependent Tc1 program with repressed IL-6/TGF-beta1 signaling. *J Immunol* 191(7):3712-3724.
652. Buchbinder SP*, et al.* (2008) Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 372(9653):1881-1893.

653. Shi M, Lin TH, Appell KC, & Berg LJ (2008) Janus-kinase-3-dependent signals induce chromatin remodeling at the Ifng locus during T helper 1 cell differentiation. *Immunity* 28(6):763-773.
654. Malek TR, Yu A, Scibelli P, Lichtenheld MG, & Codias EK (2001) Broad programming by IL-2 receptor signaling for extended growth to multiple cytokines and functional maturation of antigen-activated T cells. *J Immunol* 166(3):1675-1683.
655. Castro I, Yu A, Dee MJ, & Malek TR (2011) The basis of distinctive IL-2- and IL-15-dependent signaling: weak CD122-dependent signaling favors CD8+ T central-memory cell survival but not T effector-memory cell development. *J Immunol* 187(10):5170-5182.
656. Zerbini CA & Lomonte AB (2012) Tofacitinib for the treatment of rheumatoid arthritis. *Expert Rev Clin Immunol* 8(4):319-331.
657. Ichii H, Sakamoto A, Kuroda Y, & Tokuhisa T (2004) Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells. *J Immunol* 173(2):883-891.
658. Johnston RJ*, et al.* (2009) Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325(5943):1006-1010.
659. Cassese G*, et al.* (2007) Bone marrow CD8 cells down-modulate membrane IL-7Ralpha expression and exhibit increased STAT-5 and p38 MAPK phosphorylation in the organ environment. *Blood* 110(6):1960-1969.
660. Kemp RA, Pearson CF, Cornish GH, & Seddon BP (2010) Evidence of STAT5-dependent and -independent routes to CD8 memory formation and a preferential role for IL-7 over IL-15 in STAT5 activation. *Immunology and cell biology* 88(2):213-219.
661. Gebhardt T*, et al.* (2009) Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* 10(5):524-530.
662. Masopust D*, et al.* (2010) Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med* 207(3):553-564.
663. Shin H & Iwasaki A (2013) Tissue-resident memory T cells. *Immunol Rev* 255(1):165-181.
664. Schenkel JM*, et al.* (2016) IL-15-Independent Maintenance of Tissue-Resident and Boosted Effector Memory CD8 T Cells. *J Immunol* 196(9):3920-3926.
665. Mackay LK*, et al.* (2015) T-box Transcription Factors Combine with the Cytokines TGF-beta and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* 43(6):1101-1111.
666. Cui W, Liu Y, Weinstein JS, Craft J, & Kaech SM (2011) An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. *Immunity* 35(5):792-805.
667. Ostiguy V, Allard EL, Marquis M, Leignadier J, & Labrecque N (2007) IL-21 promotes T lymphocyte survival by activating the phosphatidylinositol-3 kinase signaling cascade. *J Leukoc Biol* 82(3):645-656.

Appendices

Annex. 1. Review article

Cytokines and persistent viral infections

Jean-Christophe Beltra[1,2], Hélène Decaluwe[1,2,3,*]

This review article is published in the journal “Cytokine”, vol. 82, issue “Cytokines in inflammation, aging, cancer and obesity” doi: 10.1016/j.cyto.2016.02.006 (2016) PMID 26907634.

I performed the bibliographic research and wrote the entire manuscript with corrections from H. Decaluwe.

Cytokine 82 (2016) 4–15

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Review article

Cytokines and persistent viral infections

Jean-Christophe Beltra [a,b], Hélène Decaluwe [a,b,c,*]

[a] Cytokines and Adaptive Immunity Laboratory, CHU Sainte-Justine Research Center, Montreal, Quebec, Canada
[b] Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada
[c] Immunology and Rheumatology Division, Department of Pediatrics, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada

A R T I C L E I N F O

Article history:
Received 31 October 2015
Received in revised form 11 February 2016
Accepted 11 February 2016
Available online 20 February 2016

Keywords:
Cytokine
Chronic viral infection
Exhaustion
T cell

A B S T R A C T

Intracellular pathogens such as the human immunodeficiency virus, hepatitis C and B or Epstein–Barr virus often cause chronic viral infections in humans. Persistence of these viruses in the host is associated with a dramatic loss of T-cell immune response due to functional T-cell exhaustion. Developing efficient immunotherapeutic approaches to prevent viral persistence and/or to restore a highly functional T-cell mediated immunity remains a major challenge. During the last two decades, numerous studies aimed to identify relevant host-derived factors that could be modulated to achieve this goal. In this review, we focus on recent advances in our understanding of the role of cytokines in preventing or facilitating viral persistence. We concentrate on the impact of multiple relevant cytokines in T-cell dependent immune response to chronic viral infection and the potential for using cytokines as therapeutic agents in mice and humans.

© 2016 Elsevier Ltd. All rights reserved.

Contents

1. Introduction 5
2. Immuno-regulatory cytokines 5
   2.1. IL-10 5
   2.2. Transforming growth factor β (TGF-β) 6
3. Inflammatory cytokines 7
   3.1. Type I interferons (IFNs) 7
   3.2. Glycoprotein 130 (gp130)-dependent cytokines 8
4. Common gamma (γ_c) chain-dependent cytokines 9
   4.1. IL-21 9
   4.2. IL-7 9
   4.3. IL-2 10
5. Concluding remarks 10
Conflict of interest 11
Acknowledgements 11
References 11

Abbreviations: Ag, antigen; AIDS, acquired immune deficiency syndrome; APC, antigen presenting cell; Arm, armstrong; Cl-13, clone-13; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DC, dendritic cell; EBV, Epstein–Barr virus; FDC, follicular dendritic cell; γ_c-chain, common gamma chain; GCB, germinal center B; Gp130, glycoprotein 130; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IFN, interferon; IFNAR1, interferon alpha receptor 1; IgG, immunoglobulin G; IL-2/IL2R, interleukin-2/interleukin 2 receptor; IL-6/IL-6R, interleukin-6/interleukin 6 receptor; IL-7/IL7R, interleukin-7/interleukin 7 receptor; IL-10/IL10R, interleukin-10/interleukin 10 receptor; IL-21/IL21R, interleukin-21/interleukin 21 receptor; IL-27/IL27Rα, interleukin-27/interleukin 27 receptor alpha; ISG, interferon-stimulated genes; JAK, janus tyrosine kinase; LAG-3, lymphocyte activation gene 3; LAP, latency-associated protein; LCMV, lymphocytic choriomeningitis virus; LIF, leukemia inhibitory factor; LLC, large latent complex; LTBP, latent TGFβ binding protein; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex; NK, natural killer; OSM, oncostatin M; PD1, programmed cell death 1; PDL1, programmed death ligand 1; p.i., post infection; PI3K, phosphatidylinositide-3 kinase; SIV, simian immunodeficiency virus; SLC, small latent complex; SOCS3, suppressor of cytokine signaling 3; STAT, signal transducer and activator of transcription; Tfh, T follicular helper; Tg, transgenic; TGFβ/TGFBR, transforming growth factor beta/transforming growth factor beta receptor; Th, T helper; Tim3, T cell Ig- and mucin-domain-containing molecule-3; TNFα, tumor necrosis factor alpha; Tyk2, tyrosine kinase 2.

* Corresponding author at: CHU Sainte-Justine, 3175 Cote-Sainte-Catherine, Montreal, Quebec H3T 1C5, Canada.
E-mail address: helene.decaluwe@umontreal.ca (H. Decaluwe).

http://dx.doi.org/10.1016/j.cyto.2016.02.006
1043-4666/© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Anti-viral T-cell response to acute viral infections is a tightly regulated process. After activation, antigen (Ag)-specific T cells undergo massive clonal expansion and acquire cardinal effector functions essential for the eradication of virally-infected cells. Following resolution of infection, the majority of expanded T cells are rapidly eliminated during the contraction phase to prevent excessive immune activation. A small fraction of these effectors survive to form a long-lasting protective memory pool [1]. However, chronic viruses such as the human immunodeficiency virus (HIV), hepatitis C and B viruses (HCV and HBV) and Epstein–Barr virus (EBV) often manage to avoid immune surveillance and establish a persistent infection in the host. In these particular conditions, the immune system elaborates inherent suppressive strategies to prevent uncontrolled T-cell expansion that might cause severe pathology to the host. However, these immune-derived suppressive functions, while being host protective, further facilitate viral persistence and lead to the gradual loss of T-cell immunity by a process called T-cell exhaustion [2,3]. During that exhaustion process, CD8 T cells are progressively deprived of their cardinal effector functions (i.e. cytokine production, proliferation potential, killing ability) because of increased expression of multiple inhibitory receptors including PD1, LAG-3, 2B4, Tim3 and CD160 [4–6]. In addition, commitment to exhaustion suppresses the development of memory cells precluding the establishment of long-term protective immunity [7,8]. Similarly, CD4 T cells also become exhausted as evidenced by a rapid loss of effector cytokine production (i.e. IL-2, TNFα and IFNγ), sustained expression of PD1, CTLA4 and increased production of IL-10 and/or IL-21 [9–11]. While exhausted T cells continue to display some level of antiviral functions, the effector T-cell pool progressively erodes as the infection persists, which ultimately leads to the complete loss of T-cell immunity [12]. Consequently, identifying the immune-derived factors that favor viral persistence and T-cell dysfunction with the goal of developing new therapeutic strategies to prevent or reverse exhaustion has been the subject of intense research during the last decades. Among the immune-derived factors, there has been a particular focus on cytokines because of their capacity to drive essential stimulatory and suppressive functions on both innate and adaptive immune cells. Based on observations in humans and mice, we discuss recent advances in the understanding of the role of cytokines in preventing or facilitating viral persistence. Our review focuses on the impact of several relevant cytokines on T-cell responses and their potential use as therapeutic agents for humans and mice.

2. Immuno-regulatory cytokines

2.1. IL-10

Several hematopoietic cell types produce IL-10 including dendritic cells (DCs), B cells, monocytes, macrophages, CD4 T cells, CD8 T cells and regulatory T cells [13]. IL-10 signals via a heterodimeric class II cytokine receptor composed of an inducible IL10R1-chain and an IL10R2-chain constitutively expressed on both hematopoietic and non-hematopoietic cell types that are also shared by the class II cytokines IL-22, IL-26, IL-28 and IL-29 [14,15]. Engagement of IL-10 to its cognate receptor triggers activation of the janus tyrosine kinases Jak1 and Tyk2 and phosphorylation of the signal transducers and activators of transcription STAT3, STAT1 and also STAT5 [16–20]. Subsequent signals allow IL-10 to mediate its immuno-suppressive functions that affect multiple hematopoietic cell types.

In several persistent infections, notably in patients with HIV, HBV, HCV or EBV infections, IL-10 levels were reported to be elevated [13,21–25]. Concomitantly, polymorphisms within the IL-10 promoter region that reduce the secretion of the cytokine were correlated with enhanced control of these viruses [26–32]. Moreover, numerous viruses including EBV, human cytomegalovirus (HCMV) and some poxviruses produce their own IL-10 homologs to dampen antiviral immunity and favor their persistence in the hosts [33]. Interestingly, IL-10 blockade potently increases the proliferation and cytokine secretion of T cells isolated from chronically infected HIV or HCV patients [34–38]. On the contrary, engagement of PD1 with its ligand PDL1 can trigger IL-10 expression, thus contributing to functional T-cell exhaustion [37,39]. Together, these cumulative evidence suggest a potential immunosuppressive role of IL-10 during chronic viral infections in humans.

Two concomitant studies of Brooks and Ejrnaes modeled chronic viral infection in mice and unravelled an essential role of IL-10 in promoting viral persistence [40,41]. Similar to what was observed in persistent human infections, both teams reported superior IL-10 levels after infection with the variant lymphocytic choriomeningitis virus (LCMV) strain clone 13 (Cl-13), which induces a 2–3 month viremia in the host, compared to the LCMV Armstrong (Arm) strain, which is rapidly cleared [42,43]. Remarkably, infection of IL-10-deficient mice by LCMV Cl-13 led to rapid viral control by day 9 post infection (p.i.). Early blockade of IL-10 signals with anti-IL10R specific antibodies similarly resulted in accelerated clearance of Cl-13 infection [40,41]. Rapid elimination of the Cl-13 virus was associated with enhanced magnitude and functionality of CD4 and CD8 T cells. Importantly, IL-10 blockade at later time points also led to enhanced viral control and T-cell responses. However, in both studies, these effects were less impressive than what was observed in IL-10$^{-/-}$ mice or mice treated early with anti-IL10R specific antibodies. Hence, IL-10 acts essentially as an early determinant of viral persistence, while it continuously dampens immune functions throughout chronicity. This latter point was emphasized in a study that demonstrated that IL10R-antibody treatment of mice with a well-established persistent Cl-13 infection still improved T-cell immunity and substantially decreased viral loads [44]. More importantly, this study revealed that IL-10 blockade was even more effective when combined with anti-PDL1 treatment, suggesting that this dual regimen might be effective in combating established persistent infections.

Remaining is the question of how IL-10 favors viral persistence (Fig. 1). IL-10 was previously shown to alter DC maturation by reducing the expression of MHC class I and II as well as the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) [13,16,45,46]. Hence, IL-10-primed DCs or DCs isolated from HIV patients are poor inducers of CD4 T-cell proliferation [45–47]. Further, DCs from chronically-infected mice diverge naïve CD4 T cells from a Th1 IFNγ^+ profile to an IL-10 secretion profile (Fig. 1) [41]. That effect was not observed when DCs were isolated from anti-IL10R treated mice [41]. This is in support of a direct impact of IL-10 on DCs' priming abilities that favor the accumulation of suppressive IL-10$^+$ CD4 T cells (Fig. 1). Thus, IL-10 indirectly promotes CD4 T-cell exhaustion by altering DC maturation leading to dampened priming of the cells and divergence to a suppressive IL-10 producing profile. Conversely, IL-10 can behave as a proliferative factor for CD8 T cells *in vitro* and as a growth factor *in vivo* during primary response to *Listeria monocytogenes* [48–50]. However, IL-10 restrains HIV-specific CD8 T-cell proliferation *in vitro* [34]. Hence, the impact of IL-10 on CD8 T-cell response is likely context-dependent and preferentially inhibiting during chronic viral infections (Fig. 1). Those assertions define IL-10 as an overall suppressor of T-cell response during persistent viral infections. However, whether IL-10 favors viral persistence by directly promoting T-cell exhaustion remains to be determined.

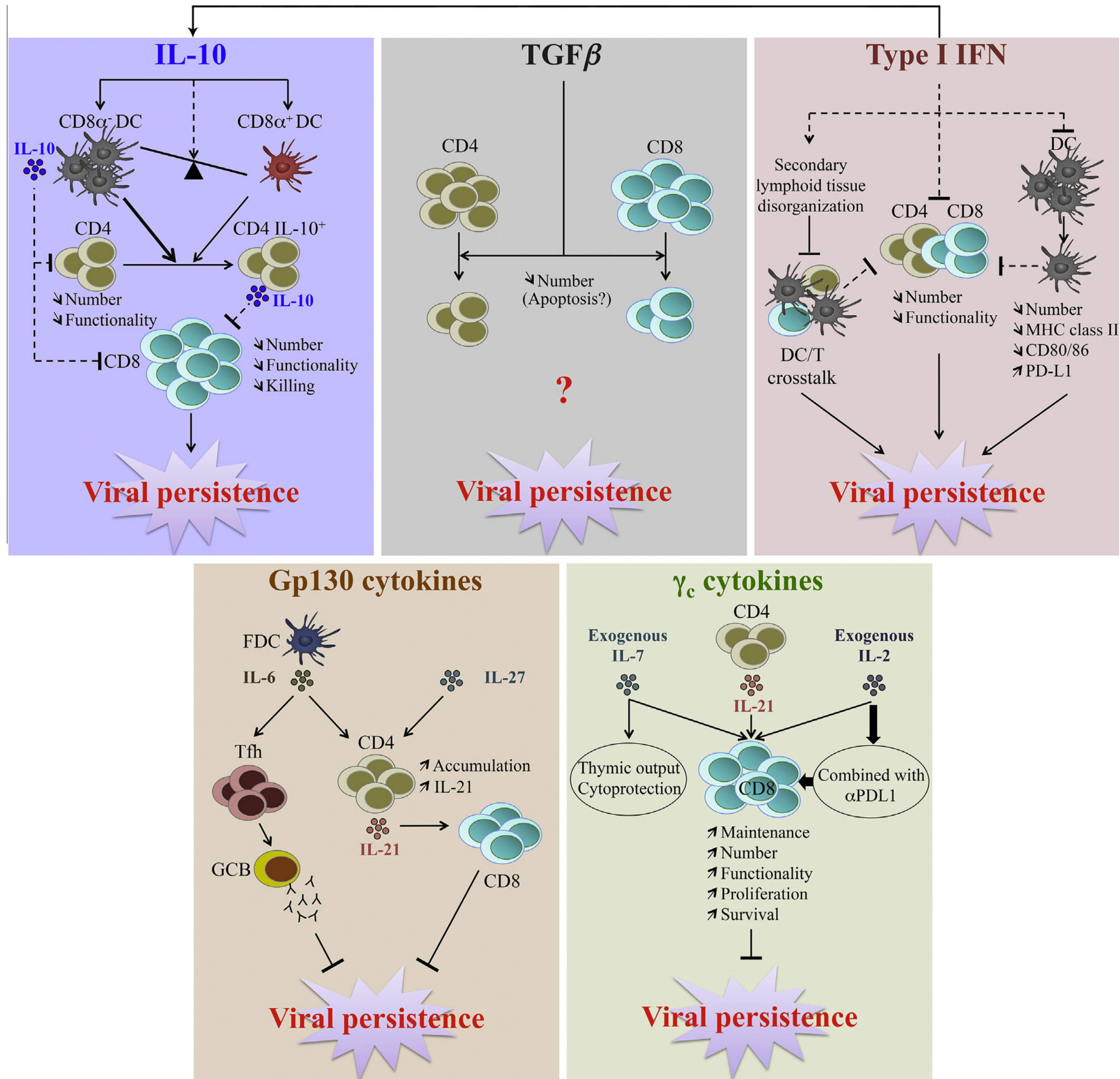


Fig. 1. Cytokines regulate T-cell responses during chronic viral infection. ***IL-10***: Infection of mice with LCMV Cl-13 causes a gradual decline in the number of CD8α⁺ DCs, shifting the ratio in favor of the suppressive CD8α⁻ DCs. This imbalance during chronic viral infection is sustained by IL-10, which supports the subsequent differentiation of suppressive IL-10⁺ CD4 T cells. IL-10 produced by CD8α⁻ DCs, CD4 T cells as well as macrophages and monocytes (not depicted) directly and/or indirectly reduces T-cell numbers and hinders their functions, which lead to viral persistence. However, a cell-intrinsic impact of IL-10 on T-cell dysfunction and exhaustion remains to be established. ***TGFβ***: TGFβ directly reduces the number of Ag-specific CD4 and CD8 T cells likely through increased Bim-mediated apoptosis. However, TGFβ neutralization *in vivo* has minimal to no impact on viral control during a chronic infection. ***Type I IFNs***: Type I IFNs cause a dramatic disorganization of secondary lymphoid tissues that limits efficient cross-talk between APCs and T cells. Type I IFNs further limit DCs number and impair their maturation by decreasing MHC Class II and CD80/86 levels while promoting PDL1 expression. These cytokines also increase circulating levels of IL-10. These combined alterations likely hamper the magnitude of CD4 and CD8 T-cell responses, reducing their functionality and favoring viral persistence. ***Gp130-dependent cytokines***: During the chronic phase of LCMV Cl-13 infection, follicular dendritic cells (FDC) produce the highest amounts of IL-6. IL-6 promotes the development of Tfh cells that in turn support GCB cells accumulation and anti-LCMV antibody production necessary for viral control. IL-6 and IL-27 redundantly promote IL-21 production by Ag-specific CD4 T cells. IL-21 maintains the number of Ag-specific CD8 T cells leading to better viral control, while IL-27 has the unique capacity to support the maintenance Ag-specific CD4 T cells during chronicity. **γ_c-dependent cytokines**: CD4-derived IL-21 provides cell-intrinsic signals that are essential for the maintenance of a functional Ag-specific CD8 T-cell population. This critical cytokine is essential to contain a chronic viral infection. Prolonged IL-7 therapy augments the number of polyfunctional CD8 T cells through increased proliferation and survival leading to accelerated viral clearance. IL-7 also displays other beneficial effects including the improvement of thymic output and prevention of organ pathology through IL-22 induction. Low-dose IL-2 regimen in chronically infected mice increases the number of polyfunctional CD8 T cells, especially when combined with αPDL1 treatment, to sustain viral decline. However, IL-2 administration into CD4-depleted Cl-13 mice adversely increases viral loads (not depicted).

2.2. Transforming growth factor β (TGF-β)

The TGFβ superfamily in mammals includes three isoforms, TGFβ1, TGFβ2 and TGFβ3, that are encoded by distinct genes [51]. The dominant TGFβ1 form can be selectively produced by activated T cells, macrophages, DCs as well as non-immune cell types including stromal cells from lymphoid tissues [51–55]. TGFβ1 is first secreted in an inactive "latent form", trapped by the so-called latency-associated protein (LAP) that form the small latent complex (SLC). Eventually other latent TGFβ binding proteins (LTBP) can bind the SLC to form a large latent complex (LLC) [56,57]. Once freed from these accessory proteins, TGFβ1 homodimers bind to their cognate receptor composed of two subunits of TGFβRI and two subunits of TGFβRII [58–60]. Engagement

of TGFβ1 activates the kinase activity of TGFβRI and II. This allows for recruitment and phosphorylation of SMADs proteins notably SMAD2 and SMAD3 that complex with SMAD4 to access the nucleus and achieve the transcription of SMAD-responsive genes [58–65]. Thereby, TGFβ1 exerts several regulatory functions in almost all immune cell types and particularly affects the T-cell compartment [66].

TGFβ has long been associated with a plethora of regulatory functions in multiple hematopoietic cell types. Indeed, mice deficient for TGFβ1 (TGFβ1$^{-/-}$) develop a lethal inflammatory syndrome associated with massive infiltration of immune cells including lymphocytes in several organs [67,68]. Interestingly, depletion of CD4 or CD8 T cells alleviates this overwhelming inflammatory syndrome suggesting that TGFβ directly suppresses T-cell auto-activation [69,70]. Accordingly, mice with a T-cell restricted deficiency for TGFβRII develop uncontrolled expansion and activation of peripheral CD4 and mostly CD8 T cells associated with severe organ lesions and rapid demise [71,72]. Consistent with these observations, TGFβ was shown to directly suppress several primary T-cell functions that are also lost during chronic viral infection including proliferation, antiviral cytokine production (IFNγ) and cytotoxicity [43,66,73–77]. Besides its suppressive functions, TGFβ also greatly influences the differentiation of activated T cells. Hence, TGFβ restrains Th1 and Th2 CD4 T-cell differentiation while directing regulatory T-cell development *in vitro* [66]. TGFβ-mediated alteration of Th1 CD4 T-cell differentiation is at least partially due to its capacity to repress the expression of the T-box transcription factor T-bet [78]. Of note, T-bet directly represses PD1 expression on CD8 T cells, restrains terminal CD8 T-cell exhaustion and sustains CD4 T-cell response during chronic viral infection [9,12,79]. Thus, we may speculate that TGFβ directly aggravates T-cell exhaustion through repression of T-bet, although this has not been demonstrated to date (see below).

In mice, Tinoco et al. examined the impact of cell-intrinsic TGFβ in the silencing of T-cell responses during a chronic viral infection (Fig. 1) [80]. In accordance with the heightened TGFβ production often observed in humans during chronic viral infections – although no clear correlations have been established between TFGβ1 levels and disease progression in humans [81–83] – TGFβ production and signaling were increased in T cells isolated from chronically infected Cl-13 mice compared to Arm mice. This confirmed that elevated and persistent levels of TGFβ are a hallmark of chronic viral infections. However, attenuation of TGFβ signaling in T cells by the transgenic expression of a dominant negative form of the TGFBRII receptor (dnTGFBRII) mainly limited the Bim-dependent apoptosis of effector T cells (Fig. 1) [80,84,85]. This resulted in a greater accumulation of Ag-specific CD8 T cells that appeared less exhausted as they produced more cytokines, expressed lower levels of PD1 and displayed enhanced killing functions [80]. However, these functional enhancements reflected the more rapid viral clearance in mice with attenuated TGFβ signaling rather than a cell-intrinsic effect of TGFβ. Indeed, dnTGFBRII mice rapidly cleared a Cl-13 infection without presenting organ pathologies. This allowed for the development of a competent memory pool that fails to develop normally in that model [7,8,80].

The demonstration by Tinoco and colleagues that a T-cell-restricted attenuation of TGFβ signals led to the rapid clearance of Cl-13 infection suggested that *in vivo* neutralization of TGFβ might represent a relevant therapeutic option for purging a chronic viral infection [80]. Unfortunately, several groups tested the impact of TGFβ blockade *in vivo* with disappointing results. In fact, TGFβ blockade during priming or during the chronic phase of Cl-13 infection only slightly increased the number of CD4 and CD8 T cells [86,87]. Yet, the functionality of these effectors was barely improved and no reduction in inhibitory receptor expression (i.e. PD1, 2B4) was reported. Importantly, the modest effects of TGFβ neutralization had no impact on viral control regardless of the timing of treatment [86,87]. These results imply that targeting TGFβ alone is neither sufficient to prevent the establishment of chronic viral infection nor to promote the clearance of an established chronic viral infection (Fig. 1). However, it remains possible that combining TGFβ blockade with other regimens might efficiently improve the control of chronic viral infections.

3. Inflammatory cytokines

3.1. Type I interferons (IFNs)

Type I IFNs are a large family of soluble mediators comprising the most described IFNα and IFNβ and the less defined classes IFNκ, ω, ε, δ, τ and ζ. Plasmacytoid dendritic cells are a major source of IFNα while several other cell types can produce type I IFNs after viral sensing by pathogen recognition receptors [88,89]. Once secreted, type I IFNs engage a unique heterodimeric receptor composed of the IFNAR1 and IFNAR2 subunits; activate the janus tyrosine kinases Jak1 and Tyk2 and trigger several downstream molecules (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) and signaling pathways (PI3K and MAPK) [89,90]. Activation of those signaling pathways leads to the transcription of hundreds of interferon-stimulated genes (ISGs) creating a typical type I IFN signature [91,92]. ISGs encode proteins with direct antiviral functions and are responsible for type I IFN-mediated enhancement of innate and adaptive immune functions and maturation.

While often being associated with positive antiviral functions, type I IFNs recently received attention regarding their potential deleterious effects during persistent viral infections. Indeed, heightened inflammation and sustained type I IFN signature often correlate with disease progression in chronic viral infections. For example, HIV infection is associated with chronic immune activation and unremitting type I IFN responses, which correlate with disease severity and reduction of CD4 T-cell counts [93–96]. Concomitantly, comparative transcriptional analysis of CD4 and CD8 T cells between HIV progressor patients and viral "elite" controllers revealed an elevated level of ISGs in the former, implying that type I IFN signals may contribute to disease progression [97,98]. Similarly, the relative resistance of African green monkeys or sooty mangabeys to simian immunodeficiency virus (SIV)-induced immunodeficiency is associated with a restrained type I IFN signature as opposed to SIV-infected rhesus macaques who often progress to AIDS and present persistent high levels of ISGs [99,100]. Hence, levels and persistence of type I IFN signals have become a relevant biological determinant of viral persistence and disease progression during chronic viral infections. In addition to those correlative observations, type I IFNs exert a set of immunosuppressive functions intimately linked to viral persistence. Indeed, type I IFNs are critical inducers of IL-10 and PDL1 expression, two major components that promote viral persistence [101–103]. In addition, priming of naïve CD8 T cells with type I IFNs reduced their proliferative potential to cognate Ag-encounter [104]. IFNα also enhanced the Fas/FasL-mediated antigen-induced cell death in human T cells [105]. Further, type I IFNs potently damped IFNγ secretion by murine T and NK cells in a STAT1-dependent manner [106]. Hence, sustained inflammatory responses, induction of immuno-suppressive factors and attenuation of T-cell responses through PD1 ligation represent three distinct pathways by which type I IFNs can mediate virus persistence.

Two studies by Teijaro et al. and Wilson et al. examined more thoroughly the role of IFNα/β during a chronic viral infection using the LCMV Cl-13 mouse model [107,108]. As expected, persistent infection with Cl-13 led to superior levels of IFNα and IFNβ compared to mice infected with LCMV Arm. In addition, viral

persistence in Cl-13-infected mice was associated with prolonged expression of ISGs, STATs and IFN-I regulatory elements, in accordance with the sustained type I IFN response observed in humans with HIV and HCV [94–96,109]. Importantly, both studies reported that early IFNAR1 blockade, which prevents ligation of all type I IFNs to their common receptor chain, although initially permissive for viral replication, led to enhanced control of viremia and accelerated viral clearance at later time points. Moreover, blockade of IFNAR1 when chronicity was well established similarly decreased viral titers. Conversely, Wang and colleagues demonstrated that providing exogenous IFNα/β efficiently boosted T-cell responses and accelerated Cl-13 elimination when given at the time of T-cell priming, while providing IFNα/β at earlier time points or after the peak of the T-cell expansion was ineffective [110]. These observations suggest that type I IFNs modulation needs to be finely tuned since divergent outcomes may arise depending on the timing since infection. This latter point is of critical importance for the treatment of chronic viral infections in humans. Indeed, IFNα has been successfully used for years as a first-line treatment of HCV in combination with the antiviral drug ribavirin [111]. However a significant number of patients are refractory to IFNα therapy. In fact, treatment outcome appear to correlate with the extent of type I IFN signature in HCV patients, with refractory patients presenting the strongest signature [111,112]. Hence, defining the optimal time frame for type I IFNs modulation to achieve a positive antiviral response in patients remains to be studied.

Disrupting type I IFNs signaling with anti-IFNAR1 antibodies revealed how these cytokines suppressed antiviral responses during chronic viral infection (Fig. 1). Early IFNα/β burst sustained the secretion of multiple pro-inflammatory cytokines and caused a dramatic disorganization of secondary lymphoid tissues (Fig. 1) [107,108]. IFNAR1 blockade efficiently prevented secondary lymphoid tissue disorganization and increased the number of several innate and adaptive immune cells including DCs, NK cells, B cells and CD4 T cells. Levels of suppressive factors such as IL-10 and PDL1 were also largely reduced with IFNAR1 blockade, while DC maturation and CD4 T-cell functions were readily improved (Fig. 1) [107,108]. Notably, the rapid control of viremia with early IFNAR1 blockade strictly required the presence of CD4 T cells. Interestingly, neutralization of IFNβ-signals alone – although to a lesser extent than IFNAR1 blockade – also enhanced CD4 T-cell responses, preserved lymphoid tissue architecture and accelerated viral clearance while IFNα blockade did not [113]. Rather, IFNα likely dispensed early antiviral effects while being dispensable for viral clearance thereafter. However, IFNβ blockade did not alter IL-10 and PDL1 levels. In addition, while IFNAR1 blockade had little impact on CD8 T-cell responses, IFNβ neutralization greatly improved their number and functionality [107,108,113]. Together, these results point to a dominant role for IFNβ over IFNα in mediating suppressive functions during a chronic viral infection, but also suggest that type I IFNs use redundant and unique pathways to impair anti-viral immune responses and favor viral persistence.

3.2. Glycoprotein 130 (gp130)-dependent cytokines

The gp130 family of cytokines is a subclass of type I cytokines composed of the prototypical IL-6 as well as IL-11, IL-27, leukemia inhibitory factor (LIF), cardiotrophin-1, ciliary neurotrophic factor and oncostatin M (OSM) [114]. These soluble mediators bind to and signal through unique multimeric receptors composed of one (IL-27, LIF, OSM) or two (IL-6, IL-11) common gp130 subunits associated with distinct α-chains inherent to each cytokine [114,115]. The gp130 receptor subunit is essential for signal transduction and predominantly activates JAK/STAT molecules (notably JAK1, JAK2, Tyk2 and STAT1, STAT3, STAT5) but also the PI3K/AKT and MAPK pathways [116–119]. Gp130 is ubiquitously expressed within hematopoietic and non-hematopoietic tissues [120]. Thus, cytokines that signal through the gp130 receptor chain mediate a broad spectrum of actions in the development of innate and adaptive immune responses.

Several aspects of the biology of gp130-dependent cytokines have suggested a role for these cytokines in the control of chronic viral infection. First, numerous studies uncovered the dominant role of gp130-dependent cytokines in the regulation of inflammatory responses. Particularly, IL-6 displays key pro-inflammatory properties essential for the initiation of inflammatory responses and subsequent recruitment of innate and adaptive mediators to inflamed tissues [114]. These functions of IL-6, besides being associated to multiple inflammatory autoimmune diseases, are also essential to sustain immune responses to pathogenic infections [121]. Indeed, several reports have underlined the incapacity of IL-6$^{-/-}$ mice to control fungal, parasitic, bacterial and viral infections as a result of defective innate cells recruitment and suboptimal T-cell responses [122–128]. Conversely, gp130-dependent cytokines, notably IL-6 and IL-27, also display anti-inflammatory properties [114,129]. For instance, IL-6 prevents uncontrolled accumulation of neutrophils to inflamed tissues and limits severe lung pathology in a mouse model of influenza infection [130–133]. Further, IL-27 tempers overwhelming T-cell activation during infection and prevents the development of pathogenic Th17 cells in multiple autoimmune diseases, in contrast to IL-6 [114,133,134]. Thus, not only gp130-dependent cytokines have the potency to sustain adaptive responses during infection but also prevent T-cell hyperactivation and limit organ immunopathologies.

These observations suggest that gp130-dependent cytokines sustain T-cell responses during chronic viral infection while limiting exacerbated responses. Of importance, IL-6 is known for its direct impact on the differentiation of T follicular helper (Tfh) cells [129,135,136]. Interestingly, a higher proportion of Tfh cells was reported in mice infected with LCMV Cl-13 compared to LCMV Arm, and that CD4 T-cell subset was critical to support B-cell responses and viral control [9,137]. In line with its capacity to promote Tfh-cell differentiation, IL-6 but also IL-27 induce IL-21 production by CD4 T cells, an essential cytokine for the maintenance of CD8 T-cell responses during chronic viral infection (discussed below) [11,138–143]. These results suggest that gp130-dependent cytokines are required to sustain both cellular and humoral immune responses during chronic viral infection.

In humans, elevated serum levels of IL-6 were reported in patients with HIV, HCV and HBV infections, and in the former, disease progression was correlated with lower levels of IL-6 [144–146]. Additional studies also reported an inverse correlation between serum levels of IL-27 and markers of disease progression in HIV and HCV patients, while others did not [147–150]. Finally, patients suffering from rheumatoid arthritis (or other inflammatory autoimmune diseases) and treated with tocilizumab, a monoclonal antibody against IL6R that induces rapid and sustained clinical responses, may reactivate *Mycobacterium tuberculosis*, HBV or HCV, suggesting a role for IL-6 in the control of latent infections [121]. Taken together, these observations are highly predictive of a role for gp130-dependent cytokines in the control of adaptive responses to chronic viral infection.

Using a mouse model carrying a T-cell restricted deletion of gp130, Harker et al. uncovered an essential role for gp130-dependent signals for the control of LCMV Cl-13 [151]. Indeed, while being dispensable during the acute phase of infection (day 9 p.i.), gp130-dependent signals were essential for late accumulation of Ag-specific CD4 and CD8 T cells during viral persistence [151]. More in depth analysis revealed that the CD4 T-cell compartment was particularly affected in the absence of gp130. In fact, T-cell restricted gp130-deficiency impaired the accumulation of Tfh cells during chronicity, restrained Ag-specific CD4 T-cell sur-

vival and production of both IFNγ and IL-21 [151]. In contrast, gp130-dependent signals only modestly affected the functionality of CD8 T cells. However, as for CD4 T cells, the maintenance of Ag-specific CD8 T cells was defective during viral persistence. This observation likely resulted from the lack of CD4-derived IL-21 rather than a direct impact of gp130-dependent signals on CD8 T-cell maintenance [11,141,142,151]. Of note, defective Tfh cell generation and lack of IL-21 production correlated with a critical reduction in the number of germinal center B (GCB) cells and anti-LCMV antibody production [151]. Together, these results demonstrate that gp130-dependent signals directly and indirectly sustain T and B cell responses during chronic viral infection and are critically required for the control of viremia.

Additional work by the same group investigated the relevant gp130-dependent cytokines involved in the control of persistent viral infections [151,152]. Interestingly, the decline in T-cell numbers seen in infected gp130-deficient mice correlated with IL-6 and IL-27 being the only two gp130-dependent cytokines able to trigger the phosphorylation of STAT3 in Ag-specific CD4 T cells *ex vivo*, while IL-27 also selectively induced the phosphorylation of STAT1 [151]. Infection of either IL-6$^{-/-}$ or IL27R$\alpha^{-/-}$ mice with LCMV Cl-13 resulted in impaired viral control similar to what was observed in gp130-deficient mice [151,152]. This demonstrated that both cytokines were individually essential for the control of chronic viral infection. In IL27Rα-deficient mice, this correlated with a gradual decline in the number of Ag-specific CD4 T cells during chronicity that was not observed in IL-6$^{-/-}$ mice [151,152]. However, the proportion of Tfh cells was substantially reduced in the absence of IL-6, but not within IL27R$\alpha^{-/-}$ mice. The lack of Tfh cells in IL-6$^{-/-}$ mice impaired the development of GCB cells and led to severe reduction in anti-LCMV IgG levels, which led to uncontrolled viremia (Fig. 1) [152,153]. Of note, neither IL-6 nor IL27Rα-deficiency impaired Ag-specific CD4 T-cell capacity to produce IL-21 while this function was critically diminished in gp130-deficient mice. This indicated that IL-6 and IL-27 likely act redundantly to sustain IL-21 production by Ag-specific CD4 T cells during chronic viral infection (Fig. 1) [151,152]. This is in accordance with the fact that both IL-6 and IL-27 induce IL-21 production by Ag-specific CD4 T cells *ex vivo* [151]. Taken together, these results demonstrate that IL-6 and IL-27 display individual and redundant functions to regulate adaptive immune responses to chronic viral infection. IL-27 further inhibits HIV replication in human monocytes, macrophages, DCs and CD4 T cells *in vitro* [154–157]. These combined abilities would be of great interest in the design of novel therapeutic strategies for chronic viral infections.

4. Common gamma (γ_c) chain-dependent cytokines

The γ_c-dependent cytokines that include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are pleiotropic factors, dispensing multiple functions in several immune cell types [158]. This family of cytokines has long been associated with T-cell development. Notably, γ_c-dependent cytokines display essential signals for the generation, differentiation and homeostasis of naïve and memory T cells [159–162]. *De facto*, the potential of these cytokines as immunotherapeutic agents to boost T-cell responses during persistent viral infections and cancers has been under the scope for numerous years [163,164].

4.1. IL-21

IL-21 is the most recently discovered member of the γ_c-dependent cytokine family [165]. During viral infections, IL-21 is primarily produced by Th17 CD4 T cells [166–168]. IL-21 signals through a dimeric receptor composed of the γ_c chain (CD132) and a unique IL-21-receptor (IL21R) chain (CD360). Engagement of IL-21 to its cognate receptor activates Jak1, Jak3 and subsequently STATs notably STAT1, STAT5a, STAT5b and predominantly STAT3 [169]. In addition, IL-21 signals though the canonical PI3K and MAPK pathways. As multiple immune cell types express the IL21R, this cytokine exerts pleiotropic modulatory functions in both innate and adaptive cells including NK cells, B cells, CD4 and CD8 T cells [169].

CD4 T-cell help is essential for maintaining an effective antiviral CD8 T-cell response during chronic viral infections [170–172]. For example, loss of CD4 T cells during acute HCV infection often leads to viral persistence [173–175]. In rodents, CD4 T-cell depletion before LCMV Cl-13 inoculation causes lifelong viremia [171]. These observations were correlated with critical loss of CD8 T-cell immunity. Hence, efforts were made to identify the CD4-derived "helper" factor required to maintain a potent antiviral CD8 T-cell response during chronic viral infection. Because IL-2 production by T cells rapidly vanishes due to functional exhaustion, IL-21 became the candidate cytokine. Three independent studies used the LCMV Cl-13 model to explore the impact of IL-21 in maintaining efficient T-cell responses during chronic viral infection [11,141,142]. IL-21 was readily detected at distinct phases of chronic LCMV infection and CD4 T cells were uniformly reported to be the main source of the cytokine [11,141,142]. Interestingly, chronically stimulated CD4 T cells appeared to rapidly shift from an IL-2 to an IL-21 secretion profile [11]. The importance of IL-21 was further underlined by the fact that IL21R$^{-/-}$ or IL-21$^{-/-}$ mice failed to control chronic LCMV infection despite the maintenance of high numbers of CD4 T cells. More in depth analysis revealed that CD4-derived IL-21 critically sustained the maintenance and polyfunctionality of CD8 T cells in a cell-intrinsic manner (Fig. 1) [11,141,142]. Treatment of chronically infected mice with exogenous IL-21 improved the number and functionality of CD8 T cells, decreased viral titers, but led to animal demise in 70% of cases [141]. However, in this study, IL-21 treatment was only attempted during the acute phase of the infection and in severe conditions, when CD4 T cells were depleted. Hence, further work is needed to determine if IL-21 administration in conditions of reduced viral loads can improve CD8 T-cell responses without lethal adverse effects. Concomitantly, determining how IL-21 intrinsically sustains CD8 T-cell maintenance and effector functions is an important future research question.

4.2. IL-7

IL-7 is a central cytokine for the development of T cells and an essential component for the homeostasis of naïve and memory T cells [160,176–181]. Primarily produced by stromal cells in primary and secondary lymphoid tissues, IL-7 signals through a dimeric receptor composed of the IL7Rα-chain (CD127) and the γ_c chain (CD132). IL7R principally triggers Jak1 and Jak3 and subsequently STAT5, but can also activate the PI3K and MAPK pathways [178,182]. Hence IL-7 regulates several key functions of T-cell biology including metabolism, migration, cell cycle progression and survival [178,182].

The unique capacity of IL-7 to promote T-cell development, survival and homeostasis raised interest in using this cytokine as an immunotherapeutic agent to reverse the severe lymphopenia that often comes with chronic viral infections [183–185]. IL-7 administration to patients with HIV or primates with SIV greatly enhanced the number of circulating CD4 and CD8 T cells with a preferential peripheral expansion of naïve and central memory T cells [186–190]. Thus, IL-7 appeared as a powerful supportive agent for maintaining substantial numbers of T cells during chronic viral infection. In addition, when used as antitumor vaccine adjuvant in mice, IL-7 increases the proliferation and cytotoxicity of CD8 T cells [191,192]. Further, IL7R expression on effector CD8 T cells from

chronically infected mice was inversely correlated with the severity of exhaustion [193]. Thus, besides its capacity to enhance the number of circulating T cells, IL-7 might also prevents their exhaustion.

Studies by Nanjappa et al. and Pellegrini et al. recently examined more accurately the therapeutic benefits of prophylactic IL-7 in mice that were chronically infected with LCMV Cl-13 [194,195]. Similar to observations in humans, both studies reported that prolonged IL-7 treatment largely expanded the number of polyfunctional CD4 and CD8 T cells which led to accelerated viral elimination (Fig. 1) [194,195]. This later effect was dependent on T cells as depletion of either CD4 or CD8 T cells abrogated the beneficial effects of IL-7 [195]. In parallel, IL-7 treatment also increased the number of non-Ag specific CD8 T cells in the periphery, likely by enhancing thymic output (Fig. 1) [195]. This suggests that IL-7 treatment could be particularly interesting for HIV patients to restore a normal T-cell repertoire and prevent bystander infections associated with AIDS. Importantly, exogenous IL-7 augmented IL-22 levels, a cytoprotective cytokine that prevented liver damage that could arose from the important expansion of T cells after treatment (Fig. 1) [195–197]. Together, these observations suggest that IL-7 represents a promising immunotherapeutic agent capable of boosting Ag-specific T-cell immunity without causing collateral damage to the host.

More in depth analysis of adaptive responses also revealed that IL-7 treatment critically reduced levels of the suppressor of cytokine signaling 3 (Socs3), a known inhibitor of IL-6 signaling, within T cells [195,198]. Interestingly, IL-6 levels were increased in the serum of treated mice and IL-7-mediated viral control was hindered in IL-6-deficient animals [195]. Further, mice with T-cell restricted Socs3-deficiency recapitulated some aspects of IL-7 treatment and rapidly cleared Cl-13 infection [195]. Taken together, IL-7 therapy augments T-cell immunity directly by restoring T-cell responsiveness to IL-6 through Socs3 modulation and indirectly by augmenting IL-6 serum levels. So far, a direct impact of IL-7 in preventing and/or reversing T-cell exhaustion remains a possibility that requires further investigations.

4.3. IL-2

CD4 and CD8 T cells are the primary source of IL-2 during viral infection [199–201]. IL-2 signals on T cells via a trimeric receptor composed of a unique IL2Rα chain (CD25); an IL2Rβ chain (CD122) that is also shared with IL-15; and the γ_c chain (CD132) [201]. As other members of the γ_c-dependent cytokine family, IL-2 binding to its cognate receptor engages JAK/STAT molecules, particularly STAT5a and STAT5b and also triggers the PI3k and MAPK pathways [202]. Since its first description as a powerful growth factor for T cells *in vitro*, IL-2 was shown to mediate a broad spectrum of positive and negative functions on T-cell immunity [201,202].

Numerous *in vitro* and *in vivo* studies using IL-2$^{-/-}$ or IL2R$\alpha^{-/-}$ transgenic (Tg) mouse models have elucidated multiple positive functions of IL-2 in the enhancement of CD8 T-cell responses. During acute viral infection, IL-2 augments the late proliferation of effector CD8 T cells, especially at non-lymphoid locations, magnifying the overall antiviral T-cell response [203–206]. In addition to the quantity, IL-2 also fine-tunes the quality of primary effectors by promoting the secretion of cytolytic and antiviral molecules (i.e. granzyme, perforin, IFNγ) [207,208]. During chronic viral infection, numbers of those functions decrease due to functional exhaustion and T cells rapidly cease to produce IL-2 [43]. Hence, providing exogenous IL-2 to compensate for this early loss was considered a relevant therapeutic option.

Recent studies examined both the therapeutic potential of providing low-dose exogenous IL-2 in mice chronically infected with LMCV Cl-13 and the effects of such treatment on Ag-specific CD8 T-cell responses [209,210]. Daily low-dose IL-2 regimen for eight days in mice with established Cl-13 infection greatly improved the number of polyfunctional Ag-specific CD8 T cells at multiple lymphoid and non-lymphoid anatomical locations (Fig. 1) [210]. This increased number of CD8 effectors correlated with both a reduction in plasma viral titers assessed directly after treatment, and a subsequent reduction in inhibitory receptor levels, a typical signature of exhaustion [4,5,209,210]. Importantly, combining IL-2 therapy with PDL1 blockade exponentially increased the number of Ag-specific CD8 T cells and led to viral clearance in a majority of mice (Fig. 1) [210]. Those exciting results provided a new therapeutic opportunity for treating established chronic viral infections in humans.

There are however serious concerns about the use of IL-2 as a treatment for boosting CD8 T-cell responses in humans. First, a plethora of clinical trials in HIV patients demonstrated the limited impact of IL-2 for increasing the number of functional CD8 T cells and decreasing HIV titers [211–215]. Second, providing IL-2 alone in mice with severe life-long LCMV Cl-13 infection adversely increased viral titers [210]. Also, CD8 effectors expanded *in vivo* by exogenous IL-2 during an Arm infection were less efficient in containing a subsequent infection by the more virulent Cl-13 strain [209]. *In vitro*, IL-2-expanded CD8 T cells were not maintained after transfer into tumor-bearing mice and displayed modest anti-tumor effects [216]. Hence, care must be taken when designing therapeutic regimens with this cytokine. Finally, while IL-2 delivers critical signals to shape the differentiation of CD8 T cells during an acute viral infection [162,206–208,217], the possible contribution of this cytokine in the deflection toward T-cell exhaustion in chronic settings remains unknown. Hence, a better understanding of the physiological functions of IL-2 in that process should decipher the positive and potential suppressive functions of this cytokine on CD8 T-cell responses to chronic viral infection.

5. Concluding remarks

It has now become clear that cytokines have a major impact in immune responses to persistent viral infections. At early time points following a viral infection, these soluble mediators, in particular IL-10 and type I IFNs, dispense direct and indirect suppressive signals, essential for the persistence of the virus in the host. Evenmore, cytokines contribute to the continuous silencing of immune functions, which can be reversed by antibody blocking approaches [40,41,107,108]. Conversely, gp130-dependent cytokines deliver key signals to T cells to support continuous cellular and humoral immune responses [129,143,151,152]. Concomitantly, γ_c-dependent cytokines are essential at a physiological level for preserving a stable adaptive T-cell pool and can be modulated to reinvigorate these responses and achieve viral elimination [11,141,142,194,195,209,210]. It has also become evident that the functional impact of a cytokine can be strikingly distinct and even opposite in acute versus chronic contexts of infection. For instance, IL-21 is of negligible importance during both primary and secondary T-cell responses to acute Arm infection in mice [142,218], while it is vital during Cl-13 infection [11,141,142]. Still more, blockade of type I IFNs precludes rapid clearance of LCMV Arm, while it accelerates the elimination of the chronic strain Cl-13 [107]. These ascertainments reinforce the need to revisit some of our certitudes on the role of cytokines in immune responses using chronic infection models. With this newer understanding, we will be enabled to design better immunotherapeutic strategies through the usage of appropriate cytokines, cytokine-blocking antibodies or Jakinibs, for combating chronic viral infections in humans.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

We are grateful to members and collaborators of the Cytokines and Adaptive Immunity laboratory for their technical and scientific input on this subject. We thank Judith Kashul for critical reading of the manuscript. H. Decaluwe is supported by grants from the Canadian Institutes of Health Research – Canada, the National Institutes of Health – United States, the Cole Foundation – Canada, the Fonds de Recherche du Québec – Santé – Canada, and the Canadian Child Health Clinician Scientist Program – Canada. JC. Beltra has a research scholarship from The American Association of Immunologists – United States.

References

[1] S.M. Kaech, W. Cui, Transcriptional control of effector and memory CD8+ T cell differentiation, Nat. Rev. Immunol. 12 (2012) 749–761.
[2] E.J. Wherry, T cell exhaustion, Nat. Immunol. 12 (2011) 492–499.
[3] E.J. Wherry, M. Kurachi, Molecular and cellular insights into T cell exhaustion, Nat. Rev. Immunol. 15 (2015) 486–499.
[4] E.J. Wherry, S.J. Ha, S.M. Kaech, W.N. Haining, S. Sarkar, V. Kalia, et al., Molecular signature of CD8+ T cell exhaustion during chronic viral infection, Immunity 27 (2007) 670–684.
[5] S.D. Blackburn, H. Shin, W.N. Haining, T. Zou, C.J. Workman, A. Polley, et al., Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection, Nat. Immunol. 10 (2009) 29–37.
[6] P.M. Odorizzi, E.J. Wherry, Inhibitory receptors on lymphocytes: insights from infections, J. Immunol. 188 (2012) 2957–2965.
[7] H. Shin, S.D. Blackburn, J.N. Blattman, E.J. Wherry, Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection, J. Exp. Med. 204 (2007) 941–949.
[8] E.J. Wherry, D.L. Barber, S.M. Kaech, J.N. Blattman, R. Ahmed, Antigen-independent memory CD8 T cells do not develop during chronic viral infection, Proc. Natl. Acad. Sci. USA 101 (2004) 16004–16009.
[9] A. Crawford, J.M. Angelosanto, C. Kao, T.A. Doering, P.M. Odorizzi, B.E. Barnett, et al., Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection, Immunity 40 (2014) 289–302.
[10] D.G. Brooks, L. Teyton, M.B. Oldstone, D.B. McGavern, Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection, J. Virol. 79 (2005) 10514–10527.
[11] H. Elsaesser, K. Sauer, D.G. Brooks, IL-21 is required to control chronic viral infection, Science 324 (2009) 1569–1572.
[12] M.A. Paley, D.C. Kroy, P.M. Odorizzi, J.B. Johnnidis, D.V. Dolfi, B.E. Barnett, et al., Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection, Science 338 (2012) 1220–1225.
[13] E.B. Wilson, D.G. Brooks, The role of IL-10 in regulating immunity to persistent viral infections, Curr. Top. Microbiol. Immunol. 350 (2011) 39–65.
[14] R.P. Donnelly, F. Sheikh, S.V. Kotenko, H. Dickensheets, The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain, J. Leukoc. Biol. 76 (2004) 314–321.
[15] S.V. Kotenko, C.D. Krause, L.S. Izotova, B.P. Pollack, W. Wu, S. Pestka, Identification and functional characterization of a second chain of the interleukin-10 receptor complex, EMBO J. 16 (1997) 5894–5903.
[16] K.W. Moore, R. de Waal Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.
[17] D.S. Finbloom, K.D. Winestock, IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes, J. Immunol. 155 (1995) 1079–1090.
[18] C.F. Lai, J. Ripperger, K.K. Morella, J. Jurlander, T.S. Hawley, W.E. Carson, et al., Receptors for interleukin (IL)-10 and IL-6-type cytokines use similar signaling mechanisms for inducing transcription through IL-6 response elements, J. Biol. Chem. 271 (1996) 13968–13975.
[19] J. Wehinger, F. Gouilleux, B. Groner, J. Finke, R. Mertelsmann, R.M. Weber-Nordt, IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes, FEBS Lett. 394 (1996) 365–370.
[20] R.M. Weber-Nordt, J.K. Riley, A.C. Greenlund, K.W. Moore, J.E. Darnell, R.D. Schreiber, Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain, J. Biol. Chem. 271 (1996) 27954–27961.
[21] R.P. Woitas, U. Petersen, D. Moshage, H.H. Brackmann, B. Matz, T. Sauerbruch, et al., HCV-specific cytokine induction in monocytes of patients with different outcomes of hepatitis C, World J. Gastroenterol.: WJG 8 (2002) 562–566.
[22] H. Hofer, J.B. Neufeld, C. Oesterreicher, P. Grundtner, F. Wrba, A. Gangl, et al., Bi-allelic presence of the interleukin-10 receptor 1 G330R allele is associated with cirrhosis in chronic HCV-1 infection, Genes Immun. 6 (2005) 242–247.
[23] C. Graziosi, G. Pantaleo, K.R. Gantt, J.P. Fortin, J.F. Demarest, O.J. Cohen, et al., Lack of evidence for the dichotomy of TH1 and TH2 predominance in HIV-infected individuals, Science 265 (1994) 248–252.
[24] M. Clerici, M.L. Fusi, S. Ruzzante, S. Piconi, M. Biasin, D. Arienti, et al., Type 1 and type 2 cytokines in HIV infection – a possible role in apoptosis and disease progression, Ann. Med. 29 (1997) 185–188.
[25] S. Swaminathan, Molecular biology of Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus, Semin. Hematol. 40 (2003) 107–115.
[26] J.Y. Cheong, S.W. Cho, I.L. Hwang, S.K. Yoon, J.H. Lee, C.S. Park, et al., Association between chronic hepatitis B virus infection and interleukin-10, tumor necrosis factor-alpha gene promoter polymorphisms, J. Gastroenterol. Hepatol. 21 (2006) 1163–1169.
[27] M. Helminen, N. Lahdenpohja, M. Hurme, Polymorphism of the interleukin-10 gene is associated with susceptibility to Epstein–Barr virus infection, J. Infect. Dis. 180 (1999) 496–499.
[28] N. Paladino, H. Fainboim, G. Theiler, T. Schroder, A.E. Munoz, A.C. Flores, et al., Gender susceptibility to chronic hepatitis C virus infection associated with interleukin 10 promoter polymorphism, J. Virol. 80 (2006) 9144–9150.
[29] H.D. Shin, C. Winkler, J.C. Stephens, J. Bream, H. Young, J.J. Goedert, et al., Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10, Proc. Natl. Acad. Sci. USA 97 (2000) 14467–14472.
[30] S. Miyazoe, K. Hamasaki, K. Nakata, Y. Kajiya, K. Kitajima, K. Nakao, et al., Influence of interleukin-10 gene promoter polymorphisms on disease progression in patients chronically infected with hepatitis B virus, Am. J. Gastroenterol. 97 (2002) 2086–2092.
[31] M. Persico, M. Capasso, E. Persico, M. Masarone, A. Renzo, D. Spano, et al., Interleukin-10 – 1082 GG polymorphism influences the occurrence and the clinical characteristics of hepatitis C virus infection, J. Hepatol. 45 (2006) 779–785.
[32] S. Knapp, B.J. Hennig, A.J. Frodsham, L. Zhang, S. Hellier, M. Wright, et al., Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection, Immunogenetics 55 (2003) 362–369.
[33] B. Slobedman, P.A. Barry, J.V. Spencer, S. Avdic, A. Abendroth, Virus-encoded homologs of cellular interleukin-10 and their control of host immune function, J. Virol. 83 (2009) 9618–9629.
[34] M.A. Brockman, D.S. Kwon, D.P. Tighe, D.F. Pavlik, P.C. Rosato, J. Sela, et al., IL-10 is up-regulated in multiple cell types during viremic HIV infection and reversibly inhibits virus-specific T cells, Blood 114 (2009) 346–356.
[35] M. Clerici, T.A. Wynn, J.A. Berzofsky, S.P. Blatt, C.W. Hendrix, A. Sher, et al., Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus, J. Clin. Invest. 93 (1994) 768–775.
[36] A.L. Landay, M. Clerici, F. Hashemi, H. Kessler, J.A. Berzofsky, G.M. Shearer, In vitro restoration of T cell immune function in human immunodeficiency virus-positive persons: effects of interleukin (IL)-12 and anti-IL-10, J. Infect. Dis. 173 (1996) 1085–1091.
[37] E.A. Said, F.P. Dupuy, L. Trautmann, Y. Zhang, Y. Shi, M. El-Far, et al., Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection, Nat. Med. 16 (2010) 452–459.
[38] E.I. Rigopoulou, W.G. Abbott, P. Haigh, N.V. Naoumov, Blocking of interleukin-10 receptor – a novel approach to stimulate T-helper cell type 1 responses to hepatitis C virus, Clin. Immunol. 117 (2005) 57–64.
[39] H. Dong, G. Zhu, K. Tamada, L. Chen, B7–H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion, Nat. Med. 5 (1999) 1365–1369.
[40] D.G. Brooks, M.J. Trifilo, K.H. Edelmann, L. Teyton, D.B. McGavern, M.B. Oldstone, Interleukin-10 determines viral clearance or persistence in vivo, Nat. Med. 12 (2006) 1301–1309.
[41] M. Ejrnaes, C.M. Filippi, M.M. Martinic, E.M. Ling, L.M. Togher, S. Crotty, et al., Resolution of a chronic viral infection after interleukin-10 receptor blockade, J. Exp. Med. 203 (2006) 2461–2472.
[42] R. Ahmed, A. Salmi, L.D. Butler, J.M. Chiller, M.B. Oldstone, Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence, J. Exp. Med. 160 (1984) 521–540.
[43] E.J. Wherry, J.N. Blattman, K. Murali-Krishna, R. van der Most, R. Ahmed, Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment, J. Virol. 77 (2003) 4911–4927.
[44] D.G. Brooks, S.J. Ha, H. Elsaesser, A.H. Sharpe, G.J. Freeman, M.B. Oldstone, IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection, Proc. Natl. Acad. Sci. USA 105 (2008) 20428–20433.
[45] K. Steinbrink, M. Wolfl, H. Jonuleit, J. Knop, A.H. Enk, Induction of tolerance by IL-10-treated dendritic cells, J. Immunol. 159 (1997) 4772–4780.
[46] K. Kajino, I. Nakamura, H. Bamba, T. Sawai, K. Ogasawara, Involvement of IL-10 in exhaustion of myeloid dendritic cells and rescue by CD40 stimulation, Immunology 120 (2007) 28–37.
[47] C. Carbonneil, V. Donkova-Petrini, A. Aouba, L. Weiss, Defective dendritic cell function in HIV-infected patients receiving effective highly active antiretroviral therapy: neutralization of IL-10 production and depletion of CD4+CD25+ T cells restore high levels of HIV-specific CD4+ T cell responses

induced by dendritic cells generated in the presence of IFN-alpha, J. Immunol. 172 (2004) 7832–7840.
[48] K.E. Foulds, M.J. Rotte, R.A. Seder, IL-10 is required for optimal CD8 T cell memory following Listeria monocytogenes infection, J. Immunol. 177 (2006) 2565–2574.
[49] H. Groux, M. Bigler, J.E. de Vries, M.G. Roncarolo, Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells, J. Immunol. 160 (1998) 3188–3193.
[50] S.S. Kang, P.M. Allen, Priming in the presence of IL-10 results in direct enhancement of CD8+ T cell primary responses and inhibition of secondary responses, J. Immunol. 174 (2005) 5382–5389.
[51] R. Govinden, K.D. Bhoola, Genealogy, expression, and cellular function of transforming growth factor-beta, Pharmacol. Ther. 98 (2003) 257–265.
[52] J.H. Kehrl, L.M. Wakefield, A.B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, et al., Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth, J. Exp. Med. 163 (1986) 1037–1050.
[53] J.J. Letterio, A.B. Roberts, Regulation of immune responses by TGF-beta, Annu. Rev. Immunol. 16 (1998) 137–161.
[54] L. Lagneaux, A. Delforge, C. Dorval, D. Bron, P. Stryckmans, Excessive production of transforming growth factor-beta by bone marrow stromal cells in B-cell chronic lymphocytic leukemia inhibits growth of hematopoietic precursors and interleukin-6 production, Blood 82 (1993) 2379–2385.
[55] A. Filer, C. Pitzalis, C.D. Buckley, Targeting the stromal microenvironment in chronic inflammation, Curr. Opin. Pharmacol. 6 (2006) 393–400.
[56] J.P. Annes, J.S. Munger, D.B. Rifkin, Making sense of latent TGFbeta activation, J. Cell Sci. 116 (2003) 217–224.
[57] I. Nunes, R.L. Shapiro, D.B. Rifkin, Characterization of latent TGF-beta activation by murine peritoneal macrophages, J. Immunol. 155 (1995) 1450–1459.
[58] Y. Shi, J. Massague, Mechanisms of TGF-beta signaling from cell membrane to the nucleus, Cell 113 (2003) 685–700.
[59] J. Massague, TGF-beta signal transduction, Annu. Rev. Biochem. 67 (1998) 753–791.
[60] J. Massague, How cells read TGF-beta signals, Nat. Rev. Mol. Cell Biol. 1 (2000) 169–178.
[61] G.J. Inman, F.J. Nicolas, C.S. Hill, Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity, Mol. Cell 10 (2002) 283–294.
[62] Y. Shi, Y.F. Wang, L. Jayaraman, H. Yang, J. Massague, N.P. Pavletich, Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling, Cell 94 (1998) 585–594.
[63] L. Zawel, J.L. Dai, P. Buckhaults, S. Zhou, K.W. Kinzler, B. Vogelstein, et al., Human Smad3 and Smad4 are sequence-specific transcription activators, Mol. Cell 1 (1998) 611–617.
[64] L. Xu, Y.G. Chen, J. Massague, The nuclear import function of Smad2 is masked by SARA and unmasked by TGFbeta-dependent phosphorylation, Nat. Cell. Biol. 2 (2000) 559–562.
[65] L. Xu, Y. Kang, S. Col, J. Massague, Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus, Mol. Cell 10 (2002) 271–282.
[66] M.O. Li, Y.Y. Wan, S. Sanjabi, A.K. Robertson, R.A. Flavell, Transforming growth factor-beta regulation of immune responses, Annu. Rev. Immunol. 24 (2006) 99–146.
[67] A.B. Kulkarni, C.G. Huh, D. Becker, A. Geiser, M. Lyght, K.C. Flanders, et al., Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death, Proc. Natl. Acad. Sci. USA 90 (1993) 770–774.
[68] M.M. Shull, I. Ormsby, A.B. Kier, S. Pawlowski, R.J. Diebold, M. Yin, et al., Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease, Nature 359 (1992) 693–699.
[69] S. Kobayashi, K. Yoshida, J.M. Ward, J.J. Letterio, G. Longenecker, L. Yaswen, et al., Beta 2-microglobulin-deficient background ameliorates lethal phenotype of the TGF-beta 1 null mouse, J. Immunol. 163 (1999) 4013–4019.
[70] J.J. Letterio, A.G. Geiser, A.B. Kulkarni, H. Dang, L. Kong, T. Nakabayashi, et al., Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression, J. Clin. Invest. 98 (1996) 2109–2119.
[71] J.C. Marie, D. Liggitt, A.Y. Rudensky, Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor, Immunity 25 (2006) 441–454.
[72] P.J. Lucas, S.J. Kim, S.J. Melby, R.E. Gress, Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor, J. Exp. Med. 191 (2000) 1187–1196.
[73] J.T. Lin, S.L. Martin, L. Xia, J.D. Gorham, TGF-beta 1 uses distinct mechanisms to inhibit IFN-gamma expression in CD4+ T cells at priming and at recall: differential involvement of Stat4 and T-bet, J. Immunol. 174 (2005) 5950–5958.
[74] M.J. Smyth, S.L. Strobl, H.A. Young, J.R. Ortaldo, A.C. Ochoa, Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood CD8+ T lymphocytes. Inhibition by transforming growth factor-beta, J. Immunol. 146 (1991) 3289–3297.
[75] M. Ahmadzadeh, L.A. Johnson, B. Heemskerk, J.R. Wunderlich, M.E. Dudley, D. E. White, et al., Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired, Blood 114 (2009) 1537–1544.
[76] M.L. Chen, M.J. Pittet, L. Gorelik, R.A. Flavell, R. Weissleder, H. von Boehmer, et al., Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo, Proc. Natl. Acad. Sci. USA 102 (2005) 419–424.
[77] T.R. Mempel, M.J. Pittet, K. Khazaie, W. Weninger, R. Weissleder, H. von Boehmer, et al., Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation, Immunity 25 (2006) 129–141.
[78] L. Gorelik, S. Constant, R.A. Flavell, Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation, J. Exp. Med. 195 (2002) 1499–1505.
[79] C. Kao, K.J. Oestreich, M.A. Paley, A. Crawford, J.M. Angelosanto, M.A. Ali, et al., Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection, Nat. Immunol. 12 (2011) 663–671.
[80] R. Tinoco, V. Alcalde, Y. Yang, K. Sauer, E.I. Zuniga, Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo, Immunity 31 (2009) 145–157.
[81] G. Malherbe, H.C. Steel, S. Cassol, T. de Oliveira, C.J. Seebregts, R. Anderson, et al., Circulating biomarkers of immune activation distinguish viral suppression from nonsuppression in HAART-treated patients with advanced HIV-1 subtype C infection, Mediat. Inflamm. 2014 (2014) 198413.
[82] J. Kekow, W. Wachsman, J.A. McCutchan, W.L. Gross, M. Zachariah, D.A. Carson, et al., Transforming growth factor-beta and suppression of humoral immune responses in HIV infection, J. Clin. Invest. 87 (1991) 1010–1016.
[83] D.R. Nelson, R.P. Gonzalez-Peralta, K. Qian, Y. Xu, C.G. Marousis, G.L. Davis, et al., Transforming growth factor-beta 1 in chronic hepatitis C, J. Viral Hepatitis 4 (1997) 29–35.
[84] J.M. Grayson, A.E. Weant, B.C. Holbrook, D. Hildeman, Role of Bim in regulating CD8+ T-cell responses during chronic viral infection, J. Virol. 80 (2006) 8627–8638.
[85] P.D. Hughes, G.T. Belz, K.A. Fortner, R.C. Budd, A. Strasser, P. Bouillet, Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity, Immunity 28 (2008) 197–205.
[86] T. Boettler, Y. Cheng, K. Ehrhardt, M. von Herrath, TGF-beta blockade does not improve control of an established persistent viral infection, Viral Immunol. 25 (2012) 232–238.
[87] L. Garidou, S. Heydari, S. Gossa, D.B. McGavern, Therapeutic blockade of transforming growth factor beta fails to promote clearance of a persistent viral infection, J. Virol. 86 (2012) 7060–7071.
[88] M. Colonna, G. Trinchieri, Y.J. Liu, Plasmacytoid dendritic cells in immunity, Nat. Immunol. 5 (2004) 1219–1226.
[89] F. McNab, K. Mayer-Barber, A. Sher, A. Wack, A. O'Garra, Type I interferons in infectious disease, Nat. Rev. Immunol. 15 (2015) 87–103.
[90] L.B. Ivashkiv, L.T. Donlin, Regulation of type I interferon responses, Nat. Rev. Immunol. 14 (2014) 36–49.
[91] J.W. Schoggins, S.J. Wilson, M. Panis, M.Y. Murphy, C.T. Jones, P. Bieniasz, et al., A diverse range of gene products are effectors of the type I interferon antiviral response, Nature 472 (2011) 481–485.
[92] N. Yan, Z.J. Chen, Intrinsic antiviral immunity, Nat. Immunol. 13 (2012) 214–222.
[93] A. Boasso, G.M. Shearer, Chronic innate immune activation as a cause of HIV-1 immunopathogenesis, Clin. Immunol. 126 (2008) 235–242.
[94] G.A. Hardy, S. Sieg, B. Rodriguez, D. Anthony, R. Asaad, W. Jiang, et al., Interferon-alpha is the primary plasma type-I IFN in HIV-1 infection and correlates with immune activation and disease markers, PLoS ONE 8 (2013) e56527.
[95] A.R. Sedaghat, J. German, T.M. Teslovich, J. Cofrancesco Jr., C.C. Jie, C.C. Talbot Jr., et al., Chronic CD4+ T-cell activation and depletion in human immunodeficiency virus type 1 infection: type I interferon-mediated disruption of T-cell dynamics, J. Virol. 82 (2008) 1870–1883.
[96] E. Stylianou, P. Aukrust, K. Bendtzen, F. Muller, S.S. Froland, Interferons and interferon (IFN)-inducible protein 10 during highly active anti-retroviral therapy (HAART)-possible immunosuppressive role of IFN-alpha in HIV infection, Clin. Exp. Immunol. 119 (2000) 479–485.
[97] M.D. Hyrcza, C. Kovacs, M. Loutfy, R. Halpenny, L. Heisler, S. Yang, et al., Distinct transcriptional profiles in ex vivo CD4+ and CD8+ T cells are established early in human immunodeficiency virus type 1 infection and are characterized by a chronic interferon response as well as extensive transcriptional changes in CD8+ T cells, J. Virol. 81 (2007) 3477–3486.
[98] M. Rotger, J. Dalmau, A. Rauch, P. McLaren, S.E. Bosinger, R. Martinez, et al., Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque, J. Clin. Invest. 121 (2011) 2391–2400.
[99] B. Jacquelin, V. Mayau, B. Targat, A.S. Liovat, D. Kunkel, G. Petitjean, et al., Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response, J. Clin. Invest. 119 (2009) 3544–3555.
[100] J.D. Estes, S.N. Gordon, M. Zeng, A.M. Chahroudi, R.M. Dunham, S.I. Staprans, et al., Early resolution of acute immune activation and induction of PD-1 in SIV-infected sooty mangabeys distinguishes nonpathogenic from pathogenic infection in rhesus macaques, J. Immunol. 180 (2008) 6798–6807.
[101] Y. Arimori, R. Nakamura, H. Yamada, K. Shibata, N. Maeda, T. Kase, et al., Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice, Antiviral Res. 99 (2013) 230–237.
[102] B. Schreiner, M. Mitsdoerffer, B.C. Kieseier, L. Chen, H.P. Hartung, M. Weller, et al., Interferon-beta enhances monocyte and dendritic cell expression of B7–H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance

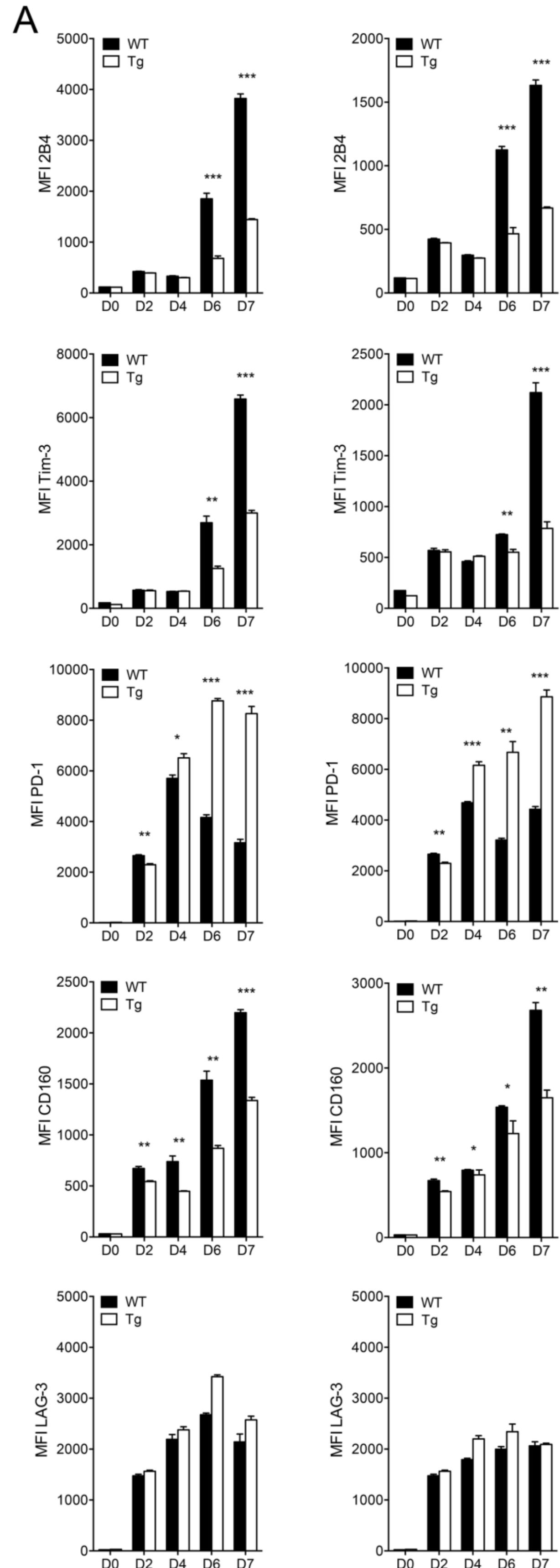
for the immune modulatory effect in multiple sclerosis, J. Neuroimmunol. 155 (2004) 172–182.
[103] R.A. Rudick, D.E. Goodkin, L.D. Jacobs, D.L. Cookfair, R.M. Herndon, J.R. Richert, et al., Impact of interferon beta-1a on neurologic disability in relapsing multiple sclerosis. 1997, Neurology 57 (2001) S25–S30.
[104] H.D. Marshall, S.L. Urban, R.M. Welsh, Virus-induced transient immune suppression and the inhibition of T cell proliferation by type I interferon, J. Virol. 85 (2011) 5929–5939.
[105] A. Kaser, S. Nagata, H. Tilg, Interferon alpha augments activation-induced T cell death by upregulation of Fas (CD95/APO-1) and Fas ligand expression, Cytokine 11 (1999) 736–743.
[106] K.B. Nguyen, L.P. Cousens, L.A. Doughty, G.C. Pien, J.E. Durbin, C.A. Biron, Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox, Nat. Immunol. 1 (2000) 70–76.
[107] J.R. Teijaro, C. Ng, A.M. Lee, B.M. Sullivan, K.C. Sheehan, M. Welch, et al., Persistent LCMV infection is controlled by blockade of type I interferon signaling, Science 340 (2013) 207–211.
[108] E.B. Wilson, D.H. Yamada, H. Elsaesser, J. Herskovitz, J. Deng, G. Cheng, et al., Blockade of chronic type I interferon signaling to control persistent LCMV infection, Science 340 (2013) 202–207.
[109] C.R. Bolen, M.D. Robek, L. Brodsky, V. Schulz, J.K. Lim, M.W. Taylor, et al., The blood transcriptional signature of chronic hepatitis C virus is consistent with an ongoing interferon-mediated antiviral response, J. Interferon Cytokine Res.: Off. J. Int. Soc. Interferon Cytokine Res. 33 (2013) 15–23.
[110] Y. Wang, M. Swiecki, M. Cella, G. Alber, R.D. Schreiber, S. Gilfillan, et al., Timing and magnitude of type I interferon responses by distinct sensors impact CD8 T cell exhaustion and chronic viral infection, Cell Host Microbe 11 (2012) 631–642.
[111] M.H. Heim, 25 years of interferon-based treatment of chronic hepatitis C: an epoch coming to an end, Nat. Rev. Immunol. 13 (2013) 535–542.
[112] E.B. Wilson, D.G. Brooks, Decoding the complexity of type I interferon to treat persistent viral infections, Trends Microbiol. 21 (2013) 634–640.
[113] C.T. Ng, B.M. Sullivan, J.R. Teijaro, A.M. Lee, M. Welch, S. Rice, et al., Blockade of interferon Beta, but not interferon alpha, signaling controls persistent viral infection, Cell Host Microbe 17 (2015) 653–661.
[114] J.S. Silver, C.A. Hunter, Gp130 at the nexus of inflammation, autoimmunity, and cancer, J. Leukoc. Biol. 88 (2010) 1145–1156.
[115] T. Taga, T. Kishimoto, Gp130 and the interleukin-6 family of cytokines, Annu. Rev. Immunol. 15 (1997) 797–819.
[116] N. Stahl, T.G. Boulton, T. Farruggella, N.Y. Ip, S. Davis, B.A. Witthuhn, et al., Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components, Science 263 (1994) 92–95.
[117] C. Lutticken, U.M. Wegenka, J. Yuan, J. Buschmann, C. Schindler, A. Ziemiecki, et al., Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130, Science 263 (1994) 89–92.
[118] M. Takahashi-Tezuka, Y. Yoshida, T. Fukada, T. Ohtani, Y. Yamanaka, K. Nishida, et al., Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase, Mol. Cell. Biol. 18 (1998) 4109–4117.
[119] P.C. Heinrich, I. Behrmann, S. Haan, H.M. Hermanns, G. Muller-Newen, F. Schaper, Principles of interleukin (IL)-6-type cytokine signalling and its regulation, Biochem. J. 374 (2003) 1–20.
[120] M. Saito, K. Yoshida, M. Hibi, T. Taga, T. Kishimoto, Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo, J. Immunol. 148 (1992) 4066–4071.
[121] X. Yao, J. Huang, H. Zhong, N. Shen, R. Faggioni, M. Fung, et al., Targeting interleukin-6 in inflammatory autoimmune diseases and cancers, Pharmacol. Ther. 141 (2014) 125–139.
[122] L. Romani, A. Mencacci, E. Cenci, R. Spaccapelo, C. Toniatti, P. Puccetti, et al., Impaired neutrophil response and CD4+ T helper cell 1 development in interleukin 6-deficient mice infected with Candida albicans, J. Exp. Med. 183 (1996) 1345–1355.
[123] S.A. Dalrymple, R. Slattery, D.M. Aud, M. Krishna, L.A. Lucian, R. Murray, Interleukin-6 is required for a protective immune response to systemic *Escherichia coli* infection, Infect. Immun. 64 (1996) 3231–3235.
[124] Y. Suzuki, S. Rani, O. Liesenfeld, T. Kojima, S. Lim, T.A. Nguyen, et al., Impaired resistance to the development of toxoplasmic encephalitis in interleukin-6-deficient mice, Infect. Immun. 65 (1997) 2339–2345.
[125] M. Kopf, H. Baumann, G. Freer, M. Freudenberg, M. Lamers, T. Kishimoto, et al., Impaired immune and acute-phase responses in interleukin-6-deficient mice, Nature 368 (1994) 339–342.
[126] H. Jebbari, C.W. Roberts, D.J. Ferguson, H. Bluethmann, J. Alexander, A protective role for IL-6 during early infection with Toxoplasma gondii, Parasite Immunol. 20 (1998) 231–239.
[127] C.H. Ladel, C. Blum, A. Dreher, K. Reifenberg, M. Kopf, S.H. Kaufmann, Lethal tuberculosis in interleukin-6-deficient mutant mice, Infect. Immun. 65 (1997) 4843–4849.
[128] T. van der Poll, C.V. Keogh, X. Guirao, W.A. Buurman, M. Kopf, S.F. Lowry, Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia, J. Infect. Dis. 176 (1997) 439–444.
[129] C.A. Hunter, S.A. Jones, IL-6 as a keystone cytokine in health and disease, Nat. Immunol. 16 (2015) 448–457.
[130] V. Modur, Y. Li, G.A. Zimmerman, S.M. Prescott, T.M. McIntyre, Retrograde inflammatory signaling from neutrophils to endothelial cells by soluble interleukin-6 receptor alpha, J. Clin. Invest. 100 (1997) 2752–2756.
[131] R.M. McLoughlin, S.M. Hurst, M.A. Nowell, D.A. Harris, S. Horiuchi, L.W. Morgan, et al., Differential regulation of neutrophil-activating chemokines by IL-6 and its soluble receptor isoforms, J. Immunol. 172 (2004) 5676–5683.
[132] S.M. Hurst, T.S. Wilkinson, R.M. McLoughlin, S. Jones, S. Horiuchi, N. Yamamoto, et al., Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation, Immunity 14 (2001) 705–714.
[133] S.N. Lauder, E. Jones, K. Smart, A. Bloom, A.S. Williams, J.P. Hindley, et al., Interleukin-6 limits influenza-induced inflammation and protects against fatal lung pathology, Eur. J. Immunol. 43 (2013) 2613–2625.
[134] A. Villarino, L. Hibbert, L. Lieberman, E. Wilson, T. Mak, H. Yoshida, et al., The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection, Immunity 19 (2003) 645–655.
[135] Y.S. Choi, D. Eto, J.A. Yang, C. Lao, S. Crotty, Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation, J. Immunol. 190 (2013) 3049–3053.
[136] D. Eto, C. Lao, D. DiToro, B. Barnett, T.C. Escobar, R. Kageyama, et al., IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation, PLoS ONE 6 (2011) e17739.
[137] L.M. Fahey, E.B. Wilson, H. Elsaesser, C.D. Fistonich, D.B. McGavern, D.G. Brooks, Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells, J. Exp. Med. 208 (2011) 987–999.
[138] A. Suto, D. Kashiwakuma, S. Kagami, K. Hirose, N. Watanabe, K. Yokote, et al., Development and characterization of IL-21-producing CD4+ T cells, J. Exp. Med. 205 (2008) 1369–1379.
[139] O. Dienz, S.M. Eaton, J.P. Bond, W. Neveu, D. Moquin, R. Noubade, et al., The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells, J. Exp. Med. 206 (2009) 69–78.
[140] F. Eddahri, S. Denanglaire, F. Bureau, R. Spolski, W.J. Leonard, O. Leo, et al., Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities, Blood 113 (2009) 2426–2433.
[141] J.S. Yi, M. Du, A.J. Zajac, A vital role for interleukin-21 in the control of a chronic viral infection, Science 324 (2009) 1572–1576.
[142] A. Frohlich, J. Kisielow, I. Schmitz, S. Freigang, A.T. Shamshiev, J. Weber, et al., IL-21R on T cells is critical for sustained functionality and control of chronic viral infection, Science 324 (2009) 1576–1580.
[143] E.D. Wojno, C.A. Hunter, New directions in the basic and translational biology of interleukin-27, Trends Immunol. 33 (2012) 91–97.
[144] D.L. Birx, R.R. Redfield, K. Tencer, A. Fowler, D.S. Burke, G. Tosato, Induction of interleukin-6 during human immunodeficiency virus infection, Blood 76 (1990) 2303–2310.
[145] N.E. Spanakis, G.A. Garinis, E.C. Alexopoulos, G.P. Patrinos, P.G. Menounos, A. Sklavounou, et al., Cytokine serum levels in patients with chronic HCV infection, J. Clin. Lab. Anal. 16 (2002) 40–46.
[146] D. Torre, C. Zeroli, M. Giola, G. Ferrario, G.P. Fiori, G. Bonetta, et al., Serum levels of interleukin-1 alpha, interleukin-1 beta, interleukin-6, and tumor necrosis factor in patients with acute viral hepatitis, Clin. Infect. Dis.: Off. Publ. Infect. Dis. Soc. Am. 18 (1994) 194–198.
[147] A. Ashrafi Hafez, A. Ahmadi Vasmehjani, R. Baharlou, S.D. Mousavi Nasab, M. H. Davami, A. Najafi, et al., Analytical assessment of interleukin – 23 and - 27 cytokines in healthy people and patients with hepatitis C virus infection (genotypes 1 and 3a), Hepatitis Month. 14 (2014) e21000.
[148] L. He, J. Zhao, M.H. Wang, K.K. Siu, Y.X. Gan, L. Chen, et al., Interleukin-27 is differentially associated with HIV viral load and CD4+ T cell counts in therapy-naive HIV-mono-infected and HIV/HCV-co-infected Chinese, PLoS ONE 9 (2014) e96792.
[149] C. Guzzo, W.M. Hopman, N.F. Che Mat, W. Wobeser, K. Gee, Impact of HIV infection, highly active antiretroviral therapy, and hepatitis C coinfection on serum interleukin-27, Aids 24 (2010) 1371–1374.
[150] S. Swaminathan, Z. Hu, A.W. Rupert, J.M. Higgins, R.L. Dewar, R. Stevens, et al., Plasma interleukin-27 (IL-27) levels are not modulated in patients with chronic HIV-1 infection, PLoS ONE 9 (2014) e98989.
[151] J.A. Harker, A. Dolgoter, E.I. Zuniga, Cell-intrinsic IL-27 and gp130 cytokine receptor signaling regulates virus-specific CD4(+) T cell responses and viral control during chronic infection, Immunity 39 (2013) 548–559.
[152] J.A. Harker, G.M. Lewis, L. Mack, E.I. Zuniga, Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection, Science 334 (2011) 825–829.
[153] A. Bergthaler, L. Flatz, A. Verschoor, A.N. Hegazy, M. Holdener, K. Fink, et al., Impaired antibody response causes persistence of prototypic T cell-contained virus, PLoS Biol. 7 (2009) e1000080.
[154] T. Imamichi, J. Yang, D.W. Huang, T.W. Brann, B.A. Fullmer, J.W. Adelsberger, et al., IL-27, a novel anti-HIV cytokine, activates multiple interferon-inducible genes in macrophages, Aids 22 (2008) 39–45.
[155] T. Greenwell-Wild, N. Vazquez, W. Jin, Z. Rangel, P.J. Munson, S.M. Wahl, Interleukin-27 inhibition of HIV-1 involves an intermediate induction of type I interferon, Blood 114 (2009) 1864–1874.
[156] L. Dai, K.B. Lidie, Q. Chen, J.W. Adelsberger, X. Zheng, D. Huang, et al., IL-27 inhibits HIV-1 infection in human macrophages by down-regulating host factor SPTBN1 during monocyte to macrophage differentiation, J. Exp. Med. 210 (2013) 517–534.
[157] Q. Chen, S. Swaminathan, D. Yang, L. Dai, H. Sui, J. Yang, et al., Interleukin-27 is a potent inhibitor of cis HIV-1 replication in monocyte-derived dendritic cells via a type I interferon-independent pathway, PLoS ONE 8 (2013) e59194.

[158] Y. Rochman, R. Spolski, W.J. Leonard, New insights into the regulation of T cells by gamma(c) family cytokines, Nat. Rev. Immunol. 9 (2009) 480–490.
[159] H. Decaluwe, M. Taillardet, E. Corcuff, I. Munitic, H.K. Law, B. Rocha, et al., Gamma(c) deficiency precludes CD8+ T cell memory despite formation of potent T cell effectors, Proc. Natl. Acad. Sci. USA 107 (2010) 9311–9316.
[160] C.D. Surh, J. Sprent, Homeostasis of naive and memory T cells, Immunity 29 (2008) 848–862.
[161] G.X. Masse, E. Corcuff, H. Decaluwe, U. Bommhardt, O. Lantz, J. Buer, et al., Gamma(c) cytokines provide multiple homeostatic signals to naive CD4(+) T cells, Eur. J. Immunol. 37 (2007) 2606–2616.
[162] C. Mathieu, J.C. Beltra, T. Charpentier, S. Bourbonnais, J.P. Santo, A. Lamarre, et al., IL-2 and IL-15 regulate CD8 memory T-cell differentiation but are dispensable for protective recall responses, Eur. J. Immunol. 45 (2015) 3324–3338.
[163] N.M. Fewkes, C.L. Mackall, Novel gamma-chain cytokines as candidate immune modulators in immune therapies for cancer, Cancer J. 16 (2010) 392–398.
[164] M. Diallo, Y. Zheng, X. Chen, Y. He, H. Zhou, Z. Chen, Prospect of IL-2, IL-7, IL-15 and IL-21 for HIV immune-based therapy, Zhong Nan Da Xue Xue Bao Yi Xue Ban. 36 (2011) 1037–1045.
[165] H. Asao, C. Okuyama, S. Kumaki, N. Ishii, S. Tsuchiya, D. Foster, et al., Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex, J. Immunol. 167 (2001) 1–5.
[166] C. Holm, C.G. Nyvold, S.R. Paludan, A.R. Thomsen, M. Hokland, Interleukin-21 mRNA expression during virus infections, Cytokine 33 (2006) 41–45.
[167] T. Korn, E. Bettelli, W. Gao, A. Awasthi, A. Jager, T.B. Strom, et al., IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells, Nature 448 (2007) 484–487.
[168] R. Nurieva, X.O. Yang, G. Martinez, Y. Zhang, A.D. Panopoulos, L. Ma, et al., Essential autocrine regulation by IL-21 in the generation of inflammatory T cells, Nature 448 (2007) 480–483.
[169] R. Spolski, W.J. Leonard, Interleukin-21: basic biology and implications for cancer and autoimmunity, Annu. Rev. Immunol. 26 (2008) 57–79.
[170] M. Battegay, D. Moskophidis, A. Rahemtulla, H. Hengartner, T.W. Mak, R.M. Zinkernagel, Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice, J. Virol. 68 (1994) 4700–4704.
[171] M. Matloubian, R.J. Concepcion, R. Ahmed, CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection, J. Virol. 68 (1994) 8056–8063.
[172] R.D. Aubert, A.O. Kamphorst, S. Sarkar, V. Vezys, S.J. Ha, D.L. Barber, et al., Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection, Proc. Natl. Acad. Sci. USA 108 (2011) 21182–21187.
[173] J.T. Gerlach, H.M. Diepolder, M.C. Jung, N.H. Gruener, W.W. Schraut, R. Zachoval, et al., Recurrence of hepatitis C virus after loss of virus-specific CD4 (+) T-cell response in acute hepatitis C, Gastroenterology 117 (1999) 933–941.
[174] A. Grakoui, N.H. Shoukry, D.J. Woollard, J.H. Han, H.L. Hanson, J. Ghrayeb, et al., HCV persistence and immune evasion in the absence of memory T cell help, Science 302 (2003) 659–662.
[175] S. Smyk-Pearson, I.A. Tester, J. Klarquist, B.E. Palmer, J.M. Pawlotsky, L. Golden-Mason, et al., Spontaneous recovery in acute human hepatitis C virus infection: functional T-cell thresholds and relative importance of CD4 help, J. Virol. 82 (2008) 1827–1837.
[176] A. Puel, S.F. Ziegler, R.H. Buckley, W.J. Leonard, Defective IL7R expression in T (-)B(+)NK(+) severe combined immunodeficiency, Nat. Genet. 20 (1998) 394–397.
[177] J.T. Tan, E. Dudl, E. LeRoy, R. Murray, J. Sprent, K.I. Weinberg, et al., IL-7 is critical for homeostatic proliferation and survival of naive T cells, Proc. Natl. Acad. Sci. USA 98 (2001) 8732–8737.
[178] K. Takada, S.C. Jameson, Naive T cell homeostasis: from awareness of space to a sense of place, Nat. Rev. Immunol. 9 (2009) 823–832.
[179] T.W. Hand, M. Morre, S.M. Kaech, Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection, Proc. Natl. Acad. Sci. USA 104 (2007) 11730–11735.
[180] L.M. Bradley, L. Haynes, S.L. Swain, IL-7: maintaining T-cell memory and achieving homeostasis, Trends Immunol. 26 (2005) 172–176.
[181] K.S. Schluns, W.C. Kieper, S.C. Jameson, L. Lefrancois, Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo, Nat. Immunol. 1 (2000) 426–432.
[182] C. Kittipatarin, A.R. Khaled, Interlinking interleukin-7, Cytokine 39 (2007) 75–83.
[183] J. Sprent, J.H. Cho, O. Boyman, C.D. Surh, T cell homeostasis, Immunol. Cell Biol. 86 (2008) 312–319.
[184] Q. Jiang, W.Q. Li, F.B. Aiello, R. Mazzucchelli, B. Asefa, A.R. Khaled, et al., Cell biology of IL-7, a key lymphotrophin, Cytokine Growth Factor Rev. 16 (2005) 513–533.
[185] S. Wojciechowski, P. Tripathi, T. Bourdeau, L. Acero, H.L. Grimes, J.D. Katz, et al., Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis, J. Exp. Med. 204 (2007) 1665–1675.
[186] Y. Levy, C. Lacabaratz, L. Weiss, J.P. Viard, C. Goujard, J.D. Lelievre, et al., Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment, J. Clin. Invest. 119 (2009) 997–1007.
[187] I. Sereti, R.M. Dunham, J. Spritzler, E. Aga, M.A. Proschan, K. Medvik, et al., IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection, Blood 113 (2009) 6304–6314.
[188] T.J. Fry, M. Moniuszko, S. Creekmore, S.J. Donohue, D.C. Douek, S. Giardina, et al., IL-7 therapy dramatically alters peripheral T-cell homeostasis in normal and SIV-infected nonhuman primates, Blood 101 (2003) 2294–2299.
[189] M.T. Nugeyre, V. Monceaux, S. Beq, M.C. Cumont, R. Ho Tsong Fang, L. Chene, et al., IL-7 stimulates T cell renewal without increasing viral replication in simian immunodeficiency virus-infected macaques, J. Immunol. 171 (2003) 4447–4453.
[190] S. Beq, M.T. Nugeyre, R. Ho Tsong Fang, D. Gautier, R. Legrand, N. Schmitt, et al., IL-7 induces immunological improvement in SIV-infected rhesus macaques under antiviral therapy, J. Immunol. 176 (2006) 914–922.
[191] Y.W. Chu, S.A. Memon, S.O. Sharrow, F.T. Hakim, M. Eckhaus, P.J. Lucas, et al., Exogenous IL-7 increases recent thymic emigrants in peripheral lymphoid tissue without enhanced thymic function, Blood 104 (2004) 1110–1119.
[192] M. Pellegrini, T. Calzascia, A.R. Elford, A. Shahinian, A.E. Lin, D. Dissanayake, et al., Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies, Nat. Med. 15 (2009) 528–536.
[193] K.S. Lang, M. Recher, A.A. Navarini, N.L. Harris, M. Lohning, T. Junt, et al., Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation, Eur. J. Immunol. 35 (2005) 738–745.
[194] S.G. Nanjappa, E.H. Kim, M. Suresh, Immunotherapeutic effects of IL-7 during a chronic viral infection in mice, Blood 117 (2011) 5123–5132.
[195] M. Pellegrini, T. Calzascia, J.G. Toe, S.P. Preston, A.E. Lin, A.R. Elford, et al., IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology, Cell 144 (2011) 601–613.
[196] S. Radaeva, R. Sun, H.N. Pan, F. Hong, B. Gao, Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation, Hepatology 39 (2004) 1332–1342.
[197] L.A. Zenewicz, G.D. Yancopoulos, D.M. Valenzuela, A.J. Murphy, M. Karow, R. A. Flavell, Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation, Immunity 27 (2007) 647–659.
[198] B.A. Croker, D.L. Krebs, J.G. Zhang, S. Wormald, T.A. Willson, E.G. Stanley, et al., SOCS3 negatively regulates IL-6 signaling in vivo, Nat. Immunol. 4 (2003) 540–545.
[199] K. Pfizenmaier, P. Scheurich, W. Daubener, M. Kronke, M. Rollinghoff, H. Wagner, Quantitative representation of all T cells committed to develop into cytotoxic effector cells and/or interleukin 2 activity-producing helper cells within murine T lymphocyte subsets, Eur. J. Immunol. 14 (1984) 33–39.
[200] W.N. D'Souza, L. Lefrancois, Frontline: an in-depth evaluation of the production of IL-2 by antigen-specific CD8 T cells in vivo, Eur. J. Immunol. 34 (2004) 2977–2985.
[201] W. Liao, J.X. Lin, W.J. Leonard, Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy, Immunity 38 (2013) 13–25.
[202] O. Boyman, J. Sprent, The role of interleukin-2 during homeostasis and activation of the immune system, Nat. Rev. Immunol. 12 (2012) 180–190.
[203] W.N. D'Souza, K.S. Schluns, D. Masopust, L. Lefrancois, Essential role for IL-2 in the regulation of antiviral extralymphoid CD8 T cell responses, J. Immunol. 168 (2002) 5566–5572.
[204] W.N. D'Souza, L. Lefrancois, IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion, J. Immunol. 171 (2003) 5727–5735.
[205] M.A. Williams, A.J. Tyznik, M.J. Bevan, Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells, Nature 441 (2006) 890–893.
[206] J.J. Obar, M.J. Molloy, E.R. Jellison, T.A. Stoklasek, W. Zhang, E.J. Usherwood, et al., CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses, Proc. Natl. Acad. Sci. USA 107 (2010) 193–198.
[207] V. Kalia, S. Sarkar, S. Subramaniam, W.N. Haining, K.A. Smith, R. Ahmed, Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo, Immunity 32 (2010) 91–103.
[208] M.E. Pipkin, J.A. Sacks, F. Cruz-Guilloty, M.G. Lichtenheld, M.J. Bevan, A. Rao, Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells, Immunity 32 (2010) 79–90.
[209] J.N. Blattman, J.M. Grayson, E.J. Wherry, S.M. Kaech, K.A. Smith, R. Ahmed, Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo, Nat. Med. 9 (2003) 540–547.
[210] E.E. West, H.T. Jin, A.U. Rasheed, P. Penaloza-Macmaster, S.J. Ha, W.G. Tan, et al., PD-L1 blockade synergizes with IL-2 therapy in reinvigorating exhausted T cells, J. Clin. Invest. 123 (2013) 2604–2615.
[211] Y. Levy, C. Capitant, S. Houhou, I. Carriere, J.P. Viard, C. Goujard, et al., Comparison of subcutaneous and intravenous interleukin-2 in asymptomatic HIV-1 infection: a randomised controlled trial. ANRS 048 study group, Lancet 353 (1999) 1923–1929.
[212] G.A. Hardy, N. Imami, M.R. Nelson, A.K. Sullivan, R. Moss, M.M. Aasa-Chapman, et al., A phase I, randomized study of combined IL-2 and therapeutic immunisation with antiretroviral therapy, J. Immune Therap. Vacc. 5 (2007) 6.
[213] J.A. Kovacs, S. Vogel, J.M. Albert, J. Falloon, R.T. Davey Jr., R.E. Walker, et al., Controlled trial of interleukin-2 infusions in patients infected with the human immunodeficiency virus, N. Engl. J. Med. 335 (1996) 1350–1356.
[214] Group I-ES, Committee SS, D. Abrams, Y. Levy, M.H. Losso, A. Babiker, et al., Interleukin-2 therapy in patients with HIV infection, N. Engl. J. Med. 361 (2009) 1548–1559.

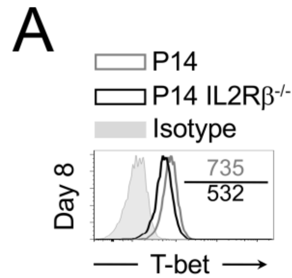
[215] S.L. Pett, A.D. Kelleher, S. Emery, Role of interleukin-2 in patients with HIV infection, Drugs 70 (2010) 1115–1130.
[216] C.S. Hinrichs, R. Spolski, C.M. Paulos, L. Gattinoni, K.W. Kerstann, D.C. Palmer, et al., IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy, Blood 111 (2008) 5326–5333.
[217] D.M. Mitchell, E.V. Ravkov, M.A. Williams, Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells, J. Immunol. 184 (2010) 6719–6730.
[218] J.S. Yi, J.T. Ingram, A.J. Zajac, IL-21 deficiency influences CD8 T cell quality and recall responses following an acute viral infection, J. Immunol. 185 (2010) 4835–4845.

Annex. 2.

Blimp-1 deficiency alters inhibitory receptors expression *in vitro.* A) 2.10^4 OT1 CD8 T cells from Blimp-$1^{+/+}$ E81-cre (WT) and Blimp-$1^{fl/fl}$ E81-cre (Tg) mice were activated 48h *in vitro* with 2.10^4 peptide loaded DCs ($OVA_{257-264}$ SIINFEKL peptide; 0,1µM). At days 2, 4 and 6, WT and Tg CD8 T cells were numbered, normalized and cultured back in complete medium supplemented with 10U/ml of IL-2 (left panels) or IL-15 (right panel). Graphs represent the MFI of indicated inhibitory receptors on WT (black bars) and Tg (white bars) CD8 T cells throughout the culture. Data are representative of two independent experiments with all conditions realized in triplicates. error bars, mean± s.e.m.

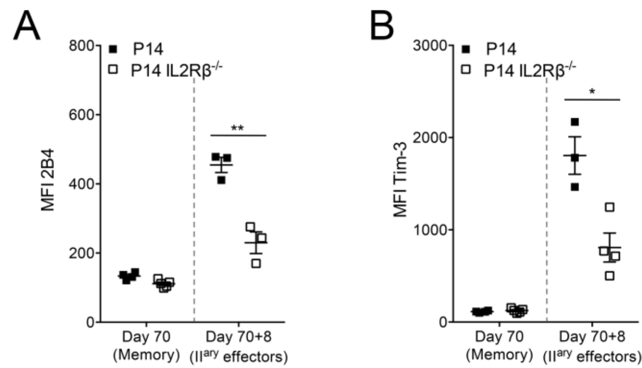


Annex. 3.



IL2Rβ-deficiency reduces T-bet expression at day 8p.i. Tg P14 or P14 IL2Rβ$^{-/-}$ (CD45.2) cells were adoptively transferred into recipient mice (CD45.1.2) prior to LCMV Cl-13 infection. A) Intracellular T-bet expression in P14 (grey histograms) and P14 IL2Rβ$^{-/-}$ (black histograms) cells at day 8 p.i.. Values indicate the MFI; grey filled curves are isotype controls. Data are representative of two independent experiments (three mice per group in each).

Annex. 4.



IL2Rβ-deficiency prevents 2B4 and Tim-3 expression on secondary effectors. 10^5 Tg P14 or P14 IL2Rβ$^{-/-}$ (CD45.2) cells were adoptively transferred into recipient mice (CD45.1.2) prior to LCMV Arm infection. At day 70 p.i., memory P14 and P14 IL2Rβ$^{-/-}$ were sorted and transferred into new naive recipient mice (CD45.1.2) 20 hours before re-challenge with 2.10^6 PFU of LCMV Cl-13. A-B) Cumulative MFI of A) 2B4 and B) Tim-3 on P14 (filled squatters) and P14 IL2Rβ$^{-/-}$ (opened squatters) memory (left) and secondary effectors (right) at day 8 after secondary infection. Data are pooled from one experiment with 3 to 4 mice per groups. error bars (*A*, *B*) mean± s.e.m.

Annex. 5. Article

IL-2 and IL-15 regulate CD8 memory T cell differentiation but are dispensable for protective recall responses

Cédric Mathieu[1,2], Jean-Christophe Beltra[1,2], Tania Charpentier[3], Sara Bourbonnais[1], James P. Di Santo[4], Alain Lamarre[3], and Hélène Decaluwe[1,2,5,*]

This article is published in the journal "European Journal of Immunology", vol. 45, issue 12; doi: 10.1002/eji.201546000 (2015) PMID 26426795.

In this article for which I am second author, I performed all the experiments related to the chronic strain of LCMV (entire figure 3).

European Journal of
Immunology

IL-2 and IL-15 regulate CD8+ memory T-cell differentiation but are dispensable for protective recall responses

Cédric Mathieu[1,2], *Jean-Christophe Beltra*[1,2], *Tania Charpentier*[3], *Sara Bourbonnais*[1], *James P. Di Santo*[4], *Alain Lamarre*[3] *and Hélène Decaluwe*[1,2,5]

[1] Cytokines and Adaptive Immunity Laboratory, CHU Sainte-Justine Research Center, Montreal, Quebec, Canada
[2] Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada
[3] Immunovirology Laboratory, INRS-Institut Armand-Frappier, Laval, Quebec, Canada
[4] Innate Immunity Unit, INSERM U668, Institut Pasteur, Paris, France
[5] Immunology and Rheumatology Division, Department of Pediatrics, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada

The ability to mount effective secondary responses is a cardinal feature of memory CD8$^+$ T cells. An understanding of the factors that regulate the generation and recall capacities of memory T cells remains to be ascertained. Several cues indicate that two highly related cytokines, IL-2 and IL-15, share redundant functions in this process. To establish their combined roles in memory CD8$^+$ T-cell development, maintenance, and secondary responses, we compared the outcome of adoptively transferred IL2Rβ$^{+/-}$ or IL2Rβ$^{-/-}$ CD8$^+$ T cells after an acute viral infection in mice. Our results demonstrate that both IL-2 and IL-15 signals condition the differentiation of primary and secondary short-lived effector cells by altering the transcriptional network governing lineage choices. These two cytokines also regulate the homeostasis of the memory T-cell pool, with effector memory CD8$^+$ T cells being the most sensitive to these two interleukins. Noticeably, the inability to respond to both cytokines limits the proliferation and survival of primary and secondary effectors cells, whereas it does not preclude potent cytotoxic functions and viral control either initially or upon rechallenge. Globally, these results indicate that lack of IL-2 and IL-15 signaling modulates the CD8$^+$ T-cell differentiation program but does not impede adequate effector functions.

Keywords: CD8$^+$ T cells · IL-2 · IL-15 · Memory · T-cell differentiation

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

CD8$^+$ T lymphocytes are the cornerstones of antiviral immunity and the critical pawns in the chess game against intracellular pathogens. Development of functional CD8$^+$ memory T cells is a

Correspondence: Dr. Hélène Decaluwe
e-mail: helene.decaluwe@umontreal.ca

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

tightly regulated process during which cells pass through an effector state before establishing long-term immune protection [1]. The recognition of pathogen-encoded peptides and their stimulation through costimulatory and cytokine signals initiate a specific transcriptional program critical for $CD8^+$ T-cell lineage choices and functions [2, 3]. As the memory T-cell pool is the direct progeny of these effectors cells, understanding the cellular and molecular mechanisms involved in the transition from cytotoxic T lymphocytes (CTLs) to memory cells constitutes an area of intense investigation.

In the quest to dissect these mechanisms, two effector $CD8^+$ T-cell populations were identified, based on their distinct expression of CD127 and KLRG1, and correlated with their propensity to persist and become memory [4–6]. While $KLRG1^{hi}$ $CD127^{lo}$ Short-Lived Effector Cells (SLECs) are destined to become terminal effectors, $KLRG1^{lo}$ $CD127^{hi}$ Memory Precursor Effector Cells (MPECs) are fated to develop into long-lived memory cells. Since, multiple factors were shown to modulate the lineage choices between these two effector subsets. Limited antigenic stimulation preferentially commits effector $CD8^+$ T cells toward a MPEC phenotype while prolonged antigen encounter promotes terminal SLEC differentiation [7, 8]. Similarly, inflammatory cytokines (in particular IL-12) enhance SLEC generation in a T-bet-dependent manner, whereas low levels of inflammation favor memory formation [6, 9]. Finally, cytokines that signal through the common gamma (γ_c) chain (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) modify the $CD8^+$ T-cell differentiation program upon infection and are indispensable for the generation of $CD8^+$ memory T cells [10]. While IL-7 promotes MPEC and $CD8^+$ memory T-cell survival [11], IL-2 and IL-15 cooperate to sustain SLEC proliferation, differentiation, and survival [9, 10, 12–15]. IL-15 is also a pivotal cytokine involved in the maintenance and homeostatic proliferation of the $CD8^+$ memory T-cell pool [16, 17]. Interestingly, IL-21, which is critical during chronic viral infection to limit exhaustion, has a minimal role on SLEC/MPEC lineage choices and $CD8^+$ memory T-cell formation during an acute viral infection in help-independent infection models [18].

While the role of γ_c-dependent signals on $CD8^+$ memory T-cell development is indisputable, the biological impact of IL-2 and IL-15 on $CD8^+$ memory T-cell functions remains controversial. Studies have demonstrated a critical role for IL-2 in the regulation of Blimp-1 and Eomes transcription [9, 12], two transcription factors that have an opposite effect on the development of $CD8^+$ memory T cells [19–21]. Moreover, some authors suggest that IL-2 present at the time of priming sustain the expansion of secondary effectors, while others do not [9, 14, 22, 23]. Conversely, IL-15, a γ_c-cytokine closely related to IL-2, appears dispensable for secondary effector expansion, while it may affect secondary effector memory (T_{EM}) differentiation [15, 24]. Thus, while IL-2 and IL-15 are known to modify the differentiation of effectors, their impact on the generation and maintenance of central memory (T_{CM}) and T_{EM} $CD8^+$ T cells, and ultimately on their capacity to mount protective responses, remains ill-defined.

IL-2 and IL-15 not only share the γ_c and IL2Rβ chains, they similarly activate JAK1, JAK3, and STAT5. Moreover, upon ligation of the appropriate cytokine, their quaternary receptor structure is nearly indistinguishable from each other [25, 26]. We thus hypothesized that, in light of their common receptor components and signaling pathways, IL-2 and IL-15 likely co-regulate similar genes and share redundant functions during an immune response. To circumvent the compensatory impact of one cytokine in the absence of the other, we decided to evaluate the role of combined IL-2 and IL-15 signals on $CD8^+$ memory T-cell function. To do so, we used a P14 TCR transgenic mice model deficient for the IL2Rβ chain and found that IL2Rβ signals condition the expansion and differentiation of primary SLECs in a more profound manner than what was reported in the absence of either IL-2 or IL-15 [9, 12, 14–16]. This supports the concept of biological redundancy of these cytokines in vivo. We also found that IL-2 and IL-15 were pivotal for the generation of T_{EM} and secondary SLECs. However, these cytokines were dispensable for adequate effector functions, by both primary and secondary effectors, despite significantly lower numbers of effector cells and reduced granzyme levels. Hence, our findings attest of the central role for IL-2 and IL-15 in the proliferation, survival and differentiation of primary and secondary effectors during an acute viral infection, and in the maintenance of the $CD8^+$ T_{EM} pool.

Results

IL-2 and IL-15 sustain survival and proliferation of effectors and their differentiation in SLECs

To abrogate specifically combined IL-2 and IL-15 signals in $CD8^+$ T cells, we generated a mouse model carrying a deletion in the IL2Rβ chain. This TCR transgenic mouse (P14) on the Rag2-deficient background harbors a monoclonal population of naïve $CD8^+$ T cell specific for the envelope glycoprotein epitope 33–41 ($GP_{33\text{-}41}$) of the lymphocytic choriomeningitis virus (LCMV) (Supporting Information Fig. 1). 10^5 P14 $IL2R\beta^{-/-}$ or $IL2R\beta^{+/-}$ $CD8^+$ T cells were adoptively transferred into naïve C57BL/6 recipients 20 h prior to LCMV Armstrong infection. This strain of virus causes an acute infection in mice, with pathogen clearance in 7–10 days [27]. Although $IL2R\beta^{+/-}$ and $IL2R\beta^{-/-}$ cells proliferated initially with the same kinetics, the peak of expansion was significantly reduced in $IL2R\beta^{-/-}$ T cells by day 7 ($38,6.10^6$ versus $10,9.10^6$ antigen-specific cells respectively, $p = 0,0001$) (Fig. 1A). This hampered expansion could be explained by a reduced proliferation rate, as depicted by the lower percentage of $Ki\text{-}67^+$ $IL2R\beta^{-/-}$ cells (80 versus 90% in $IL2R\beta^{+/-}$ cells), and an increased apoptosis, both in terms of active caspase-3 and Annexin V/7-AAD expression (14 and 27% of $IL2R\beta^{-/-}$ being caspase-3^+ or Annexin V^+ 7-AAD^+ as opposed to 7 and 17% of the $IL2R\beta^{+/-}$ cells at day 7, $p = 0,0033$) (Fig. 1B upper panel, Supporting Information Fig. 2A). This enhanced apoptosis was correlated to a slight increase in the expression of Bim, a pro-apoptotic molecule, while the levels of Bcl-2, its anti-apoptotic counterpart, remained unchanged (Fig. 1B, lower panel) [28]. Despite reduced cell expansion, the dynamics of activation (as assessed by

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

monitoring cell surface markers) were largely unchanged in the absence of IL2Rβ (Supporting Information Fig. 2B). However, differences were noted in terms of KLRG1, CD27, CD62L, and CD127 expression at day 7 (Fig. 1C, Supporting Information Fig. 2C). Whereas IL2R$\beta^{+/-}$ cells presented a terminally differentiated phenotype (CD62L^{low} CD127^{low} KLRG1^{high}), a fraction of IL2R$\beta^{-/-}$ cells was already expressing markers of memory T cells at day 7 (CD62L^{high} CD127^{high} KLRG1^{low} CD27^{high}). In fact, the proportion of KLRG1^{high} CD127^{low} SLECs at day 7 was significantly reduced in the absence of IL-2 and IL-15 signaling, while KLRG1^{low} CD127^{high} MPEC differentiation was accelerated (Fig. 1D left panel, Supporting Information Fig. 2D). A preferential accumulation of KLRG1^{high} CD127^{low} SLECs over KLRG1^{low} CD127^{high} MPECs was seen in control mice, while SLEC and MPEC numbers were equivalent in IL2R$\beta^{-/-}$ mice (Fig. 1D, right panel). These data demonstrate a preponderant role for IL-2 and IL-15 signals in the development of SLECs, as demonstrated for IL-2 by others [9, 12, 15]. We then analyzed the intracellular expression of T-bet and Eomes, key transcription factors implicated in effector and memory lineage choices [6, 29]. While IL2R$\beta^{-/-}$ cells presented slightly reduced levels of T-bet at day 5 and 7, Eomes levels were significantly increased at day 7 of infection, but not at day 5 (Fig. 1E). Even more, the absence of IL2Rβ signals increased the proportion of cells expressing both Eomes and T-bet, thus shifting the balance in favor of MPEC differentiation. (Fig. 1F). Of note, the expression level of CD127 at the surface of the cells did not explain the difference seen in Eomes expression, as MPECs and SLECs, as well as CD127^{high} and CD127^{low} subsets, both expressed higher levels of Eomes in the absence of IL2Rβ signaling (Supporting Information Fig. 2E–F). This accelerated MPEC differentiation correlated with increased *Klf2*, *Bcl6,* and *Eomes* mRNA levels and reduced *Prdm1* and *Tbx21* levels (Fig. 1G), in accordance with the known role of these transcription factors in memory and effector T-cell differen-

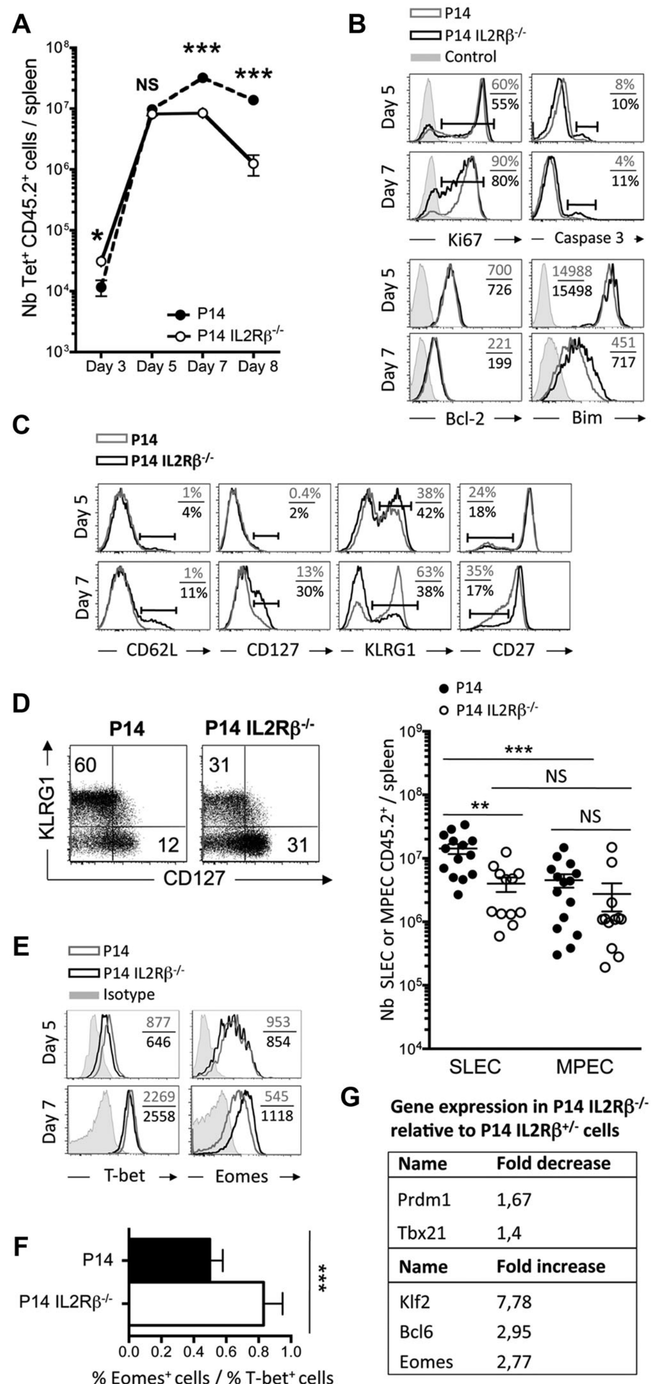


Figure 1. IL2Rβ-dependent cytokines condition the survival, proliferation and terminal differentiation of KLRG1^{high} CD127^{low} SLECs via transcriptional network modulation. 10^5 P14 IL2R$\beta^{+/-}$ or P14 IL2R$\beta^{-/-}$ CD45.2$^+$ CD8$^+$ T cells were adoptively transferred into naive CD45.1.2$^+$ mice that were subsequently infected with 2×10^5 PFU of LCMV Armstrong. Expansion and contraction of antigen-specific splenocytes were analyzed by flow cytometry at specific time points. (A) Total number of antigen-specific CD8$^+$ T cells was calculated based on GP$_{33\text{-}41}$ tetramer and CD45.2 congenic marker staining. Data represent the mean ± SEM of eight mice per time point, pooled from four separate experiments (B) Intracellular staining of Ki67, active Caspase 3, Bcl-2, and Bim by P14 IL2R$\beta^{+/-}$ (gray line histogram) or P14 IL2R$\beta^{-/-}$ (black line histogram) effector CD8$^+$ T cells at day 5 and 7 post-infection (pi). Isotype control in shaded gray histogram. Values indicate the respective frequency of positive cells for each marker or its mean fluorescence intensity (MFI). Results are representative of nine mice per genotype pooled from three independent experiments. (C) Cell surface expression of the indicated differentiation markers by P14 IL2R$\beta^{+/-}$ (gray line histogram) or P14 IL2R$\beta^{-/-}$ (black line histogram) at day 5 and 7 pi. Values indicate the frequency or MFI from one representative experiment ($n = 14$ per genotype pooled from three independent experiments). (D) Dot plot (left) and absolute numbers (right) of SLECs or MPECs, based on KLRG1 and CD127 expression from P14 IL2R$\beta^{+/-}$ (filled circle) or P14 IL2R$\beta^{-/-}$ (opened circle) CD8$^+$ T cells at day 7 pi. Values in dot plot indicate the percentage of each correspondent population from a representative experiment. SLEC and MPEC numbers represent the total samples pooled from four independent experiments ($n = 12$). (E) Intracellular expression of T-bet and Eomes in P14 IL2R$\beta^{+/-}$ (gray line histogram) or P14 IL2R$\beta^{-/-}$ (black line histogram) cells at day 5 and 7 pi. Isotype control is shown in shaded gray and numbers indicate MFI ($n = 6$ per genotype from two separate experiments). (F) Ratio of the frequency of Eomes$^+$ T cells over the frequency of T-bet$^+$ T cells among P14 IL2R$\beta^{+/-}$ (black bar) or P14 IL2R$\beta^{-/-}$ (white bar) CD8$^+$ T cells at day 7 pi (mean ± SEM pooled from two separate experiments, $n = 6$). (G) Expression of the indicated genes was evaluated by RTqPCR. Values indicate the fold decrease (top) or increase (bottom) in each expression level in P14 IL2R$\beta^{-/-}$ versus P14 IL2R$\beta^{+/-}$ CD8$^+$ T cells at day 7 pi ($n = 5$ per genotype from two independent experiments). $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, NS $p \geq 0.05$; two-tailed unpaired Student's *t*-test.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

tiation, respectively [3, 30]. Together, our data indicate that IL-2 and IL-15 condition the expansion and differentiation of SLECs, by regulating the survival and proliferation of $CD8^+$ effectors and modulating the transcriptional network governing lineage choices.

IL2Rβ signals are dispensable for potent T-cell effector functions

We next evaluated the consequences of abrogated IL2Rβ-dependent signals on the functional capacities of P14 $CD8^+$ T cells. At day 7 of infection, similar proportions of IL2R$\beta^{-/-}$ or IL2R$\beta^{+/-}$ $CD8^+$ effector T cells produced IFN-γ or TNF-α following ex vivo restimulation, while two-fold more IL2R$\beta^{-/-}$ cells secreted IL-2 (Fig. 2A). This could be related to the increased frequency of MPECs among IL2R$\beta^{-/-}$ cells, which are known to secrete more IL-2 than SLECs [4]. The absence of IL-2 and IL-15 also slightly increased the proportion of triple producers (IFN-γ^+ TNF-α^+ IL-2^+) (16% of IL2R$\beta^{-/-}$ cells versus 11% of IL2R$\beta^{+/-}$ cells) (Fig. 2B), suggesting that the presence of these cytokines may somewhat limit T-cell function. To test the cytotoxic functions of $CD8^+$ T cells in the presence or absence of IL2Rβ, we performed an in vivo killing assay using $GP_{33\text{-}41}$-loaded target cells in adoptively transferred perforin-deficient (Pfp$^{-/-}$) mice infected 7 days prior to the assay. These recipient mice are incapable of controlling a LCMV infection due to defective granule-dependent cytotoxicity [31]. It serves as a good tool to evaluate specifically the functionality of the adoptively transferred P14 cells. This assay demonstrated that although IL2R$\beta^{-/-}$ T cells presented a slight delay in their capacity to eliminate $GP_{33\text{-}41}{}^+$ target cells (Supporting Information Fig. 3A), they were as potent killers as their IL2R$\beta^{+/-}$ counterparts (Fig. 2C). These results were unexpected considering the significant reduction in the number of effectors in the absence of IL2Rβ (Fig. 1A). To confirm these findings, we further performed an adoptive transfer of one-log less naïve cells prior to infection. Yet, IL2R$\beta^{-/-}$ effectors cells were still able to eliminate antigen-specific targets cells (Supporting Information Fig. 3B). This was also in contrast with the significant reduction in granzyme levels in the absence of IL2Rβ signaling (Fig. 2D, Supporting Information Fig. 3C), in accordance with previous reports [15, 32]. However, potent effector functions correlated with similar expression of the cytotoxic molecule perforin and the degranulation marker CD107a (Fig. 2D), and equivalent viral loads at day 7 of infection (Fig. 2E). To refine the evaluation of effector functions, we then assessed if IL2R$\beta^{-/-}$ P14 cells could prevent LCMV-induced hemophagocytic lymphohistiocytosis (HLH) in Pfp$^{-/-}$ hosts [33]. Pfp$^{-/-}$ recipients receiving

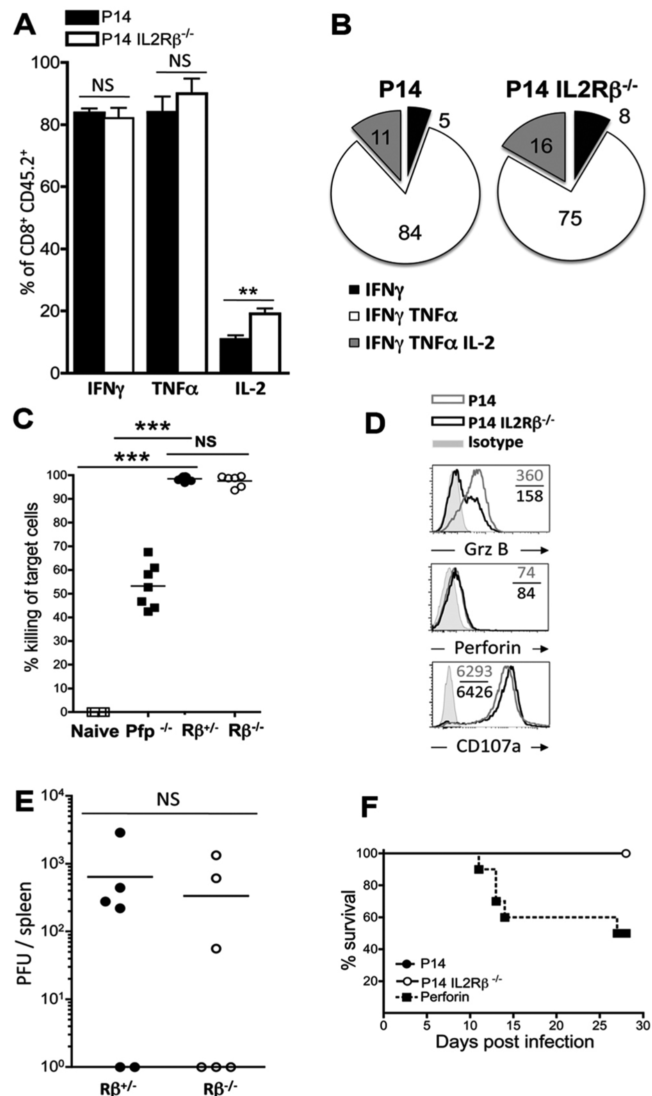


Figure 2. IL2Rβ-deficient $CD8^+$ T cells are potent cytotoxic cells despite a significant reduction in granzyme levels. P14 IL2R$\beta^{+/-}$ and P14 IL2R$\beta^{-/-}$ chimeric mice were generated as described in Fig. 1. (A) Frequency of P14 IL2R$\beta^{+/-}$ (black bars) or P14 IL2R$\beta^{-/-}$ (white bars) $CD8^+$ T cells expressing IFN-γ, TNF-α, or IL-2 at day 7 pi after 4 h ex vivo restimulation with $GP_{33\text{-}41}$ peptide (mean ± SEM pooled from two separate experiments; $n = 11$ per genotype). (B) Pie charts representing the percentage of splenic P14 IL2R$\beta^{+/-}$ (left) and P14 IL2R$\beta^{-/-}$ (right) effector $CD8^+$ T cells secreting multiple cytokines simultaneously. Values indicate percentage of the correspondent phenotype from $n = 6$ per genotype pooled from two separate experiments. (C) In vivo cytotoxic assay testing the killing function in 3 h of day 7 P14 IL2R$\beta^{+/-}$ (filled circle) and P14 IL2R$\beta^{-/-}$ (opened circle) $CD8^+$ effector T cells compared with that of infected (filled square) and uninfected/naïve (opened square) Pfp$^{-/-}$ cells. Target cell killing was calculated by dividing the percentage of peptide-loaded target cells divided by the percentage of unloaded target cells in infected mice by the percentage of peptide-loaded target cells divided by the percentage of unloaded target cells in uninfected mice. Data are shown as mean ± SEM from six mice per genotype pooled from two separate experiments. (D) Intracellular Granzyme B (Grz B), perforin and CD107a expression of day 7 infected P14 IL2R$\beta^{+/-}$ (gray line histogram) or P14 IL2R$\beta^{-/-}$ (black line histogram) $CD8^+$ T cells. Isotype control in shaded gray histogram. Values on histograms indicate MFI from a representative experiment ($n = 8$ per genotype pooled from three separate experiments). (E) Viral titers in spleen of P14 IL2R$\beta^{+/-}$ (filled circle) or P14 IL2R$\beta^{-/-}$ (opened circle) recipient mice at day 7 pi (mean ± SEM pooled from two independent experiments; $n = 6$ per genotype). (F) Naive perforin knock-out (Pfp$^{-/-}$) mice were infected with 2×10^5 PFU of LCMV Armstrong 24 h after adoptive transfer of 2×10^5 P14 IL2R$\beta^{+/-}$ (filled circle) and P14 IL2R$\beta^{-/-}$ (opened circle) T cells. Survival and clinical signs were followed over time. Kaplan-Meier curve of six to eight mice per genotype pooled from two separate experiments. $^{**}p < 0.005$, $^{***}p < 0.0005$, NS $p \geq 0.05$; two-tailed unpaired Student's t-test.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

either IL2Rβ$^{+/-}$ or IL2Rβ$^{-/-}$ CD8^{+} T cells survived equally well (Fig. 2F) and did not demonstrate clinical signs of HLH, including weight loss, hypothermia, and pancytopenia (Supporting Information Fig. 3D–E), confirming viral control by IL2Rβ$^{-/-}$ cells.

We then questioned if the lack of functional impact in the absence of IL2Rβ-signals was related to the low viral load at that time of the response. To answer this question, we took advantage of the LCMV clone 13 strain that generates a chronic infection in mice [34], and compared the response of IL2Rβ$^{+/-}$ and IL2Rβ$^{-/-}$ cells at day 8 of infection in this context. It is important to note that, in this chronic infection model, the number of effector cells at day 8 post infection is reduced by two-logs compared to the acute infection model, giving us also the opportunity to compare cytotoxic functions in the presence of significantly lower number of effectors (3,9.10^{7} IL2Rβ$^{+/-}$ in acute versus 4,1.10^{5} IL2Rβ$^{+/-}$ in chronic infection, 1,1.10^{7} IL2Rβ$^{-/-}$ in acute versus 7,1.10^{4} IL2Rβ$^{-/-}$ cells in chronic infection, $p = 0.0001$). IL2Rβ$^{+/-}$ CD8^{+} T cells produced significantly less TNF-α (16 versus 84%, $p<0.0001$) and IL-2 (5 versus 11%, $p = 0.003$) during a chronic rather than an acute infection, as previously described [34], while their capacity to secrete IFN-γ was preserved (Fig. 3A, left panel). In contrast, in the context of a chronic infection, the abrogation of signaling through the IL2Rβ chain limited the capacity of the cells to secrete the three cytokines, with again an increased sensitivity for TNF-α (8 versus 84%, $p<0.0001$) and IL-2 (9 versus 16%, $p = 0.0004$) (Fig. 3A, right panel). Interestingly, as demonstrated during an acute infection, under this constant inflammatory and antigenic stimulation, IL2Rβ$^{-/-}$ CD8^{+} effector T cells secreted slightly more IL-2 than their IL2Rβ$^{+/-}$ counterparts (Fig. 3B). The capacity of IL2Rβ$^{-/-}$ cells to secrete IFN-γ was also restricted during this chronic infection, while TNF-α secretion, already severely hampered in this context, could not be further diminished by the absence of IL2Rβ signals (Fig. 3B). Nevertheless, these minor differences were not associated with significant changes in the proportion of cells secreting one, two or three cytokines simultaneously (Fig. 3C). As during an acute viral infection, granzyme B expression was severely hindered in the absence of IL2Rβ-dependent signals (Fig. 3D). Despite this, the capacity to degranulate was mostly preserved, as assessed by the expression of CD107a after 5 h of in vitro stimulation (Fig. 3D). To test if the differences in cytokine production and granzyme B levels would limit cytotoxic functions in vivo, we performed a 6-h functional assay in Pfp$^{-/-}$ mice, as described above. Interestingly, IL2Rβ$^{-/-}$ cells were as efficient as IL2Rβ$^{+/-}$ cells to eliminate target cells (Fig. 3E), even though in this context the number of effector cells present was significantly reduced, as already mentioned. This reinforces the idea that IL2Rβ$^{-/-}$ cells are highly effective cytotoxic effectors. Collectively, our results confirm that IL-2 and IL-15 sustain the expression of cytotoxic granzymes but demonstrate that

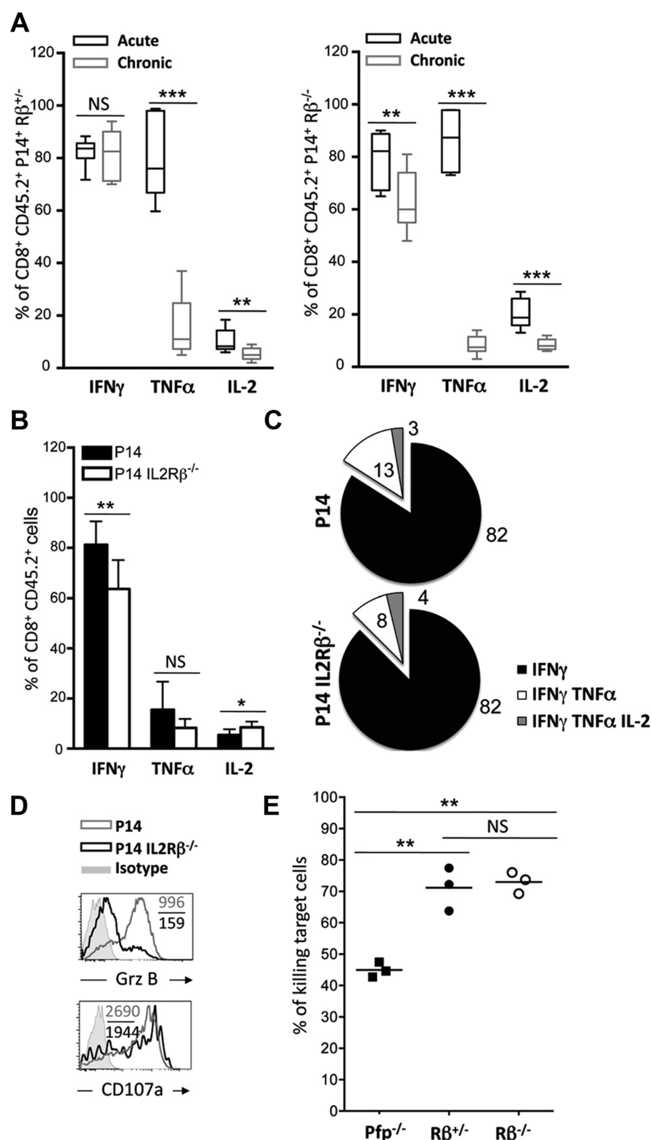


Figure 3. IL-2 and IL-15 have minimal impact on effector functions during the acute phase of a chronic viral infection. 10^{4} P14 IL2Rβ$^{+/-}$ or P14 IL2Rβ$^{-/-}$ CD45.2^{+} CD8^{+} T cells were adoptively transferred into naive CD45.1.2^{+} mice that were subsequently infected with 2×10^{6} PFU of LCMV Clone 13. (A) The respective production of IFN-γ, TNF-α, and IL-2 by P14 IL2Rβ$^{+/-}$ (left panel) and P14 IL2Rβ$^{-/-}$ (right panel) cells at the peak of the response following infection with LCMV Armstrong (black boxes) or LCMV Clone 13 (gray boxes). Box-and-whiskers plots represent the mean ± SEM pooled from two to five independent experiments; n = 8–12 per genotype. (B) IFN-γ, TNF-α, and IL-2 production by P14 IL2Rβ$^{+/-}$ (black bars) and P14 IL2Rβ$^{-/-}$ (white bars) cells at day 8 after LCMV Clone 13 infection (n = 9–12 per genotype pooled from five separate experiments). (C) The frequency of splenic P14 IL2Rβ$^{+/-}$ (upper panel) and P14 IL2Rβ$^{-/-}$ (lower panel) effector CD8^{+} T cells secreting multiple cytokines simultaneously are represented. Values in pie charts indicate percentage of the correspondent phenotype from nine to twelve mice per group pooled from five separate experiments. (D) Granzyme B (Grz B) and CD107a levels of P14 IL2Rβ$^{+/-}$ (gray line histogram) and P14 IL2Rβ$^{-/-}$ (black line histogram) cells at day 8 pi. Isotype control is shown in shaded gray. Values represent the MFI from a representative experiment (n = 9–10 per genotype pooled from three to four independent experiments). (E) Cytotoxic function of P14 IL2Rβ$^{+/-}$ (filled circle) and P14 IL-2Rβ$^{-/-}$ (opened circle) effector cells at day 8 pi in Pfp$^{-/-}$ recipients was evaluated in a 6-h in vivo CTL assay. Target cell killing was calculated as described in Fig. 2C. Each symbol represents an individual mouse and bars represent the mean of one of two representative experiments (n = 6 per genotype). *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, NS $p \geq 0.05$; two-tailed unpaired Student's t-test.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

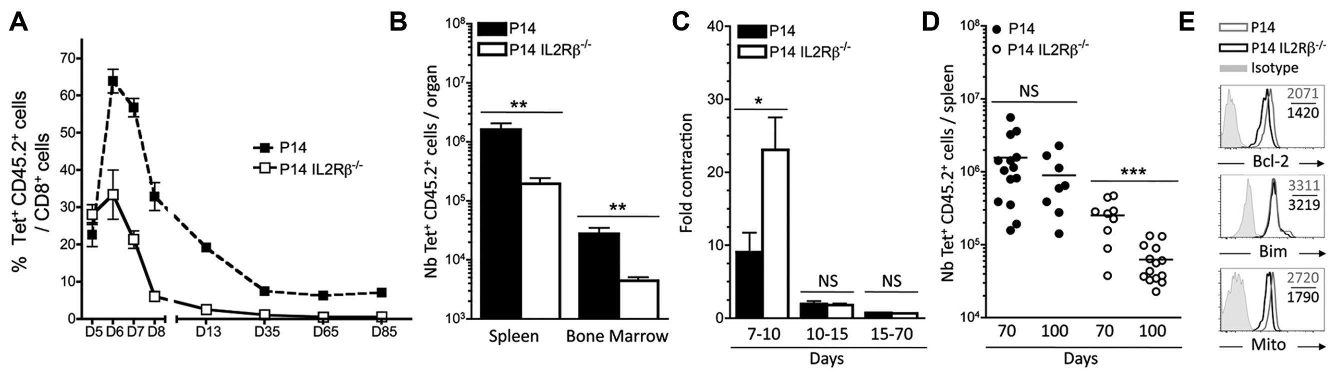


Figure 4. IL-2 and IL-15 condition the generation and maintenance of the memory $CD8^+$ T-cell pool. P14 IL2R$\beta^{+/-}$ and P14 IL2R$\beta^{-/-}$ chimeric mice were generated as described in Fig. 1. (A) The frequency of $GP_{33\text{-}41}$ Tetramer $(Tet)^+$ $CD45.2^+$ $CD8^+$ T cells from the blood of P14 IL2R$\beta^{+/-}$ (filled circle) and P14 IL2R$\beta^{-/-}$ (opened circle) mice was evaluated over a 90-day time course by flow cytometry. Results are shown as mean ± SEM of five to ten mice per genotype pooled from three independent experiments. (B) Absolute numbers of Tet^+ $CD45.2^+$ in spleen and bone marrow of P14 IL2R$\beta^{+/-}$ (black bar) and P14 IL2R$\beta^{-/-}$ (white bar) $CD8^+$ T cells in mice at day 70 pi. Data are shown as mean ± SEM pooled from three independent experiments, $n = 9$ mice per genotype. (C) The fold contraction of P14 IL2R$\beta^{+/-}$ (black bar) and P14 IL2R$\beta^{-/-}$ (white bar) cells between day 7 and 10 (left), day 10 and 15 (middle), day 15 and 70 (right) pi is shown. Fold contraction was calculated by dividing the absolute number of cells at one time point by the absolute number of cells at the second time point. Results are shown as mean ± SEM pooled from five separate experiments ($n =$ 10 per genotype). (D) Absolute number of Tet^+ $CD45.2^+$ $CD8^+$ T cells from P14 IL2R$\beta^{+/-}$ (filled circle) and P14 IL2R$\beta^{-/-}$ (opened circle) mice at day 70 and 100 pi. Each symbol represents an individual mouse and bars represent the mean of 10–14 mice per genotype pooled from three independent experiments. (E) Bcl-2, Bim, and Mitotracker Deep Red staining was quantified by flow cytometry at day 70 pi. Results are representative of 7–14 mice pooled from three independent experiments. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, NS $p \geq 0.05$; two-tailed unpaired Student's i-test.

these two redundant cytokines are not mandatory for effective killing abilities in both acute and chronic contexts.

IL2Rβ-dependent signals condition T_{EM}-cell maintenance but are dispensable for T_{CM}-cell homeostasis

IL-15 plays a critical role in the generation and self-renewal capacity of the memory $CD8^+$ T pool [16, 24]. However, little is known on its impact on the different populations of memory cells and the role of its related cytokine, IL-2. We thus assessed whether the absence of combined IL-2 and IL-15 signals would affect $CD8^+$ memory T-cell differentiation by following LCMV-infected mice more than 70 days after infection. As expected, IL2R$\beta^{-/-}$ cells were present in much lower proportions than IL2R$\beta^{+/-}$ cells throughout the response (Fig. 4A). The spleen and bone marrow were similarly diminished in the number of IL2R$\beta^{-/-}$ $CD8^+$ memory T cells generated ($1{,}6.10^6$ IL2R$\beta^{+/-}$ versus $0{,}2.10^6$ IL2R$\beta^{-/-}$ cells in spleens, $p = 0{,}006$; $2{,}8.10^4$ IL2R$\beta^{+/-}$ versus $0{,}4.10^4$ IL2R$\beta^{-/-}$ cells in bone marrows, $p = 0{,}002$) (Fig. 4B). This was associated with an accentuated contraction in the absence of IL2Rβ, as measured by the higher fold-contraction of IL2R$\beta^{-/-}$ cells compared to their IL2R$\beta^{+/-}$ counterparts (Fig. 4C). The long-term maintenance of $CD8^+$ memory T cells was also influenced by the absence of IL2Rβsignals, as depicted by the significant reduction in IL2R$\beta^{-/-}$ T-cell numbers between day 70 and 100 cells (Fig. 4D). This could be correlated with a reduced expression of Bcl-2 and a noticeable diminution in mitochondrial mass in the absence of IL2Rβ, as depicted by a mitotracker staining (Fig. 4E). These findings suggest that IL-2 and IL-15 contribute to the Bcl-2-dependent survival of $CD8^+$ memory T cells, likely by increasing mitochondrial numbers. This hypothesis would be in accordance with recent in vitro experiments demonstrating that mitochondrial fatty acid oxidation is a cardinal feature of memory $CD8^+$ T cells and sustains memory T-cell survival [35]. Together, our data emphasize that the absence of IL-2 and IL-15 signals, while it significantly decreases the number of memory $CD8^+$ T cells generated, does not abrogate memory formation. However, IL-2 and IL-15 regulate the size of the $CD8^+$ memory T-cell pool by (i) controlling the magnitude of effector cell expansion, (ii) governing the extent of T-cell contraction, (iii) promoting long-term T-cell survival, and (iv) possibly sustaining oxidative metabolism and mitochondrial biogenesis.

As IL-2Rβ-dependent signals specifically regulate effector lineage choices, we then questioned if IL-2 and IL-15 would influence the composition of the memory $CD8^+$ T-cell pool. We thus compared the expression of CD62L, CD127, CCR7, CXCR3, CD43, and CD27 on $CD8^+$ memory T cells. While IL2R$\beta^{+/-}$ and IL2R$\beta^{-/-}$ cells had uniformly re-expressed CD127, they presented striking differences in their expression of CD62L, CCR7, KLRG1, CD27, and CXCR3 (Fig. 5A, Supporting Information Fig. 4A). In fact, most IL2R$\beta^{-/-}$ cells exhibited phenotypic features characteristic of T_{CM} differentiation ($CD62L^{high}$ $CCR7^{high}$ $CD127^{high}$ $KLRG1^{low}$) (Fig. 5B and C). Moreover, the absence of IL-2 and IL-15 signaling restrained the development of memory cells with effector characteristics defined as $CD27^{low}$ $CD43^{low}$ $CXCR3^{low}$ $KLRG1^{high}$ and accelerated the development of $CD27^{high}$ $CD43^{low}$ memory T cells, known for their superior recall responses (Fig. 5B and C) [36]. Interestingly, IL2Rβ deficiency did not abort the generation of T_{EM} cells, as demonstrated by the similar frequency of these cells at day 15 and day 30 post infection (Supporting Information Fig. 4B left panel), but severely hampered their maintenance, as shown by their attrition over time, in contrast to IL2R$\beta^{+/-}$ cells (Fig. 5D

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

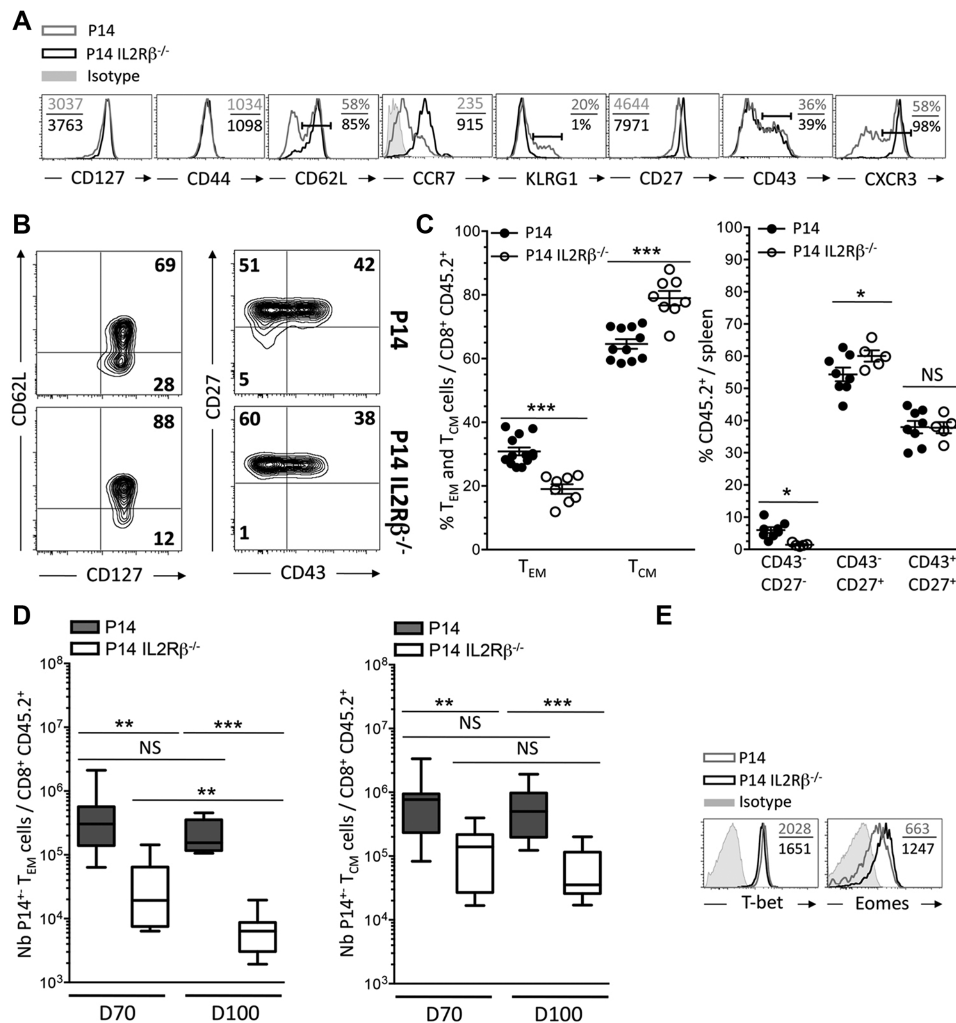


Figure 5. T_{EM} cells are highly dependent on IL-2 and IL-15 signals for their maintenance. P14 IL2Rβ$^{+/-}$ and P14 IL2Rβ$^{-/-}$ chimeric mice were generated as described in Fig. 1. (A) Cell surface expression of the indicated molecules by P14 IL2Rβ$^{+/-}$ (gray line histogram) and P14 IL2Rβ$^{-/-}$ (black line histogram) cells at day 70 pi was evaluated by flow cytometry. Values on histograms indicate the respective frequency or MFI. Data are representative of 7–14 mice pooled from four independent experiments. (B) The expression of CD62L, CD127, CD27 and CD43 in the spleen by P14 IL2Rβ$^{+/-}$ (top) and P14 IL2Rβ$^{-/-}$ (bottom) memory CD8$^+$ T cells. Numbers indicate the percentage of cells in each quadrant (representative of eight mice per genotype pooled from three independent experiments). (C) Frequency of T_{EM} cells (CD127^{hi} CD62L^{lo}) and T_{CM} cells (CD127^{hi} CD62L^{hi}) (left panel) and frequency of CD43$^-$ CD27$^-$, CD43$^-$ CD27$^+$ and CD43$^+$ CD27$^+$ cells (right panel) from P14 IL2Rβ$^{+/-}$ (filled circle) and P14 IL2Rβ$^{-/-}$ (opened circle) CD8$^+$ T cells. Each symbol represents an individual mouse and bars represent the mean of 8–14 mice per genotype pooled from three separate experiments. (D) Numbers of T_{EM} cells (left panel) and T_{CM} cells (right panel) at day 70 and day 100 pi, from P14 IL2Rβ$^{+/-}$ (gray boxes) and P14 IL2Rβ$^{-/-}$ (white boxes) mice. Results are shown as mean ± SEM pooled from three separate experiments (n = 8–14 per genotype). (E) Intracellular expression of T-bet and Eomes in P14 IL2Rβ$^{+/-}$ (gray line histogram) and P14 IL2Rβ$^{-/-}$ (black line histogram) memory CD8$^+$ T cells. Numbers indicate respective MFI (n = 6 per genotype from two separate experiments). $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, NS $p \geq 0.05$; two-tailed unpaired Student's t-test.

left panel). Noticeable, and in clear contrast to T_{EM} cells, T_{CM} cells were better sustained in the absence of IL2Rβ signals (Fig. 5D and Supporting Information Fig. 4B, right panels). This was correlated with a higher expression of Eomes and lower expression of T-bet in IL2Rβ$^{-/-}$ cells (Fig. 5E), in accordance with the fundamental importance of Eomes in T_{CM} differentiation [19]. Altogether, our findings indicate that IL-2 and IL-15 are key regulators of the maintenance of the effector memory T-cell pool, whereas their absence accelerates T_{CM} development while it does not preclude its homeostasis.

IL-2 and IL-15 promote secondary SLEC expansion but do not regulate secondary effector functions

We next questioned whether these phenotypic changes would impact the functional capacity of CD8$^+$ memory T cells. To do so, 10^4 P14 IL2Rβ$^{+/-}$ or P14 IL2Rβ$^{-/-}$ FACS-sorted memory T cells were adoptively transferred in naïve congenic hosts prior to re-infection with LCMV Armstrong. Although IL2Rβ$^{-/-}$ CD8$^+$ memory T cells demonstrated a sustainable expansion between day 0 and day 5 after viral rechallenge, their expansion was

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

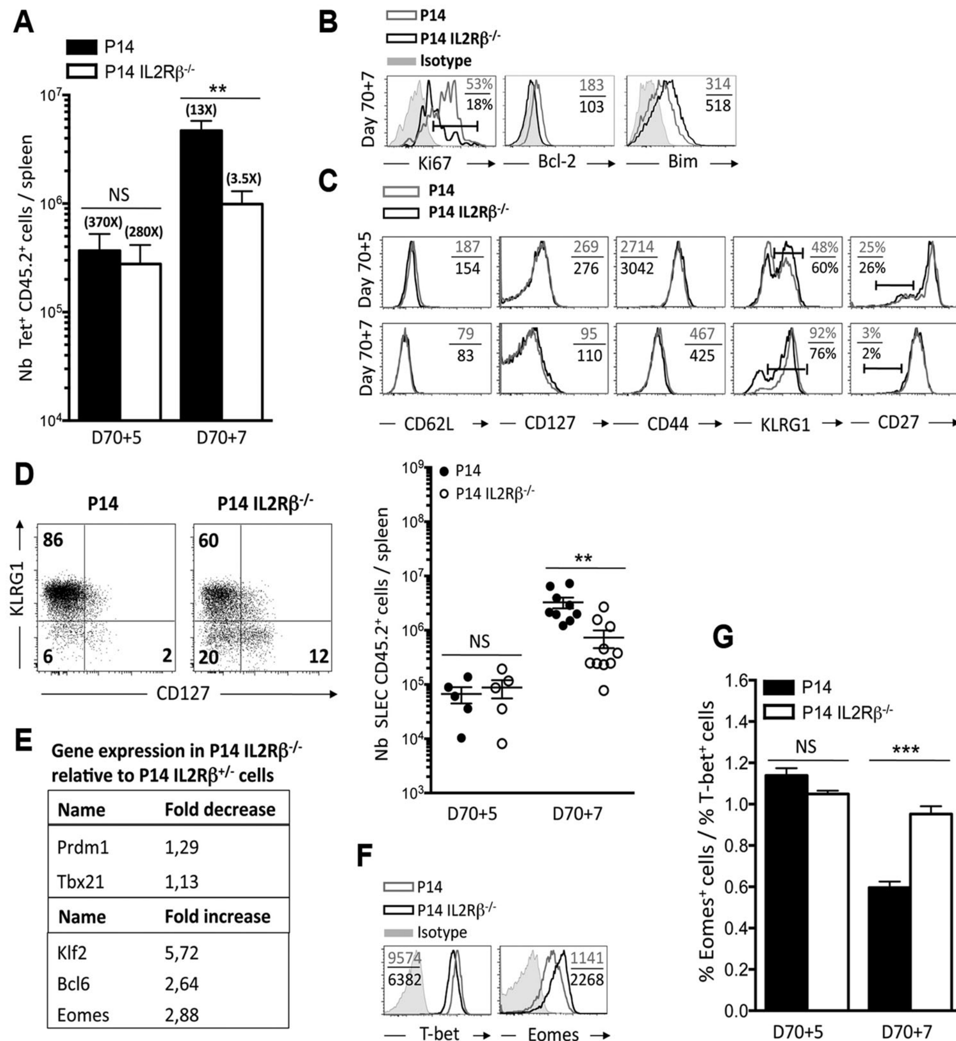


Figure 6. IL2Rβ-deficiency restricts the expansion of secondary SLECs. 10^4 P14 IL2Rβ$^{+/-}$ or P14 IL2Rβ$^{-/-}$ CD45.2$^+$ memory CD8$^+$ T cells were cell-sorted at day 70 pi and transferred into naive CD45.1.2$^+$ mice subsequently infected with 2×10^5 PFU of LCMV Armstrong. Expansion of secondary effector CD8$^+$ T cells was analyzed by flow cytometry at day 5 (day 70 + 5) and day 7 (day 70 + 7) post challenge. (A) Absolute numbers of P14 IL2Rβ$^{+/-}$ (black bars) and P14 IL2Rβ$^{-/-}$ (white bars) CD8$^+$ T cells in the spleen at day 5 and 7 pi. Data represent the mean ± SEM of six to nine mice per time point, pooled from three experiments. Values in brackets indicate the fold expansion for each cell population. Day 70+5 fold expansion was calculated by taking into account a 90% mortality rate of transferred cells. (B) Intracellular staining of Ki67, Bcl-2 and Bim by P14 IL2Rβ$^{+/-}$ (gray line histogram) or P14 IL2Rβ$^{-/-}$ (black line histogram) effector CD8$^+$ T cells at day 5 and 7 pi. Values indicate respective frequency of positive cells or MFI for each marker. Histograms are representative of nine mice per genotype pooled from three experiments. (C) Cell surface expression of the indicated differentiation markers by P14 IL2Rβ$^{+/-}$ (gray line histogram) and P14 IL2Rβ$^{-/-}$ (black line histogram) secondary effectors at day 5 and 7 pi. Numbers on histograms indicate the frequency or MFI. Histograms are representative of ten mice pooled from three independent experiments. (D) Dot plot at day 7 pi (left panel) and absolute numbers of SLECs at day 5 and 7 pi (right panel) based on KLRG1 and CD127 expression from P14 IL2Rβ$^{+/-}$ (filled circle) or P14 IL2Rβ$^{-/-}$ (opened circle) CD8$^+$ T cells. Values in dot plot indicate the percentage of each correspondent population from one of two to three separate experiments. Individual SLEC numbers are pooled from two to three independent experiments and bars represent the mean of five to ten mice per genotype. (E) Expression of the indicated genes was evaluated by RTqPCR. Values indicate the fold decrease (top) or increase (bottom) in each expression level in P14 IL2Rβ$^{-/-}$ versus P14 IL2Rβ$^{+/-}$ CD8$^+$ T cells at day 7 pi ($n = 5$ per genotype from two independent experiments). (F) Intracellular expression of T-bet and Eomes in P14 IL2Rβ$^{+/-}$ (gray line histogram) and P14 IL2Rβ$^{-/-}$ (black line histogram) T cells. Values indicate respective MFI ($n = 6$ per genotype from two independent experiments). Isotype control is shown in shaded gray. (G) Ratio of the frequency of Eomes$^+$ cells over the frequency of T-bet$^+$ cells among P14 IL2Rβ$^{+/-}$ (black bar) or P14 IL2Rβ$^{-/-}$ (white bar) T cells at day 7 pi (mean ± SEM pooled from two separate experiments, $n = 6$ per genotype). $^{**}p < 0.005$, $^{***}p < 0.0005$, NS $p \geq 0.05$; two-tailed unpaired Student's *t*-test.

severely limited thereafter (reaching a maximum of $0.9.10^6$ IL2Rβ$^{-/-}$ cells at day 70+7 compared to $4.7.10^6$ IL2Rβ$^{+/-}$ cells, $p = 0{,}003$) (Fig. 6A). This was correlated with an almost complete arrest of cell proliferation and an increased expression of Bim relative to Bcl2 (Fig. 6B). Assessment of cell surface markers further demonstrated that IL2Rβ$^{-/-}$ memory T cells could develop into secondary effectors upon re-infection (Fig. 6C). However, the number and proportion of secondary SLECs at day 7 were significantly reduced in the absence of IL2Rβ signals, while they were equivalent at day 5 (Fig. 6D). This was correlated as during the primary infection with reduced *Prdm1* and *Tbx21* mRNA levels, increased *Klf2, Bcl6,* and *Eomes* levels and a preferential

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

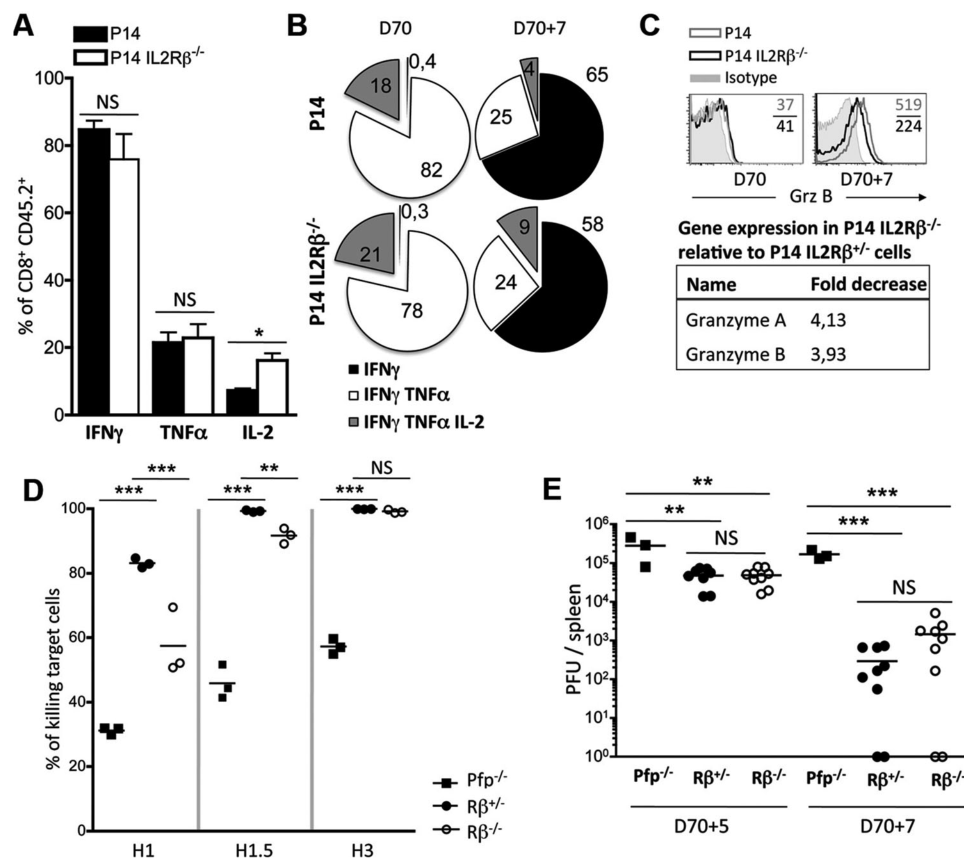


Figure 7. IL2Rβ-deficient secondary effectors efficiently control a viral infection. Memory P14 IL2Rβ$^{+/-}$ and P14 IL2Rβ$^{-/-}$ chimeric mice were generated as described in Fig. 6. (A) Intracellular cytokine staining for IFN-γ, TNF-α, and IL-2, at day 7 post-challenge, from splenic P14 IL2Rβ$^{+/-}$ (black bars) and P14 IL2Rβ$^{-/-}$ (white bars) CD8$^+$ T cells. Data are shown as mean ± SEM of $n = 6$ samples pooled from two independent experiments. (B) The frequency of cells secreting multiple cytokines simultaneously at day 70 (prior to challenge, left), and at day 70+7 (7 days after challenge, right) among splenic P14 IL2Rβ$^{+/-}$ (top) and P14 IL2Rβ$^{-/-}$ (bottom) cells. Values in pie charts indicate the percentage of each correspondent phenotype from one representative experiment ($n = 6$ per genotype pooled from two separate experiments). (C) Intracellular Grz B expression (top panel) in splenic P14 IL2Rβ$^{+/-}$ (gray line histogram) or P14 IL2Rβ$^{-/-}$ (black line histogram) at day 70 pi and day 7 post challenge (D70+7). Isotype control is shown in shaded gray. Values on histograms indicate MFI ($n = 10$ per genotype from three separate experiments). Expression of the indicated genes was evaluated by RTqPCR (bottom panel). Values indicate the fold decrease between P14 IL2Rβ$^{-/-}$ versus P14 IL2Rβ$^{+/-}$ CD8$^+$ T cells at day 7 pi ($n = 4$ from two separate experiments). (D) Target cell killing after in vivo transfer of $GP_{33\text{-}41}$-loaded splenocytes by P14 (filled circle) and P14 IL-2Rβ$^{-/-}$ (opened circle) effectors at day 7 pi in Pfp$^{-/-}$ recipients (filled square) and calculated as described in Fig. 2C. Values below the x-axis indicate the number of hours since target cells were injected. Each symbol represents an individual mouse and bars represent the mean of three mice per genotype from one representative experiment. (E) Viral titers in P14 IL2Rβ$^{+/-}$ or P14 IL2Rβ$^{-/-}$ spleens at day 5 and 7 pi were evaluated by viral plaque assays. Data are shown individually and bars represent the mean ± SEM from $n = 3$–9 mice per genotype pooled from one to three separate experiments. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, NS $p \geq 0.05$; two-tailed unpaired Student's t-test.

expression of Eomes over T-bet in IL2Rβ$^{-/-}$ cells (Fig. 6E–G). Collectively, these data highlight the decisive contribution of IL-2 and IL-15 in the proliferation, survival, and terminal differentiation of late-phase secondary SLECs (between day 5 and 7 after viral rechallenge).

By controlling secondary effector differentiation, IL2Rβ signals may affect the cytotoxic functions of these cells. To assess this question, we evaluated the capacity of memory CD8$^+$ T cells and secondary effectors to secrete cytokines and eliminate target cells. Interestingly, IL2Rβ$^{+/-}$ and IL2Rβ$^{-/-}$ memory cells had similar cytokine profiles prior to re-infection (Fig. 7B). Upon viral challenge, more than 80% of the cells could produce IFN-γ (Fig. 7A). IL2Rβ$^{+/-}$ and IL2Rβ$^{-/-}$ secondary effectors also presented the same functional characteristics, differentiating away from being a double-cytokine producer (IFN-γ, TNF-α) to secreting mostly IFN-γ after re-infection (Fig. 7B). In addition, a higher proportion of triple-cytokine producers was seen in the absence of IL2Rβ ($p = 0.03$). The cytotoxic functions of the cells were then further assessed. While IL2Rβ$^{-/-}$ memory cells presented similar granzyme B levels at baseline, the granzyme B content was reduced when compared to their IL2Rβ$^{+/-}$ counterparts once challenged (Fig. 7C). Similarly, the amount of granzyme A and B mRNA was reduced in IL2Rβ-deficient cells (Fig. 7C). Interestingly, these differences were less drastic then what they were in primary effectors (Fig. 2D). IL2Rβ$^{-/-}$ secondary effectors also retained the ability to eliminate target cells in vivo, despite a slight delay in the killing kinetic that can easily be explained by the reduced number of secondary effectors in the absence of IL2Rβ signals (Fig. 7D). Furthermore, we detected similar viral loads in the presence or absence of the IL2Rβ chain (Fig. 7E). Thus, our

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

findings indicate that IL-2 and IL-15 condition the differentiation of secondary effectors but are dispensable for potent cytotoxic functions and viral control.

We finally questioned if the absence of IL2Rβ signaling during the first week of infection would impact secondary effectors. In fact, it was previously suggested that IL-2 availability during the initial infection would program the expansion capacity of memory cells upon rechallenge [14, 23]. To revisit this question, we treated WT mice with an anti-CD122 antibody (or a mock antibody as control) during either the first week of infection or reinfection or both (Fig. 8A). This approach allows testing precisely the combined impact of IL-2 and IL-15 on the initial programming of the cells and on their cytokine requirements during re-expansion. Of note, the anti-CD122 blocking antibody efficiently blocked the phosphorylation of STAT5 upon in vivo treatment (Fig. 8B). As expected, it reduced both the frequency and number of primary SLECs (Fig. 8C) while it did not modify the number and phenotype of the $CD8^+$ memory T-cell pool when given only during the first week of infection (data not shown). Of particular interest, $CD8^+$ memory T cells generated in the initial absence of IL2Rβ signaling efficiently expanded upon rechallenge ($1.4.10^6$ versus $1.1.10^6$ secondary effectors in CD122-treated and nontreated mice, respectively, Fig. 8D group 2 versus group 1, respectively). This was not the case if IL2Rβ signaling was interrupted during reinfection (Fig. 8D, group 3). Moreover, anti-CD122 treatment during secondary infection impeded secondary SLEC expansion, as depicted by the reduction in the frequency of secondary SLECs in this group, while it did not if IL2Rβ signaling was restored at the time of challenge (Fig. 8E, group 3 versus group 2). Altogether, these results demonstrate that IL-2 and IL-15 signals must be present to sustain secondary SLEC development and that transient absence of these cytokines during the initial phase does not impact the expansion capacities of $CD8^+$ memory T cells.

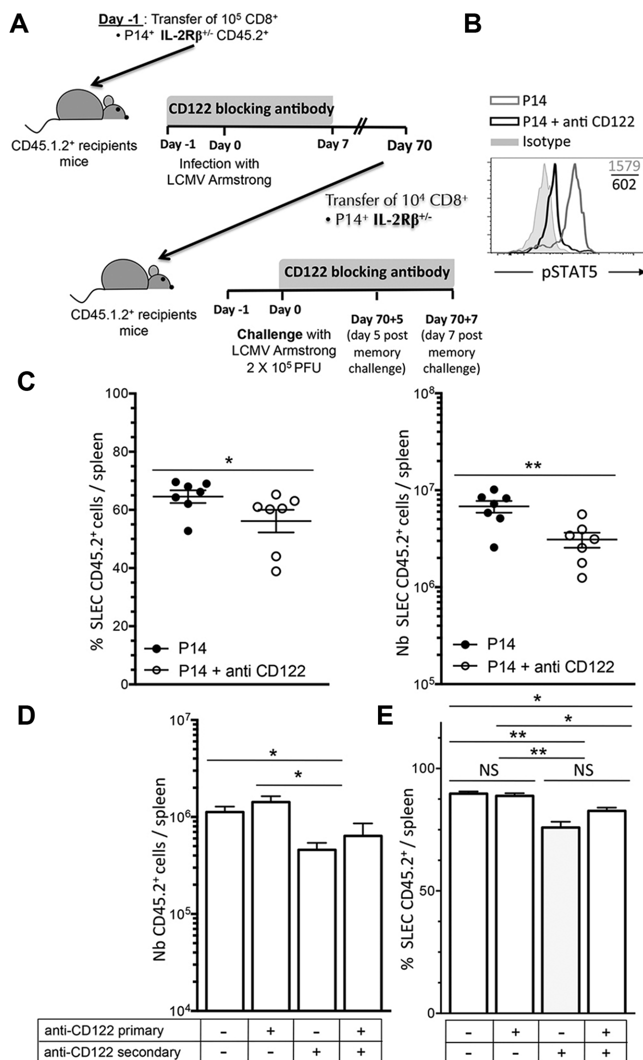


Figure 8. Blocking CD122 signals during viral rechallenge restrains the expansion of secondary SLECs. (A) P14 IL2Rβ$^{+/-}$ and P14 IL2Rβ$^{-/-}$ chimeric mice were generated as described in Fig. 1, but recipient mice received 200 μg of anti-CD122 (TM-β1) or LTF-2 (control antibody) ip every other day starting 1 day prior to cell transfer until day 6 post infection or post challenge. (B) Intracellular staining of phospho STAT5 (Y694) in P14 IL2Rβ$^{+/-}$ $CD45.2^+$ effectors $CD8^+$ T cells treated (black line histogram) or not (gray line histogram) with the anti-CD122 antibody. Isotype control in shaded gray histogram. Values indicate the MFI (n = 3 per genotype). (C) Frequency (left panel) and absolute numbers (right panel) of primary SLECs in P14 IL2Rβ$^{+/-}$ (filled circle) and P14 IL2Rβ$^{+/-}$ anti-CD122 treated (opened circle) mice. Each symbol represents an individual mouse and bars represent the mean of seven mice per genotype pooled from three independent experiments. (D-E) 10^4 P14 IL2Rβ$^{+/-}$ $CD45.2^+$ memory $CD8^+$ T cells were cell-sorted at day 70 and transferred into naive $CD45.1.2^+$ mice subsequently infected with 2×10^5 PFU of LCMV Armstrong. (D) Absolute numbers of $CD45.2^+$ cells at day 7 post-challenge in P14 IL2Rβ$^{+/-}$ and P14 IL2Rβ$^{+/-}$ anti-CD122 treated during primary or secondary infection or both (n = 3 per genotype). (E) SLEC frequencies of $CD45.2^+$ cells at day 7 post-challenge in P14 IL2Rβ$^{+/-}$ and P14 IL2Rβ$^{+/-}$ anti-CD122 treated during primary or secondary infection or both (n = 3 per genotype). $^*p < 0.05$, $^{**}p < 0.005$, NS $p \geq 0.05$; two-tailed unpaired Student's t-test for (C) and Kruskal–Wallis ANOVA for (D-E).

Discussion

γ_c-dependent cytokines have multiple and diverse roles in immunity and are an essential component of the adaptive immune response to infections. While they are central to the development of mature T cells in the thymus, γ_c cytokines influence the survival and homeostasis of naïve and memory T cells in the periphery. Moreover, we have previously demonstrated that combined γ_c-dependent signals regulate the $CD8^+$ T-cell differentiation process during an acute viral infection and are critical for the generation of $CD8^+$ memory T cells, in a Bcl-2 independent manner [10]. The complete abrogation of memory development in the absence of γ_c suggests that these cytokines share essential overlapping and redundant functions during infection, despite the presence of inflammatory signals and potent antigenic stimulation. In this context, IL-2 and IL-15 exhibit the highest degree of redundancy by presenting close structural similarities of their receptor, by signaling through the same receptor chains (IL2Rβ, γ_c) and molecules (JAK1/JAK3/STAT5, PI3K/AKT, RAS/MAPK), and by inducing the transcription of similar genes [26, 37]. To address the combined

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

contribution of IL-2 and IL-15 signals in vivo, we have designed a novel TCR transgenic mouse model that abrogates specifically IL2Rβ-dependent signals while preserving signaling by other γ_c-dependent cytokines. This model has a significant advantage over previous models that attempted to elucidate the combined contribution of both cytokines to $CD8^+$ T-cell differentiation [15]. Not only it precludes signaling through the intermediate affinity IL2Rβγ receptor, a known limitation of approaches using $IL2R\alpha^{-/-}$ cells, it also preserves adequate dendritic cell survival, maturation, and antigen presentation, which are known to be altered in $IL\text{-}15^{-/-}$ hosts [38, 39].

In the absence of IL2Rβ signaling, we demonstrate that combined IL-2 and IL-15 signals modulate both the proliferation and survival of $CD8^+$ late effectors. With a significant reduction in the number of antigen-specific effector cells at the peak of the response, our results are consistent with previous reports showing that IL-2 signals sustain T-cell expansion in vivo [22, 40, 41]. However, by accounting for the redundant contribution of IL-2 during T-cell expansion, we clarify the role of IL-15 during this phase of the response, since some authors had demonstrated that the absence of IL-15 limits T-cell expansion [13] while others did not [42, 43]. Even more, the impact of both cytokines is not only redundant but likely additive since IL2Rβ deficiency reduces the number of effectors by 3.5-fold while others had previously demonstrated that IL2Rα deficiency leads to a two-fold reduction in effector numbers in the same infectious context [14, 15]. Thus, our data suggest that IL-15 contributes to the expansion of late effectors, even before the contraction phase, as IL-2 does. Interestingly, we confirm that the initial expansion of T cells is surprisingly independent of IL-2 and IL-15 signals, although both receptor chains are rapidly expressed at the surface of the cells upon viral challenge. This is consistent with the notion that $CD8^+$ T-cell expansion is programmed upon a short encounter with the pathogen, thus hindering requirements for prolonged IL-2 and IL-15 stimulation initially [44, 45]. It also highlights that other inflammatory cytokines present at the time of priming likely provide accessory redundant signals to T cells for their expansion [46].

The impact of IL-2 and IL-15 during the expansion phase is particularly critical for SLECs, during both primary and secondary responses, while MPECs are preserved in the absence of IL2Rβ-signaling. This is in accordance with the notion that IL-2 and IL-15 regulate the proliferation and survival of SLECs, as previously demonstrated by others [12, 13, 15, 22], but expands this notion to secondary effectors. It also reinforces the concept that SLECs and MPECs respond to distinct signals during an infection and that similar cytokinic signals are intergraded by these effector cells for their proper differentiation, independently of the time of infection, being initially or upon rechallenge. We further demonstrate that $CD8^+$ T-cell differentiation per se is severely hampered in the absence of IL2Rβ signaling, and that both cytokines are likely redundant for this function. Our results suggest that IL-2 and IL-15 influence lineage choices by modifying the transcriptional program of the cells, favoring the transcription of *Tbx21* and *Prdm1* over *Eomes, Bcl6* and *Klf2*. Although we did not evaluate per se the exact contribution of each cytokine on the transcriptional network of the cells, IL-2 and IL-15 likely mediate their transcriptional effect through phosphorylation of Foxo1 in an Akt-dependent manner, since Foxo1 was shown to restrain T-bet mediated terminal effector differentiation and sustain the transcription of several memory-associated genes such as *Eomes*, *IL7Ra*, *Klf2,* and *Ccr7* [47, 48]. Further studies would be required to explore this hypothesis.

We had previously demonstrated that γ_c-dependent cytokines were mandatory for the generation of memory T cells in a Bcl-2-independent manner [10]. This was surprising knowing that IL-7 and IL-15 promote T-cell survival through enhanced expression of anti-apoptotic Bcl2 family members [49–51]. It suggested that IL-7 and/or IL-15 were indispensable for T-cell survival, independently of Bcl2, as suggested by others [49, 50, 52]. In this report, we emphasize the critical role of IL2Rβ-dependent cytokines on the survival of $CD8^+$ effector T cells, as suggested by the reduced survival and accelerated contraction of $IL2R\beta^{-/-}$ effector cells. This is in accordance with the prosurvival function of IL-15, especially during the contraction phase [6, 11, 13, 15, 43], although the role of Bcl-2 in this context remains debatable, since Bcl-2 overexpression does not rescue T-cell contraction [10]. We also demonstrate that, in the absence of IL-2 and IL-15, memory cell development was severely restrained, with significant reduction in the number of $CD8^+$ memory T cells generated and slow attrition of these cells over time. These findings highlight not only the critical importance of IL-7 in the generation of $CD8^+$ memory T cells [53, 54], but also its incapacity to completely compensate the lack of IL-15-signaling [4, 16]. It also confirms that memory T cells are maintained through both IL-7 and IL-15-dependent homeostatic mechanisms [16, 53]. One of these mechanisms could be through the regulation of fatty acid oxidation, a cardinal metabolic pathway involved in memory T-cell development and survival [35]. This is suggested by our demonstration of a reduced memory T-cell mitochondrial mass in the absence of IL2Rβ-signals. Further work remains to be performed to establish the γ_c-dependent downstream pathways that regulate cell-cycle proteins, pro- and anti-apoptotic molecules, and mitochondrial proteins decisive for $CD8^+$ memory T-cell homeostasis.

Distinct populations of memory cells likely respond differentially to IL-2 and IL-15. Interestingly, memory cells with effector-like characteristics ($CD62L^{low}$ $CD27^{low}$ $CD43^{low}$ cells) [36] were much more sensitive to IL-2 and IL-15 signals than other memory subsets. This is in accordance with the accelerated T_{CM} development in the absence of IL2Rα signaling and the loss of $CD27^{low}$ $CD43^{low}$ cells in $IL\text{-}15^{-/-}$ hosts [15, 22, 24]. In addition, we show that T_{EM} cells were not only significantly reduced in numbers in the absence of IL2Rβ-signaling, their maintenance was severely hampered over time. In contrast, IL-2 and IL-15 were largely dispensable for the maintenance of T_{CM} cells, although the presence of CD127 on MPEC did not prevent contraction of these cells [55]. These critical findings suggest that memory subsets are differentially regulated and possess distinct transcriptional networks that control their development and maintenance. Thus, IL-2 and IL-15 likely sustain effector-like memory differentiation by limiting

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

the activation of the Foxo1 pathway (and Eomes transcription), while promoting mTORC1 activation, in an AKT-dependent manner [56], as suggested above for SLEC differentiation. Similarly IL-2 and IL-15 may promote Blimp-1 expression, thus favoring not only the quantity of memory T cells generated but also the phenotypic characteristics of these cells [20, 57]. Alternatively, IL-2 and IL-15 might hamper T_{CM} differentiation by limiting, directly or indirectly, Klf2, Eomes or Bcl6 transcription, as suggested by our findings [19, 58, 59]. Altogether, these data suggest that IL-2 and IL-15 induce multiple signaling pathways and regulate multiple transcription factors to ultimately dictate the fate of $CD8^+$ T cells.

As one of the specificities of memory cells is their ability to rapidly proliferate upon rechallenge, the question of the impact of IL-2 and IL-15 on secondary expansion is of particular importance, in light of the numerous conflicting reports available [14, 15, 22–24, 42]. While some authors suggest that IL-2 programs future T-cell expansion [14, 15, 23], others do not [22]. Conversely, few reports suggest a trivial contribution of IL-15 on secondary expansion [14, 24, 42]. However, because previous work by Bevan's team had revealed the key contribution of IL2Rβ-signaling in the programming of secondary T-cell expansion [14], and knowing the redundant functions of IL-2 and IL-15 on primary effectors, we further tackled this question. To clarify the situation, we revisited the contribution of IL-2 and IL-15 during secondary infection by (i) preventing any possible redundancy of the two cytokines, (ii) abolishing any possible signaling through the IL2Rβ/γ_c heterodimer, (iii) eliminating the contribution of paracrine IL-2 secretion on effector cells, (iv) conserving proper lymphoid organ architecture and antigen presentation, and (v) testing secondary responses to the same infectious agent in a naïve environment. We demonstrate that not only secondary effectors can substantially expand, they are readily functional and cytotoxic. Although the expansion is considerable, it does present some restriction in late phases of the response, as it did during the primary response. Though this underscores that primary and secondary effectors largely rely on similar cytokinic signals for their proper expansion, as they did for their differentiation, secondary effectors appear to be slightly more sensitive to the absence of IL-2 and IL-15 signals since the difference between WT and IL2Rβ$^{-/-}$ cells numbers after the second infection is slightly more important than after the initial infection (4.7-fold versus 3.5-fold difference, respectively). We then deciphered whether reduced expansion among secondary IL2Rβ$^{-/-}$ effectors resulted from an abnormal programming initially, as suggested by Bevan, or if IL-2 and IL-15 signals were required during reinfection. Using an anti-CD122 blocking antibody during either the first week of infection or reinfection, we demonstrated that IL-2 and IL-15 needed to be present at the time of the challenge in order to sustain secondary T-cell expansion. This suggests that these two redundant cytokines do not permanently imprint a specific differentiation and expansion program to effector T cells and that transient IL-2 and IL-15 deficiency would not impact long-term memory responses. Further studies will be required to tackle the genetic and epigenetic changes regulated by combined IL-2 and IL-15 signals to control recall responses.

All together our data highlight the critical role for combined IL-2 and IL-15 signals in the proliferation and survival of primary and secondary effectors and their differentiation into SLECs. It also demonstrates that IL2Rβ signaling condition the maintenance of a memory T-cell pool with effector characteristics. Unexpectedly, IL-2 and IL-15 are dispensable for potent effector functions and viral clearance, suggesting that multiple redundant signals are in place to preserve effective cytotoxic functions upon viral challenge.

Materials and methods

Mice

$CD45.2^+$ Rag2$^{-/-}$ P14 TCR Transgenic (Tg) mice (expressing a TCR specific for the LCMV H2-D^b-restricted $GP_{33\text{-}41}$ epitope) [60] were provided by A. Freitas (Institut Pasteur, Paris, France) and backcrossed onto the C57BL/6 (B6) background. P14 mice, with or without IL2Rβ chain, were then produced by breeding P14 mice with IL2Rβ$^{-/-}$ mice (Jackson Laboratory). P14 IL2Rβ$^{+/-}$ or P14 IL2Rβ$^{-/-}$ chimeric mice were generated by adoptive transfer of 7- to 10- week-old 10^5 naïve $CD45.2^+$ TCR Tg $CD8^+$ T cells into 8- to 10-week-old naïve $CD45.1.2^+$ B6 recipient mice. 10^4 naïve P14 IL2Rβ$^{+/-}$ or IL2Rβ$^{-/-}$ cells were used in the chronic infection model. To prevent rejection of adoptively transferred cells, $CD45.1.2^+$ B6 recipient mice were generated by crossing Tg$^-$ $CD45.2^+$ mice with $CD45.1^+$ B6 mice (B6.SJL, Jackson Laboratory). For secondary infection, 10^4 P14 or P14 IL2Rβ$^{-/-}$ $CD45.2^+$ memory $CD8^+$ T cells (Day 70 post-infection) were adoptively transferred into 8- to 10-week-old naïve $CD45.1.2^+$ B6 recipient mice. For in vivo killing assays, Pfp$^{-/-}$ mice (B6$^{Prf1tm1sdz/J}$, Jackson Laboratory) were used as recipients. All donor and virus-free recipient mice were housed in specific pathogen-free facilities at the CHU Sainte-Justine Research Center. Experiments were conducted in accordance with institutional guidelines.

Virus, viral titers, and mice infection

LCMV Armstrong and LCMV clone 13 were obtained from Rolf M.Zinkernagel (Zurich University Hospital, Switzerland) and were grown respectively on BHK-21 and L929 cells, as previously described [61]. Virus titers were determined by focus-forming assay on MC57G cells [62]. Mice were infected 20 h after adoptive transfer of naïve or memory T cells. For acute infections and recall responses, mice were injected ip with 2×10^5 PFU of LCMV Armstrong. For chronic infection, mice were injected iv with 1×10^6 PFU of LCMV Clone 13. All infected mice were housed in specific infectious facilities at the INRS-IAF Experimental Biology Center.

Blocking antibody treatment

In some experiments, an anti-CD122 blocking antibody (TM-β1, 200 μg/injection ip) was injected one day prior to adoptive cell transfer and every other day thereafter, until day 6 post-infection. Other groups of mice received the same treatment during the

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

re-infection phase exclusively, or during both the primary or secondary infection. Control mice were similarly injected with a mock antibody (LTF-2). Both anti-CD122 and LTF-2 antibodies were purchased from BioXcell.

Cell isolation, flow cytometry analysis, and cell sorting

Peripheral blood (100 μL) was obtained retro-orbitally, by the saphenous vein or from the facial artery in tubes containing 100 μL of PBS 1× EDTA (5 mM). Bone marrow was obtained by flushing both femurs. Single cell suspensions were generated from spleen and bone marrow by pressing the organs and filtering the cells through a 100-μM nylon mesh, prior to erythrocyte lysis (NH_4Cl or BD FACS lysing buffer). Cell suspensions were stained in 50–100 μL of PBS with 2% FBS during 15 min on ice. Prior to staining, cells were Fc-blocked. Monoclonal antibodies were purchased from eBioscience and BD Bioscience except for Granzyme B (Molecular Probes) and CD43 (BioLegend). CCR7 staining was performed by incubating cells 40 min at 37°C with CCR7-biotin (eBioscience). Ultra-avidin-R-phycoerythrin (Leinco) was used to reveal CCR7-biotin staining. MHC class I peptide tetramers were produced by F. Lemaitre (Institut Pasteur, Paris), coupled to ultra-avidin-R-phycoerythrin (Leinco) and used simultaneously with other extracellular antibodies. Dead cells were excluded using Live/Dead Fixable Aqua Dead Cell stain kit prior to fixation (Molecular Probes). Mitotracker Deep Red (Invitrogen) and Annexin V / 7-AAD (eBioscience) staining was performed according to manufacturer's instructions and was processed before dead cell and monoclonal antibodies staining. Data were acquired using an FACS LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar). For recall responses, alive $CD8^+$ $CD3^+$ $CD45.1^-$ $CD45.2^+$ TCR Tg cells were cell-sorted on a BD FACSAria II (98% purity).

Intracellular staining and in vivo cytotoxic assay

Intracellular staining was done on freshly isolated splenocytes according to manufacturer's instructions, with the fixation and permeabilization kits from eBioscience or BD Bioscience. IFN-γ, TNF-α, and IL-2 quantification was evaluated after ex vivo restimulation with $GP_{33\text{-}41}$ peptide (0.2μg/mL) in the presence of brefeldin A (10μg/mL) for 4 h. Degranulation was similarly assessed after 5 h of ex vivo restimulation by staining for CD107a (BD Bioscience). Granzyme B and perforin staining were performed without stimulation. Bim (Cell Signaling), Bcl2 (BD Biosciences), active-caspase 3 (BD Biosciences), Ki-67 (eBioscience), T-bet (BD Bioscience), Eomes (eBioscience), and pSTAT5 Y694 (Cell Signaling) antibodies were used as suggested by manufacturers. FITC conjugated donkey anti-rabbit was used as secondary antibody for intracellular Bim staining (Jackson ImmunoResearch). In vivo cytotoxic assay was performed by transferring 10^6 $CFSE^{high}$-labeled (1 μM) $GP_{33\text{-}41}$-loaded splenocytes and 10^6 $CFSE^{low}$-labeled (0.1 μM) unloaded splenocytes in $Pfp^{-/-}$ mice at day 7 post-infection, as previously described [10]. Percentage of target cell killing was calculated as follows: 100 – (100 × ((% peptide-loaded infected/% unloaded infected)/(% peptide-loaded control/% unloaded control))).

RNA extraction and real-time quantitative PCR analysis

RNA was isolated from 2 × 10^4 cell-sorted P14 $IL2R\beta^{+/-}$ or P14 $IL2R\beta^{-/-}$ at distinct time-points post-infection by mixing with TRIzol reagent (Invitrogen) on ice. RNA was next purified with RNeasy Micro Kit (Qiagen) and cDNA was synthesized with SuperScript VILO Kit (Invitrogen). Real-time quantitative RT-PCR was performed on a Stratagene Mx3000P (Agilent Technologies) with SYBR Select Master mix (Applied Biosystems) using the following primer sets: Prdm1 forward, 5′-AC ACACAGGAGAGAAGCCACATGA-3′ and reverse, 5′-TCGAAGGT GGGTCTTGAGATTGCT-3′; Tbx21 forward 5′-AGGGGACACTCGC AACAGA-3′ and reverse, 5′-AGGGGGCTTCCAACAATG-3′; Eome sodermin forward, 5′-CCGCCCACTACAATGTTTTC-3′ and reverse GAAATCTCCTGCCTCATCCA-3′; Klf2 forward, 5′-TGTGAGAA ATGCCTTTGAGTTTACTG-3′ and reverse, 5′-CCCTTATAGAAAT ACAATCGGTCATAGTC-3′; Bcl6 forward 5′-GGGACATCTTGAC GGACGTT-3′ and reverse, 5′- TCACGGGAGGTTTAAGTGC; Granz yme A forward, 5′TTTCATCCTGTAATTGGACTAA-3′ and reverse, 5′-GCGATCTCCACACTTCTC-3′; Granzyme B forward, 5′-CCTCC TGCTACTGCTGAC-3′ and reverse, 5′-GTCAGCACAAAGTCCTCT C-3′; Hprt forward, 5′-CTCCTCAGACCGCTTTTTGC-3′, and reverse, 5′-TAACCTGGTTCATCATCGCTAATC-3′.

Statistical analysis

Statistical significance was determined by two-tailed unpaired Student's t-test and Kruskal–Wallis ANOVA using ABI Prism 6. Significance was set as any p value less than 0.05.

Acknowledgments: We thank A. Freitas for P14 mice; Rolf M. Zinkernagel for LCMV viruses, F. Le Deist for flow cytometry and cell sorting; F. Lemaitre, I. Boufaied, RM. Brito, E. Michaud, N. Cotta-Grand and E. Tarrab for technical assistance; M. Perez Marcogliese, H. Orlando, D. Carrier, A. Delisle, V. Pellerin, S. Lespérance, E. Pelletier, H. Slimani for animal care. We are grateful to V. Abadie, N. Heveker, H. Soudeyns, C. Beauséjour and N. Labrecque, and members of their labs, for helpful discussions. H.D. is supported by grants from the Canadian Institutes of Health Research (CIHR MOP-130469), the Fonds de Recherche du Québec – Santé, the Cole Foundation and the Canadian Child Health Clinician Scientist Program. J.P.D.S. is supported by grants from the Institut Pasteur and the INSERM. A.L. acknowledges support from the CIHR grant MOP-89797 and from the Jeanne and J.-Louis Lévesque Research Chair in Immunovirology from the J.-Louis Lévesque Foundation. C.M. and JC.B. have research scholarships from the University of Montreal and the CHU Sainte-Justine Foundation.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Gerlach, C., van Heijst, J. W., Swart, E., Sie, D., Armstrong, N., Kerkhoven, R. M., Zehn, D. et al.**, One naive T cell, multiple fates in CD8+ T cell differentiation. *J. Exp. Med.* 2010. **207**: 1235–1246.

2 **Zhang, N. and Bevan, M. J.**, CD8(+) T cells: foot soldiers of the immune system. *Immunity* 2011. **35**: 161–168.

3 **Kaech, S. M. and Cui, W.**, Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol* 2012. **12**: 749–761.

4 **Kaech, S. M., Tan, J. T., Wherry, E. J., Konieczny, B. T., Surh, C. D. and Ahmed, R.**, Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 2003. **4**: 1191–1198.

5 **Sarkar, S., Kalia, V., Haining, W. N., Konieczny, B. T., Subramaniam, S. and Ahmed, R.**, Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J. Exp. Med.* 2008. **205**: 625–640.

6 **Joshi, N. S., Cui, W., Chandele, A., Lee, H. K., Urso, D. R., Hagman, J., Gapin, L. et al.**, Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 2007. **27**: 281–295.

7 **Prlic, M., Hernandez-Hoyos, G. and Bevan, M. J.**, Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. *J. Exp. Med.* 2006. **203**: 2135–2143.

8 **Henrickson, S. E., Mempel, T. R., Mazo, I. B., Liu, B., Artyomov, M. N., Zheng, H., Peixoto, A. et al.**, T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nat Immunol* 2008. **9**: 282–291.

9 **Pipkin, M. E., Sacks, J. A., Cruz-Guilloty, F., Lichtenheld, M. G., Bevan, M. J. and Rao, A.**, Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 2010. **32**: 79–90.

10 **Decaluwe, H., Taillardet, M., Corcuff, E., Munitic, I., Law, H. K., Rocha, B., Riviere, Y. et al.**, Gamma(c) deficiency precludes CD8+ T cell memory despite formation of potent T cell effectors. *Proc Natl Acad Sci USA* 2010. **107**: 9311–9316.

11 **Rubinstein, M. P., Lind, N. A., Purton, J. F., Filippou, P., Best, J. A., McGhee, P. A., Surh, C. D. et al.**, IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response. *Blood* 2008. **112**: 3704–3712.

12 **Kalia, V., Sarkar, S., Subramaniam, S., Haining, W. N., Smith, K. A. and Ahmed, R.**, Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity* 2010. **32**: 91–103.

13 **Sanjabi, S., Mosaheb, M. M. and Flavell, R. A.**, Opposing effects of TGF-beta and IL-15 cytokines control the number of short-lived effector CD8+ T cells. *Immunity* 2009. **31**: 131–144.

14 **Williams, M. A., Tyznik, A. J. and Bevan, M. J.**, Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 2006. **441**: 890–893.

15 **Mitchell, D. M., Ravkov, E. V. and Williams, M. A.**, Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells. *J Immunol* 2010. **184**: 6719–6730.

16 **Becker, T. C., Wherry, E. J., Boone, D., Murali-Krishna, K., Antia, R., Ma, A. and Ahmed, R.**, Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J. Exp. Med.* 2002. **195**: 1541–1548.

17 **Wherry, E. J., Becker, T. C., Boone, D., Kaja, M. K., Ma, A. and Ahmed, R.**, Homeostatic proliferation but not the generation of virus specific memory CD8 T cells is impaired in the absence of IL-15 or IL-15Ralpha. *Adv. Exp. Med. Biol.* 2002. **512**: 165–175.

18 **Yi, J. S., Ingram, J. T. and Zajac, A. J.**, IL-21 deficiency influences CD8 T cell quality and recall responses following an acute viral infection. *J. Immunol* 2010. **185**: 4835–4845.

19 **Banerjee, A., Gordon, S. M., Intlekofer, A. M., Paley, M. A., Mooney, E. C., Lindsten, T., Wherry, E. J. et al.**, Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. *J. Immunol* 2010. **185**: 4988–4992.

20 **Rutishauser, R. L., Martins, G. A., Kalachikov, S., Chandele, A., Parish, I. A., Meffre, E., Jacob, J. et al.**, Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 2009. **31**: 296–308.

21 **Shin, H. M., Kapoor, V. N., Guan, T., Kaech, S. M., Welsh, R. M. and Berg, L. J.**, Epigenetic modifications induced by Blimp-1 Regulate CD8(+) T cell memory progression during acute virus infection. *Immunity* 2013. **39**: 661–675.

22 **Obar, J. J., Molloy, M. J., Jellison, E. R., Stoklasek, T. A., Zhang, W., Usherwood, E. J. and Lefrancois, L.**, CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc. Natl. Acad. Sci. USA* 2010. **107**: 193–198.

23 **Bachmann, M. F., Wolint, P., Walton, S., Schwarz, K. and Oxenius, A.**, Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections. *Eur. J. Immunol* 2007. **37**: 1502–1512.

24 **Sandau, M. M., Kohlmeier, J. E., Woodland, D. L. and Jameson, S. C.**, IL-15 regulates both quantitative and qualitative features of the memory CD8 T cell pool. *J. Immunol* 2010. **184**: 35–44.

25 **Boyman, O. and Sprent, J.**, The role of interleukin-2 during homeostasis and activation of the immune system. *Nat. Rev. Immunol* 2012. **12**: 180–190.

26 **Ring, A. M., Lin, J. X., Feng, D., Mitra, S., Rickert, M., Bowman, G. R., Pande, V. S. et al.**, Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat. Immunol* 2012. **13**: 1187–1195.

27 **Butz, E. A. and Bevan, M. J.**, Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* 1998. **8**: 167–175.

28 **Kurtulus, S., Tripathi, P., Moreno-Fernandez, M. E., Sholl, A., Katz, J. D., Grimes, H. L. and Hildeman, D. A.**, Bcl-2 allows effector and memory CD8+ T cells to tolerate higher expression of Bim. *J. Immunol* 2011. **186**: 5729–5737.

29 **Intlekofer, A. M., Takemoto, N., Wherry, E. J., Longworth, S. A., Northrup, J. T., Palanivel, V. R., Mullen, A. C. et al.**, Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* 2005. **6**: 1236–1244.

30 **Crotty, S., Johnston, R. J. and Schoenberger, S. P.**, Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* 2010. **11**: 114–120.

31 **Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K. J., Podack, E. R. et al.**, Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 1994. **369**: 31–37.

32 **Ye, W., Young, J. D. and Liu, C. C.**, Interleukin-15 induces the expression of mRNAs of cytolytic mediators and augments cytotoxic activities in primary murine lymphocytes. *Cell Immunol* 1996. **174**: 54–62.

33 **Jordan, M. B., Hildeman, D., Kappler, J. and Marrack, P.**, An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood* 2004. **104**: 735–743.

34 **Wherry, E. J., Blattman, J. N., Murali-Krishna, K., van der Most, R. and Ahmed, R.**, Viral persistence alters CD8 T-cell immunodominance and

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

tissue distribution and results in distinct stages of functional impairment. *J. Virol.* 2003. **77**: 4911–4927.

35 **van der Windt, G. J., Everts, B., Chang, C. H., Curtis, J. D., Freitas, T. C., Amiel, E., Pearce, E. J. et al.**, Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. *Immunity* 2012. **36**: 68–78.

36 **Hikono, H., Kohlmeier, J. E., Takamura, S., Wittmer, S. T., Roberts, A. D. and Woodland, D. L.**, Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J. Exp. Med.* 2007. **204**: 1625–1636.

37 **Arneja, A., Johnson, H., Gabrovsek, L., Lauffenburger, D. A. and White, F. M.**, Qualitatively different T cell phenotypic responses to IL-2 versus IL-15 are unified by identical dependences on receptor signal strength and duration. *J. Immunol* 2014. **192**: 123–135.

38 **Dubois, S. P., Waldmann, T. A. and Muller, J. R.**, Survival adjustment of mature dendritic cells by IL-15. *Proc. Natl. Acad. Sci. USA* 2005. **102**: 8662–8667.

39 **Ohteki, T., Tada, H., Ishida, K., Sato, T., Maki, C., Yamada, T., Hamuro, J. et al.**, Essential roles of DC-derived IL-15 as a mediator of inflammatory responses in vivo. *J. Exp. Med.* 2006. **203**: 2329–2338.

40 **D'Souza, W. N. and Lefrancois, L.**, IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. *J. Immunol* 2003. **171**: 5727–5735.

41 **Blattman, J. N., Grayson, J. M., Wherry, E. J., Kaech, S. M., Smith, K. A. and Ahmed, R.**, Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo. *Nat. Med.* 2003. **9**: 540–547.

42 **Yajima, T., Nishimura, H., Sad, S., Shen, H., Kuwano, H. and Yoshikai, Y.**, A novel role of IL-15 in early activation of memory CD8+ CTL after reinfection. *J. Immunol* 2005. **174**: 3590–3597.

43 **Yajima, T., Yoshihara, K., Nakazato, K., Kumabe, S., Koyasu, S., Sad, S., Shen, H. et al.**, IL-15 regulates CD8+ T cell contraction during primary infection. *J. Immunol* 2006. **176**: 507–515.

44 **Kaech, S. M. and Ahmed, R.**, Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol* 2001. **2**: 415–422.

45 **van Stipdonk, M. J., Lemmens, E. E. and Schoenberger, S. P.**, Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol* 2001. **2**: 423–429.

46 **Kolumam, G. A., Thomas, S., Thompson, L. J., Sprent, J. and Murali-Krishna, K.**, Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J. Exp. Med.* 2005. **202**: 637–650.

47 **Michelini, R. H., Doedens, A. L., Goldrath, A. W. and Hedrick, S. M.**, Differentiation of CD8 memory T cells depends on Foxo1. *J. Exp. Med.* 2013. **210**: 1189–1200.

48 **Rao, R. R., Li, Q., Gubbels Bupp, M. R. and Shrikant, P. A.**, Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8(+) T cell differentiation. *Immunity* 2012. **36**: 374–387.

49 **Akbar, A. N., Borthwick, N. J., Wickremasinghe, R. G., Panayoitidis, P., Pilling, D., Bofill, M., Krajewski, S. et al.**, Interleukin-2 receptor common gamma-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-xL) but not pro-apoptotic (bax, bcl-xS) gene expression. *Eur. J. Immunol* 1996. **26**: 294–299.

50 **Berard, M., Brandt, K., Bulfone-Paus, S. and Tough, D. F.**, IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. *J. Immunol* 2003. **170**: 5018–5026.

51 **Lai, Y. G., Hou, M. S., Lo, A., Huang, S. T., Huang, Y. W., Yang-Yen, H. F. and Liao, N. S.**, IL-15 modulates the balance between Bcl-2 and Bim via a Jak3/1-PI3K-Akt-ERK pathway to promote CD8alphaalpha+ intestinal intraepithelial lymphocyte survival. *Eur. J. Immunol* 2013. **43**: 2305–2316.

52 **Kim, E. H., Sullivan, J. A., Plisch, E. H., Tejera, M. M., Jatzek, A., Choi, K. Y. and Suresh, M.**, Signal integration by Akt regulates CD8 T cell effector and memory differentiation. *J. Immunol* 2012. **188**: 4305–4314.

53 **Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J. and Surh, C. D.**, Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J. Exp. Med.* 2002. **195**: 1523–1532.

54 **Osborne, L. C., Dhanji, S., Snow, J. W., Priatel, J. J., Ma, M. C., Miners, M. J., Teh, H. S. et al.**, Impaired CD8 T cell memory and CD4 T cell primary responses in IL-7R alpha mutant mice. *J. Exp. Med.* 2007. **204**: 619–631.

55 **Hand, T. W., Morre, M. and Kaech, S. M.**, Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc. Natl. Acad. Sci. USA* 2007. **104**: 11730–11735.

56 **Kim, M. V., Ouyang, W., Liao, W., Zhang, M. Q. and Li, M. O.**, The transcription factor Foxo1 controls central-memory CD8+ T cell responses to infection. *Immunity* 2013. **39**: 286–297.

57 **Kallies, A., Xin, A., Belz, G. T. and Nutt, S. L.**, Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity* 2009. **31**: 283–295.

58 **Ichii, H., Sakamoto, A., Hatano, M., Okada, S., Toyama, H., Taki, S., Arima, M. et al.**, Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells. *Nat Immunol* 2002. **3**: 558–563.

59 **Liao, W., Spolski, R., Li, P., Du, N., West, E. E., Ren, M., Mitra, S. et al.**, Opposing actions of IL-2 and IL-21 on Th9 differentiation correlate with their differential regulation of BCL6 expression. *Proc. Natl. Acad. Sci. USA* 2014. **111**: 3508–3513.

60 **Pircher, H., Burki, K., Lang, R., Hengartner, H. and Zinkernagel, R. M.**, Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature* 1989. **342**: 559–561.

61 **Dutko, F. J. and Oldstone, M. B.**, Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *J. Gen. Virol.* 1983. **64**: 1689–1698.

62 **Battegay, M., Cooper, S., Althage, A., Banziger, J., Hengartner, H. and Zinkernagel, R. M.**, Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. *J. Virol. Methods* 1991. **33**: 191–198.

Abbreviations: **Akt**: AK-transforming/protein kinase B, · **Bcl-2**: B cell lymphoma 2 · **Bcl6**: B cell lymphoma 6 · **Bim**: Bcl-2-interacting mediator of cell death · **Blimp-1**: B lymphocyte-induced maturation protein-1 · **Eomes**: Eomesodermin · **Foxo**: forkhead-box O · γ_c: common gamma chain · **Grz B**: Granzyme B · **$GP_{33\text{-}41}$**: glycoprotein 33-41 · **IL2Rβ**: IL-2 receptor beta chain · **LCMV**: lymphocytic choriomeningitis virus · **MPEC**: memory precursor effector cell · **SLEC**: short-lived effector cell · **STAT5**: Signal transducer and activator of transcription 5 · **T_{CM} cell**: central memory T cell · **T_{EM} cell**: effector memory T cell

Full correspondence: Dr. Hélène Decaluwe, CHU Sainte-Justine, 3175 Cote-Sainte-Catherine, Montreal, Quebec, H3T 1C5, Canada
Fax: +1-514-345-4897
e-mail: helene.decaluwe@umontreal.ca

Received: 11/8/2015
Revised: 11/8/2015
Accepted: 25/9/2015
Accepted article online: 1/10/2015

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim