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Negative Co-signaling in the Expansion and Function of Human Antigen-Specific T-cells for Adoptive Cell Therapy

By

Shirin Lak

Département de Biochimie et Médecine Moléculaire, Faculté de médecine

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En Biochimie

Résumé

Le transfert adoptif de cellules T et le traitement par le blocage des points de contrôle immunologiques ont révolutionné le traitement du cancer. En particulier, l'utilisation de cellules T antigène-spécifiques en thérapie cellulaire adoptive a facilité le développement d'immunothérapies anticancéreuses plus précises et personnalisées. Les résultats de la thérapie cellulaire adoptive (TCA) sont liés à la qualité des cellules T spécifiques expansées *ex vivo*, telles que leur état de différenciation et leur diversité clonale. Cependant, le pré-conditionnement des produits de thérapie cellulaire adoptive et les traitements d'immunothérapie combinatoire sont de nouveaux concepts en développement de la thérapie du cancer pour optimiser l'immunothérapie du cancer dans un plus grand groupe de patients.

Nous avons formulé l'hypothèse que le blocage d'un ou plusieurs récepteurs inhibiteurs au cours de l'expansion *ex vivo* favorise une meilleure expansion et une meilleure fonction des cellules T destinées à la TCA.

Pour étudier l'expansion et la différenciation de cellules T antigène-spécifiques lors d'un blocage des points de contrôle, nous avons adopté une méthode qui nous permet de stimuler et expanser de rares cellules T antigène-spécifiques à partir de cellules mononuclées du sang périphérique (PBMCs) via de multiples stimulations utilisant des cellules dendritiques chargées avec l'antigène d'intérêt. Nous avons utilisé deux approches pour supprimer l'activité des récepteurs régulateurs négatifs, les anticorps bloquants des points de contrôle et la délétion génique via CRISPR/Cas9.

Nous démontrons que le blocage combiné de PD-L1 et TIM3 améliore considérablement la l'expansion de cellules T $CD8^+$ spécifiques à des antigènes viraux et tumoraux. De plus, le blocage des points de contrôle a entraîné la génération de cellules T spécifiques fonctionelles tel que démontré par la production de cytokines et la cytotoxicité in vitro. En utilisant de séquençage de l'ARN en cellule unique (scRNA-seq) et de séquençage des récepteurs des lymphocytes T (TCP-seq), nous avons remarqué que les cellules T spécifiques sont très fortment oligoclonales. Nous avons également identifié quelques clones dominants partagés entre les donneurs. L'application de l'inhibition des points de controles ne confère pas de signatures transcriptionelles particulières mais pourrait affecter certains clones provenant de certains donneurs davantage que d'autres. De plus, le peu de changements dans la composition clonale des cellules expandues suggèrent que le

blocage de ces points de contrôle immunologiques n'altère pas de façon significative le produit cellulaire obtenu.

Des données récentes soutiennent également un rôle du CD5 dans la régulation de l'activation des cellules T naïves et leur état fonctionnel. Cependant, dans un contexte compatible avec la TCA, nos données montrent que la suppression de CD5 via CRISPR/Cas9 n'a que des effets modestes sur la génération de cellules T antigène-spécifiques. Par contre, la combinaison éventuelle avec l'inhibition de d'autres points de contrôle immunologiques pourrait être envisagée.

En conclusion, nos travaux fournissent une nouvelle méthode pour générer des cellules T spécifiques pour la TCA et la caractérisation à plus haute résolution de cellules T spécifiques expansées *ex vivo*. Nous avons donc démontré que le blocage combiné de plusieurs points de contrôle peut optimiser de manière synergique la production de cellules T spécifiques sans compromettre la spécificité de la réponse. Il s'agit là d'une stratégie rapidement applicable pour améliorer le nombre et la qualité des cellules T antigène-spécifiques expansées *ex vivo* pour l'immunothérapie.

Mots clés : Thérapie cellulaire adoptive, Point de contrôle, PD-1, PD-L1, TIM3, CD5, Cellule T spécifique de l'antigène

Abstract

Immunotherapy, especially the adoptive transfer of T cells and immune checkpoint blockade therapy, have revolutionized cancer therapy. In particular, utilizing antigen-specific T cells for adoptive cell therapy has enabled the development of specific and effective strategies. It has paved the way for developing more accurate and personalized cancer immunotherapies. Adoptive cell therapy (ACT) results depend on the characteristics of *ex vivo* expanded T cells, such as their differentiation and clonal diversity. However, e*x vivo* expanded specific T cells often express several inhibitory receptors involved in T-cell exhaustion and markers of terminal effector differentiation. Accordingly, we hypothesized that blocking one or several inhibitory receptors during the *ex vivo* expansion could improve the expansion and differentiation of antigen-specific T cells.

Preconditioning the ACT products and combinatorial immunotherapy approaches are newly developed concepts in cancer therapy to optimize cancer immunotherapy for a larger group of patients. To study the development of antigen-specific T-cells in combination with checkpoint blockade, we have adopted a method that allows the expansion of rare antigen-specific T cell precursors from PBMCs via multiple stimulations, using antigen-pulsed dendritic cells. In the current study, we utilized our protocol to generate and expand antigen-specific $CD8^+$ T cells targeting the oncogenic Epstein-Barr virus (EBV)-LMP2 and a tumor-associated antigen (TAA) from the Wilms Tumor 1 (WT1) protein. We employed two approaches to abolish the negative regulatory receptors, antibody-mediated blockade and deletion via CRISPR/Cas9. We evaluated the impact of checkpoint blockade on antigen-specific T cells development, proliferation, and function. Additionally, TCR clonality and transcriptomic changes were assessed by genomic studies, including single-cell RNA (scRNA) sequencing and T-cell receptor sequencing.

Supporting our hypothesis, we observed that blocking both PD-L1 and TIM3 (not any of them alone) significantly enhanced LMP2 and WT1-specific T cell generation and expansion. Additionally, checkpoint blockade resulted in higher specific T cell function, including cytokine production and *in vitro* targeted cytotoxicity. Using scRNA-seq and TCR sequencing approaches, we first remarked that the specific T cells are highly oligoclonal and identified a few dominant shared clones between donors. Immune checkpoint blockade did not confer consistent transcriptional signatures but may have a clonotype and donor-specific impact on the expression

of activation and exhaustion-related genes. Overall, immune checkpoint blockade did not markedly alter the clonal composition of the T-cell product.

We also evaluated the impact of CD5 deletion in antigen-specific T cell priming and expansion as an inhibitory receptor and a part of the immune response synapse. However, in a human ACT setting, our data show that the CRISPR/Cas9 mediated CD5 deletion only has modest effects on antigen-specific T-cell generation. However, future combinations with the blockade of other immune checkpoint may be warranted.

Conclusion We demonstrated that blocking PD-L1 and TIM3 during the *ex vivo* expansion improves antigen-specific T-cell yield. We show that blocking multiple checkpoints can synergistically optimize specific T-cell production without compromising the response's specificity. It is a rapidly implementable strategy to enhance the number and quality of *ex vivo* expanded antigen-specific T cells for immunotherapy.

Keywords: Adoptive cell therapy, checkpoint, PD-1, PD-L1, TIM3, CD5, Antigen-specific T cell

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List of Abbreviations

A

ACT	Adoptive Cell Therapy
Ads	Human Adenovirus
AICD	Activation Induced Cell Death
ALL	Acute Lymphoblastic Leukemia
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
ATF	Activating Transcription Factor

B

B2M	β2 Microglobulin
Bcl-xL	B-cell lymphoma-extra large
BCR	B Cell Receptor
BTLA	B and T Lymphocyte Attenuator

C

C	Constant
CAR	Chimeric Antigen Receptor
Caspase	Cysteine-aspartic proteases
CBL	Casitas B-lineage Lymphoma
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CDR	Complementarity Determining Region
CLIP	Class II-associated Ii Peptide
CLL	Chemokine (C-C motif) Ligand
Csk	C-Terminal Src Kinase
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T-lymphocytes
CTV	Cell Trace Violet
CTY	Cell Trace Yellow

	CXCR	Chemokine (C-X-C motif) Receptor
D		
	D	Diversity ???
	DAG	Diacylglycerol
	DAMPs	Danger Associated Molecular Patterns
	DC	Dendritic Cell
	DMSO	Dimethylsulfoxide
	DNA-PK	DNA-dependent protein kinase
E		
	EBV	Epstein-Barr virus
	ELIZA	Enzyme-linked immunosorbent assay
	ER	Endoplasmic reticulum
	ERK	Extracellular signal-Regulated Kinase
F		
	FBS	Fetal Bovine Serum
	FDA	Food and Drug Administration
	FOXP3	Forkhead Box P3
G		
	GATA3	GATA binding protein 3
	Gal-9	Galectin-9
	GLUT1	Glucose Transporter 1
	GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
	GrzB	GranzymeB
	GvHD	Graft-versus-host disease
	GvL	Graft-versus-leukemia
H		
	HER2	Human Epidermal Growth Factor Receptor 2
	HLA	Human Leukocyte Antigen
	HMGB1	High Mobility Group Protein B1
	HSCT	Hematopoietic stem cell transplantation
	HVEM	Herpesvirus Entry Mediator

I

ICAM	Intercellular Adhesion Molecule
ICOS	Inducible T-cell COStimulator
ILCs	Innate Lymphocyte Cells
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant Chain
IKβ	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
irAEs	Immune-related adverse events
IP3	Inositol trisphosphate
IP3K	Inositol trisphosphate Kinase
IRAK	IL-1R-associated kinases
IS	Immunological Synapse
ITAM	Immunoreceptor Tyrosine-based Activator Motif
ITSM	Immunoreceptor Tyrosine-based Switch Motif

J,K

J	Joining
JNK	C-Jun N-terminal protein kinas
KLRG1	Killer cell Lectin-like Receptor G1

L

LAG-3	Lymphocyte-Activation Gene 3
LAIR1	Leukocyte-associated immunoglobulin-like receptor 1
LAT	linker for activation of T cells
LCK	Lymphocyte-Specific Protein-Tyrosine Kinase
LFA	Lymphocyte Function-Associated Antigen
LN	Lymph Node
LPS	Lipopolysaccharide
LRSC	Leuko Reduction System Chamber

M

mAB	Monoclonal Antibody
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived Suppressor cells
MHC	Major Histocompatibility Complex
MICA/B	MHC class I chain-related protein A and B
moDC	Monocyte Derived Dendritic Cell
mTOR	Mammalian Target of Rapamycin

N

NFAT	Nuclear factor of activated T-cells
NF-Kb	Nuclear factor kappa B
NK	Natural Killer

P

PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered saline
PD-1	Programmed Cell Death-1
PDK1	Phosphoinositide-Dependent Kinase-1
PD-L1	Programmed Death Ligand-1
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
PI3K	Phosphoinositide-3 kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PK	Protein Kinase
PKCθ	Protein kinase C-θ
PLCγ-1	Phosphoinositide phospholipase Cγ-1
PMA	Phorbol 12-myristate 13-acetate
PRF1	Perforin1
PRMs	Pattern recognition molecules
PRR	Pattern Recognition Receptor
PS	Phosphatidyl Serin

	PTKs	Protein Tyrosine Kinases
	PTPs	Protein Tyrosine Phosphatases
R		
	RAG 1/2	Recombination activating gene ½
	ROS	Reactive Oxygen Species
	RSS	Recombination Signal Sequence
	RT	Room Temperature
S		
	ScFV	Single Chain Variable Fragment
	SC-RNA	Single Cell RNA
	SH2	Src Homology 2
	SNP	Single nucleotide polymorphism
	STAT	Signal transducer and activator of transcription
T		
	TAA	Tumor-Associated Antigen
	TAP	Transporter associated with Antigen Processing
	T-bet	T-box transcription factor TBX21
	Tcm	Central Memort T cell
	TCR	T-cell Receptor
	TdT	Terminal deoxynucleotidyl transferase
	Teff	Effector T cell
	Tem	Effector Memory T cell
	TF	Transcription Factor
	TfH	Follicular T Helper Cells
	TGF-β	Transforming Growth Factor-β
	Th cells	Helper-T Lymphocytes
	TIGIT	T cell immunoreceptor with Ig and ITIM domains
	TILs	Tumor-Infiltrating Lymphocytes
	TIM-3	T-cell Immunoglobulin and Mucin-domain Containing-3
	TLR	Toll-like Receptor
	TME	Tumor Microenvironment

TNF	Tumor Necrosis Factor
TNFRSF1	TNF Receptor Superfamily1
TRAF	TNF-receptor-associated factor
TRAIL	Tumour Necrosis Factor-related Apoptosis-Inducing Ligand
Treg	Regulatory T cell
TSA	Tumor-Specific Antigen
Tscm	Stem-cell Like T cells

V, Z, W

V	Variable
VEGF	Vascular endothelial growth factor
VGF	Vaccinia Growth Factor
ZAP-70	Zeta-chain-associated protein kinase 70
WT1	Wilms' tumor suppressor gene1

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Overview

Adoptive transfer of donor-derived, *ex vivo* expanded, antigen-specific T cells can eradicate virus-infected cells after transplantation of hematopoietic cells (HCT) and have shown great promise in cancer. Several groups have documented tumor eradication or transient immune responses in cancer patients using similar approaches. The various benefits of using antigen-specific T cells for adoptive therapy include the possibility of targeting several antigens, the capacity to rely on native T-cell receptors hence avoiding antigenic redirection through gene engineering, and having multiple clones targeting the same antigen. However, increasing levels of specific signaling to induce expansion and activation of the cells during their manufacturing leads to high and persistent expression of multiple inhibitory receptors associated with T-cell exhaustion. Exhausted T cells and/or terminally differentiated effector T cells have limited *in vivo* expansion and functional capacity. Accordingly, long-lasting and improved immune responses are expected when the transferred cells are in the early stages of differentiation. Several improvements are needed to extend the efficiency of immunotherapy to a more significant number of patients and a more comprehensive range of cancers. We surmise that the use of combined immunotherapy strategies applied to T-cell therapy is a promising approach to overcome common hurdles in adoptive T-cell immunotherapy (ACT).

The present study proposes a novel combinatorial immunotherapy approach to improve current T cell products for adoptive cell therapy. The goal of this study was to develop an improved method for generating ACT products in which the repertoire diversity of the parent cells is maintained, but the function and proliferation potential is improved to facilitate efficient specific immune responses. Here we suggest pretreatment of *ex vivo* expanded specific T cells with checkpoint blockades to limit exhaustion and dysfunctionality of the cells. We considered two approaches to abolish the negative regulatory receptors, checkpoint blockade antibodies, and deletion via CRISPR/Cas9. The advantages of such a protocol are (1) the array of clonal diversity of the parent cell population is captured, (2) limiting the possibility of exhaustion driven by the multiple stimulations or prolonged culture.

The results from this work are presented in four chapters. The first chapter (Introduction) is an overview of the T cell immune responses, the regulatory mechanisms, and various immunotherapy methods. The results from this study are collectively presented and discussed in two original

research papers (second and third chapter). The first manuscript (Chapter two) describes our results of blocking PD-L1 and TIM3 inhibitory receptors during antigen-specific T cell expansion in order to maximize T-cell yield while preserving functionality and specificity despite multiple stimulations. We demonstrate the significantly enhanced generation of functional antigen-specific CD8+ T cells under checkpoint blockade. We also evaluated the impact of blocking PD-L1 and TIM3 on the clonal diversity and transcriptome of the specific T cells via TCR VDJ sequencing and single-cell sequencing alone or together. We assessed the genes that could be altered by checkpoint blockade to limit exhaustion. These included the T cell activation signaling pathway components, proliferation, and specific functions such as cytokine production and cytotoxicity. We concluded that although checkpoint blockade during T cell *ex vivo* expansion can limit T-cell exhaustion, it is not associated with a consistent pattern of gene expression or clonality.
The second manuscript (Chapter three) focuses on the impact of CD5 on specific T cell expansion as an inhibitory receptor and as a member of the TCR signaling synapse. We show that CD5 elimination has a modest positive impact on specific T cell expansion at the cost of inducing more terminal differentiation and exhaustion receptor expression.

Our study describes how different immunotherapies can be combined to achieve the best therapeutic outcome. Immune checkpoint therapy and adoptive cell therapy have both been successful in cancer, but only a small portion of patients benefit from currently available products. Moreover, this study highlights the impact and importance of each inhibitor's expression pattern and physiology on blockade outcome. We assessed the importance of inhibitory receptor hierarchy and their synergistic function by comparing the inhibition of one or multiple inhibitory receptors together and at different contexts. A comprehensive understanding of pathways controlling inhibitory receptors and T cell activation is necessary to facilitate the development of next-generation immunotherapies. Lastly, we provide a novel approach relying on TCR and single-cell sequencing to control and assess the quality and safety of adoptive cell therapy products.

Chapter One

1. Introduction

1.1 Properties and overview of the immune response

1.1.1 General features of the innate and adaptive immunity

The immune system is broadly classified into two major clusters: the innate immune system and the adaptive immune system; each consists of various components/ functions and takes on different tasks, yet they act closely together.

Innate immunity is a collection of defense mechanisms primarily responsible for generating rapid responses to signals from pathogens. It has fundamentally evolved to prevent, detect, and eliminate pathogen invasion within the first few days through stepwise defense strategies and elements. These include (1) physical barriers such as skin and mucus layer that overlays the epithelium in the respiratory, gastrointestinal, and genitourinary tracts, (2) soluble factors such as complement system, and most importantly, (3) phagocytic cells including neutrophils, monocytes, macrophages, and a family of lymphocytes including natural killer (NK) cells, innate lymphocyte cells (ILCs). Innate mechanisms cannot always eliminate the pathogen; however, they have an essential role in initiating and directing the adaptive immune response(1). For instance, the innate immune system's dendritic cells (DCs) provide the signals needed to start adaptive immune responses. The role of DC in connecting innate and adaptive immunity will be discussed later.

The adaptive immune response, also known as acquired or specific immunity, is the body’s defense system tailored to target specific pathogens. In principle, the adaptive immune response can respond to a vast array of antigens, including proteins, glycoproteins, and polysaccharides of pathogens, using variable but highly specific receptors.

Specificity, immunological memory, and self/non-self recognition are the defining characteristics of immune responses. In healthy, immune-competent individuals, acquired immunity won’t respond to self-components (self/nonself recognition), and it generates a lasting protective effect that increases in magnitude and defensive capabilities with each successive exposure to a particular pathogen (immunological memory).

Off note, recent studies suggest that innate immunity can also provide some memory function but differs from adaptive memory in major aspects. Live vaccine studies in mouse models revealed that monocytes, macrophages, and NK cells could reject the graft weeks after through memory-like characteristics. This likely occurs through epigenetic changes, but the development of innate cell memory leading to secondary responses remains incompletely understood (2).

There are two types of adaptive immune responses: cellular or cell-mediated immunity and humoral or antibody-mediated immunity. These are carried out by different cell classes, express distinct antigen receptors, and have quite different roles in the immune system.

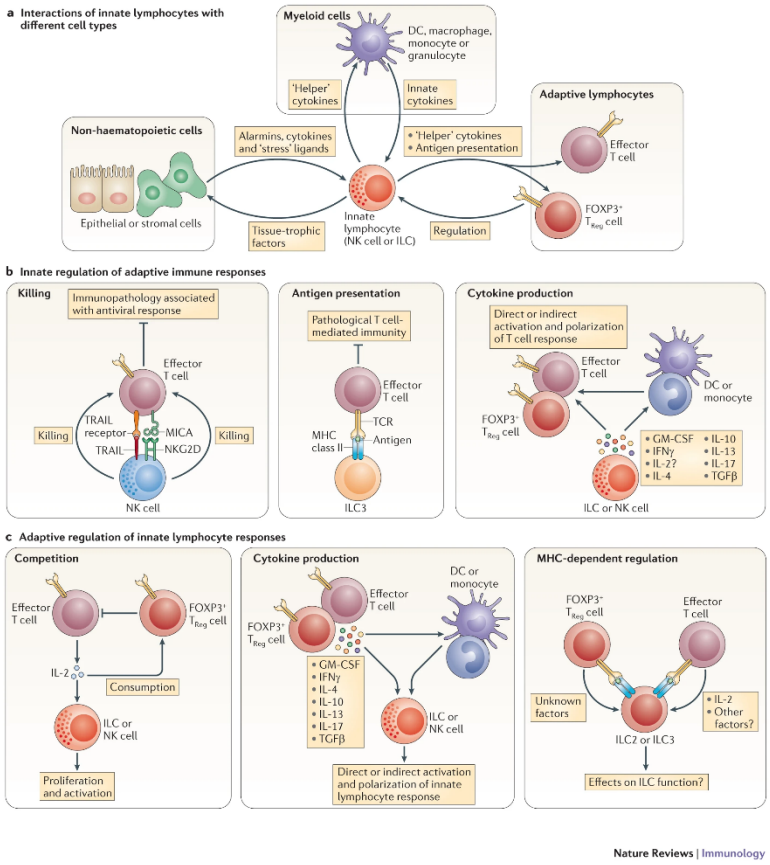

Figure 1 Different types of Immune response. There are two types of immune system responses: Innate immunity and adaptive immunity. The innate immune response is consists of physical, chemical, and cellular defense mechanisms, and it is in tight connection with adaptive immunity. Adaptive immune responses are mediated by various types of T cells and B cells.(Reproduced with permission from: Nature Reviews(3))

1.1.2 The principal cells of the adaptive immune system

1.1.2.1 Lymphocytes:

Lymphocytes and their products are the main elements of adaptive immunity. Adaptive immune response mainly relies on two types of lymphocytes: T cells and B cells. Both originate from bone marrow sited progenitors but mature in different organs and then reside in various locations. Lymphocytes bear special receptors, each with unique specificity for a different single antigen. Considering millions of lymphocytes carrying different receptors collectively, a person possesses a vast repertoire against various pathogens. Following the antigen recognition, B cells and T cells are activated, divided, and generate multiple lymphocytes with the same specificity known as a "clone."(1)

1.1.2.1.1 B cells

B cells provide humoral immunity, and their effector response is delivered through antibody secretion. They originate from hematopoietic stem cells, and their development and maturation start and continue in the bone marrow. B cells use immunoglobulin in a membrane-bound form as the antigen-recognizing receptor known as B cell receptor (BCR). B cell receptor (BCR) reacts to a broad range of epitopes and binds to the intact surface of free extracellular pathogens or their products. BCR structure is constructed in the same way as corresponding antibodies, a pair of heavy (H) and light (L) polypeptide chains with two identical antigen-binding sites, except for a small portion of the carboxy terminus of the heavy chain that anchors BCR to the cell membrane. Each polypeptide chain consists of a constant (C- carboxy terminus) and variable (V- N terminus) region making the antibody binding site(1).

By antigen encounter, B cells are activated. Following receiving some help from T cells, B cells undergo differentiation and several rounds of proliferation, resulting in a heterogeneous population, including antibody-secreting plasma cells and memory B cells. B cell function has been reviewed by other groups(4) and will not be discussed further in this thesis.

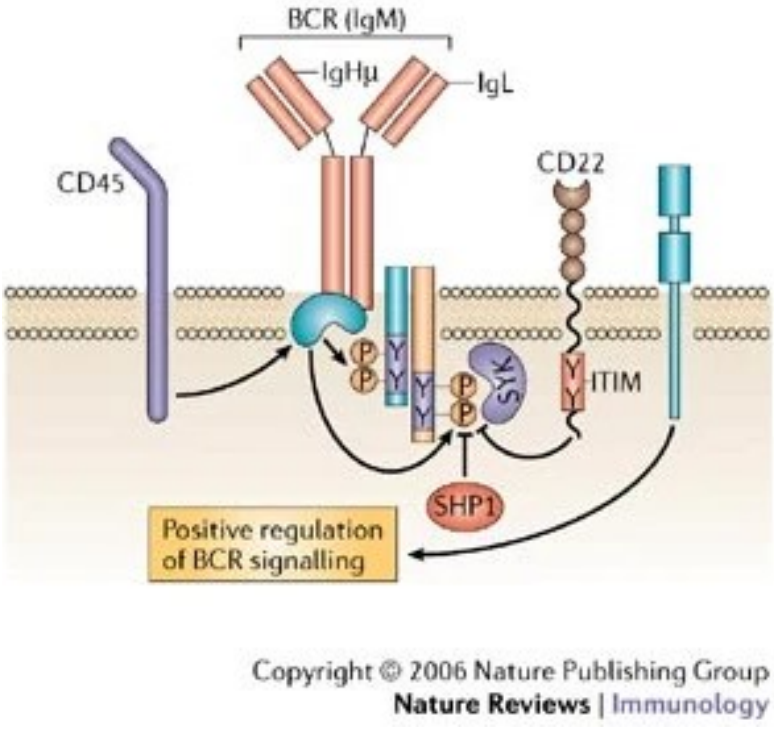

Figure 2 B cell receptor (BCR) Structure, BCR general structure includes four polypeptide chains, two heavy chains and two light chains. BCR has two distinctive antigen binding sites recognizing the surface of the epitope. (Reproduced with permission from: Nature Reviews(5))

1.1.2.1.2 T cells

T cells play a central role in the cell-mediated immune response. They respond to intracellular pathogens such as viruses and intracellular bacteria or their products that have been internalized by endocytosis/phagocytosis. T lymphocytes originate from hematopoietic stem cells in bone marrow, but then T cell precursors migrate to the thymus to undergo their maturation process. Various thymic microenvironment cues direct T cell maturation and differentiation. Through a series of maturation steps, including positive and negative selection, T cells develop their antigen recognition receptor known as T cell receptor (TCR) and other T cell-specific cell markers, including CD4 and CD8. T cell receptor (TCR) is a heterodimer of two different polypeptide chains. TCRα and TCRβ chains construct the major TCR population, but a minority of T cells bear an alternative (TCRγ and TCRδ chains) but structurally similar receptor. TCR has only one antigen-binding site, responding to a short amino acid sequence presented in the form of antigen-major histocompatibility (MHC) complex(1). TCR structure and signaling will be discussed in detail later.

During the positive selection, immature T cells that can successfully bind to the presented self-antigen receive the survival signals and migrate from the thymus cortex to the medulla. In the medulla, strongly self-reactive T cells will be eliminated in the negative selection or become regulatory T cells. Collectively, T cell maturation results in the development of mature T cells capable of antigen recognition with appropriate affinity, proliferation potential, and survival in the periphery.

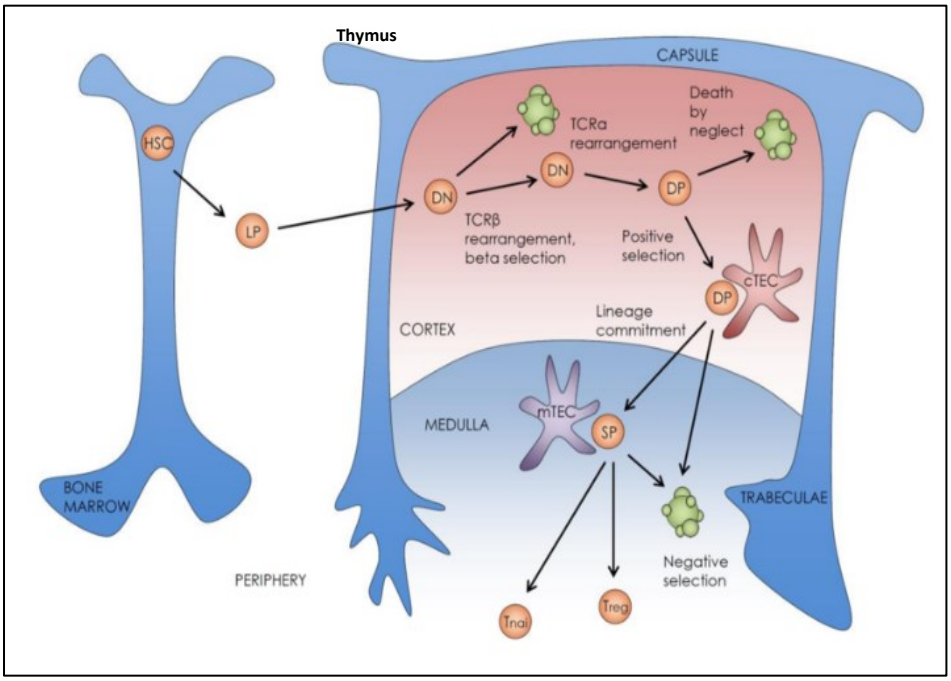

Figure 3 T cell maturation. Progenitors migrate to thymus and go through the maturation and selection process in different parts of thymus (figure adapted from thesis by Dr. Aleksey V. Belikov)

T cells are a heterogeneous population of cells with highly diverse functions, and each type performs a distinct role during an immune response. T cells could be mainly divided into two types based on expressing CD4 or CD8 cell surface markers, including CD4+ helper T cells (Th) and CD8 + cytotoxic T cells (CTL). CD4 and CD8 identifying markers are essential in determining T cell interaction with other cells and antigen recognition.

There is also a third type of T cells called regulatory T cells (Tregs), which are a CD4+ T cell subpopulation with entirely different functions. Tregs suppress or prevent the immune response

when needed by various mechanisms such as suppressive cytokines production or cell-cell interactions.

Unlike B cells, T cells cannot encounter free antigens and only react to processed protein antigens presented by Major histocompatibility antigen (MHC). Antigen presentation is an essential step in T cell response, by which protein antigens are detected, engulfed, processed, and presented to T lymphocytes by antigen-presenting cells (APCs). T cells that recognize and bind successfully to the antigen are activated and differentiated into different subtypes of effector and memory cells. T cell activation, subpopulations, and their functions will be discussed in the following pages.

1.1.2.2 Antigen-presenting cells

Antigen-presenting cells (APCs) are the cells with the machinery to process and present antigens to T lymphocytes. Dendritic cells, Macrophages, and B cells are all professional APCs and can activate T cells. Three signals have been defined for naïve T cells activation. The primary signal is successful antigen recognition through TCR and could be provided by all APCs. The second signal, known as positive co-signal or co-stimulatory signal, is especially needed to activate naïve T cells and ensures optimal and complete activation. The co-stimulatory signals are provided by mature DCs, B cells, and macrophages. However, since mature DCs can provide both first and second signals to initiate an immune response, they are considered professional APCs(1). Antigen presentation and the process to start an immune response will be discussed in detail in the following. Cytokines produced by APCs and helper T cells are the third signal needed for T cell activation and differentiation.

As mentioned, adaptive immune responses are highly specific, and lymphocytes respond to each pathogen differently. One purpose is that antigens from different types of pathogens are presented by different classes of the major histocompatibility complex (MHC) molecules and cells. MHCs in animals and HLAs in humans are essential cell surface molecules in antigen presentation and self, non-self-recognition process. There are two classes of MHC molecules (MHC class I and MHC class II) and six HLA molecules. Human MHC I comprises HLA-A, HLA-B, HLA-C molecules, and MHC II molecules are HLA-DR, HLA-DQ, and HLA-DP. MHC molecules are transmembrane proteins consisting of heterodimer polypeptide chains. MHC I molecule contains a heavy chain, α-chain, and a light chain, β2 macroglobulin, but the peptide-binding groove is only constructed by α-chain. β2 macroglobulin (β2M) is to stabilize the MHC complex on the cell surface. MHC II molecules are also composed of an α-chain and β-chain but form the peptide-binding groove together(6).

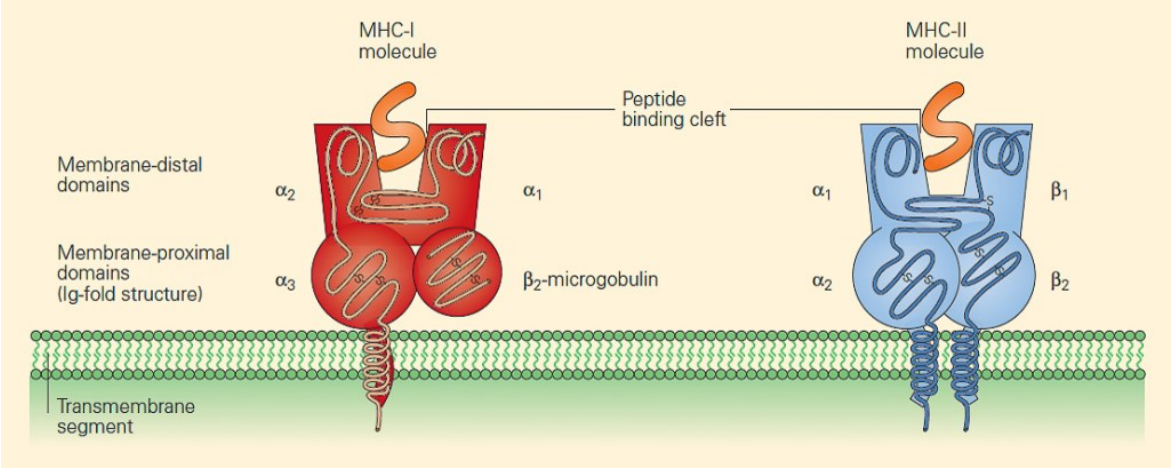

Figure 4 Major histocompatibility (MHC) complex structure, Two types of MHC molecules present antigens to T cells. Each MHC molecule has a peptide binding groove to present antigen to T cells (from open-source Dr. Bellanti's Immunology I, II, and III texts published in 1971, 1978, and 1985.)

APCs can express both MHC classes on their surface. They use MHC class I to present peptide fragments of intracellular pathogens to CD8+ T cells, and MHC class II presents extracellular antigens to CD4+ T cells. There are some exceptions to this canonical rule, as "cross-presentation" mostly refers to the presentation of extracellular antigens through MHC I by DCs, and is mainly for the maintenance of peripheral tolerance(7).

CD4 and CD8 bind to the MHC II and MHC I molecule and are a part of the TCR complex, but as TCR binds to the antigen-presenting groove, CD4 and CD8 bind to the invariant constant region of MHC molecules and help to stabilize the MHC-TCR interaction(6).

1.2 T cell subset differentiation and their related immune response

TCR signaling plays a significant role in T cell lineage specifications and the development of various subsets. CD4 and CD8 T cells are divided into multiple subsets with diverse functions. T cell subsets reside in different tissue/organs to ensure maximum coverage and adjust the immune response against various pathogens. Notably, studies on T cell subsets and plasticity have shed light on new perspectives for translational medicine and immune-modulating therapies for multiple human diseases and cancers.

1.2.1 Helper T cells (Th)

CD4+T helper cells are critical for the immune system and have a central role in directing immune responses and inducing other immune cells. In addition, T helper cells maintain self-tolerance as Tregs and help cytotoxic T cell activation and B cells in the activation process. CD4+ T cells mostly display helper activity on other immune cells by secreting a vast array of cytokines and affecting the different cells. They can recruit natural killer cells, instruct phagocytes to destroy infected cells, and further activate CTLs by secreting IFN-γ. They also can help B cell maturation and CTL and macrophage activation through IL-4 secretion and promote T cell expansion and proliferation by IL-2. Of note, there are some reports of cytotoxic CD4+T cells in antiviral immunity(1, 8).

Initially, CD4+ T helper cells were categorized into only two major subsets, Th1, and Th2 T cells, in charge of intracellular and extracellular pathogens. Further studies revealed the plasticity of CD4 T cells and a wide range of heterogeneity. Since then, five major subtypes of CD4 T cells, including conventional Th1 and Th2, inflammatory Th17, Tregs, and follicular helper T cells (Tfh), each with multiple minor subtypes, have been defined(9).

Naïve T cells have stem cell-like characteristics and can go through different differentiation paths based on their microenvironment signals. Upon priming in the presence of lineage-specifying

cytokines and transcriptional programs, subset characteristics are epigenetically imprinted and preserved upon cellular division. However, T cell subsets are not terminally differentiated, and recent studies show memory helper cells can acquire different characteristics by re-stimulation(10).

IFN-γ and IL-4 induce Th1/Th2 specific characteristics and inhibit the opposite lineage differentiation. IFN-γ and T-bet master transcription factors induce Th1 cell development in response to intracellular pathogens. The hallmark cytokine inducing Th2 differentiation is IL-4, and the master regulator is GATA3, secrets IL-4, IL-5, IL-10, and IL-13, but not IFN-γ (11).

TGF-β plays an essential role in both Th17 and Treg differentiation. In Th17 development, the combination of TGF- and IL-6 signals activate SATA3, which upregulates lineage-defining RORγt transcription factor. Th17 cells produce IL-22 and IL-17 effector cytokines in response to extracellular pathogens and fungi. On the other hand, in Tregs, TGF alone stimulates Foxp3 transcription, which has a crucial role in Treg generation(9).

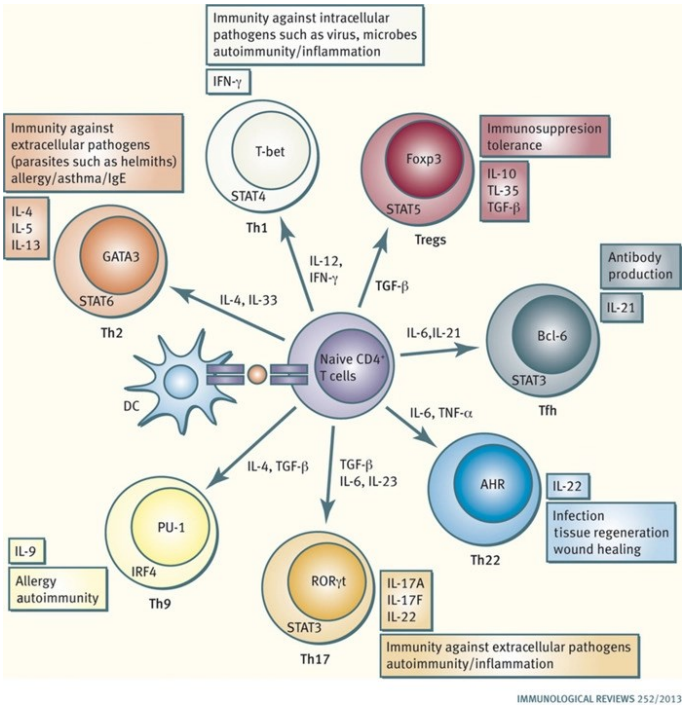

Figure 5 Helper CD4+ T cell subsets Cytokine milieu and various gene expressions drive the lineage specification of T cells (Reproduced with permission from: Wiley(12))

1.2.2 Regulatory T Cells (Tregs)

Regulatory T cells (Tregs) are T cells that regulate or suppress other cells in the immune system. They are recognized by expressing CD4 and CD25 cell surface markers and FOXP3 transcription factor. Tregs control the immune response to self and foreign antigens and help prevent autoimmune diseases. They help maintain homeostasis through different means, such as secreting suppressive IL-10 and TGF-β cytokines(8).

1.2.3 Cytotoxic T cells (CTL)

Effector CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs), execute two main functions: (1) direct killing of infected cells (through granzymes and perforin) and (2) secretion of inflammatory cytokines such as interferon-γ (IFNγ) and tumor necrosis factor (TNF)(1). There are three phases in CD8 T cell response to infection or tumor cells: 1-the clonal expansion, 2-homeostasis following the virus clearance or tumor elimination, and 3-long-lived memory T-cell generation.

Following the activation, cytotoxic T lymphocytes mainly use effector molecules like perforin (PRF1) and granule-associated enzymes (granzymes; GZM) to abolish their target. The granule exocytosis pathway releases effector molecules. Perforin, granzyme, and PRF1 containing cytolytic granules are mobilized toward the immunological synapse (IS) to release their content. Once released, perforins will form pores on the membrane of target cells and allow the GZMs to enter and kill the target cells by cleaving critical intracellular substrates controlling cell survival(1).

Effector T cells can also induce apoptosis in tumor cells through the death ligand/death receptor (FasL/Fas) system. CTLs express TNF-α receptors, Fas ligand (FasL), and TRAIL death ligands that bind the TNF members superfamily and trigger target cell death. Death ligands also help in controlling T cell homeostasis through activation-induced cell death (AICD) (1).

1.2.4 Effector CD8 T cell VS. memory T cells

Memory formation is the hallmark of the adaptive immune response. After pathogen clearance, most of the effector T cells die via apoptosis (the contraction phase), except about 5-10% of cells

that survive and mature into memory CD8+ T cells(13). The long-lived memory T cells can survive in an antigen-independent/ cytokine-dependent manner and quickly generate effector functions upon secondary stimulation. They share some characteristics with naïve and effector T cells, including the self-regenerating potential and producing effector molecules. However, they can persist and circulate in the periphery for a lifetime and respond to lower antigens concentrations compared to naïve T cells(14). Memory T cells consist of a heterogeneous pool of cells with varying functions and survival potential, including stem cell memory T (T_{scm}), central memory (Tcm), effector memory (Tem), and tissue-resident memory T cells (T_{TRM}). These subpopulations can be distinguished by certain cell surface markers, proliferation and cytotoxicity potential, and anatomic localization(15). Of note, characterizing and defining memory cell subsets based on only their cell surface markers could be misleading. There are essential characteristics that accurately distinguish T cell populations, such as trafficking, localization, effector function, and durability.

Central memory (T_{cm}) cells are characterized by the $CD45RO^{+}CCR7^{+}CD27^{+}CD28^{+}CD62L^{hi+}$ phenotype, show high proliferative potential, and reside in secondary lymphoid organs through expressing various homing and chemokine receptors but have limited immediate cytotoxic response. Central memory T cells express superior antigen recognition potential thus mainly resides and circulates through the sites with a higher chance of antigen encounters like LNs, spleen, and peripheral blood. Compared to T_{cm} cells, effector memory (Tem) cells express low levels of CD62L, CCR7 and comparably receive fewer survival signals but are highly specialized in effector functions, including cytotoxicity and cytokine production. In order to handle the infections efficiently and quickly, T_{em} cells localize predominantly in peripheral nonlymphoid tissues like the lung, liver, and intestine(15, 16).

Further lineage studies revealed two distinct memory cell subsets, including $T_{scm,}$ a naïve-like subpopulation, and T_{TRM}, the non-circulating memory cells in peripheral tissue. T_{scm} is recognized by increased proliferation potential and levels of CD95, IL-2Rβ, CXCR3, LFA-1 compared to naïve T cells ($CD45RA^{+}CD45RO^{-}CCR7^{+}CD62L^{+}CD27^{+}CD28^{+}IL\text{-}7R\alpha^{+}$) and are believed to differentiate into other memory subtypes and eventually effector cells(15).

The memory generation process is not random, and not all the effector T cells qualify to progress into long-lived memory cells(17). Understanding the developmental fate of memory T cells and

defining the lineage relationships between naive T cells, effector T cells, and memory T cells seem essential for the rational design of vaccines and immunotherapy strategies. Additionally, the high number of memory T cells in the tumor microenvironment significantly correlates with tumor elimination and positive clinical outcome. Stem cell and central memory T cells have shown superior functional efficiency in preclinical and clinical immunotherapy studies. Furthermore, they produce higher cytokines and effector molecules *in vitro* and show significantly more tumor eliminating function *in vivo* than effector memory T cells. Thus, a thorough understanding of their differentiation mechanisms and determining which effector cell dies and which qualifies as a memory precursor will allow the development of more effective immunotherapies(14, 18).

Three models of memory T cell formation have been described, including (1) linear model, (2) circular model, and (3) self-renewing effector model(19). In the linear model, naïve T cells go through differentiation progressively (Naïve→T_{eff}→T_{scm}→T_{cm}→T_{em}→T_{eff}), which is led by the strength and duration of TCR stimulation. Persistent and intense antigen stimulation triggers the acquisition of effector characteristics and terminal effector differentiation(T_{eff}). Terminally differentiated cells are believed to be end-stage and proceed to apoptosis during the homeostasis phase. Based on this model, memory T cells contain gene expression profiles and phenotype similarities with both effector and naïve T cells. They gradually lose memory-associated gene expression and gain effector-associated gene expression.

The linear differentiation model was accepted as the only memory generation explanation until memory cells with fewer rounds of cell division were discovered. Based on the circular differentiation model, effector T cells may proceed by divergent differentiation fates, either undergo apoptosis or differentiate into memory T cells. This results from a circular pattern of transcriptional and epigenetic changes over time(on-off–on or off–on-off), and memory cells are developed during the contraction phase (Naïve→T_{eff}→T_{scm}→T_{cm}→T_{em}→T_{eff}).

Some naïve cells become committed to memory fate precursors in the self-renewing effector model, while others differentiate into effector T cells during the clonal expansion(20, 21).

Notably, the transition to memory T cells requires proper priming and activation signals in all these models. In addition, weak or persistent strong antigen stimulation could lead to T cell anergy or terminal differentiation, respectively, discussed in detail in the following sections.

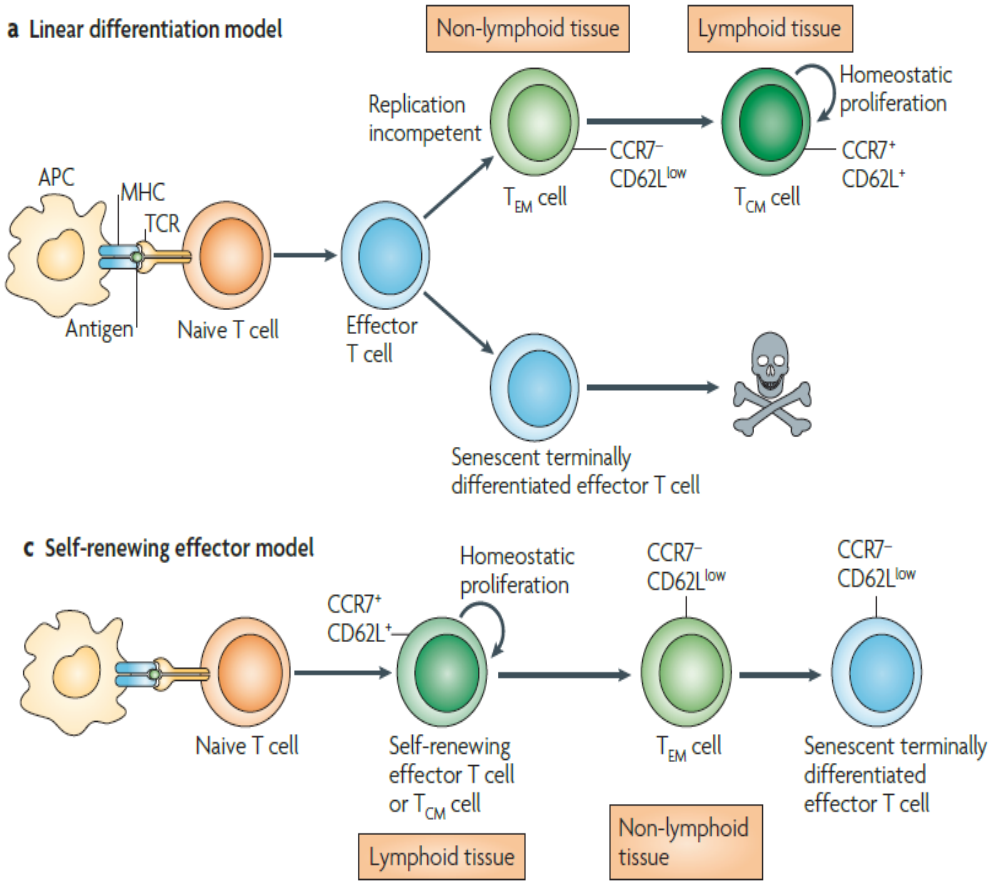

Figure 6 memory T cells, their precursors and cell surface markers (Reproduced with permission from: Nature Reviews(18))

1.3 T cell receptor structure and characteristics

Mature T cells recirculate between the blood and peripheral lymphoid tissues until they encounter and successfully identify their specific antigen. T cells that have not yet encountered the antigen are known as **naïve T cells** and, in order to participate in adoptive immune response, need to be primed. T cell functional development from a naïve/resting state to fully activated, proliferating cells is a critical step in the adaptive immune response known as "priming"(1, 22).

Numerous receptors and signaling molecules are involved in naïve T cell priming, including the T cell receptor (TCR): CD3 complex, co-stimulatory receptors, and adaptor proteins. The molecular interactions underlying TCR signaling require a stable T cell: APC junction termed immunological synapse (IS). IS is a multimolecular organization that integrates three different classes of receptors: (1) adhesion molecules, (2) MHC-TCR, and (3) co-stimulatory/inhibitory receptors. IS formation will stabilize the interactions and enhance the interaction interval, ensuring the T cell's full activation despite the low affinity of TCR or low antigen concentration. Moreover, the receptors and co-receptors involved in IS are tied to a diverse network of signal transduction pathways directing the transcriptional and metabolic programs that determine lymphocyte function(23, 24).

Adhesion molecules are needed to pull cells together and generate an adhesion domain to form the IS. For instance, LFA-1 is the major integrin on T cells meditating adhesion by binding to ICAMs on APCs and other cells. Besides mediating the connection, adhesion molecules increase the sensitivity to pMHC, activate signal transduction to Ca^{2+} channels, and activate downstream signaling pathways such as RAS and MAPK(24). Adhesion molecules play a pivotal role in regulating all aspects of the immune response, but here, we will only discuss the TCR complex and co-signaling; how they are structured and united into an effective immune response.

T cell receptor (TCR) is a glycosylated heterodimer expressed at the surface of T cells. The majority of TCRs are composed of the αβ polypeptide chain, with extracellular variable (V) and constant domain (C), a transmembrane region, and a short cytoplasmic tail. The gene locus of α and β chain contains variable (V), joining (J), and constant (C) gene segments, but the TCRβ locus contains a diversity (D) gene segment in addition to V and J segments.

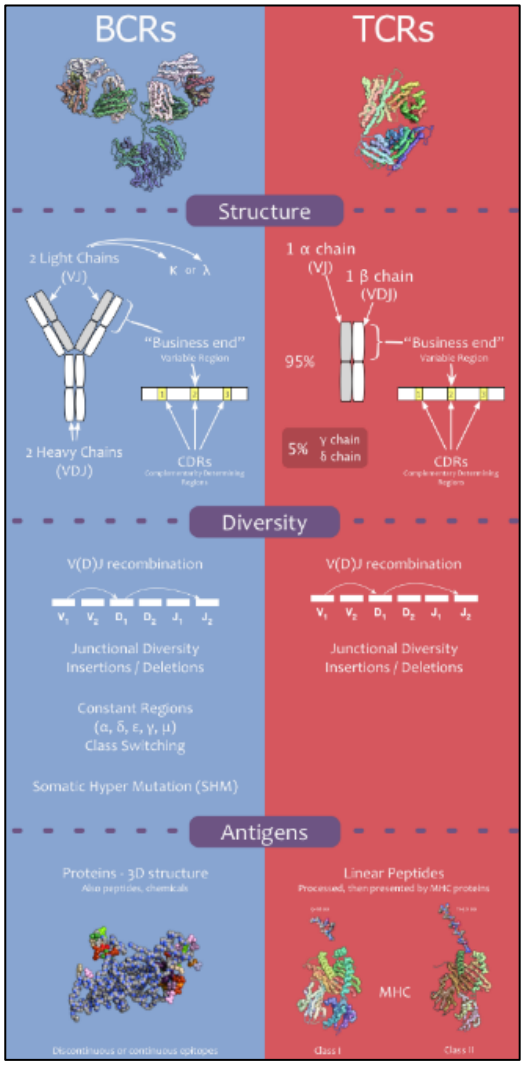

Figure 7 TCR and BCR structure similarities and differences Both TCR genes and general structures have significant similarities to immunoglobulin. They both are multichain complexes (as shown) however, recognize different type of antigens. (From open-source: Digital World Biology)

The TCR gene segment goes through a lymphocyte-specific recombination process, called V(D)J recombination, during the early stages of development in the thymus to assemble a final TCR sequence from many potential pieces. Several enzymes direct the V(D)J recombination, most importantly lymphoid-specific recombinase (RAG1/2) and ubiquitously expressed DNA repair proteins(1, 25). RAG1/RAG2 are restriction endonuclease, creating a double-strand break in DNA at recombination signal sequence (RSS). RSS serves as a recognition site for recombination

machinery and flanks all TCR gene segments. The double-strand break results in forming a closed hairpin recognized by Artemis, an endonuclease that opens the hairpins at the coding ends. The broken ends are brought together and repaired by a DNA repair process referred to as the "nonhomologous end-joining." Multiple enzymes are involved in this process, including DNA-PK, lymphoid-specific TdT, and ubiquitous factors(25, 26). Notably, terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase that adds nontemplate nucleotides to the ends enabling the TCR diversity. TCR diversity is shaped by multiple possible combinations of V, J, and D gene segments and, most importantly, junctional diversity. Junctional diversity is defined as the "removal or addition of nucleotides at the junctions of the V and D, D and J, or V and J segments at the time these segments are joined."(1)

The "antigen-binding site" of the TCR complex is formed by six highly variable regions known as complementarities determining regions (CDRs) loops, three from the Vα domain and three from the Vβ domain. TCR βchain CDR3 is the most variable loop and in direct contact with the peptide-MHC complex. It is formed by D and J gene segments containing junctional segment and non-templated nucleotides (P- and N-nucleotides), significantly determining the TCR specificity(25-27).

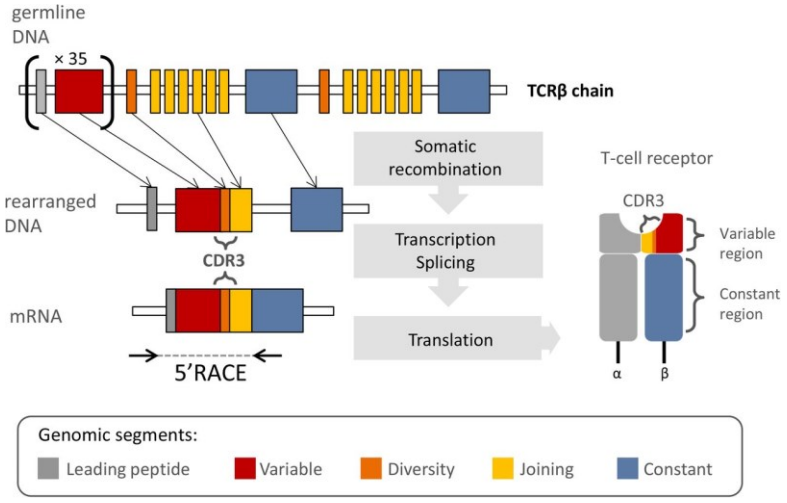

Figure 8 Schematic representation of the TCRβ chain rearrangement. Sevaral available combinations of VDJ segments facilitate the TCR diversity. Additionally the non template adding of nucleotides at the junctions creating CDRs adds on more diversity to the antige binding site. (Reproduce with permission from: Scientific reports(28))

TCRs do not go through affinity maturation(29); however, they recognize and respond to antigens with great sensitivity. Three major TCR characteristics determine the sensitivity and successful antigen recognition, including (1) TCR affinity, (2) TCR avidity, and (3) functional avidity. TCR affinity is a factor in assessing TCR sensitivity to a specific antigen and is defined by measuring the strength of interaction between a single TCR and pMHC. Simultaneously, avidity is associated with the strength of multiple TCR-pMHC interactions, and other molecules like co-signaling receptors can affect it. Functional avidity is the outcome of TCR-pMHC strength, antigen concentration, and co-signaling molecules' expression levels. The binding affinity of a TCR for its target antigen largely determines the avidity of the T cell carrying that TCR (30, 31).

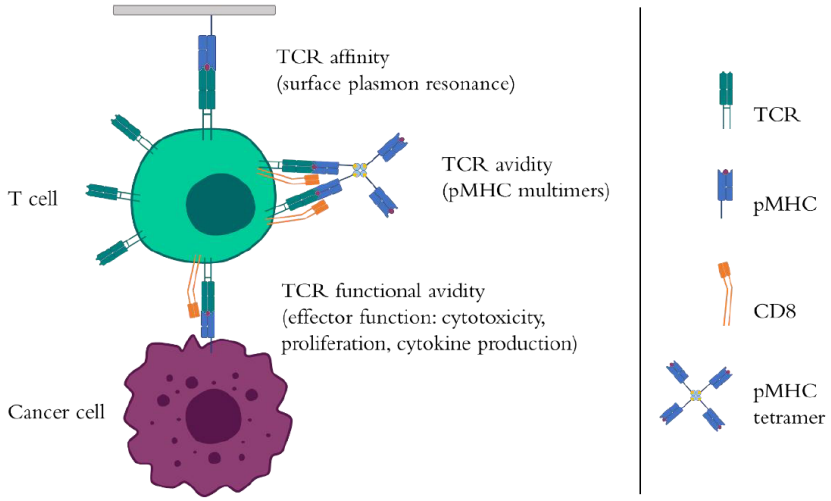

Figure 9 Antigen recognition and TCR affinity, TCR with the same specificity recognize and target antigens with different strength. TCR affinity, avidity and functional affinity determine the strength of TCR: peptide connection. (From open source: MDPI (31))

1.4 T cell activation

Naïve T cells need at least two signals provided by TCR and co-stimulatory receptors to be fully activated. The TCR stimulation determines the specificity of the T cell response, and the co-stimulatory signals amplify TCR downstream signaling and ensure T cell activation. Later studies also revealed a third signal provided by T cells and APCs secreted cytokines. Cytokines regulate the immune response, T cell differentiation, and activate other immune cells. They are essential for antigen-activated T cells to avoid apoptosis and tolerance(32).

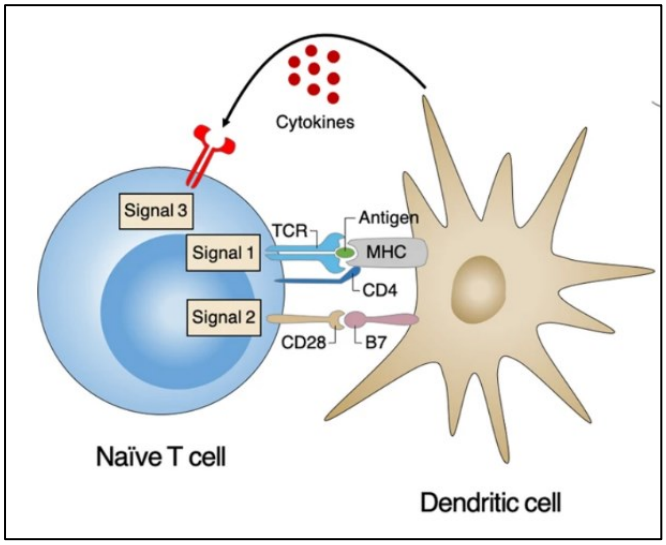

Figure 10 T cells need three signals to be fully activated 1-antigen recognition via TCR, 2- Co stimulatory signals to intensify activation signals, 3- cytokines for proliferation and clonal expansion (Reproduced from: Experimental and Molecular Medicine- Open source (33))

1.4.1 First signal: Antigen recognition and TCR signaling

The primary mediator of T cell activation is antigen recognition. Antigen-presenting cells such as dendritic cells constantly scan the peripheral tissue and present a wide range of antigens using multiple cell surface receptors. DCs are activated upon antigen encounter and recognition and migrate to T cell zones in secondary immune organs to present the processed antigen to T cells and activate them(34). Before the antigen encounter, DCs are in an immature state and express

low MHC molecules and co-signaling receptors. As a result, they are inefficient in antigen presentation. However, due to their high endocytosis potential and high expression of antigen recognition receptors, immature DCs are incredibly efficient in antigen recognition and capture.

During an infection, immature DCs recognize the pathogen-associated-molecular pattern (PAMPs), small molecules conserved in pathogens, through their pattern-recognition receptors (PRRs) such as toll-like receptors (TLR). Several PAMPs have been discovered so far, such as bacterial lipopolysaccharide (LPS) found on the outer cell wall of gram-negative bacteria. These immunogenic molecules transfer the "danger signal" to the immature DCs and turn on metabolic, cellular, and gene transcription programs leading to DC maturation. Mature DCs upregulate MHC molecules and several co-stimulatory receptors, including B7 molecules, and efficiently process and present antigens(34, 35).

Extracellular antigens are first internalized and then degraded into small fragments by different proteolytic enzymes stored in endolysosomes such as glycosidases, proteases, and sulfatases. Through the degradation, antigens will be processed into short peptide fragments that fit into the MHC molecule peptide groove. To form the final peptide-MHC complex, newly synthesized MHC II molecules in ER are transported to endo/lysosomal compartments. It is essential to ensure the precise peptide loading to the MHC molecule groove and proper folding. As a result, the MHC molecule protein structure is first stabilized using an invariant chain (Ii) that is later degraded and replaced by class II-associated invariant chain peptide (CLIP). Subsequently, CLIP is swapped with pathogen-derived peptide fragments via the chaperon HLA-DM. The peptide-bound MHC II molecules are transferred to the cell surface and stay till recognized by specific TCR.

Intracellular pathogen-derived antigens such as virus products undergo the same degradation process, and proteasome breaks them into 8-15 amino acid-length peptide fragments. The fragments are transported to the endoplasmic reticulum (ER), where the two polypeptide chains of the MHC I molecule are synthesized and kept. TAP, the transporter associated with the antigen processing system, is consists of two subunits TAP1 and TAP2 and loads the antigen fragments onto the peptide-binding groove of the MHC I molecule in ER. The peptide-MHC I molecule complexes are transferred to the cell surface via secretory vesicles after the peptide loading(1, 35).

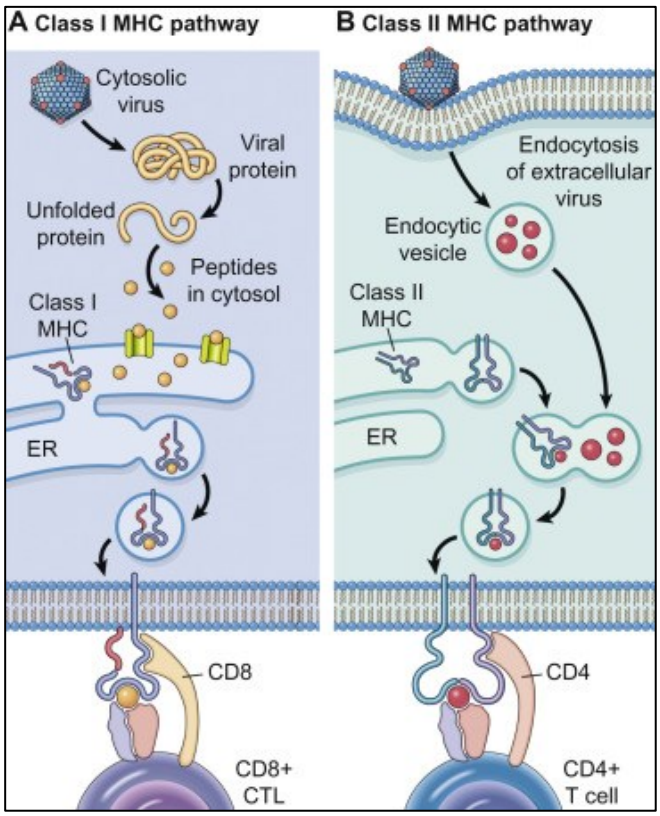

Figure 11 Intracellular and extracellular antigen presentation process In order to differentiate the antigens DCs process them through different methods and present them by different classes of MHC molecules as shown
(Reproduced with permission from Nature Reviews(36))

Of note, antigen presentation and recognition are determining factors in anti-tumor immune responses. In cancer, tumor cells present the tumor antigens by the MHC molecules, which are either directly recognized by CD8+T cells or are taken up by DCs and cross presented to prime CD8+ T cells(37). Different types of tumor antigens have been discovered so far, including (1) tumor-specific antigens (TSAs), antigens expressed exclusively by tumor cells, and (2) tumor-associated antigens (TAAs)- antigens with a distinct expression on tumor cells which may also be expressed by normal cells. Neoantigens are TSA resulting from point mutations and other mechanisms such as gene fusions and play an important role in cancer immunotherapy(38).

In order to fully engage with antigen and stabilize the connection, TCR binds to both antigen peptide and the MHC molecule. T cells can only bind and recognize antigens presented by self MHC molecules, known as the "MHC restriction" rule. T cells get committed to MHC restriction during their positive selection process in the thymus. It ensures the specificity of T cell response to foreign antigens presented by self-cells(2).

Bound to the pMHC complex, TCR initiates an intracellular signaling cascade of phosphorylation that results in T cell activation, proliferation, and differentiation. TCR has a short intracellular domain with no signaling motifs, thereby serves as antigen recognition and the specificity of the response. The TCR transfers the activation signal through the invariant CD3 complex. CD3 is a protein complex involved in T cell activation and is composed of four different (γ, δ, ε, and ζ) subunits. The CD3 subunits form dimers (CD3εγ, CD3εδ, and CD3ζζ) each subunit comprises a single extracellular Ig-like domain and a long cytoplasmic tail. All CD3 subunits have signal transduction motifs named immune receptor tyrosine-based activation motifs (ITAMs) required for intracellular signaling(39, 40). CD4 and CD8 are other members of the TCR complex, and their intracellular domains are associated with Lck protein tyrosine kinase. CD4 and CD8 are essential for initiating and facilitating TCR signaling.

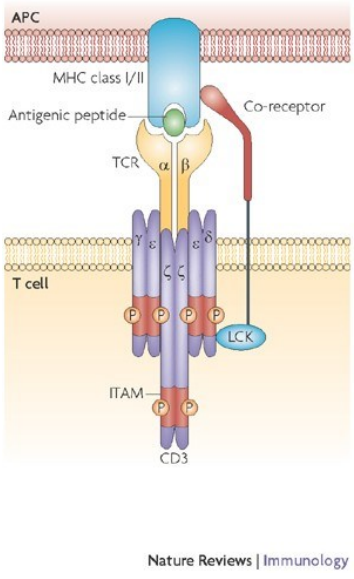

Figure 12 TCR:CD3 complex expressed by T cells TCR complex encludes TCR, CD3, CD4/8 and The ζ chain. The complex is to first recognize and bind to the presented antigen and secondly to relay the signal to downstream signaling pathways and activate T cell. (Reproduced with permissions from Nature reviews(41))

The signals initiated by stimulated TCR lead to phosphorylation of ITAM motifs of CD3 subunits and activation signal transfer to T cells. How the signals are transferred from the TCR to the CD3 subunits is still debated. The three-dimensional structure studies have shed some light on the process, but our understanding still has gaps. Accordingly, several models have been proposed; The most accepted model is the conformation theory, suggesting that the TCR-pMHC engagement induces conformational changes in the associated CD3 chains, bringing ITAMs next to co-receptor associated kinases phosphorylases and leading to ITAM phosphorylation(23, 27).

The next step is to convert the antigen recognition into intracellular chemical components involved in downstream signaling complexes, which determine T cell fate, cytokine production, survival, proliferation, and differentiation. Multiple proteins, including enzymes, adaptor proteins, and scaffolds, are involved in transducing and orchestrate antigen-binding signals. Of note, the scaffolding and adaptor proteins lack any enzymatic activity and act as an assembly site and recruit other proteins to signaling complexes.

Receptor-associated protein tyrosine kinases (PTKs) are actively involved in the initial stages of T cell activation to the point that their inhibition eradicates most if not all the TCR downstream events(32). PTKs catalyze tyrosine phosphorylation, a typical reversible process for transferring signals to reverse phosphatases. Lck, an src family protein tyrosine kinase member, is a crucial regulator of TCR signaling initiation(42). Lck phosphorylates CD3 ITAMs, which become subject to binding by the SH2 domain of ZAP70. Next is a series of phosphorylation events and the formation of multi-subunit adapter protein complexes. ZAP70 is phosphorylated and activated by Lck; once activated, it phosphorylated and recruits LAT and SLP-76 to form an activating complex to recruit signaling effector molecules. LAT-SLP-76 complex promotes phosphorylation and activation of several effector molecules, including phospholipase C gamma 1 (PLC-γ1) and phosphatidylinositol-4,5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol triphosphate (IP3). Following that, downstream signaling pathways, including the PI3K, NF-κB, MAPK, and GTPase-dependent pathways, are activated and contribute to optimal T cell activation and transcription of T cell genes involved in T cell function and differentiation(1, 43, 44).

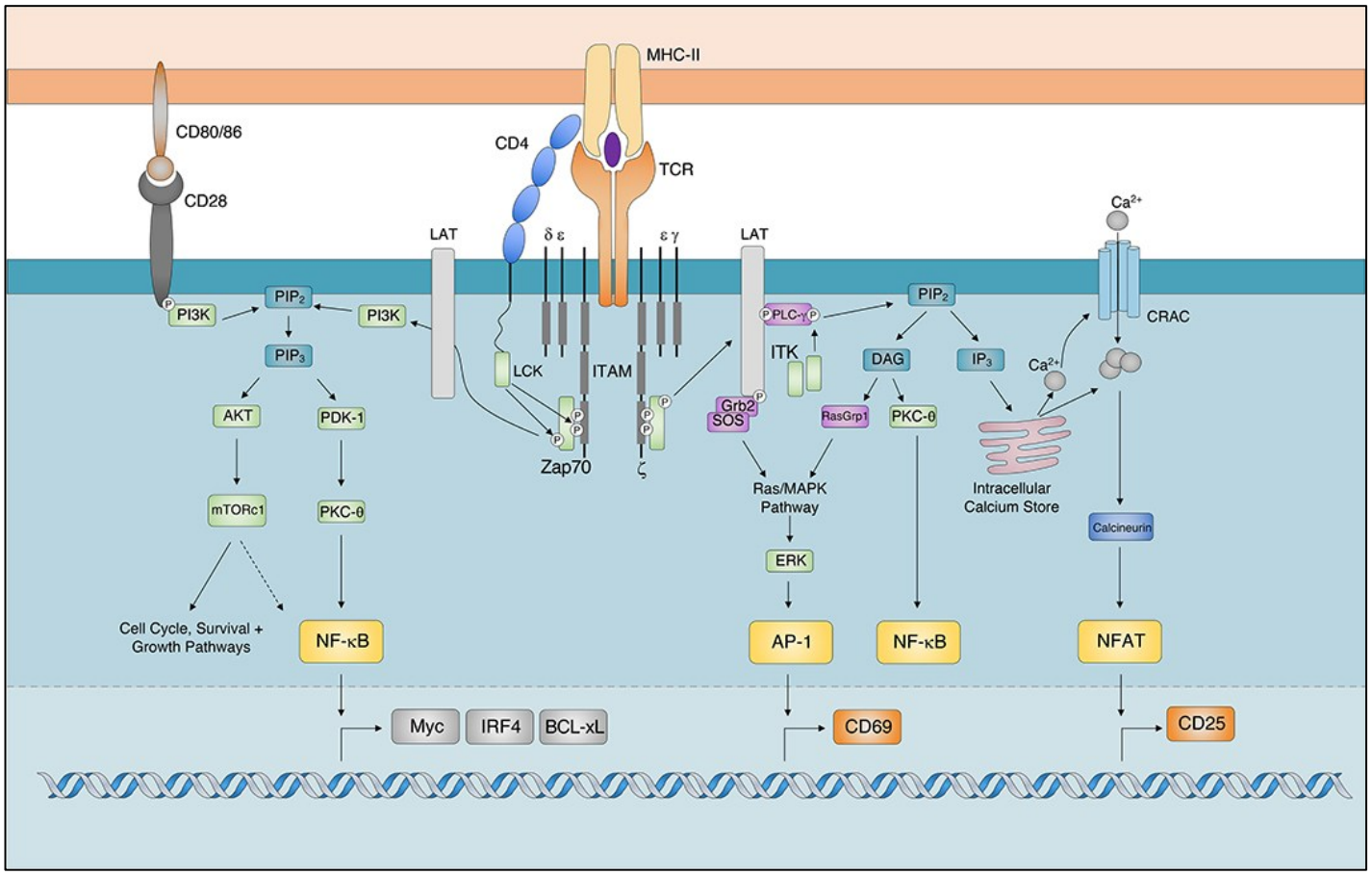

Figure 13 TCR downstream signaling pathways. Sevarl signaling pathways envolved in cell activation, proliferation and survival are in close contact with TCR. Following the TCR stimulation a cascade of phosphorylation and activation starts. The shown pathways are explained in the manuscript. The figure shows the cascade of events following the TCR stimulation. (Reproduced with permission from Nature Reviews(45))

1.4.1.1 TCR signaling regulation and protein tyrosine kinases

T cell proper activation depends on receiving all three signals and their regulation and balance. T cell receptor (TCR) signaling is subject to extrinsic and intrinsic regulatory processes, including the concentration and availability of antigen, TCR characteristics, translational and post-translational mechanisms, and co-signaling receptors.

Antigen presentation initiates a cascade of phosphorylation and activation of multiple adaptor proteins via PTKs and PTPs. Depending on the phosphorylation site and downstream signaling, they can activate or deactivate the target and determine T cell fate(46). For instance, following TCR engagement and stimulation, if Lck is phosphorylated and activated by Csk, TCR signaling could lead to positive downstream signaling and T cell activation(27). On the contrary, if CD45 takes action and dephosphorylates Lck, T cell will maintain homeostasis. Any mutation in CD45 or Csk could cause severe immunodeficiency in humans, suggesting their importance in T cell activation(47). In general, PTPs and PTKs are involved in several T cell signaling pathways and regulate diverse T cell functions such as cytokine production and response to extracellular signals. Due to their central role in regulating T cells, they are also under study as the therapeutic targets in cancer(48).

T cell co-signaling receptors are vital regulators of T cell signaling, but they also need to be regulated to prevent overexpression or dysfunction. Different cell surface expression patterns, transcriptional and posttranscriptional modifications, and induced expression are examples of co-signaling receptor regulations. Additionally, most co-signaling receptors bind to more than one ligand, and some even share ligands with other receptors, leading to another regulation level(49). One most studied example is the competition between CD28 and CTLA-4 to bind B7-1/2 ligands, which will be explained later.

T cell signaling is also regulated by post-translational mechanisms like ubiquitination of TCR signaling-related effector molecules. The E3 ubiquitin-protein ligases CBL, CBL- B, RNF128 (also known as GRAIL), and Itchy homolog (ITCH) target multiple proximal effectors in T cells and are critical for the regulation of TCR signaling and the prevention of autoimmunity(50). ZAP70 ubiquitylation, another post-translational regulation of TCR signaling, targets multiple effector molecules in TCR signaling critical for T cell activation(27).

Together, studies on T cell activation suggest a complex network of signaling pathways and multiple levels of regulations highly affecting all aspects of T cell. Regulation of T cell activation has been reviewed by various groups and will not be discussed further(27).

1.4.1.2 Transcriptional Activation downstream of TCR signals

Multiple gene expression changes are required to activate T cells to proliferate and produce cytokines fully. Following the TCR signaling, the scaffold needed to recruit downstream signaling molecules is formed. Next, co-stimulatory molecules join this primary scaffold to amplify the signals and overcome the TCR activation threshold(27). NF-kB, NFAT, and AP-1 are three transcription factors playing the most critical role in TCR signaling-induced changes(32) (Figure 12)

NF-κB is an inducible transcription factor involved in regulating proinflammatory responses via modulating an extensive array of genes. Its activation following TCR signaling modulates naïve T cell activation and differentiation. NF-κB resides in its inactive form bound to IκB in the cytoplasm. Following CD28 positive co-signals, IκB is degraded, and Ikk complex is activated, allowing NF-κB activation and its translocation to nucleolus to initiate transcription of T cell activation-related genes(51).

AP-1 is a leucine zipper transcription factor composed of homo/heterodimers of Jun, Fos, and ATF. It can bind to AP-1-binding sites in activated T cells and modulate differentiation through regulating gene expression like IL-2(27).

NFAT transcription factor involved in Ca2+ influx and long-term Ca2+ signaling, which induces transcription of a wide range of genes in T cells. Ca2+ efflux is sensed by Ca2+-related proteins, which activate NFAT after TCR stimulation(27).

Overall, TCR signal transduction concludes the transcription of genes involved in many aspects of T cells like activation, proliferation, differentiation, and survival. Therefore, it is tightly regulated to ensure the optimum immune response against various pathogens and tumor cells. Dysregulation and mutations in TCR-related genes and signals could affect the T cell functions extensively, leading to autoimmunity and disturbing immune homeostasis. As a result, considerable efforts have been made to understand TCR signaling and the related pathways to improve T cell-based therapies over the years.

1.4.2 Second signal: Co-stimulatory and co-inhibitory signals

"Second signal" and "co-signaling receptors" were first described by experiments showing failed or attenuated T-cell activation without APCs, suggesting that APCs express molecules or receptors which help to transfer or intensify T cell activation signals(52). Further monoclonal antibody experiments revealed multiple receptors on T cells binding to their ligand on APCs along with TCR, now known as co-signaling receptors.

Stimulating or inhibitory signals of co-signaling receptors regulate T cell responses specifically. Co-signaling receptors can regulate the initiation of T cell activation through stimulatory signals (co-stimulatory receptors) and the termination via negative signals (co-inhibitory receptors). Stimulatory receptors act to amplify the TCR activation signal following ligand binding. Co-stimulatory signals are needed the most during the T cell priming and can influence T cell activation and differentiation. Antigen recognition by the TCR alone is insufficient for T-cell priming. In the absence of co-stimulatory signals resulting from signal disruption, antigen presentation by immature DCs, or high inhibitory signals, the T cell will enter a state of anergy, in which the lymphocyte is functionally inactivated but remains alive for an extended period in an irreversible hyporesponsive state(53).

The outcome of TCR stimulation, to become activated or stay unresponsive, depends on the balance between co-stimulatory and co-inhibitory receptors engagement. Co-signaling receptors interacting with their appropriate ligand can initiate a cascade of events affecting T cell function, proliferation, survival, and differentiation but, most importantly, ensure T cell activation when it's needed(53, 54).

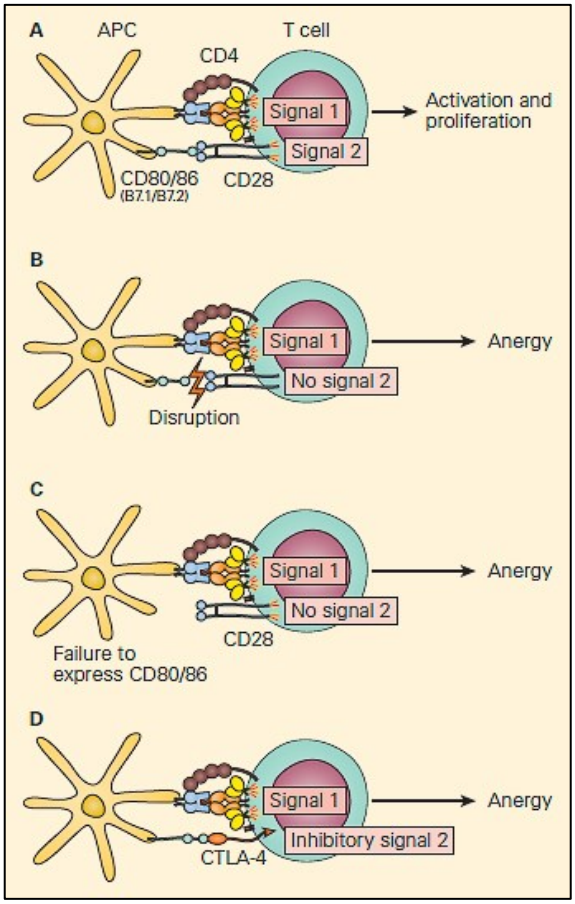

Figure 14 Importance of second signal so called co stimulatory signal in T cell activation Co-stimulatory signals are the second most impostant signal needed for proper T cells activation and are provided by APCs. Elimination or disruption of the co-stimulatory signals specially through the priming can lead to T cell anergy and unresponsiveness as shown. (From open source: immunopedia.com https://www.immunopaedia.org.za/immunology/advanced/2-central-peripheral-tolerance/)

Co-signaling receptors are structurally classified into two superfamilies, including CD28 and TNF receptor (TNFR) superfamily and several small subfamilies. Four distinct families of co-signaling receptors has been identified so far, (1) B7-CD28 family including CD28, cytotoxic T-lymphocyte antigen-4 (CTLA-4; CD152), programmed death-1 (PD-1; CD279), inducible costimulatory molecule (ICOS; CD278), and B and T-lymphocyte attenuator (BTLA; CD272); (2) CD2/signaling lymphocyte activation molecule (SLAM) family including SLAM (CD150), 2B4

(CD244), and CD48; (3) Ig family including T-cell immunoglobulin mucin-3 (TIM-3), CD160, and Lymphocyte-activation gene 3 (Lag-3); and (4) TNF-receptor superfamily including CD27(49).

Co stimulatory receptors of different families

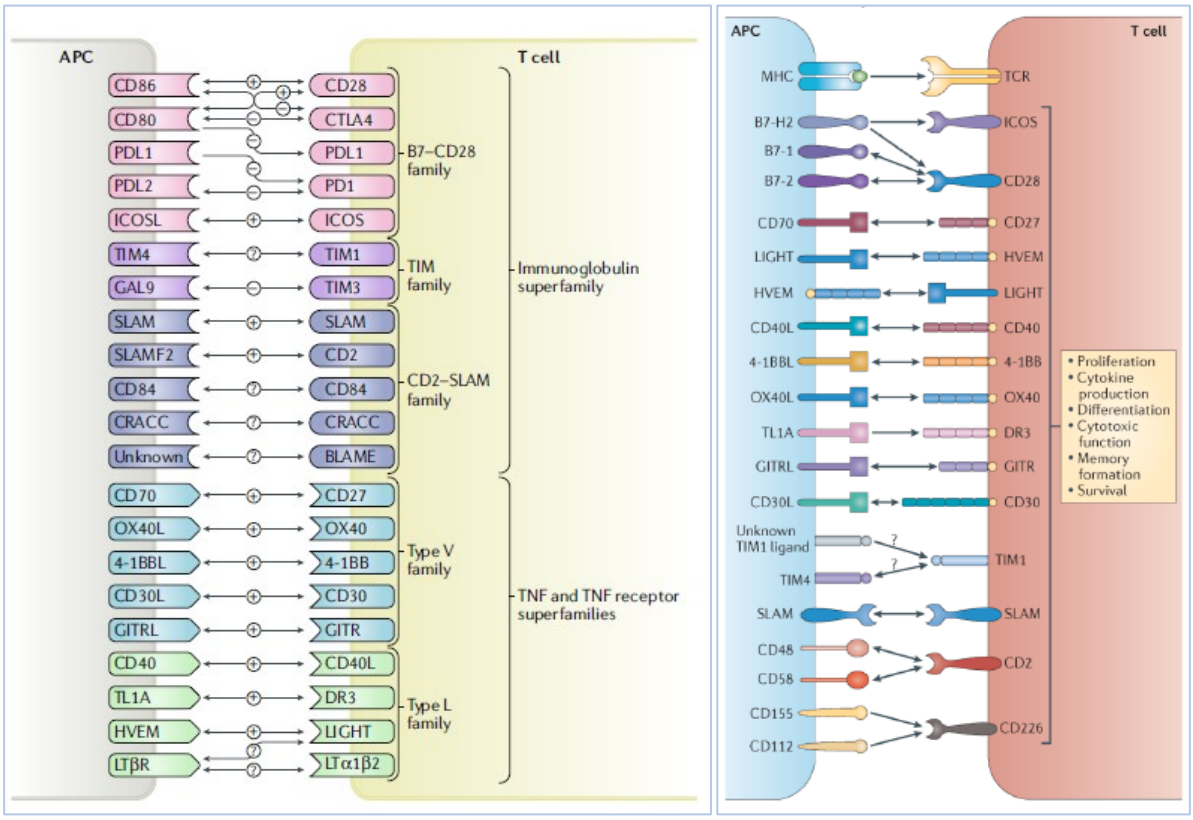

Figure 15 Two superfamily of co-signaling receptors and their inhibitory and stimulatory members
(Reproduced with permission from Nature Reviews(55))

1.4.2.1 CD28 co-signaling receptor superfamily

CD28 family members share a common structure consisting of an extracellular variable immunoglobulin-like domain attached to transmembrane and intracellular domains. CD28, CTLA-4, Inducible T-cell co-stimulator (ICOS), PD-1, and T cell immunoreceptor with Ig and ITIM domains (TIGIT) are the most studied members of this superfamily and are targeted for therapeutic purposes by multiple groups(56). CD28 is the primary and most essential co-stimulatory receptor

needed along with TCR stimulation to prime naïve and resting T cells. Its signaling is also vital in restimulating memory CD8+T cells and is needed to reach maximum cell expansion and pathogen clearance(57). CD28 is constitutively expressed by T cells, and the signal is critical not only for T cell activation and growth but also for its survival. CD28 deficient mice have proved the importance of CD28 signaling in naïve T cell activation. CD28 knock-out mice are immune-compromised and illustrate reduced T cell-related immune responses, defect T cell differentiation, and significantly reduced proliferation and survival(58). As a result, CD28 signaling has been considered the primary regulator of T cell activation, ensuring maximum TCR activation. It drives highly essential activities, including IS formation, TCR downstream phosphorylation, transcription factor gene expression modifications, and actin cytoskeleton remodeling(5).

CD28 family members bind to more than one ligand and act differently depending on the immune response context. CD28 primarily binds to B7-1 (CD80) and B7-2 (CD86), mainly expressed by DCs(5). Mature DCs express the highest CD80 and CD86 among APCs. CD86 is expressed constitutively and is vital to initiate the T cell activation, but CD80 is expressed later and adds another level of regulation(59). CD80 and CD86 have overlapping functions but are not redundant; studies show reduced CD28 dependent signals in the absence of each (60). Cytokines, including IFN-α, IFN-γ, and GM-CSF, along with other stimuli such as microbial-derived products (e.g., LPS), can upregulate the expression of CD80 and CD86 on APCs. Both CD80 and CD86 can bind to other receptors with different affinities and functions. For instance, CTLA-4, another member of the CD28 family, binds to CD80/CD86 with higher binding affinity and inhibits T cell activation(61, 62). As a result, CTLA-4 competes with CD28 in binding to CD80/CD86, but unlike CD28, its expression on T cell surface is significantly lower and depends on TCR activation(59).

CD28 intracellular domain has no enzymatic activity but presents signaling motifs that bind to SH2 and SH3 domain-containing signaling proteins. It has two cytoplasmic motifs involved in signaling: YMNM and PYAP. YMNM is associated with PI3K, a standard signaling intermediate, to initiate the targeting of AKT. The CD28–PI3K–AKT pathway meditates several downstream signaling, including NF-κB, NFAT, BCL-XL, mammalian target of rapamycin (mTOR), glucose transporter type 1 (GLUT1), promoting T cell proliferation and survival. Both the YMNM and PYAP motifs can also recruit protein kinase C θ (PKCθ) to the IS and induce its activation(63). CD28 is involved in T cell survival via up-regulating IL-2 production and Bcl-xL expression level.

Mutations in CD28 signaling motifs resulted in decreased CD28-dependent IL-2 secretion in T cells, leading to markedly less proliferation and impaired survival in the periphery(64).

As mentioned, based on the ligand and the context of the immune response, CD28 can act as both pro and anti-inflammatory. For instance, CD28 plays a vital role in Treg's survival and homeostasis in the periphery. CD28 deficient mice experiments revealed decreased Treg suppressing function and promoted auto immunity(59, 65).

ICOS is another stimulatory member of the CD28 family and is mainly expressed by activated CD4+ T cells. Its exclusive ligand, ICOSL, is only expressed by professional APCs, especially DCs of the germinal center presenting to follicular helper T cells. Other than the same Ig-like structure, ICOS has the similar but non-redundant role as CD28. It recruits PIP3 concluding in Akt activation, but ICOS-mediated Akt activating signals are more robust than CD28. ICOS deficient mice exhibit enhanced Th1 immune response, T cell proliferation, and effector cell differentiation are also affected(66).

On the other hand, ICOS signaling promotes Th2 response, and anti-ICOS antibodies reduced Th2 cytokines such as IL-4 and IL-5 in response to infection. As a result, ICOS is considered a Th1/Th2 immunity modulator with more importance in Th2 responses than Th1 immunity. ICOS signaling induces IL-4 and IL-21 secretion through the C-MAF pathway, and it's crucial for CXC-chemokine receptor 5 (CXCR5) expression, a Tfh specific cell surface marker(59).

Overall, it has become clear that CD28 family members and ligands not simply function as TCR signal amplifiers but deliver unique signals that control intracellular biochemical events, from post-translational protein modification (e.g., phosphorylation) to epigenetic changes that alter the gene expression program of T cells(63, 64). Considering their potential in regulating T cell immune responses, understanding their underlying mechanisms, characterizing their ligands, biological activity, and pathological interactions may also pave the way for their clinical use as a therapeutic target.

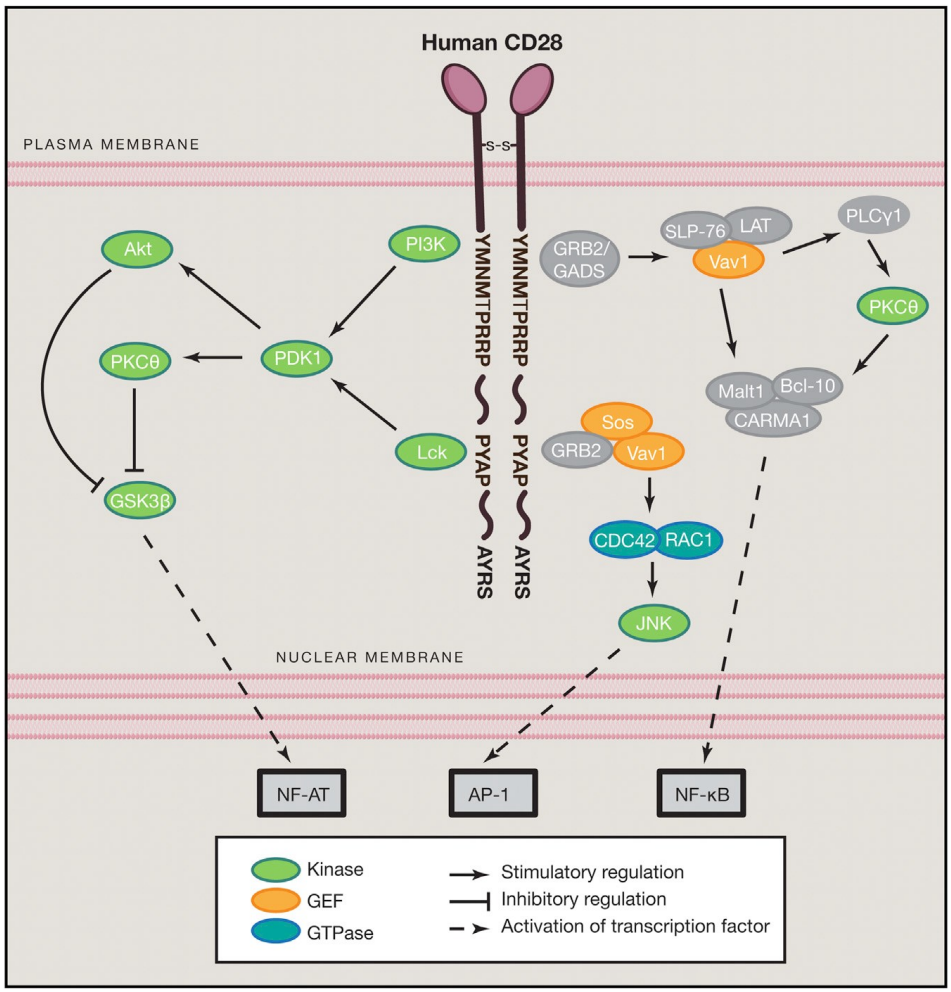

Figure 16 CD28 signaling and its downstream pathways CD28 provides the co-stimulatory signals needed for T cell activation and survival. The major associated signaling pathways are shown in the figure. (Reproduced with permission from ELSEVIER(67))

1.4.2.2 TNF receptor family and co-signaling

The tumor necrosis factor receptor superfamily (TNFRSF) and their ligands direct diverse functions from meditating lymphoid tissue development and homeostasis to regulating cell proliferation, survival, and differentiation(68). TNFR signaling can modulate several different cells related to life, death, or differentiation depending on the immune response context. There are three TNFRSF receptors based on their signaling motif, including those utilizing the death domain, those engaging the TRAF family of ubiquitin E3 ligases, and those lacking a cytosolic domain and functioning as decoy receptors. Therefore, depending on the signaling domain and context of the immune response, TNFRSF members may activate cell survival or apoptosis(68, 69).

T cells express multiple numbers of TNFRSF as co-signaling molecules serving as both inhibitory and stimulatory receptors(70). CD27, OX-40, 4-1BB, and CD30 and their apposite ligand on APCs (CD70, OX-40L, 41BBL, and CD30L, respectively) function as co-stimulatory receptors to promote the survival of proliferating cells through their differentiation process and on into memory. TNFRSF members are upregulated at later stages of T cell activation and use different groups of enzymes and adaptor proteins than CD28 tyrosine phosphorylation. They utilize a cascade of ubiquitin conjugation as a scaffold to recruit adaptor proteins into signaling complexes. Many of the co-signaling TNFRSF members are expressed in activated T lymphocytes, and their specific ligands are expressed in professional APCs, neutrophils, macrophages, or stromal cells. The membrane-anchored expression pattern restricts signaling to cell-to-cell contact, whereas some ligands or receptors are shed into soluble forms that can act systemically(68).

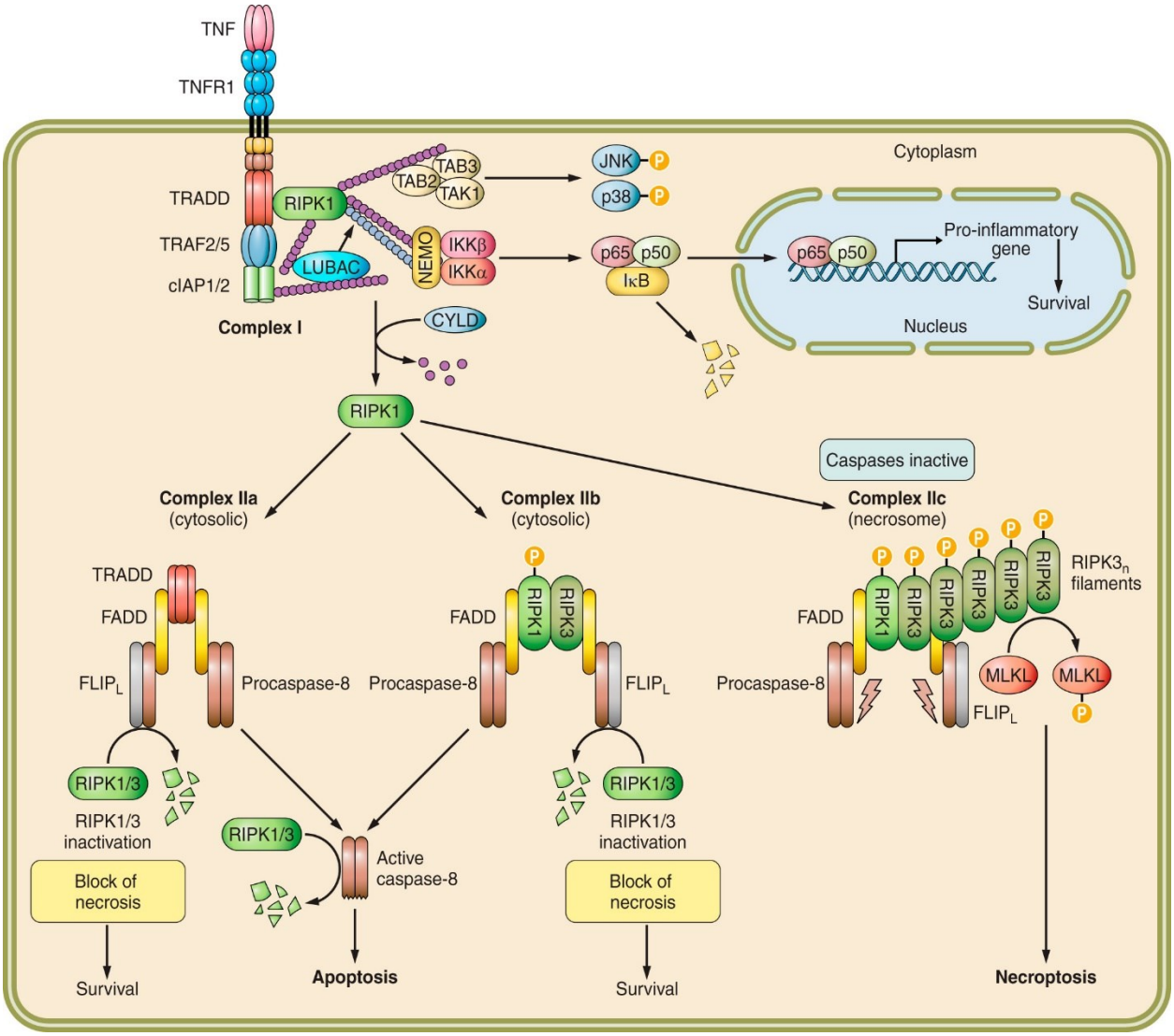

Figure 17 The signaling pathways downstream of TNFRSF Depending on the type of the signaling motif, ligand interaction of the TNF receptor family members can lead to cell survival or death. Some of the related signaling pathways are shown. (Reproduced with permission from Physiological Reviews(69))

Following their ligand binding, three TNF receptor receptors (4-1BB, OX-40, CD27) activate several downstream signaling through facilitating TNF receptor-associated factor (TRAF) adaptor proteins recruitment. Canonical and no-canonical NFκ-B, Fos/Jun transcriptional regulators, p38 mitogen-activated protein kinase (MAPK), activator protein 1 (AP1), ERK, and NFAT are TNFR downstream activated signaling molecules involved in several T cell functions. TNFRSF can also promote T cell survival by upregulating anti-apoptotic factors, including BCL-2, BCL-XL, and BFL1 (BCL2A1)(71).

OX40 promotes cell activation, proliferation, and differentiation of CD4+ and CD8+ T cells following ligand (OX40L; also known as CD252) binding and TCR signaling. OX40 utilizes TRAF2 and TRAF5 and activates canonical and non-canonical NF-kB, PI3K, and AKT. Studies in both mice and humans have proven the role of OX40 signaling in controlling viral infections and memory cell development, and it's currently a target in combination immunotherapy(72).

4-1BB is another co-stimulatory member of the TNRSF with roles in expansion, acquisition of effector function, survival, and development of T cell memory. It is transiently expressed by activated T cells following TCR while its primary ligand, 4-1BBL, is expressed predominantly on professional APCs. 4-1BB ligand binding transfer signaling through TRAF1 and TRAF2 and activates NF-kB, AKT, p38 MAPK, and ERK pathways, which induce T cell survival via increased expression of survival genes like Bcl-2, Bcl-XL, and Bfl-1 and decreases the expression of pro-apoptotic Bim. However, 4-1BB overexpression can result in apoptosis of overstimulated active T cells and can induce Treg differentiation(68).

Of note, there is also one TNFRSF member, herpesvirus entry mediator (HVEM), serving as a ligand for negative regulatory checkpoint receptors, B and T lymphocyte attenuator (BTLA), CD160, delivering negative signals(70).

Finally, agonist antibodies targeting TNFRS members, including 4-1BB, OX-40, and CD27, have received considerable attention for their therapeutic utility in enhancing anti-tumor immune responses, particularly in combination with other immuno-modulatory antibodies targeting co-inhibitory pathways in T cells. However, the design of the antibody to ensure safe activation and to minimize the overreaction of the cells have the progress of the field.

1.4.2.3 Inhibitory receptors

The second group of co-signaling receptors, co-inhibitory receptors, performs as brakes on the T cell response and delivers negative feedback to activated T cells. Activated T cells express several inhibitory receptors such as PD-1, CTLA-4, LAG-3, TIM-3, TIGIT, VISTA, BTLA, 2B4, and CD160. Inhibitory receptors from different families mediate and deliver negative signals through various ways, ranging from interrupting co-stimulatory receptor signals like PD-1 and CD28 to employing inhibitory sequence motifs such as ITIMs and ITSMs(73). As mentioned, the balance between positive co-stimulatory signals and negative co-inhibitory signals determines the direction of TCR downstream signaling. Inhibitory receptors, their signaling pathways, and their importance in T cell therapy will be discussed in detail in the following pages.

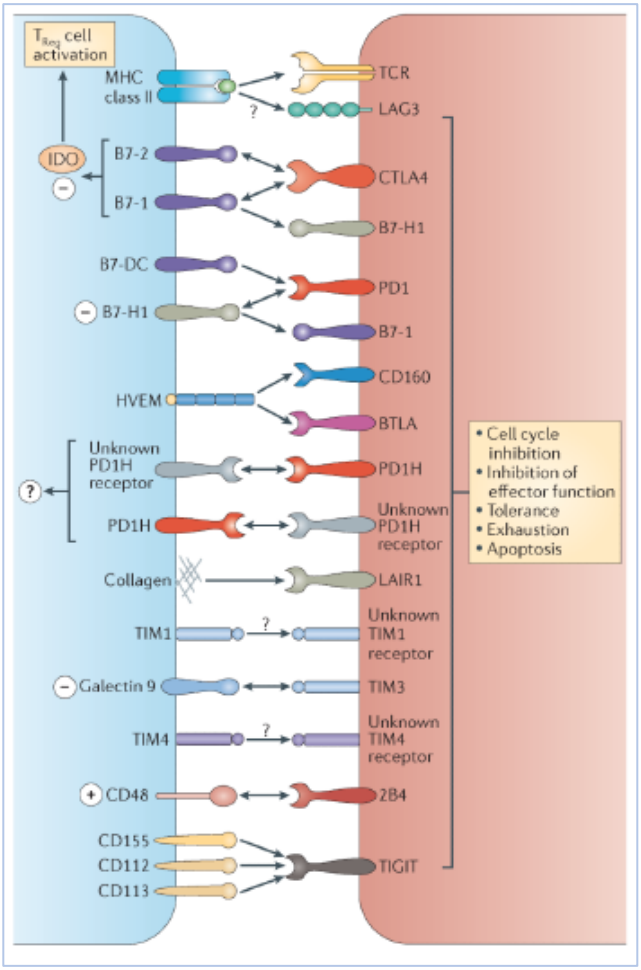

Figure 18 Inhibitory receptors of different families Inhibitory receptors negatively regulate T cells and are essential to keep the immune response under control. Some of their roles are shown in the figure. (Reproduced with permission from Nature Reviews(55))

1.4.3 Third signal: Cytokines

Evidence for the necessity of a third signal to fully activate naïve T cells comes from studies investigating T cell activation through artificial antigen-presenting cells lacking cytokine receptors(74). With only two signals, antigen presentation and co-stimulatory signals, memory T cells were successfully activated and differentiated into cytotoxic T cells; but naïve T cells showed limited clonal expansion, defect effector functions, and generation of long-lasting tolerant cells. Later addition of IL-12 or type I IFNs (IFN-α/β) resulted in strong clonal expansion and cytotoxic T cell development from naïve cells(57, 75). These experiments projected two critical characteristics of T cell activation, first cytokines are required for clonal expansion and functional maturation of naïve T cells. Second, APCs, especially mature DCs, are the source of these cytokines(74).

In vitro results were confirmed by mice lacking IL-12 and type I interferon receptors. Also, the necessity of cytokines being the third signal was assured by the fact that the cytokine-provided signals cannot be replaced or replace co-stimulatory signals and act as an independent but associated signal(76).

Gene expression evaluation in T cells activated with or without the third signal revealed that transcription factors and chromatin remodeling play a central role in related differentiation and functional development processes. IL-12 and IFN type II presence cause upregulation of multiple genes involved in cell survival (CD25, Bcl-3, serine protease inhibitor 6, etc.), effector function (perforin, granzyme B, IFN-g), and co-stimulatory signals(74).

The third signal sources a complex cascade of gene expression modulation, preparing activated T cells for differentiation, migrating to the infection site, and developing a proper immune response. Naïve T cells that receive the third signal are promoted to migrate to the site of infection or inflammation to handle the pathogen(77). In addition, they will be further activated by cytokines and chemokines released by other cells such as neutrophils, mast cells, and macrophages at the site of infection(1, 78).

1.5 Inhibitory receptors and their mechanisms of action in regulating immune responses

The immune system is required to eliminate pathogens but also to maintain self-tolerance through distinguishing the healthy tissues and cells from infected and malignant ones. Therefore, several mechanisms and checkpoints work together to uphold homeostasis and peripheral tolerance while allowing the immune system to perform safely. With this in mind, an optimal T cell activation and effector function could be defined as a specific response **at the required place** for **the desired duration**.

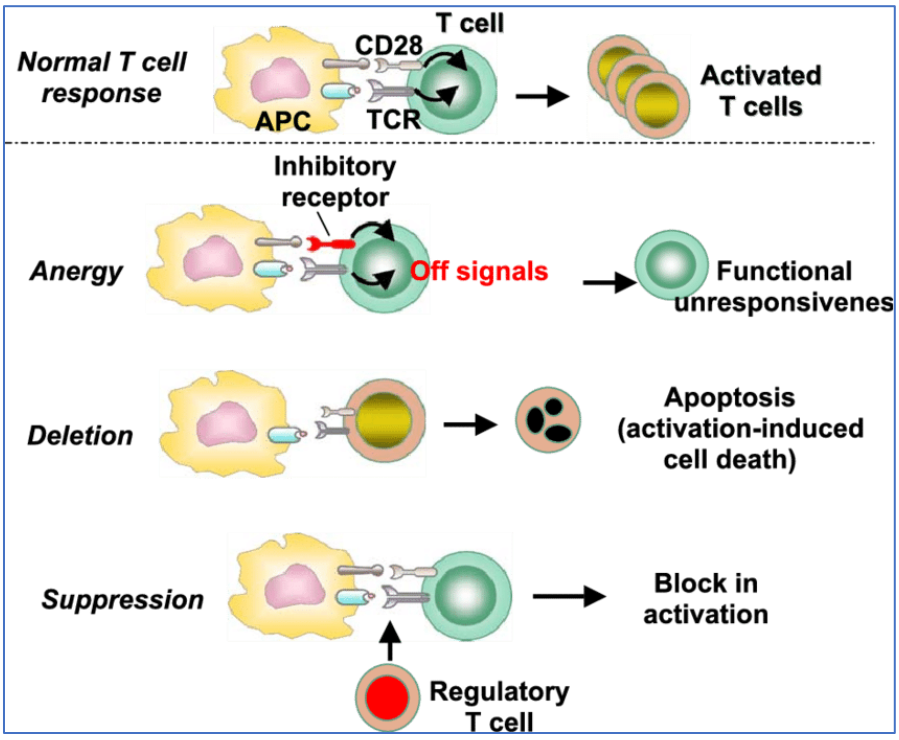

Figure 19 T cell responses are regulated by various mechanisms in periphery Anergy, a tolerance mechanism in which T cells are functionally inactive despite antigen encounter but stay alive for sometime in periphery. Peripheral deletion is the mechanism through which the potentially self-reactive T cell clones are removed. Peripheral suppression is modulated by T regulatory signals. (From open source: Wikipedia)

The immune system has developed various mechanisms to control T cell responses and avoid unnecessary and self-harming immunopathology, including eliminating autoreactive thymocytes, suppressive function of Tregs, and several other barriers. These screens the periphery constantly (as summarized in figure 19) to limit the activation and expansion of T cell responses.

Inhibitory receptors (IRs) were first revealed by investigating intracellular signaling motifs of T cell co-signaling receptors. Consequently, the presence of immunoreceptor tyrosine-based inhibitory motif (ITIM) or immunoreceptor tyrosine-based switch motif (ITSM) was documented as a standard and defining feature of the cytoplasmic domain of receptors that attenuate and negatively regulate T cell activation signals. Most co-inhibitory receptors contain the ITIM motif, and each receptor has been associated with a crucial and non-redundant role in immunoregulation(79, 80).

Contrary to the previous beliefs, co-inhibitory receptors do not play the role of an on/off switch but more like a threshold that could diminish and regulate the strength of stimulatory signals and activated T cells' effector functions. In other words, IRs should be considered "checkpoints" that effector T cells must pass to exert their full functions. Notably, loss of inhibitory signaling is often associated with autoreactivity and direct inflammatory responses, illustrating its critical role in maintaining immune homeostasis and preventing autoimmunity(81, 82).

The strength of the inhibitory signal relative to the stimulatory and activating signals determines the outcome of TCR signaling for T cells to be activated or stay unresponsive. The strength of the signal is a variable of receptor expression, ligands' availability and expression, and the affinity of the receptor/ ligand interaction. Receptor/ ligand expression varies among the tissue and cell type and depends on the cell's activation state(27, 83). Some inhibitory receptors are constitutively expressed by active T cells, such as PD-1, ensuring a certain activation level to overcome the inhibitory signal. Some are upregulated in response to T cell activation, like CTLA-4, which acts during later cellular responses(73).

There are multiple outcomes to inhibitory receptor signaling ranging from temporary inhibition and attenuation of T cell signals to permanent unresponsiveness and cell death. Inhibitory receptors can modulate T cell activation signals utilizing three major mechanisms(84). The first mechanism is **competing with co-stimulatory receptors** and preventing T cells from receiving stimulatory

signals needed for activation. The most studied example of this mechanism is CD28 and CTLA-4 receptors binding to B7 molecules. CTLA-4 binds to B7 molecules with greater affinity, so when both receptors are present, the ratio of CD28:B7 binding versus CTLA-4: B7 binding determines whether a T cell will undergo activation or anergy(85, 86).

The second mechanism involves **recruiting intracellular mediators** such as protein phosphatases by signaling motifs to dephosphorylate and **deactivate signaling molecules of TCR and co-stimulatory receptors**(87)**.** Inhibitory receptor-ligand binding results in tyrosine phosphorylation of ITIM/ITSM, often by an Src family kinase like Lck, which in return, will act as a docking site for cytoplasmic phosphatases having an Src homology 2 (SH2) domain and recruit them for further modifications in downstream molecules(88). There are two classes of SH2- containing inhibitory signaling effector molecules: 1- the tyrosine phosphatase SHP-1 and 2- the inositol phosphatase SHIP (SH2-containing inositol polyphosphate 5-phosphatase). Both could interact with multiple signaling molecules to transmit the TCR attenuating signals **via dephosphorylation.** In one mechanism, SHP-1 association with LAT disrupts LAT activation and subsequent TCR signaling(89). CTLA-4, PD-1, and LAG3 signaling are examples of IR utilizing intracellular mediators to recruit phosphatases and suppress T cell activation(90). The third group of receptors functions by upregulating genes involved in T cell dysfunction and immune inhibiting functions. IRs could use one or a combination of these mechanisms depending on their signaling motifs and expression patterns(82). Understanding IR/ligand interactions is essential for the rational design of therapeutic agents, especially targeting the IRs and their downstream signaling.

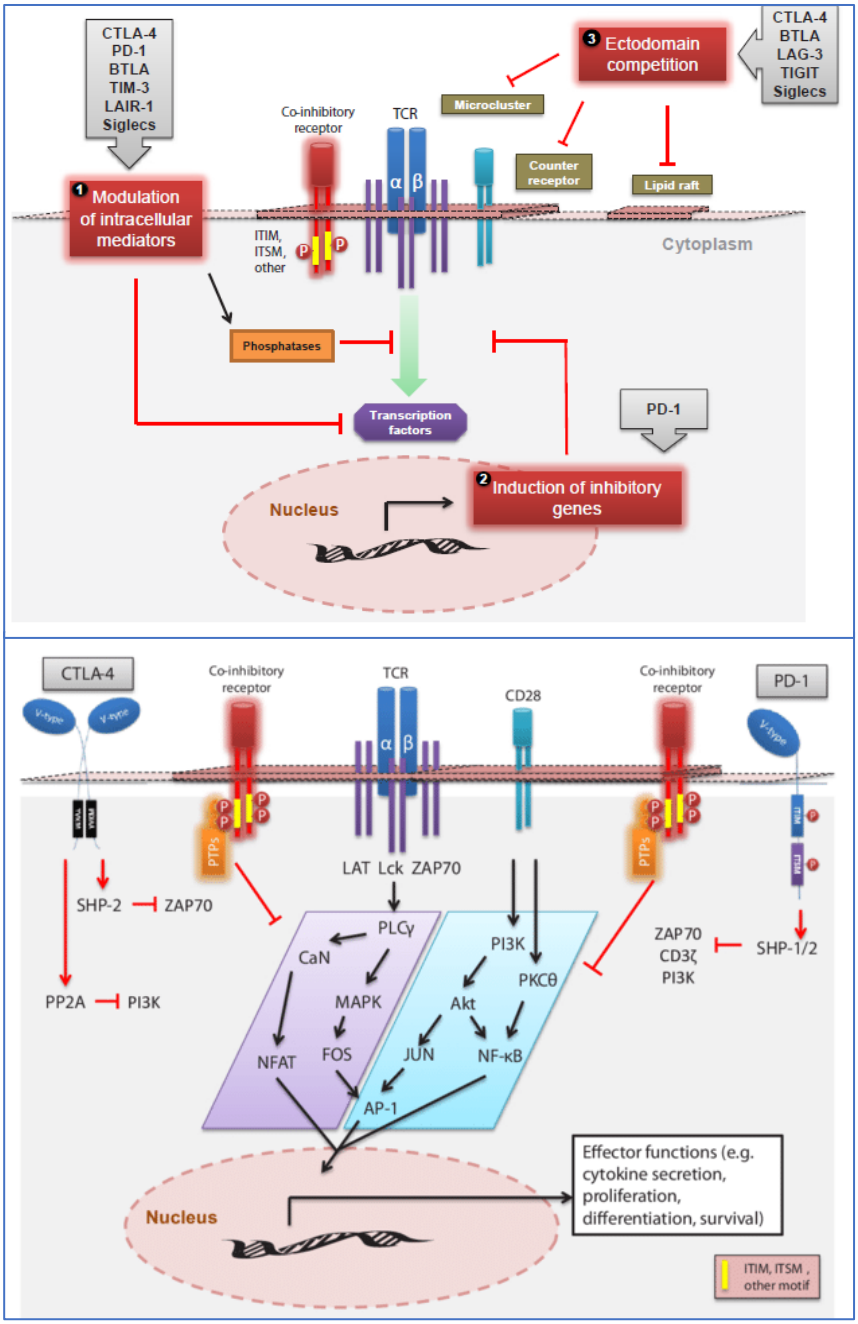

Figure 20 Inhibitory receptors use various mechanisms to negatively regulate T cells. As shown in the figure IR signaling motifs are in close contact with several signaling pathways involved in T cell function. (Reproduced with permission from Journal of Clinical & Cellular Immunology(52))

The majority of T cell co-inhibitory receptors belong to the immunoglobulin (Ig) superfamily. PD-1, CTLA-4, are the most studied inhibitory members of this family(52). TIM3 from mucin-like, LAG3, and TIGIT are other well-studied inhibitory receptors, especially in cancer and chronic

infections(82). We will primarily discuss the common aspects of inhibitory receptors: *in vivo* signaling consequences and how co-inhibitory receptors are involved in the T cell exhaustion as relevant for anti-cancer therapy.

1.5.1 PD-1/PD-L1

Programmed cell death protein 1 (PD-1), also known as CD279, is an Ig superfamily (IgSF) member and a type I transmembrane protein. PD-1 regulates T cell responses during T cell activation and maintains immune response homeostasis and self-tolerance. It is broadly expressed on multiple subsets of T cells, B cells, monocytes, DCs, and its expression is regulated by several transcription factors, including NFATC1, FOXO1, T-bet, BLIMP1, and GSK3. Recent studies have also documented epigenetic regulations controlling PD-1 expression during T cell activation and differentiation. PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are the ligands of PD-1 that belong to the B7 family. PD-L1 is constitutively expressed by a wide range of hematopoietic and non-hematopoietic cells. At the same time, PD-L2 is inducible by pro-inflammatory signals and is strictly expressed by professional APCs like DCs, macrophages, and specific B cell subsets(91-94). Inflammatory cytokines such as type I and II interferons produced by activated T cells upregulate PD-L1 expression, promoting immune resistance in the tumor microenvironment. Tumor cells overexpress PD-L1, which leads to immune escape and induces T cell suppression and exhaustion(95).

The crucial function of PD-1 signaling in normal host physiology is to modulate T cell responses and maintain them within a desired physiological range. PD-1 signaling serves as negative feedback and downregulates T cell effector functions to protect healthy tissue from an excessive immune response. PD-1 limits the overactivation and fine-tunes the TCR signaling by increasing the needed threshold to react to self-antigens. Therefore, its loss could result in autoimmunity and immunopathology during effector response against pathogens(96).

PD-1 uses several mechanisms to attenuate T cell activation and function. PD-1 cytoplasmic tail has two tyrosine motifs, ITIM and ITSM signaling motifs, which become phosphorylated following ligand binding and role as docking sites for SH domain-containing phosphatases like SHP1. PD-1 could interact with both SHP-1 and SHP-2 but mainly uses SHP-2 to transmit the

inhibitory signals. These PTPs could modulate and interrupt TCR and CD28 downstream signaling via dephosphorylating key kinases and adaptor proteins like LCK, PI3K(97).

PD-1 attenuates stimulatory signals downstream of TCR and decreases T cell activation, proliferation, survival, and cytokine production, especially IFN-γ, TNF-α, and IL-2. In addition, PD-1 signaling can reduce the expression of transcription factors essential for effector function (Gata3, Tbx21 (T-bet), and Eomes) and impair T cell survival via reducing anti-apoptotic gene expression while increasing the expression of proapoptotic genes (e.g., Bcl2l1 (Bcl-xl) and Bcl2l11 (Bim), respectively). PD-1 signaling alters T cell metabolism mainly via attenuating Akt (protein kinase B) activation and thus inhibiting mTOR (mammalian target of rapamycin) activity, switching T cell metabolism glycolysis to fatty acid oxidation(97-99).

PD-1 gained more attention because of its role in T cell exhaustion during cancer and chronic infection. However, PD-1 is not a specific exhaustion marker but, as mentioned, a T cell activation marker expressed on activated T cells involved in immune response(100). Exhausted T cells upregulate a program of genes that functions as negative regulators of TCR-driven signals and prevent the recruitment of key signaling molecules from initiating T cell activation. Exhaustion is an important phase in T cell differentiation and is discussed in detail in the following(101).

Upregulation of inhibitory receptors and their ligands to suppress and evade anti-tumor immune response is a major hallmark of cancer. Studies on IR's role in the tumor microenvironment led to the emergence of immune response-based therapies called checkpoint blockade therapy(102). Blocking inhibitory receptors to reinvigorate immune response and promote tumor regression is now an FDA-approved therapy for several types of cancer. PD-1 is an attractive checkpoint therapy target since it’s only expressed by antigen-activated T cells. As a result, blocking PD-1 will be expected to affect the ongoing immune response primarily. Treatment with anti-PD-1 agents showed successful clinical outcomes and durable immune response in various types of cancer(103, 104). Clinical evidence revealed that PD-1 blockade is most effective in tumors expressing PD-L1 and infiltrated with specific T cells. However, interestingly, some mouse studies suggest that the success of PD-1/PD-L1 checkpoint blockade does not seem to be dependent on tumor PD-L1 expression. PD-L1 expression was reported on nonmalignant cells in TME, such as platelets(105), macrophages, and dendritic cells(106). As a result, the response to PD-1/PD-L1 was subsequently

evaluated based on the PD-L1 expression on tumor cells or combining tumor and immune cell expression.

Specific T cells suppressed through PD-1/PD-L1 interaction in TME positively respond to PD-1 blockade and regain some of their antitumor function. Genomic studies have also demonstrated that PD-1 blocking could reinvigorate T cell activation and lead to changes in metabolic and proliferation genes that partially contribute to its therapeutic efficiency. Surprisingly, in some mouse models, PD-L1 blockade, but not anti-PD-1, induced tumor regression, suggesting their distinct mechanism in tumor cells and cancer therapy(107). Accordingly, currently, there are three FDA-approved PD-L1 blocking antibodies (Atezolizumab, Avelumab, and Durvalumab) available for lung cancer, and several clinical trials evaluate the efficiency of PD-L1 blockade in other cancer immunotherapies(108). Overall survival rates improved in all assessed PD-L1-blockade treated patients, and fewer adverse events were also observed(109).

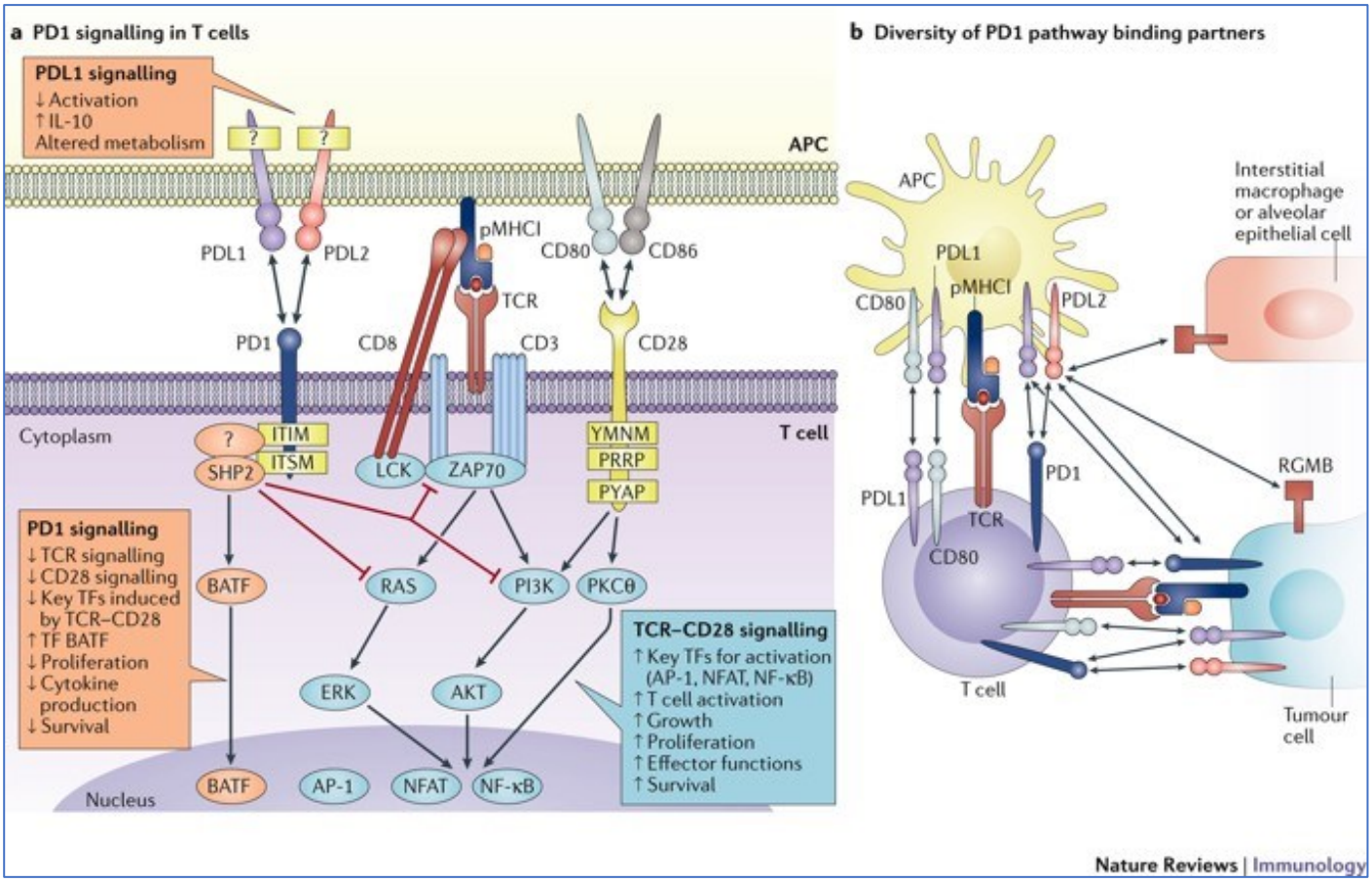

Figure 21 PD-1/PD-L1 downstream signaling pathways (Reproduced with permission from Nature Reviews(110))

PD-1 can modulate metabolism during T cell activation via inhibiting glycolysis genes. Accordingly, anti-PD-1/L1 in combination with metabolic pathways activators like rapamycin (mTOR), AMP-activated protein kinase (AMPK), and PPARγ co-activator 1α (PGC1α) signaling pathways could synergically improve immunotherapy efficiency(96).

1.5.2 TIM3

T cell immunoglobulin and mucin-domain containing-3 (TIM3), also known as CD366, is another co-inhibitory receptor expressed on activated T cells. TIM3 is a type I transmembrane protein from the immunoglobulin superfamily (IgSF) and a member of the TIM gene family expressed by both humans and mice. FoxP3+ Tregs, multiple subsets of T cells (Th1, Th17, activated CD8+ T cells), and some innate immune cells (Macrophages and Dendritic cells) express TIM3. TIM3 expression on T cells is inducible (upon activation), but it's constitutively expressed on innate immune cells(111).

So far, four ligands have been identified for TIM3; Galectin-9, Ceacam-1, HMGB1, and phosphatidyl-serine (PtdSer) to a different domain of TIM3 receptor. TIM3 has a unique protein structure making it different from other IgSF members or mucin domain-containing receptors. Its extracellular domain consists of an IgV domain with six instead of four cysteines forming a unique ligand-binding pocket. TIM3 cytoplasmic tail is different from other co-inhibitory receptors and lacks conventional signaling motifs such as ITIM and ITSM. Instead, it contains five reserved tyrosine residues functioning as SH2 domain-binding motifs and capable of recruiting signaling molecules. Multiple SH2 domain-containing kinases, including Fyn, Lck, PI3K p85, and Itk, have been documented to bind to these residues(82).

Galectin-9, a soluble protein, was identified as a TIM3 specific ligand that mainly induces calcium influx, then apoptosis in Th1 cells. The interaction between galectin-9 and TIM3 also leads to decreased cytokine production in Th subtypes and suppressing the proliferation and activation of NK T cells and some myeloid cells(112).

Recently characterized carcinoembryonic antigen cell adhesion molecule 1 (Ceacam1) has been documented as another TIM3 ligand with cis and trans interaction. Ceacam-1/TIM3 binding

inhibits T cell effector function through both types of interaction. Its co-expression and cis interaction with TIM3 are also crucial for TIM3 protein stability and inhibitory function(113).

Phosphatidylserine (PtdSer) is a molecule expressed on the surfaces of apoptotic cells and was shown to bind to TIM1, TIM3, and TIM4. This interaction is more critical in phagocytes than T cells since it facilitates the clearance of apoptotic bodies and promotes the cross-presentation of antigens by DCs(114, 115).

High mobility group protein B1 (HMGB1) is a damage-associated molecular pattern protein that senses endogenous danger signals and is also a TIM3 ligand, especially in the tumor microenvironment. HMGB1 binds to DNA released from dying cells in TME and eases their recognition by innate immune cells. However, TIM3, expressed on tumor-infiltrating DCs, actively competes with nucleic acids released from dying tumor cells to bind HMGB1 and consequently inhibits stimulation of the innate immune response by nucleic acids and attenuates the production of proinflammatory cytokines; thus, TIM3 blocking may enhance antitumor innate immune responses(116).

There are conflicting results from prior studies on the TIM3 linked signaling pathways, suggesting inhibitory and stimulatory roles. Bat3 (HLA-B associated transcript 3) and Fyn switch in binding to TIM3 cytoplasmic tail has been suggested as a critical factor in determining the inhibitory or stimulatory outcome of TIM3 signaling(117). In the absence of TIM3 ligands, Bat3 is constantly bound to cytoplasmic tail tyrosine residues and blocks the SH2 containing kinases binding sites. Following acute TCR/ CD3 and CD28 receptor stimulation, an active form of Lck is recruited by Bat3. It positively modulates the T cell signaling pathway and represses TIM3 mediated cell death and exhaustion. In this context, transient expression of TIM3 leads to its interaction with TCR signaling components like ZAP70, SLP-76 thereby, further enhancing NFAT and NF-kB signaling and TCR signaling(118-120). Also, a recent study showed that in Bat3 deficient mice, effector T cells enhance TIM3 and *prdm1* expression, leading to upregulation of other inhibitory receptors, including PD-1(121).

In contrast, following the Gal9/Ceacam-1 binding, tyrosine residues are phosphorylated, so Bat3 is released, leading to TIM3-inhibitory function by allowing SH2 domain-containing Src kinases

binding. Additionally, Fyn binds to the same site as Bat3 but recruits Csk to suppress Lck function and inhibit TCR downstream signaling(122).

Taken together, previous experiments suggest the possibility of TIM3 interaction with multiple immune cell signaling components, probably depending on the ligand and immune response context, leading to either a stimulatory or inhibitory impact on the immune response. This

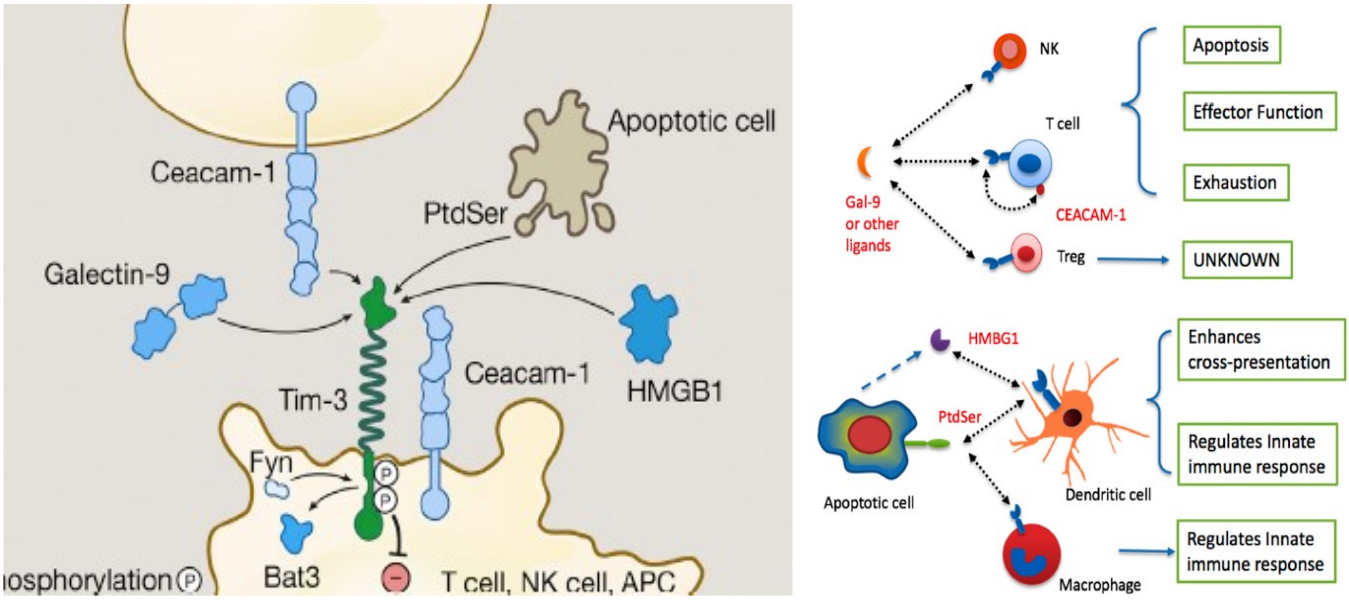

Figure 22 TIM3 can bind multiple ligands with diverse outcomes, TIM3 bind to multiple ligands which may lead to divers outcomes by envolving different downstream signaling pathways.
(Reproduced with permission from: Nature Reviews and Open source from MDPI)

highlights the importance of thorough mechanistic studies on TIM3 signaling pathways, to design TIM3 including immunotherapies.

TIM3 was initially identified as an immune response regulator in autoimmunity, blocking enhanced autoimmune responses in human and mouse experiments. It is specifically vital in autoimmunity because of its IFN-γ secretion regulating function in responding T cells. Similar observations were made in transplant and tumor microenvironments, emphasizing TIM3's role as a T cell co-inhibitory receptor(123).

Studies from both chronic viral infections and cancers suggest that the high and chronic expression of TIM3 on active T cells is associated with exhaustion and T cell dysfunction(124, 125). High levels of TIM3 expression and PD-1 on CD8+ T cells represent severe exhaustion compared to

single PD-1+ T cells and have been associated with a poor prognosis for tumor progression(126-128).

In addition to effector T cells, TIM3 is also expressed by FoxP3+ Tregs and has been shown to enhance the regulatory function of FoxP3+ Tregs in the maintenance of tolerance to the allograft. TIM3+ regulatory T cells are also abundant in the tumor microenvironment and may represent a critical factor in suppressing TIL antitumor immune response(129-131).

Of note, antigen-presenting DCs also express TIM3, which in high level has been shown to correlate with suppressed IL-12 expression, impaired maturation due to inhibited NF-kB signaling as well as diminished antigen presentation(132).

TIM3 has also been considered as a potential prognostic biomarker for cancer. It is highly expressed on tumor-specific CD8+ T cells, dysfunctional TILs, and poor prognosis in cancer patients. TILs and tumor cells also express high levels of TIM3, pointing to the great potential of TIM3 signaling in tumor progression and suppressive tumor microenvironment(133). As mentioned before, TIM3 expression is associated with PD-1 on exhausted TILs supporting the idea of their functional correlation in driving the exhaustion. Blocking on PD-1 has been successful in only a limited portion of patients and cancers; however, blocking TIM3 along with PD-1 has been remarkably more efficient in preclinical studies(127, 134). Especially in clinical and pre-clinical studies, patients who show signs of resistance or never respond to PD-1 blockade therapy, the addition of TIM3 or other checkpoint blockades may be an effective therapeutic solution.

In combining checkpoint blockade, considering each checkpoint's critical characteristics like the expression pattern and downstream signaling is of great importance. For instance, PD-1 and CTLA-4 are expressed by effector T cells, and blocking both have been associated with severe autoimmunity because of unbalanced Treg/ effector T cells ratio following the therapy(135). However, TIM3 is selectively expressed by IFN producing effector T cells and primarily on TILs suggesting that targeting TIM3 combined with PD-1 or CTLA-4 may lead to less autoimmune-like toxicities. Additionally, TIM3 downstream signaling is widely different from representative co-inhibitory receptors. Since it lacks ITIM/ITSM motifs and uses other adaptor proteins, by blocking TIM3, we may target varied aspects of exhaustion-promoting signals(82).

1.5.3 CD5

CD5 is a scavenger receptor cysteine-rich Superfamily (SRCR) recently described as a TCR signaling negative regulator. It is expressed in both humans and mice on all T cell and B-1 cells (a subtype of B cells) following the activation. CD5 is an activation marker in T cells, and its expression level correlates with TCR signaling strength and affinity. Recent studies show that it colocalizes with the TCR at the immunological synapse and negatively regulates TCR signaling through priming(136). CD5 specific endogenous ligand is still unidentified, and CD6 has been described as a possible ligand by *in vitro* studies, but it does not appear to be an exclusive ligand(137).

CD5 lacks the ITIM/ITSM signaling motifs, but its cytoplasmic tail contains two serine residues, at least three tyrosines, and two threonines, susceptible to phosphorylation and functioning docking sites for some kinases and adaptor proteins. The CD5 intracellular tail can be phosphorylated by Lck or Fyn kinases, and recent data show its association with the TCR-signaling related molecules like C-Cbl, RasGAP, or PI3K. In addition, CD5 can recruit E3 ubiquitin-protein ligases CBL and CBLB and promote ubiquitinylating colocalized signaling effectors(138-140).

Mouse model studies show that CD5 expression is correlated with the strength of TCR- signaling at specific developmental stages like the negative and positive selection of thymocytes. The impact of CD5 in thymic development remains to be fully characterized; however, it was shown that thymocytes from CD5-deleted mice are hyperresponsive to CD3 stimulation and exhibit altered positive and negative selection(141, 142). Some additional studies show that CD5 may also promote survival in normal and malignant human lymphocytes. CD5 promotes lymphocyte survival by preventing activation-induced cell death (AICD), an apoptotic pathway triggered by the death receptor CD95 (APO-1, FAS) and its ligand (CD95L, FASL) following T-cell hyperactivation. Also, CD5 prevents antigen-specific T cell apoptosis by the downregulation of FASL expression and inhibition of caspase-8 activation(143-145).

CD5 downregulation has been associated with improved T cell proliferation, allows TILs to overcome tumor evasion, and promotes apoptosis in autoreactive T cells and B-CLL cells(146, 147). As a negative regulator of TCR signaling, CD5 can also be considered an immune checkpoint blockade target(148).

1.5.4 Other co-inhibitory receptors

Identifying PD-1and CTLA-4 inhibitory receptors and their function as a checkpoint in immunotherapy led to the search and characterization of several other inhibitory receptors. Some with a prominent role in cancer and the potential to become immunotherapy targets. The following paragraphs briefly describe these receptors that are not directly related to the work presented in this thesis.

1.5.4.1 LAG3

Lymphocyte-activation gene-3 (LAG-3 or CD223) is expressed on activated CD4+ and CD8+ T cells as a checkpoint and a subset of natural killer cells. LAG-3 belongs to the immunoglobulin superfamily (IgSF) and is structurally similar to that of the CD4 and associates with the TCR complex. It attenuates T cell activation within the first hours of priming through competing with CD4 in binding to MHC II on APCs and interrupts proper activation of T cells. Crosslinking LAG-3 and CD3 can impair T cell proliferation and cytokine secretion by inhibiting calcium ion flux. Additionally, it can inhibit T cell expansion by blocking the cell cycle and controls activated and memory T cell clonal expansion and growth(82). Of note, LAG-3 shows a unique synergy with PD-1 in multiple settings to inhibit immune responses. Sustained expression of LAG-3 is associated with the exhaustion program of dysfunctional CD8+ TILs. Co-expression of LAG-3 and PD-1 on TILs shows an impaired ability to produce inflammatory cytokines such as IFN-γ and TNF-α. Conversely, the blockade of both IRs improves the proliferation and cytokine production of antigen-specific CD8+ T cells. Thus, various mAbs and bispecific antibodies targeting LAG3 interactions are investigated in clinical trials(149, 150).

1.5.4.2 CTLA-4

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a type I transmembrane glycoprotein of the Ig superfamily expressed at a low level by all T cell subsets. It is mainly found in intracellular vesicles and is immediately transferred to the cell surface following TCR-pMHC engagement. CTLA-4 is constitutively expressed by Tregs, but its expression on other T cell subsets is upregulated upon activation(151). CTLA-4 is a CD28 homolog that directly competes with CD28 to bind to the identical ligands, B7-1 and B7-2, expressed by APCs but with higher avidity and affinity. As a result, CTLA-4 blocks CD28 co-stimulatory signals and attenuates T cell

proliferation and activation primarily mediated by PI3K and AKT(152). The CTLA-4 mediated Treg suppression is necessary to maintain immunologic tolerance. Regulatory T cells require CTLA-4 to downregulate the activation and proliferation of other T cell subsets by affecting antigen presentation via APCs(153). Also, remarkable outcomes were achieved by blocking CTLA-4 alone or in combination with PD-1/PD-L1 in certain types of cancer. As such, CTLA-4 blockade is now an established approach to treat certain cancers in humans(154).

1.5.4.3 2B4

2B4, also known as CD244, is an inhibitory receptor and member of the Signaling Lymphocyte Activation Molecule (SLAM) family. It is expressed by several immunocompetent cells, including different subsets of T cell (γδ T cells and αβ CD8+ and CD4+ T cells), innate immune cells like NK cells, DCs, and basophils, as well as MDCs. It is co-expressed with PD-1, LAG3, TIM3, and CD160 on activated T cells, and recently its signaling has been associated with exhaustion in specific T cells in chronic infection and TILs. Blocking CD244 is associated with enhanced cytokine production of specific CD8+ T cells in mice and humans, making it an exciting immunotherapy target(155, 156).

1.6 T cell Exhaustion

T cell exhaustion was first described in Lymphocytic choriomeningitis virus (LCMV) chronic infection and later in cancer as a distinct state of T cell differentiation. Under constant TCR stimulation and activation like what happens in the tumor microenvironment and chronic infection, T cells become functionally impaired and unable to clean the virus or eliminate the tumor cells. Exhausted T cells have several distinct characteristics, including progressive loss of functions, such as cytokine production, cytotoxicity, reduced proliferative potential, sustained upregulation of several inhibitory receptors, metabolic and gene expression alterations, and most importantly, failure to develop long-lasting memory cells(157-159). The development of T cell exhaustion is primarily due to chronic and constant antigenic stimulation. Chronic infection leads to sustain expression of inhibitory receptors and prolonged production of type I IFNs (IFN-α/β) that paradoxically upregulate expression and production of inhibitory molecules. Thus the continuous stimulation is believed to be the main driver of exhaustion and correlates with the severity of T cell dysfunction(158, 160).

The role of continuous stimulation has been confirmed by mouse models infected with LCMV acutely and chronically. In fact, by eliminating the persisting stimulation effector, T cells could differentiate into functional memory T cells(160). They also found that after about 4 weeks of constant stimulation, exhaustion becomes established, and the cells will not recover to normal effector T cells even in the absence of antigen stimulation. This so-called "steady-state" exhaustion is maintained by epigenetic changes that install an irreversible exhaustion-related epigenetic landscape(161). The epigenetic features of exhaustion are out of the scope of this thesis and will only be mentioned briefly later.

Notably, exhaustion should not be confused with self-tolerance and anergy. T cell tolerance to self-antigens is a regulatory setting of the immune system required to prevent autoimmunity. As mentioned in previous sections, self-tolerance is established during T cell development in the thymus through the negative selection of autoreactive T cells and is maintained in the periphery through negative signaling and regulatory T cells. Alternatively, lack of co-stimulatory signals during T cell priming results in anergy and T cell unresponsiveness. However, in the context of chronic infection and cancer, T cell exhaustion represents a state of impaired function mediated by excessive antigen stimulation and sustained inhibitory signals. These conditions might have shared characteristics, like hyporesponsiveness, but further studies have revealed that distinct cellular and molecular factors regulate them(162).

Upregulation and sustained expression of inhibitory receptors is a hallmark of T cell exhaustion. Ever-increasing PD-1 expression emerged as the major but not exclusive marker of exhaustion; however, the underlying mechanisms are still not entirely known. PD-1 can modulate exhaustion through several mechanisms, including negative regulation of T cell activation, attenuation of stimulatory signals, and induction of the inhibitory genes(163, 164).

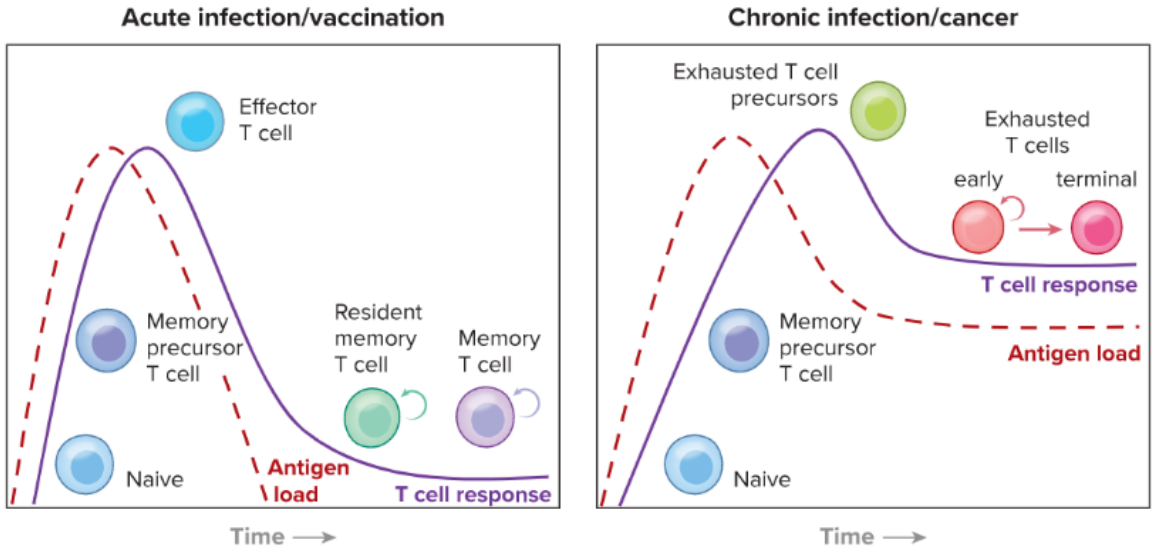

Figure 23 T cell exhaustion (Reproduces with permission from Annual Reviews(165))

It is important to notice that high expression of inhibitory receptors in a transient and regulated fashion represents normal effector T cells. In this notion, inhibitory receptors role as the TCR activation signal modulator and prevent T cell hyperactivation, activation-induced cell death, and immunopathology(101). Co-inhibitory receptors play a crucial role in restricting T cell activation and defects in their function lead to aberrant immune responses such as autoimmunity. However, modulating the inhibitory receptors during some acute infections suggest that inhibitory receptors may hinder effective immune responses and that blockade of negative regulatory pathways might improve immunity to pathogens. In contrast, disrupting inhibitory receptor pathways can also be harmful. For example, as mentioned before, although PD-1–deficient mice can more efficiently clear the pathogen, they also develop more severe pathology, likely due to an overaggressive adaptive immune response. Other studies showed that the absence or blockade of PD-L1 reduces early CD8+ T cell responses to virus in mice. In these examples, inhibitory receptors on innate immune cells, such as DCs and macrophages, play important roles in T cell activation and survival (166). Thus, tempering of T cell responses to prevent immunopathology and perhaps sustaining highly activated effector T cells by preventing activation-induced death or terminal differentiation is a beneficial aspect of inhibitory receptor pathways.

Exhausted T cell express several cell surface inhibitory receptors other that PD-1/PD-L1. PD-1 co-expression with other inhibitory receptors, including TIM3, LAG3, 2B4, and CTLA-4, has been documented as a feature of severe T cell exhaustion. The higher the number of inhibitory receptors

co-expressed by exhausted T cells, the worse the exhaustion is. These inhibitory receptors use a separate mechanisms to regulate T cells, and their role in exhaustion is not redundant. However, their co-expression is mechanically relevant and synergistically regulates T cell exhaustion. Thus there is a potential to reverse or tune the severity of exhaustion through modulating one or more of these receptors.

In addition to the negative regulation of inhibitory receptors, the lack of or impaired stimulatory signals are involved in T cell exhaustion(167). Other factors contributing to exhaustion include suppressive cytokines such as IL-10 and TGF-β, suppressive immune cells like MDSCs and Foxp3+ CD4+ Tregs during chronic infections or in the tumor microenvironment(164). This suggests not only a complex network of receptors and soluble molecules causing and regulating the T cell exhaustion but several potential candidates to reverse it.

Exhausted T cells are not inert and can proliferate, produce some effector molecules, and contain the infection. This notion has been confirmed by experiments illustrating that the depletion of exhausted T cells results in a fatal infection. However, blocking the inhibitory receptors and reinvigorating exhausted T cells during chronic viral infection leads to viral clearance(101).

Lineage tracking techniques revealed several distinct heterogeneous clusters of exhausted T cells shaping the larger pool of cells(101). Additional single-cell transcriptomic and mass cytometry profiling have revealed the progressive state of differentiation in exhaustion. Several subsets with varying levels of exhaustion have been recognized. Exhausted T cell pool includes (1) progenitor or stem cell-like cells with the most preserved function, (2) not terminally exhausted cells that are reversible(3) the fully established exhausted T cells with specific transcriptional and epigenetic signatures. Identifying the heterogeneous subsets of exhausted T cells is essential, considering that each subset reacts differently to immunotherapy, especially checkpoint blockade(168, 169). The most abundant subset determines the outcome of therapy. For instance, the PD-1^{Int} $CD44^{Hi}$ population was shown to respond strongly to the anti-PD1/PD-L1 therapy while the PD-1^{Hi} $CD44^{Int}$ were almost resistant to checkpoint therapy in mice LCMV chronic infection(170). As a result, the progenitor cell population is the main target of interventions to reverse the exhaustion.

Understanding gene regulation in exhausted T cells could help develop methods to reinvigorate them and improve the immune response in chronic infection and cancer. As mentioned, exhausted

CD8 T cells express a distinct molecular signature, yet there are several transcription factors and pathways with overlapping functions in effector/memory T cells(158). For instance, the T-box transcription factors (T-bet) and Eomesodermin (Eomes) control normal effector and memory differentiation in CD8 and CD4 T cells. However, T-bet/Eomes balance and the different expression levels of inhibitory receptors, especially PD-1, have been identified as exhaustion subpopulation markers. T-bet^{hi}PD-1^{int} CD8 T cells represent the progenitor T cell subset, which proliferates in response to persisting antigen and ultimately gives rise to Eomes^{hi} PD- 1^{hi} CD8 T cells, the terminally differentiated subset which cannot be reinvigorated(158, 160).

Accordingly, the progressive differentiation of progenitor pool leading to loss of function and increase of terminally differentiated T cells is thought to be one reason for losing immune control during chronic infections(168).

Studies focusing on network analysis of exhausted T cells have discovered valuable information regarding the involved pathways, transcription factors, and interactions. Performing whole-exome sequencing and RNA sequencing, identified T-bet, Eomes, Blimp1, IRF4, BATF1, and NFAT, as the transcription factors associated with the exhaustion phenotype. NFATC1, IRF4, BATF, and TOX upregulation are correlated with dysfunctional state acquisition. Blimp-1 is moderately expressed in normal effector T cells; however, in chronic LCMV infections, its upregulation promotes inhibitory receptors and repressing the memory differentiation genes(160).

Elevated levels of IRF4 have been associated with metabolic disruption and increased ROS production in exhausted T cells. Transcription factors interacting with TCR directly or indirectly, like NFAT and BATF1, are the most critical drivers of exhaustion. BATF1 functions as the negative regulator of AP1, a TCR signaling downstream transcription factor, and indirectly represses many T cell functions such as IFN-γ production. NFAT family members also get activated following TCR stimulation and upregulate several inhibitory receptor expressions, including PD-1, TIM3, and LAG3 on activated T cells(101, 171).

The overexpression of thymocyte selection-associated high mobility group-box protein (Tox) along with PD-1 enhanced expression is significantly correlated with exhaustion phenotype development and maintenance. TOX deficiency improves exhausted T cells' function and survival in mouse models in chronic viral infections and cancer (172).

Indeed, exhaustion can affect almost every aspect of the T cell signaling network, including the dramatic changes in TCR and cytokine signaling pathways, gene regulation, and transcription factors involved in T cells' metabolism, migration, and epigenetic state. Other metabolic changes in exhausted T cells include significant suppression of glycolysis, OXPHOS, glucose deprivation due to GLUT1 downregulation, and repression of mitochondrial biogenesis. Metabolic changes may compromise memory generation and long-term survival. Downregulation of mTOR or activation of the AMPK pathway is part of the metabolic dysregulation driven by PD-1. It can lead to the repression of the critical metabolic regulators, like the peroxisome proliferator-activated receptor γ (PPARγ) and coactivator 1α (PGC1α)(157, 168).

Overall, exhaustion compromises proper immune response in chronic infections and cancer and could induce immune surveillance evasion. However, it is partially reversible, and some early subsets could be reinvigorated via specific intervention. Hence, several studies are in progress to characterize exhausted cells and find approaches to reverse the dysfunctional state and reinvigorate immune responses.

1.6.1 Reversing the state of exhaustion

The dominant role of inhibitory receptors in T cell exhaustion led to the idea of checkpoint blockade therapy. Strategies to interrupt IRs signaling with monoclonal antibodies, generally known as checkpoint blockade, have revolutionized cancer immunotherapy(173). Blocking inhibitory receptors would enable exhausted T cells to overcome the negative regulatory mechanisms and restore some of their function, such as proliferation and cytokine production. Several inhibitory receptors are expressed on exhausted T cells, any of which could be a target of checkpoint blockade, theoretically. Blockade of CTLA-4 (ipilimumab) was the first checkpoint blockade approved by the FDA to treat advanced melanoma and was associated with increased overall survival. In clinical and preclinical studies, reduced viral load, tumor burden, and improved survival were reported following the PD-1/PD-L1 therapy. In melanoma, checkpoint therapy has enhanced the response rate up to 60%. Monoclonal antibodies blocking PD-1 (Pembrolizumab) and CTLA-4 (Ipilimumab) were FDA approved for melanoma a decade ago, and since there have been several additional blocking antibodies under study(174). Currently, ipilimumab (anti-CTLA-

4), pembrolizumab (anti-PD-1), and nivolumab (anti-PD-1) are a part of cancer patient treatment regimens(174, 175).

Therapeutic blockade of the PD-1 improves pathogen control and tumor elimination by enhancing the proliferation potential of specific T cells and the expression of effector molecules (perforin, granzymes, and cytokines).

Several new studies suggest the importance of CD28 co-stimulation signaling in responding to PD-1 checkpoint blockade appropriately. At the same time, CTLA-4 blockade induces tumor cell elimination through uninhibited positive CD28 co-stimulation and enhances anti-tumor immune response. Accordingly, combination therapies targeting both inhibitory and stimulatory receptors are being developed in cancer clinical trials. Combination of TNF receptor superfamily members like OX40 (CD134), CD27, and 4-1BB (CD137) agonists with PD-1 and CTLA-4 blockade have shown impressive results in resistance to checkpoint therapy(173, 176).

As mentioned before, the negative function of co-inhibitory receptors in T cell exhaustion is not redundant. Due to different timing and expression patterns, each receptor may impact different aspects and subpopulations of cells(164, 177). Consequently, exploring other potential checkpoints is in progress, and several new targets like TIM3, LAG3, TIGIT, and VISTA have been suggested. Additionally, targeting more than one inhibitory receptor with different signaling pathways or expressed by different types of cells is under progress in preclinical trial studies(178). For example, VISTA is expressed by M2 macrophages in PD-1/PD-L1 blocked patients and could be considered a secondary nonredundant checkpoint target(179). The combination of TIM3/ LAG3 with PD-1 and CTLA-4 has been examined, and less immune evasion and improved tumor cell-targeted cytotoxicity was achieved(177). However, our understanding of inhibitory receptors' biological role and signaling is limited, and a deeper understanding of their function is critical to prevent future therapy-related complications.

There are challenges in checkpoint therapy limiting its efficiency and applicability, including unpredictable response rates, treatment-induced alteration of the immune response, and immune-related adverse events (iRAE). iRAEs are the adverse side effects of checkpoint blocking that resemble autoimmune responses and affect the skin, gastrointestinal tract, lung, heart, and endocrine system. iRAE can be considered the off-target effect of blocking inhibitory receptors

like PD-1 and CTLA-4, which also play an essential role in immune homeostasis and self-tolerance. Adverse inflammatory events have been reported in 80% of small cell lung cancer patients following PD-1 single blockade and 90% after receiving the combination of CTLA-4 and PD-1 checkpoint blockade. However, most of these adverse effects are reversible and controlled by administrating immunosuppressives within a few weeks after the initiation(180).

Not all the patients respond positively to the checkpoint due to resistance to checkpoint blockade, and a significant portion of responders experience relapse and tumor progress in the long term. Resistance to checkpoint blockade involves complex mechanisms and could emerge at any stage of treatment. The overall response rate to PD-1/L1 checkpoint therapy has been reported at around 30%(181). In melanoma and prostate cancer, after CTLA-4 antibody treatment, tumor cells upregulated VISTA inhibitory receptors, leading to another pathway for inhibition of T cells(179). T cell exhaustion and dysfunction because of constitutive PD-L1 expression are significant factors contributing to PD-1/PD-L1 blockade resistance, leading to most patients redeveloping tumors and relapse. To overcome many of the resistance mechanisms to checkpoint therapy combinations of multiple checkpoint blocking antibodies together or with other molecules have been suggested and tested in preclinical studies(182). Blocking several inhibitory receptors like CTLA-4 and PD-1 has been tested in metastatic melanoma and significantly enhanced response rate and survival in patients with secondary resistance(183).

Studies in progress explore potential biomarkers present in the tumor microenvironment to predict the response rate and the best combination of checkpoint blockades to induce a proper immune response and achieve long-term survival. In general, the presence of targeted molecules on tumor cells and the tumor-infiltrating cells could manifest a responsive patient. However, secondary resistance to therapy and immune response evasion is still likely.

From the perspective of immunotherapy, other than blocking checkpoints, cytokine modulation like blocking IL-10 and TGF-β (immune response suppressing cytokines) or adding IL-2, IL-7, and IL-21 (T cell growth and activation inducer cytokines) would also help to improve the outcome in chronic infection and cancer. IL-2 administration was approved for metastatic renal cell carcinoma in 1992 and was associated with improved tumor cell elimination and controlled tumor

progress(184). Later, blocking IL-10 was shown to be useful at handling chronic LCMV and potential viral clearance in mice(185).

Taken together, our understanding of T-cell exhaustion is advancing at a rapid pace, and as our knowledge improves, novel, more efficient, and specific immunotherapies will be developed.

1.7 Adoptive Cell Therapy: Current challenges and opportunities

Immunotherapy harnesses the antitumor potential of immune response to eliminate tumor cells. Cancer immunotherapy has been successful in a subset of solid tumors and is developing to cover several different malignancies. There are multiple various types of immunotherapy available, including but not limited to monoclonal antibodies, cytokines, vaccines, and adoptive T cell transfer (ACT). In general, immunotherapy could be categorized into two major approaches: (1) strategies to boost or restore the endogenous immune response, including a wide range of therapies from conventional non-specific IL-2 therapy to FDA-approved immune checkpoint inhibitors (2) highly specific T-cell-based adoptive cell therapies (ACT) relying on T cell anti-cancer immune responses(176).

Adoptive transfer of autologous or allogeneic T cells to cancer patients has been a breakthrough in cancer therapy, leading to cancer immunotherapy. However, the immune response is relatively inefficient and fails to induce tumor rejection in most patients. Several reasons have been suggested so far, and there are still hurdles to address. Adoptive T cell therapy includes Tumor-infiltrating lymphocyte (TIL) treatment, endogenous specific T cell therapy, and genetically engineered cells like chimeric antigen receptor (CAR)-T cells and transgenic T cell receptor (TCR)-modified T cells(183).

1.7.1 Tumor-infiltrating lymphocytes (TILs) therapy

TILs-based therapy has been performed in melanoma for 3 decades with a current response rate of 50%, including 20% complete remission and 95% of the 20% with more than 5 years of survival rate(186). TILs therapy involves T cells' isolation from tumors, in vitro expansion with IL-2, and then infusion back to the patient. TILs are a heterogeneous population of cells with various

efficiency and specificities. Current efforts are focused on improving the final product and simplifying the protocols to isolate and expand the T cells (176).

Despite the outstanding results in selected melanoma patients, several impediments remain before TIL therapy can be applied routinely, such as the requirement for tumor tissue and methods to enrich tumor-reactive T cells. Alternatively, the transgenic transfer of tumor-specific TCR to a large number of T cells could be envisaged, but this is limited by the low number of so-called "public" neoantigens and the cell therapy infrastructure required for such advanced cell therapies(187). Of note, public neoantigens are characterized as immunogenic antigens shared in a subset of patients with a given cancer type(188).

1.7.2 Endogenous tumor antigen-specific T cell therapy

Antigen-specific T cell adoptive therapy has been used extensively and successfully following hematopoietic stem cell transplant (HSCT) to target refractory viral infections and post-transplant lymphoproliferative disease (PTLD)(189). Virus-specific T cell (VSTs) therapy offers to restore and accelerate immune response and control cytomegalovirus (CMV) and EBV infection after HSCT in 70%–90% of patients. More than 70% of the patients with EBV-associated PTLD showed complete remission with minimal toxicity following donor-derived EBV-specific T cell infusion. Additionally, VSTs are an alternative to conventional pharmacotherapies conferring protection in drug-resistant patients(190).

Success in the management of refractory infections has inspired the research beyond the context of post-transplant complications. Virus-derived antigens represent optimal targets for a T-cell immune response, as they are usually immunogenic and widely expressed on the tumor cells. 15 percent of all human cancers worldwide may be attributed to viruses. EBV is associated with ~40% of Hodgkin (HL) and non-Hodgkin lymphoma (NHL) in immunocompetent hosts and 95% of cases of nasopharyngeal cancer (NPC). Human papillomavirus (HPV) antigens have also been validated recently as targets in HPV-associated malignancies(190, 191). Endogenous tumor-reactive T cells can be detected in the peripheral blood of patients, isolated and expanded while retaining anti-tumor activity. Several tumor-associated antigens (TAAs), tumor germline antigens, and tissue differentiation antigens are tested in clinical trials. Specifically, specific T-cell therapy enables targeting a vast array of antigenic targets and broadens the applicability of T-cell therapy.

There are two approaches to generate specific T cells for ACT (1) direct selection of specific T cells from peripheral blood and (2) *ex vivo* expansion of specific T cells through multiple stimulations.

In direct selection, rare specific T cell precursors in the peripheral blood of patients are selected and isolated using HLA-peptide multimers or IFN-γ capture assays and then expanded while preserving activity and specificity to transfer to patients. Compared to nonspecific lymphocyte transfer, direct selection and isolation of specific T cells have eliminated GvHD risk and have shown a higher response rate. However, direct selection and isolation are challenging when a few number of specific circulating T cells are present or difficult to access. It needs large amounts of PBMC (harvested through apheresis)and has mainly been used for viral antigens(192).

The field has evolved rapidly, and specific T cell transfer has been more efficient than conventional cancer therapies in some early clinical trials due to their specific and accelerated immune response(193). Additionally, in contrast to TIL therapy, *ex vivo* specific T cell expansion does not require TIL-rich tumor biopsies, which may not be present or difficult to access surgically. However, challenges, such as the size of the repertoire, the amount of required stimulation, and the lack of durable response, may decrease therapy efficiency. Also, most manufacturing methodologies to generate and expand specific T-cells rely on repetitive antigenic stimulation during *ex vivo* cultures that could cause terminal T cell differentiation and exhaustion. Another problem with specific T cells is the loss of targeted antigen, a common mechanism of immune response evasion by tumor cells. Studies to improve these protocols and develop early-stage specific T-cell products that persist long-term and show enhanced function are ongoing(194).

1.7.3 Genetically engineered T cells

Gengineered T cells could be designed to express high-affinity TCRs with the possibility of inducing an efficient immune response targeting almost any protein-peptide with a known TCR sequence(195). However, TCR recognizes antigens through HLA molecules and in a co-stimulatory signals-dependent manner, which are downregulated by most tumor cells. To overcome these limits, chimeric antigen receptor (CAR)-T cells that are HLA-independent and can directly target the cell surface molecules were developed. CAR-T cells recognize antigens by a specific cell surface receptor consist of an extracellular domain linked to intracellular signaling

motifs. The extracellular domain is mostly the antibody single-chain variable fragment (scFv) specific to a particular surface antigen. This structure offers many combinations, such as adding multiple co-signaling receptor motifs, checkpoint blockers, or any positive regulator of T cell activation(176, 196).

Despite rapid advances in gene editing and T cell biology, CAR-T cells at this time have only been shown to be active against a minority of cancers (mostly of B-cell origin)(197). Relapses and toxicities due to downregulation of targeted antigen, off-tumor cytotoxicity, and the CAR-T cell disappearance due to low antigen presentation and activation signals are the significant drawbacks of CAR-T cell therapy. Later studies in patients who experienced relapse revealed that CAR-T cells could also go through exhaustion and dysfunctional CAR-T cells which may lead to their loss and relapse and tumor progress(198).

Overall, many challenges limit the therapeutic efficacy of CAR-T cells in solid tumors and hematological malignancies. As mentioned, loss of antigen is the main cause of relapse because it can lead to poor antigen recognition, loss of activation and survival signals, and in conclusion the CAR-T cell disappearance.

1.7.4 Limitations and strategies to improve adoptive cell therapy

Varying response rates among the patients, even with the same type of tumor, are challenging immunotherapy. Positive results are still limited to certain human malignancies, and ACT needs improvement (173, 198). The dynamic and constantly evolving and changing nature of the immune responses in each patient could affect the outcome of adoptive cell therapy. Considering the complicated interactions of various immune system components, particularly the priming, activation, cytotoxic activity, and regulation of antitumor immune responses, the need to understand the downstream mechanisms and involved pathways is required more than ever.

Challenges with high priority remain the (1) specificity of the therapy to avoid or minimize toxicity, (2) the primary and adaptive resistance to immune-based therapies, and (3) the identification of the optimal combination therapies. Also, the results could be further improved if the trafficking, function, and persistence of transferred T cells could be enhanced. In this regard, combinatorial ACT strategies to manufacture the most potent T cell product that efficiently target

tumor cells and evades the resistance/escape mechanisms are arguably the future of immunotherapy. Comprehensive monitoring of ACT products and trials in terms of both function and phenotype before and after infusion will chaperon the development of optimal combination therapies.

1.7.4.1 Survival and expansion

The ability to persist and exert effector function against tumor cells is central to successful immunotherapy. Several studies have confirmed the correlation between immunotherapy outcomes and the long-term persistence of the infused TILs or genetically engineered T cells(173, 176). Likewise, further studies in patients with relapse revealed poor T cell expansion in TME, which was also unable to eliminate tumor cells, especially in solid tumors(199, 200). Using T cells that are not fully differentiated effectors in melanoma patients was associated with long-term remission and objective response(201). Animal studies comparing the naïve/memory T cells to more differentiated T cell subsets also revealed superior *in vivo* expansion, persistence, and the antitumor response of the early differentiated cells. Strategies to enhance T cells' persistence and enrich the adoptive cell therapy products with the stem cell-like cells (Tscm) have been successfully tested in mouse models and await to be confirmed by clinical trials. *In vitro* production of T cells involves several factors such as the cell source, the target antigen, culture duration, and choice of cytokines; manipulating each element could impact the product's characteristics toward the improvement(202). Shortening T cell expansion protocols and optimizing them with cytokines like exposure to IL-2, IL-7, IL15, and modulating some pathways related to generate early differentiated T cells have been proposed by several groups. Additionally, enriching the final product to infuse the early stage-specific cells would enhance the long-term persistence and likelihood of tumor elimination(203).

1.7.4.2 Immune response evasion and resistance

Tumor cells develop and utilize multiple pathways to avoid immune response and develop resistance resulting in relapse or therapy failure. Immune response evasion and immune resistance are the key factors responsible for limiting overall survival rates in patients treated with

immunotherapy. These mechanisms are widely heterogeneous and vary between tumor types but may differ in patients with the same kind of malignancy.

Resistance to immune-based therapies includes (1) primary and (2) acquired mechanisms. Primary or intrinsic resistance represents the condition that patients never demonstrate a clinical response or shows signs of resistance immediately after treatment. In contrast, acquired resistance is a clinical state in which patients initially respond to immunotherapy but after weeks or months tumor relapses and progresses. Primary and acquired immune resistance driving factors could be either tumor cell-intrinsic or tumor cell-extrinsic(204).

Notably, secondary immune resistance is mainly rooted in initial immune resistance, and these mechanisms may evolve at later times following the immunotherapy, highlighting secondary resistance. Also, T cell function and phenotype could change through the course of therapy because of exhaustion or suppressive tumor microenvironment, so their killing activity and cytokine production may decline. In this scenario, a patient who responded to treatment at first possibly will develop a tumor and relapse(182, 204).

Several tumor intrinsic pathways are associated with primary resistance and are briefly mentioned in the table below. Defective antigen processing pathway, loss of antigen presentation by tumor cells, and modifications or loss of tumor cell surface antigens are the most common failure causes of specific T cell adoptive therapy. TAP deficiency has been associated with tumor progression in many cancers in preclinical and clinical studies. Enhanced PI3K pathways could result in HLA molecule downregulation and tumor cell escape from T cell recognition. Multiple clinical and preclinical studies suggest tumor-induced changes in antigen presentation machinery such as loss of B2microglobolin (B2M), downregulation of HLA molecule, and antigen loss evade immune cell recognition and response. As a result, identifying a tumor antigen to enhance specificity and efficiency but minimize immune escape and toxicity has been the biggest challenge in specific T cell-based therapies.

Targeting more than one antigen has been effective in CAR-T cells to overcome immune escape Ruella et al. (205). However, to reduce the immune escape chance, identifying the proper antigen to target has been suggested which could lead to induce specific antitumor immune responses without off-target toxicity. In-depth characterization of each tumor would result in one or even

more specific antigens to target; however, these experiments could be costly, time-consuming, and require several evaluations before being confirmed for immunotherapy.

	Mechanism	Examples
tumor cell intrinsic	absence of antigenic proteins	low mutational burden lack of viral antigens lack of cancer-testis antigens overlapping surface proteins
	absence of antigen presentation	deletion in TAP deletion in B2M silenced HLA
	genetic T cell exclusion	MAPK oncogenic signaling stabilized b-catenin mesenchymal transcriptome oncogenic PD-L1 expression
	insensibility to T cells	mutations in interferon gamma pathway signaling
tumor cell extrinsic	absence of T cells	lack of T cells with tumor antigen-specific TCRs
	inhibitory immune checkpoints	VISTA, LAG-3, TIM-3
	immunosuppressive cells	TAMs, Tregs

Figure 24 Tumor cell intrinsic and extrinsic mechanisms of checkpoint resistance
(Reproduced with permission from: Cell(206))

1.8 Rationale

Many strategies to improve immunotherapy responses have been proposed and are under evaluation. Accordingly, to develop new approaches and extend the benefit beyond a minority of patients and cancer types, the combination of the most successful therapies such as checkpoint blockade and T cell-based therapies has been contemplated.

The concept of combination strategies is not new to adoptive cellular therapy; for instance, the response rate of ipilimumab (anti-CTLA-4) combined with TIL therapy was 38.5%, and the median progression-free survival duration was 7.3 months in one study with 13 patients(207).

Another possible strategy to improve ACT outcome is to overcome T cell exhaustion during *ex vivo* expansion or within TME. With advances in gene editing technologies, it is now possible to delete inhibitory receptors or other negative T cell activation and function regulators before infusion. In this regard, a clinical trial in melanoma tests the impact of deleting both PD-1 and endogenous TCR in NY-ESO-specific-T-cells long term persistence and preventing exhaustion. However, deleting PD-1 has been revealed to be associated with even more severe exhaustion in mouse models, probably due to its function in modulating T cell activation signals and preventing overstimulation. So, more investigations are needed before such approaches become routinely used clinically (208).

In this regard, as discussed, PD-1/PD-L1 and TIM3 checkpoint therapies have shown some outstanding results in various cancer types in preclinical settings. Combination strategies involving PD-1 blockade have shown beneficial anti-tumor response(209). Although not yet validated clinically, TIM3 inhibition is a prime candidate to synergize with PD-1. As for PD-1, it is expressed on activated and severely exhausted CD8+ T cells. Consequently, their combined blockade might lead to some synergy in modulating the exhaustion and not only additive impacts.

Several significant molecules and receptors can be modulated to improve the likelihood of antigen recognition and TCR signal strength. These include inhibitory receptors present at the IS, specifically those regulating T cell priming. CD5, a negative regulator of TCR, is a part of T cell priming signaling and roles as a regulator of TCR sensitivity to antigens. Moreover, its high expression correlates with TCR's enhanced tolerance to self-antigens and boosted affinity to its specific antigen. As a result, its expression could be a predictive marker and modulator of TCR avidity. Thus, it is reasonable to assume that deleting CD5 could improve specific T cell priming and specific T cell generation.

Utilizing antigen-specific T cells as therapeutic tools is now one of the dominant approaches of adoptive cell therapy. *Ex vivo* specific T cell expansion using synthetic peptides or peptide-pulsed dendritic cells as APCs cast a broader net in prompting rare antigen-specific T cells in peripheral

blood (210). However, complications such as prolonged *in vitro* culture impact the phenotype and function of the specific T cells by pushing the cells toward exhaustion and terminal effector differentiation. Accordingly, terminally differentiated T cells show loss of function and therapeutic capacity following the transfer. There is now evidence that the infusion of cells with early differentiation characteristics results in objective responses and correlates with enhanced T cell engraftment, expansion, long-term persistence, and remission(195).

These provide the rationale to define relevant T cell generating protocols to enrich for a population of highly persistent T cells and identifying features that can be altered to develop the large numbers of non-exhausted, highly persistent, and functional specific T cells that can persist/expand *in vivo* long enough to eradicate established tumor and mediate long-term immunoprotection.

1.9 Hypothesis and Objectives

As discussed, it is challenging to produce the sufficient number of tumor-specific T cells with available *ex vivo* expansion protocols without compromising the quality and function of T cells or inducing exhaustion. In this study, we hypothesized that

Immune checkpoint blockade during *ex vivo* expansion would facilitate the efficient generation of specific T cells and improve the yield and function of human antigen-specific T cells by limiting the exhaustion phenotype.

1.9.1 General objective

This study aims to discover the combination of checkpoint blockade that improves antigen-specific T-cell development strategy for adoptive therapy, especially cancer. In this regard, we will **seek to combine the PD-L1 and TIM3 checkpoint blockade with *ex vivo* specific T cell expansion protocol targeting viral and tumor-associated antigens**. Another purpose of this study is to identify and evaluate novel inhibitory receptors as checkpoint blockade therapy candidates, especially in the ACT. In this regard, we will focus on CD5, which is present on all T cells, and for T cell with mounting evidence that it acts as a genuine immune checkpoint in pre-clinical models.

1.9.2 Specific Objectives

1. **To assess the impact of PD-L1/PD-1 and/or TIM3 blockade during *ex vivo* generation of antigen-specific T cells.** To do this, we adapted our clinically compliant protocol to generate antigen-specific T cells and added a step to treat the cells with checkpoint blockade antibodies. Using this model, we evaluate various checkpoint blockade combinations and immunophenotype cells at different time points. We aim to find the most efficient inhibitory receptor blockade combination to enrich non-differentiated T cells.

2. **Develop and characterize CD5 deleted antigen-specific T cells.** The CD5 inhibitory receptor will be deleted via CRISPR/Cas9 and tested in several settings, including in culture protocols aimed at expanding antigen-specific T cells.

Chapter Two

2. Combined PD-L1 and TIM-3 blockade improves the expansion of fit human CD8+ antigen-specific T cells for adoptive immunotherapy

Shirin Lak1, Valérie Janelle1, Anissa Djedid2, Gabrielle Boudreau1, Ann Brasey1,3, Véronique Lisi2, Cédric Carli1, Lambert Busque1,3,4,5, Vincent-Philippe Lavallée2,6,7, Jean-Sébastien Delisle1,4,5

1 Centre de recherche de l'Hôpital Maisonneuve-Rosemont, Montréal, Qc, Canada

2 Centre de recherche du CHU Sainte-Justine, Montréal, Qc, Canada

3 Biomarker Unit, Centre C3i, Montréal, Qc, Canada

4 Department of Medicine, Université de Montréal, Montréal, Qc, Canada

5 Hematology-Oncology and Cell therapy Division, Hôpital Maisonneuve-Rosemont, Montréal, Qc, Canada

6 Department of Pediatrics, Université de Montréal, Montréal, Qc, Canada

7 Hematology-Oncology Division, CHU Sainte-Justine CHU Sainte-Justine

Correspondence to:

Jean-Sébastien Delisle MD, FRCPC, PhD

Centre de recherche de l'Hôpital Maisonneuve-Rosemont

5415, Boul de L'Assomption

Montréal, Qc H1T 2M4

Canada

js.delisle@umontreal.ca

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2.1 Author's Contribution

SL: Study design, conducting all experiments except sequencing experiments, data analysis and discussion, preparation of all main and supplementary figures (except Figure 5, 6, and S3), and writing of the first draft of the manuscript. 65% of the total effort.

VJ: Contribution to the analysis of VDJ, scRNA-Sequencing experiments, preparation of figures (5,6 and S3), and all the tables

AD: Conducting and analysis of VDJ, scRNA-Sequencing experiments, preparation of figures (5,6 and S3), and all the tables

GB: Statistical analysis

AB: Conducting the VDJ short version sequencing experiment

VL: Assisting in the analysis of VDJ, scRNA-Sequencing experiments

CC: Study design and technical assistance in the cell cultures

LB: Sequencing experiments design

VPL: Study design, writing of some sections related to sequencing, general discussion, and revision

JS: Study design, data analysis, general discussion, and manuscript writing

2.2 Declarations

Ethics approval and consent to participate. Informed consent was obtained from all cell donors as detailed in the Method section.

Consent for publication. All authors have reviewed the final version and agreed with the submission. The corresponding author agrees to pay the publication charges.

Availability of data and material. As detailed in the Methods section, the genomic data have been deposited for public access and agree to share protocols. All materials used are commercially available.

Competing interest. The authors have no relevant conflict of interest to disclose.

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Authors' contribution. SL and JSD conceived the study. SL, VJ, AB, CC performed experiments. SL, VJ, AB, AD, GB, VL analyzed data. LB, VPL, JSD oversaw data analysis. SL, VJ and JSD wrote the manuscript. All authors reviewed the manuscript.

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2.3 List of Abbreviations

ACT	Adoptive Cell Therapy
APC	Antigen Presenting Cell
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
CDR	Complementarity Determining Region
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T-lymphocytes
CTV	Cell Trace Violet
CTY	Cell Trace Yellow
DC	Dendritic Cell
DMSO	Dimethylsulfoxide
EBV	Epstein-Barr virus
ELISPOT	Enzyme-linked mmune absorbent spot
FBS	Fetal Bovine Serum
FOXP3	Forkhead Box P3
Gal-9	Galectin-9
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GrzB	GranzymeB
HLA	Human Leukocyte Antigen
HMGB1	High Mobility Group Protein B1
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
KLRG1	Killer cell Lectin-like Receptor G1
LAG-3	Lymphocyte-Activation Gene 3
LRSC	Leuko Reduction System Chamber
mAB	Monoclonal Antibody
MHC	Major Histocompatibility Complex
NGS	Next-generation Sequencing
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered saline
PD-1	Programmed Cell Death-1
PD-L1	Programmed Death Ligand-1
PGE2	Prostaglandin E2
PHA	T-cell mitogen phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PS	Phosphatidyl Serin
RT	Room Temperature
SC-RNA	Singel Cell RNA
TAA	Tumor-Associated Antigen
TAP	Transporter associated with Antigen Processing
Tcm	Central Memory T cell
TCR	T-cell Receptor
Teff	Effector T cell
Tem	Effector Memory T cell

TGF-β	Transforming Growth Factor-β
TIM-3	T-cell Immunoglobulin and Mucin-domain Containing-3
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor Necrosis Factor
TSA	Tumor-Specific Antigen
Tscm	Stem-cell Like memory T cells
WT1	Wilms' tumor suppressor gene1

2.4 Abstract (350 words)

Background. The stimulation and expansion of antigen-specific T cells ex vivo enables the targeting of a multitude of cancer antigens. However, clinical scale T-cell expansion from rare precursors requires repeated stimulations ex vivo leading to T-cell terminal effector differentiation and exhaustion that adversely impact therapeutic potential. We leveraged immune checkpoint blockade relevant to antigen-specific CD8+ human T cells to improve the expansion and function of T cells targeting clinically relevant antigens.

Methods. A clinically-compliant protocol relying on peptide-pulsed monocyte-derived dendritic cells and cytokines was used to expand antigen-specific CD8+ targeting the oncogenic Epstein-Barr virus (EBV) and the tumor associated antigen (TAA) Wilms Tumor 1 (WT1) protein. The effects of antibody-mediated blockade of immune checkpoints applied to the cultures (T-cell expansion, phenotypes and function) were assessed at various time points. Genomic studies including single cell RNA (scRNA) sequencing and T-cell receptor sequencing were performed on EBV-specific T cells to inform about the impact of immune checkpoint blockade on the clonal distribution and gene expression of the expanded T cells.

Results. Several immune checkpoints were expressed early by ex vivo expanded antigen-specific CD8+ T cells, including PD-1 and TIM-3 with co-expression matching evidence of T-cell dysfunction as the cultures progressed. The introduction of anti-PD-L1 (expressed by the dendritic cells) and anti-TIM-3 antibodies in combination (but not individually) to the culture led to markedly improved antigen-specific T-cell expansion based on cell counts, fluorescent multimer staining and functional tests. This was not associated with evidence of T-cell dysfunction when compared to T cells expanded without immune checkpoint blockade. Genomics studies largely confirmed these results, showing that double blockade does not impart specific transcriptional programs or patterns on TCR repertoires. However, our data indicate that combined blockade may nonetheless alter gene expression in a minority of clonotypes and have donor-specific impacts.

Conclusions. The manufacturing of antigen-specific CD8+ T cells can be improved in terms of yield and functionality using blockade of TIM-3 and the PD-L1/PD-1 axis in combination. Overcoming the deleterious effects of multiple antigenic stimulations through PD-L1/TIM-3 blockade is a readily applicable approach for several adoptive-immunotherapy strategies.

2.5 Background

The vast majority of potentially actionable cancer antigens are major histocompatibility (MHC)-bound peptides. These include tumor-specific antigens (TSA) resulting from mutated proteins or aberrantly expressed regions of the genome, tumor associated antigens (TAA) that come from abnormally expressed self-proteins and finally, non-self peptides best described in the setting of virus-associated cancers or allogeneic stem cell transplantation1 2. While it has been possible to isolate TSA or TAA-specific T-cell receptors (TCR) and manufacture TCR-transgenic T cells for adoptive immunotherapy, this approach faces numerous technical hurdles and is currently available only for a minority of relevant cancer antigens3. Several effective adoptive immunotherapy strategies rely on the ex vivo expansion of native antigen-specific T cells, enabling the targeting of a vast array of antigens. However, the expansion of large numbers of antigen-specific T cells requires repeated antigen exposure (through co-culture with antigen-presenting cells – APC) and stimulatory cytokines, potentially leading to T-cell dysfunction (terminal effector differentiation and exhaustion) and poor performance following adoptive transfer4-6.

Alternatively, the endogenous cancer-reactive T-cell repertoire can be mobilized through the systemic administration of antibodies that prevent signaling from inhibitory co-signaling receptors present on the surface of exhausted T cells7 8. Such "immune-checkpoint" blockade, targeting most commonly Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and Programmed death-1 (PD-1) on T cells (or its corresponding primary ligand, Programmed death-ligand 1 - PD-L1), is now the cornerstone of therapeutic regimes against several types of neoplasia including advanced melanoma and lung cancer9. As dysfunctional cancer-reactive T cells often express multiple negative co-signaling molecules, a strategy has been to use combined approaches with the caveat that increased response may come with more immune-related toxicities10. Ex vivo expanded T cells express inhibitory receptors, and PD-L1/PD-L2 silenced antigen-presenting dendritic cells have been shown to improve the expansion and function of antigen-specific T cells ex vivo11, thus providing a solid rationale to leverage immune checkpoint blockade to improve T-cell manufacturing for adoptive immunotherapy.

We show herein that the ex vivo expansion of CD8+ T cells specific for a viral antigen and a TAA is enhanced by using combined PD-L1 and T-cell immunoglobulin and mucin-containing protein-3 (TIM-3) blockade, while single blockade or either receptor fails to improve T-cell expansion. The generated antigen-specific T cells did not show phenotypic and functional evidence of exhaustion or terminal effector differentiation. This was corroborated with single-cell RNA (sc-RNA) and VD(J) sequencing, which revealed that dual immune checkpoint blockade can impact T-cell gene expression in a donor and clonotype-dependent fashion. Taken together, our results show that dual PD-L1/TIM-3 blockade during ex vivo expansion can yield large quantities of fit human antigen-specific T cells for adoptive immunotherapy.

2.6 Methods

2.6.1 Donors and cellular procurement

Peripheral blood mononuclear cells (PBMCs) from HLA-A0201 expressing volunteer donors were isolated using Ficoll-Hypaque gradient (STEMCELL Technologies) from fresh whole blood (collected by venipuncture) or leukoreduction system chambers provided by Héma-Québec (LRSCs) as previously described12 13. All donors provided written informed consent, and all the experiments were approved by the Héma-Québec and Hôpital Maisonneuve-Rosemont Research Ethics Committees. Recovered PBMCs were either used immediately for experiments or resuspended in freezing media (90% fetal bovine serum and 10% dimethyl sulfoxide (DMSO)), transferred to Mr. Frosty containers (NALGENETM Cryo 1ºC freezing container), and stored in vapor-phase liquid nitrogen for future use.

2.6.2 Dendritic Cells (DCs) differentiation and antigen pulsing

Monocyte isolation and DC differentiation have been generated as previously described4. Briefly, monocytes were obtained using the adherence method whereby PBMCs were plated in adherent plastic plates (Sarstedt) in media (X-Vivo 15 medium (LONZA) supplemented with 5% human serum, 2 mM L-glutamine and 1mM Sodium Pyruvate (Gibco), 1000 U/ml (100 ng/ml) IL-4 and 800 U/ml (50ng/ml) GM-CSF (Both from STEMCELL technologies)) and incubated in a CO2, 37°C incubator for 7 days. On day 4, media was replaced with fresh media supplemented with IL-4 and GM-CSF. On day 7, DCs were matured with maturation media containing 1000 U/ml (100 ng/ml) IL-4, 800 U/ml (50ng/ml) GM-CSF (Both from STEMCELL technologies), 10 ng/ml TNF-

α (STEMCELL technologies), 1 μg/ml PGE2 (SIGMA), 10 ng/ml IL-1β (Feldan), 100 ng/ml IL-6 (Miltenyi Biotec) and loaded with desired peptide (1 μg/mL LMP2426-434 (CLGGLLTMV) or 1 μg/mL WT137-45 (VLDFAPPGA), both from JPT Peptides. Lastly, DC media was supplemented with IFN-γ 1000U/ml (Feldan) for the last 24 hours of culture.

2.6.3 Ex Vivo expansion of antigen-specific T cells

Antigen-specific T cells were stimulated using a clinically compliant protocol as previously described (4 and www.clinicaltrials.gov NCT03091933) from 15x106 PBMCs and expanded through multiple weekly stimulations using irradiated (40 Gy) autologous, peptide-loaded monocyte-derived DCs at a 1:10 (DC:PBMC) in a G-Rex6® Well Plate vessel (Wilson Wolf Manufacturing, New Brighton, MN). Our complete T-lymphocyte culture (CTL) media (Advanced RPMI 1640, 10% human serum, 1X L-glutamine(Gibco)) was supplemented with the following cytokines; week 1: IL-21 (30 ng/mL) and IL-12 (10 ng/ mL) (Both from Feldan), week 2: IL-21, IL-2 (100 IU/mL), IL-7 (10 ng/mL) and IL-15 (5 ng/mL) (STEMCELL Technologies), subsequent weeks: IL-2, IL-7 and IL-15. Medium, including cytokines, was refreshed every 3 to 4 days, and specific T cell cultures were restimulated once every week with peptide-loaded monocyte-derived DCs (moDCs). Cell concentration was adjusted to 1:10 ratio each week. When indicated, cultures were supplemented with 20 μg/ml of anti-PD-L1 blocking mAb (BioXcell, 29E.2A3) or/and 10 μg/ml of anti-TIM3 blocking mAb (Biolegend, F38-2E2) with all media changes. Respective isotype antibodies, mouse IgG1 (BioXcell, MOPC-21) and InVivoMAb mouse IgG2b (BioXcell, MPC-11), were used at the same concentration where indicated. All cell cultures were performed in monitored incubators (37°C in 5% CO2 and 5% air humidity). Cell viability and cell counts were assessed by the countess automated cell counter (Invitrogen) using trypan blue (Invitrogen) at a 1:1 ratio with the cellular suspension in cell counting chamber slides (Invitrogen-C10283).

2.6.4 Flow cytometry

The phenotype of mature DCs was assessed by cell surface expression of the following markers (antibody clone in parenthesis) CD80 (L307.4), CD86 (2331, FUN1), HLA-ABC (W6-32), CD11c (3.9), CD19 (HIB19) (all from BD Biosciences)), HLA-DR (LN3), and CD83 (HB15e) (both from Invitrogen). To detect antigen-specific CD8+ T cells, up to 106 cells were suspended in phosphate-buffered saline (PBS) plus 2% fetal bovine serum (FBS) and stained with allophycocyanin (APC)

labeled tetramers, MHC-Dextramers A*0201 (Immudex,), for 10 minutes in the dark at 4oC. For additional cell surface markers and phenotyping, cells were stained with following antibodies: CD3 (SKY7), CD3 (UCHT1), CD8 (SK1), CD45RO (UCHL1), CD45RA (5H9), CCR7 (150503), LAG3 (T47.530), and CD4 (RPA-T4) all from BD Biosciences; CD62L (DREG-56), TIM3 (F38-2E2), PD-1 (EH12.2H7), KLRG1 (2F1/KLRG1), CD57 (HCD57), and PD-L1 (29E.2A3) from Biolegend and CD8 (RPA-T8) and CD244 (eBioDM244) from Invitrogen. Staining was performed at room temperature (RT) in the dark for 30 minutes. According to the manufacturer's instructions for intracellular detection of cytokines, cells were permeabilized, fixed and stained using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience). Before the fixation step, up to 106 cells were stimulated to produce cytokines with the indicated peptide (0.5 μg/ml) (test condition) or PMA (50 ng/mL)-ionomycin (500 ng/mL) (Sigma-Aldrich) (positive control) and an irrelevant peptide (HLA-A0201 restricted but not used as a stimulator in the culture-negative control) for 4 h at 37°C. Cells are suspended in CTL media plus brefeldin A (Biolegend) to block the secretion of cytokines during the stimulation period. Cells were then harvested and stained for cell-surface markers, including CD3, CD4, and CD8 in 4 oC for 20 minutes. Next, fixed and permeabilized cells were stained with intracellular cytokine detection antibodies; IFN-γ (4S.B3), IL-2 (MQ1-17H12), and TNF-α (Mab11) all purchased from BD Biosciences) and Ki67 (ki-67) from Biolegend at RT for 20 minutes. Cell acquisition was performed on a Fortessa or LSR II flow cytometer (BD Biosciences), and data were analyzed with Flowlogic Software (inivai).

2.6.5 IFN-γ Enzyme-linked immune-spot (ELISpot)

ELISPOT assays were performed using Human IFN- ELISpotPLUS kit (MABTECH), following the manufacturer's instruction. Briefly, pre-coated wells were washed 5 times with sterile PBS (200ul/well), then incubated with CTL media (100 ul/well) for 30 minutes in a 37 oC incubator with 5% CO2. Cultured cells were added (5×104) to wells in duplicates and then stimulated with anti-CD3 mAb (positive control), irrelevant peptide (specificity control), and test peptide. After an 18-hour incubation the plates were washed with PBS (200ul/well) 5 times and incubated with detection antibody (7-B6-1-biotin, 1ug/ml, 100 ul/well) for 2 hrs at RT. Next, plates were washed with PBS (200ul/well) 5 times and incubated with streptavidin-HRP (100 ul/well) for 1 hr at room temperature. Lastly, plates were rewashed as above, and TMB substrate solution (100 ul/well) was added in darkness. Color development was stopped by washing extensively in deionized water

after distinct spots emerged. Plates were dried, and the dots were counted using ELISpot reader (vSpot Reader Spectrum, AID).

2.6.6 Flow Cytometry-based Cytotoxicity assay

Flow cytometry-based cytotoxicity assay was performed using either Cell Trace Violet (CTV) or Cell Trace Yellow (CTY) (Invitrogen Life Technologies) labeled LMP2426-434 pulsed as target cells as described before14. Briefly, target cells were prepared by stimulating autologous PBMCs with the T-cell mitogen phytohemagglutinin (PHA) (3 × 106 PBMCs/mL were incubated in T-cell media with 20 μg/mL PHA) for 3 days at 37 oC and 5% CO2. Peptide pulsed (or not pulsed) target cells were co-cultured with LMP2-specific T cells at various ratios for 4 hours in CTL plus 10% horse serum. Unpulsed target cells alone were used as control. After incubation, cells were harvested, stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Life Technologies) according to manufacturers' instructions and viable target, cells were quantified by flow cytometry using Flow Count Beads (Beckman Coulter). Cytotoxicity was calculated by comparing the percentage of viable target cells in test conditions relative to control (100 - (Target cell alive/ Target cell alone) × 100).

2.6.7 Single-cell RNA sequencing and High-throughput TCR sequencing

At indicated time points, T cells were sorted based on MHC-Dextramer and CD8 staining using FACS Aria III (DB Biosciences). Post sorted purity of the sorted population was 94%. A fraction of the sorted antigen-specific T cells was subjected to both VDJ and transcriptome sc-RNA sequencing at the Genome Quebec facilities. Briefly, cells were counted and viability assessed using a hemocytometer and Trypan Blue. The targeted cell recovery was set at 6000 cells and libraries were prepared using the kits Chromium Next GEM Single Cell 5' Kit v2 and Chromium Single Cell Human TCR Amplification Kit (10x Genomics inc.), as per the manufacturer’s recommendations. Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). Average size fragment was determined using a LabChip GX (PerkinElmer) instrument. The 10X Single cells 5’ libraries were sequenced on Illumina HiSeq 4000 PE28x98 while the 10X Single Cells VDJ (Human T) libraries were sequenced on Illumina HiSeq 4000 PE150. The Illumina control software was HCS HD 3.4.0.38,

the real-time analysis program was RTA v. 2.7.7. bcl2fastq2 v2.20 was then used to demultiplex samples and generate fastq files. Reads were aligned to GRCh38 genome assembly, gene expression matrices generated, and clonotype identification performed using CellRanger v3.0.2. The resulting gene expression matrices were normalised by total UMI counts per cell multiplied by the median UMI count per cell and natural-log transformed using Scanpy v1.415. PhenoGraph16 clustering was applied with k=20 and clusters with a median mitochondrial fraction greater than 0.2 were filtered out. Clonotypes were defined by nucleotide sequences of alpha and beta chains. In order to remove potential doublets, we restricted our analysis to cells in which exactly one alpha and one beta chain were identified and retained only the most abundant clonotype for each alpha and beta chain. The number of retained cells per sample is provided in Table S2. After filtering, individual count matrices were combined and normalized and log transformed together for downstream analyses.

For expression analysis, only abundant clonotypes in each donor were retained. Abundant clonotypes are defined as those identified in at least 10 cells in each condition and at a frequency over all cells of a donor greater than 0.01. Pathway expression is defined as the log2 of the sum of the normalized gene expression for every gene in the pathway. Genes associated with each pathway are listed in Table S3.

Significance of the changes in pathway expression between the control and double blockade conditions was assessed using a Mann-Whithney U test. The p-values returned for each pathway comparison were contrasted with those obtained when comparing the expression in the DDB and CTRL conditions to those in 1000 random gene sets with the same number of genes with identical expression distribution as the pathway of interest. For each random gene set, we computed the p-value of the comparison using the Mann-Whithney U test. We report the percentile ranking of the p-value in the true pathway compared to the p-values obtained from the random gene sets.

Bulk TCR repertoire profiling was performed from sorted multimer positive and negative CD8+ T-cell RNA preserved in Trizol reagent (Invitrogen) using Next-Generation Sequencing (NGS) targeting the hypervariable complementarity-determining region 3 (CDR3) of the T cell receptor beta chain (TCRβ). TRIzolTM extracted RNA in combination with the PureLink RNA mini and microcolumn system (Thermo Fisher), quantified by UV spectrophotometry (Tecan), and QC was completed on Bioanalyzer chip (Agilent). TCRb amplicon libraries were prepared from 25 ng total

RNA with the Oncomine™ TCR Beta-SR Assay for RNA (ThermoFisher Scientific). The TCRb libraries obtained were quantified on the ViiA 7 Real-Time PCR System with the Ion Library Taqman Quantitation kit (Thermo Fisher). NGS was completed on the Ion S5 semiconductor platform using an Ion 540 chip, prepared with the Ion Chef System (all from Thermo Fisher). TCRβ repertoire analysis was completed using the Ion Reporter Software (Thermo Fisher) and Immunarch package17 analyses using R software. Our data is available GEO.

2.6.8 Statistical Analysis

Statistical significance was analyzed were with the R software, version 4.0.4. Multiple group comparisons were performed using one-way ANOVA and Turkey post hoc test or the Kruskal-Wallis test with the Holm procedure to correct for multiple testing (if ANOVA requirements were not met). Paired Wilcoxon-Mann-Whitney tests were performed for two-group comparisons. p values of 0.05 or less were considered significant.

2.7 Results:

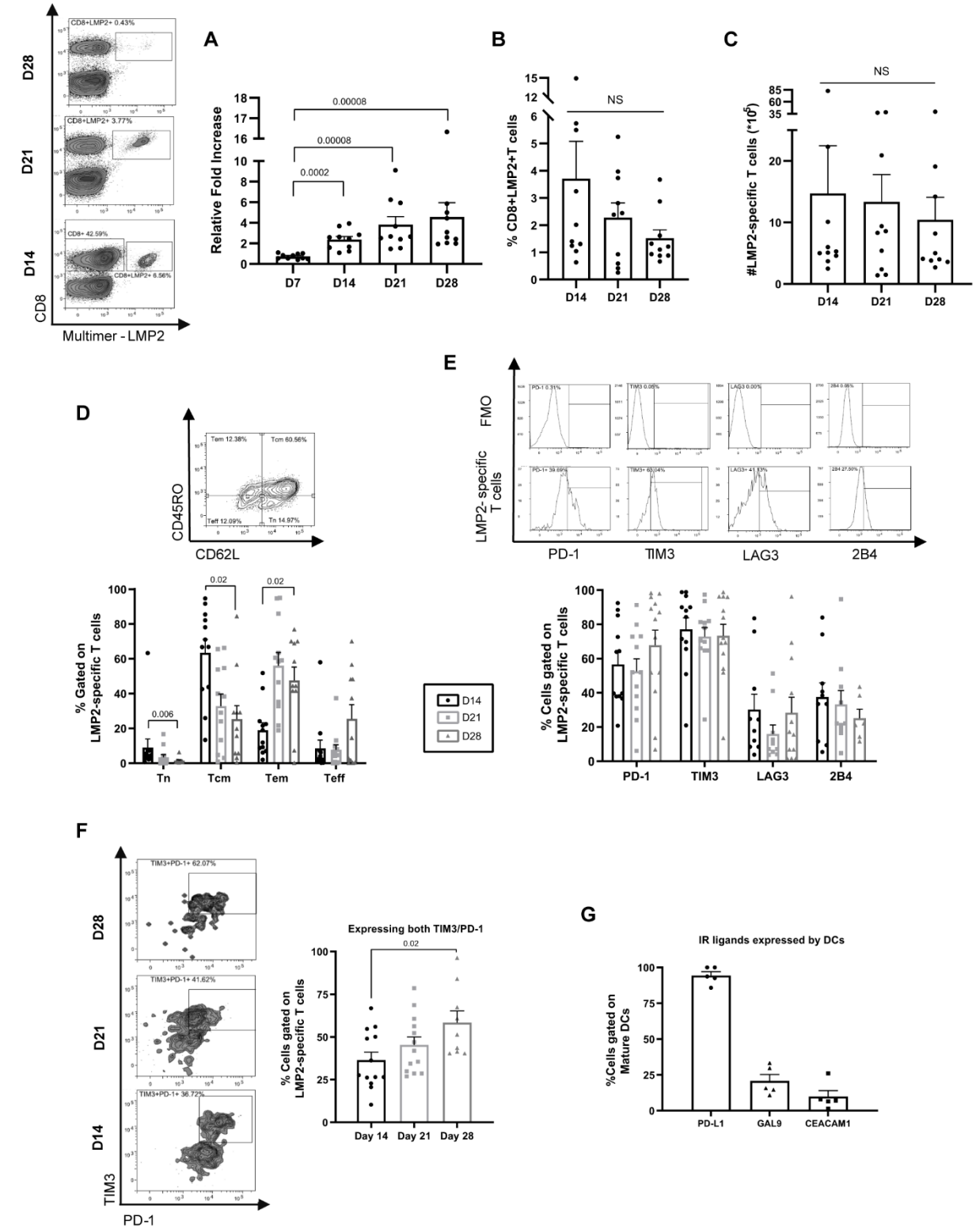
2.7.1 Multiple stimulations are detrimental to antigen-specific T-cell expansion ex vivo

Antigen-specific T cells were stimulated using moDCs loaded with the peptide LMP2426-434 (CLGGLLTMV), which is an HLA-A0201 restricted antigen from the oncogenic Epstein-Barr virus (EBV). Weekly ex vivo stimulations were performed in cytokine supplemented media, and antigen-specific expansion was assessed before each stimulation. While overall T-cell expansion progressed following each of four stimulations (albeit at limited pace after day 14 and two stimulations), the percentage and absolute number of antigen-specific CD8 T cells, as identified by fluorescent HLA-0201/ LMP2426-434 multimer, stagnated despite additional stimulations (Figure 1A-C). Further, this halted growth was matched with a gradual change in phenotype. The predominance of central memory (Tcm) differentiation profile at day 14 evolved towards an effector memory (Tem) and effector (Teff) differentiation phenotype on days 21 and 28 (Figure 1D). These results were anticipated as serial T-cell stimulations have been associated with the development of effector T cells that gradually lose their capacity to expand and persist after adoptive transfer6. The expression of the inhibitory receptors related to T-cell exhaustion (PD-1, TIM-3, and to a lesser extent LAG3 and 2B4) was substantial on a significant fraction of antigen-

specific T cells at day 14, with little modulation over time in culture (Figure 1E). However, the fraction of cells showing dual expression of PD-1 and TIM-3 increased with repeated stimulation (Figure 1F) in line with the development of CD8+ T-cell exhaustion18-20. Phenotyping of the moDCs revealed prevalent expression of corresponding PD-1 and TIM-3 ligands, (respectively PD-L1 and CEACAM-1/Galectin 9) (Figure 1G). These results confirm and extend previous data, suggesting that the early and persistent expression of PD-1 and TIM-3 by CD8+ T cells, along with the expression of their ligands by stimulating DCs, may represent a significant hurdle for the expansion of antigen-specific T cells for immunotherapy18 19.

Figure 1. Repeated antigenic encounters ex vivo leads to antigen-specific T-cell exhaustion. (A) Representative fluorescent multimer staining of LMP2426-434-specific CD8+ T cells (LMP2), (B) total cellular expansion (expressed as fold change relative to culture input – 15x106 cells) and (C) percentages, as well as calculated number of antigen-specific T cells at the indicated time points (n=10 different donors). (D) Representative staining to determine naïve (Tn), central memory (Tcm), effector memory (Tem), and effector T cells (Teff) and the percentage of HLA-A02/LMP2426-434 multimer positive CD8+ T cells within these categories at different culture time points (n= 12 different donors). (E) Representative histograms of immune checkpoint expression evaluated relative to fluorescence minus one (FMO) control in HLA-A0201-LMP2426-434 (LMP2) multimer-stained T cells and compiled results from 12 different donors. (F) Representative staining of PD-1 and TIM-3 co-expression and compiled results from 9-13 different donors. (G) Percentage expression of PD-1 and TIM-3 ligands on dendritic cells used to stimulate T cells (n=5 different donors). Significant p values are indicated on figure panels. All error bars represent standard deviation to the mean (SEM). (next page)

Figure 1


A
B
C
D
E
F
G
D28
D21
D14
CD8
Multimer - LMP2
CD8+LMP2+ 0.43%
CD8+LMP2+ 3.77%
CD8+ 42.59%
CD8+LMP2+ 6.56%
Relative Fold Increase
0.00008
0.00008
0.0002
D7
D14
D21
D28
% CD8+LMP2+T cells
NS
#LMP2-specific T cells (*10^6)
CD45RO
CD62L
Tem 12.38%
Tcm 60.56%
Teff 12.09%
Tn 14.97%
% Gated on LMP2-specific T cells
0.006
0.02
0.02
Tn
Tcm
Tem
Teff
FMO
LMP2- specific T cells
PD-1
TIM3
LAG3
2B4
% Cells gated on LMP2-specific T cells
D14
D21
D28
TIM3+PD-1+ 62.07%
TIM3+PD-1+ 41.62%
TIM3+PD-1+ 36.72%
Expressing both TIM3/PD-1
Day 14
Day 21
Day 28
IR ligands expressed by DCs
%Cells gated on Mature DCs
PD-L1
GAL9
CEACAM1

2.7.2 Combination of PD-L1/PD-1 axis and TIM-3 blockade significantly increases antigen-specific CD8+ T-cell expansion

In order to assess if immune checkpoint blockade during ex vivo expansion may improve antigen-specific T-cell yield, anti-PD-L1, anti-TIM-3, or both were added to the culture media at the beginning of culture and with all media changes. Cell counts at day 14, and even more strikingly at day 21, revealed that the double blockade condition significantly increased T-cell expansion relative to control (no checkpoint blockade) and the single blockade groups (Figure 2A). This translated into a marked increase in LMP2426-434-specific T-cell yield in the dual blockade condition despite no gain (and in fact a decline at day 14) in the percentage of multimer positive CD8+ T cells (Figure 2B-C). In contrast, the single blockade of PD-L1 or TIM-3 offered no advantage at any time point. The phenotypic assessment of LMP2426-434-specific T cells revealed no statistically significant difference in the percentages of Tcm, Tem, and Teff and no difference in PD-1 or TIM-3 expression (Figure 2D-E). However, the percentage of T cells expressing the inhibitory receptors LAG3 and 2B4 was lower in the combined relative to the control condition. Hence, dual PD-L1 and TIM-3 blockade increased T-cell growth without conferring phenotypic changes associated with increased T-cell dysfunction.

It is known that TIM-3 has a dual function. TIM-3 is transiently upregulated at intermediate levels on activated T cells and confers activation signals21. However, in settings of chronic stimulation in the presence of its ligands, TIM-3 inhibits T-cell activation and behaves as a bona fide immune checkpoint. We, therefore, slightly modified our protocol to introduce TIM-3 blockade at day 7, a week after the first stimulation (hereafter designated as delayed double blockade-DDB), with the expectation that it would further improve antigen-specific T-cell yield. Compared to the dual blockade started on day 0, DDB slightly increased total T-cell expansion at day 21 and more convincingly following an additional antigenic stimulation (day 28) (Figure 3A). Moreover, the DDB approach increased the percentage of LMP2426-434-specific T cells at all time points relative to the control condition (no blocking antibodies used), including at day 14 which was not the case when PD-L1 and TIM-3 blockade were both applied at day 0 (Figure 3B). Absolute antigen-specific counts were also increased in the DDB relative to the control condition (significant on days 21 and 28) (Figure 3C). Finally, DDB did not impact T-cell differentiation or

exhaustion marker expression (Figure 3D-E). Independent cultures using isotype control antibodies confirmed the specific effects of anti-PD-L1 and anti-TIM-3 antibodies on the antigen-specific T-cell expansion (Supplementary Figure 1). We conclude that combined blockade of the PD-L1/PD-1 axis and TIM-3 can be incorporated in ex vivo cultures to increase antigen-specific CD8+ T-cell yield for adoptive immunotherapy without altering T-cell phenotypes.

Figure 2

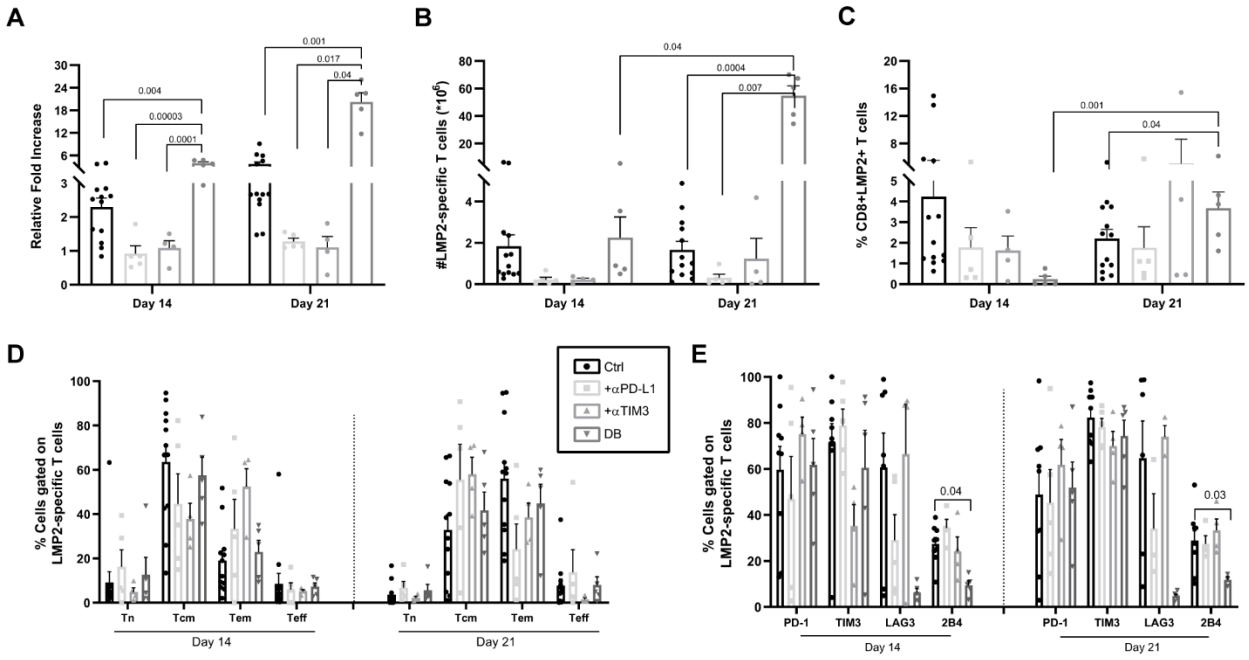


Figure 2. Dual but not single PD-L1 and TIM-3 blockade improves T-cell expansion. (A) Total cell expansion relative to input at the beginning of the culture in function of time and culture condition; no blocking antibodies (ctrl), anti-PD-L1 (α-PD-L1), anti-TIM3 (α-TIM3) or both (double blockade - DB). Absolute count (B) and percentage (C) of HLA-A0201-LMP2426-434 (LMP2) multimer positive T cells in the same conditions and at the same time-points. (D) T-cell differentiation phenotypes; naïve (Tn), central memory (Tcm), effector memory (Tem) and effector T cells (Teff) and (E) percentages of LMP2 multimer positive T cells expressing immune checkpoints in the function of culture conditions and time-points (n=4-10 different donors). Significant (<0.05) p-values are indicated on the figure panels. All error bars represent SEM.

Figure 3

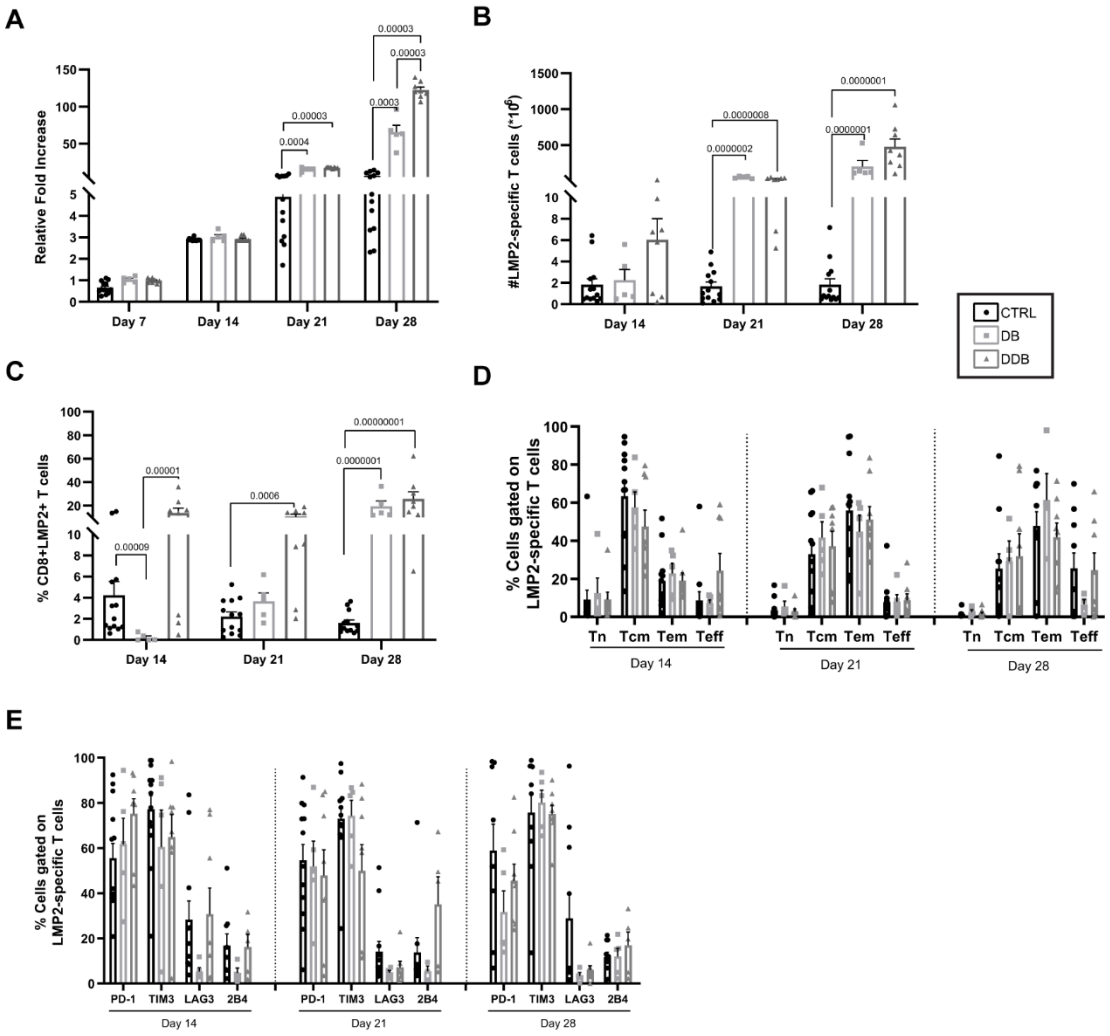


Figure 3. Delayed double blockade (DDB) further improves T-cell yield. (A) Total T-cell expansion relative to input at day 0 in the function of time and culture condition; no blocking antibodies (Ctrl), double anti-PD-L1 and anti-TIM-3 applied at culture initiation (double blockade – DB), anti-PD-L1 introduced at day 0 and anti-TIM-3 introduced at day 7 (delayed double blockade – DDB). (B) Percentages and (C) absolute counts of HLA-A0201-LMP2426-434 (LMP2) multimer positive T cells from the same cultures. (D) T-cell differentiation phenotypes and (E) immune checkpoint expression of LMP2 multimer positive cells in the same culture conditions and time-points (n=8 different donors). Significant (<0.05) p-values are indicated on the figure panels. All error bars represent SEM.

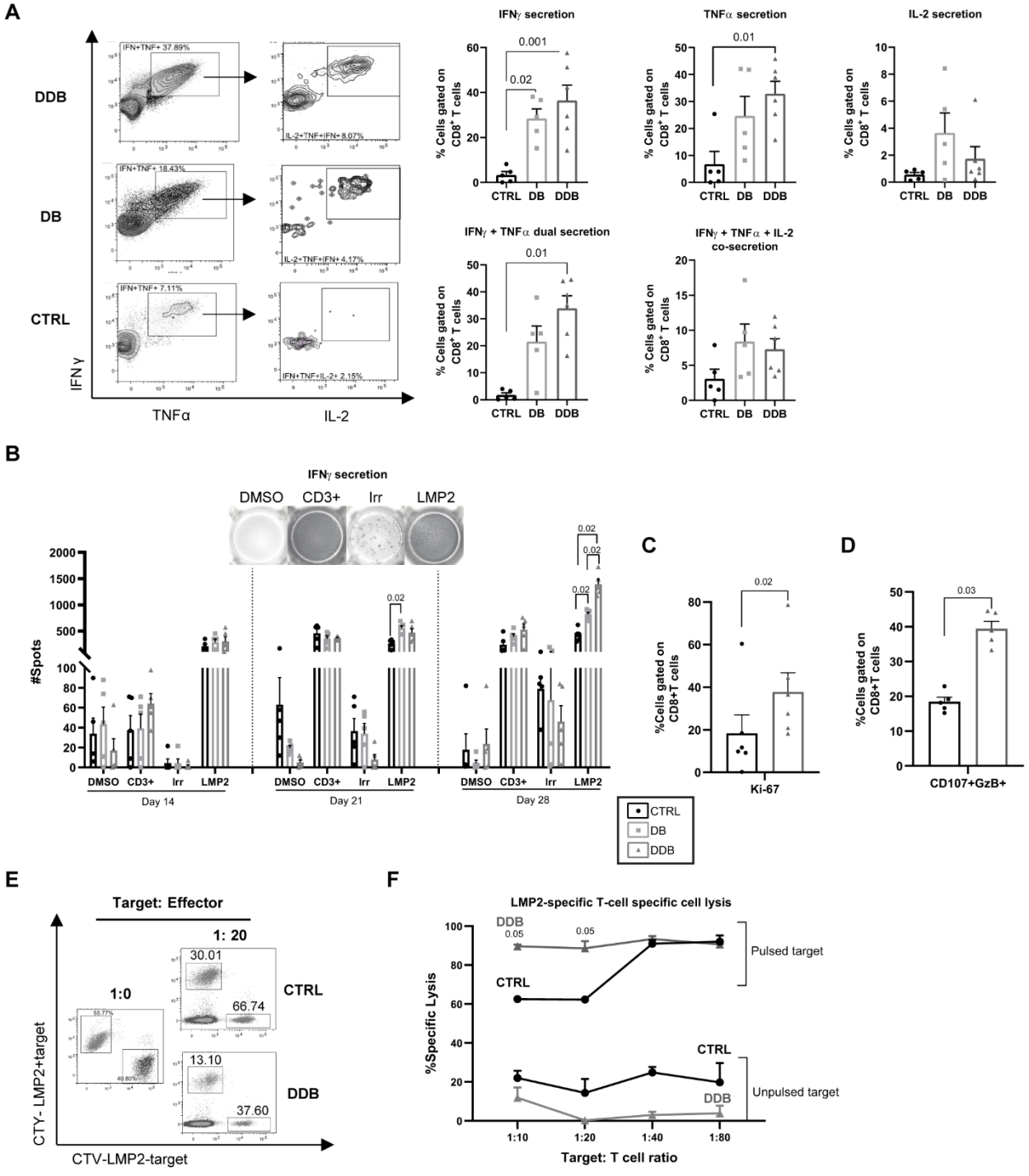
2.7.3 Double immune checkpoint blockade generates functional antigen-specific T cells

Dual PD-L1 and TIM-3 blockade increases antigen-specific CD8+ T-cell expansion in culture without altering T-cell phenotypes suggesting comparable functionality. Intracellular cytokine secretion measurements and ELISpot assays at day 28 confirmed that a higher proportion of T cells were reactive upon LMP2426-434 peptide re-exposure in the DDB condition relative to control (no checkpoint inhibition) (Figure 4A-B). This was also generally the case when DDB was compared to double immune checkpoint blockade administered at day 0 (statistically significant in ELISPOT data at day 28). We thus pursued our functional assessments comparing DDB with the control condition. As further indication of increased functionality, a greater fraction of antigen-specific T cells expanded in the DDB condition relative to control expressed the proliferation marker Ki-67 and upon peptide re-exposure, more DDB exposed T cells displayed evidence of cytotoxic potential (surface CD107a and intracellular granzyme B expression) (Figure 4C-D). This was corroborated with cytotoxicity assays showing that T cells from the DDB condition were highly effective, especially at low effector:target ratios (Figure 4E-F). Importantly, in all our assays, combined PD-L1 and TIM-3 blockade did not lead to increased non-specific (off-target or spontaneous) cytokine release or cytotoxicity. Thus, dual immune checkpoint inhibition expands T-cell products with specific and augmented antigen reactivity.

Figure 4. DDB expands a high proportion and number of functional antigen-specific T cells. (A) Representative dot plots showing intracellular cytokine secretion following $LMP2_{426-434}$ antigenic re-stimulation at day 28 and compiled results from 5-6 independent donor cultures demonstrating the percentage of $CD8^+$ T cells secreting IFNγ, TNFα, IL-2 and multiple cytokines; expansion with no blocking antibodies (CTRL), anti-PD-L1 and anti-TIM3 antibodies both introduced at the beginning of the culture (DB) or both antibodies but anti-TIM3 introduced at day 7 (DDB). (B) IFNγ ELISpot results using 50,000 cells per condition harvested from the cultures at the indicated time-points and using the following stimulating conditions: vehicle only (DMSO), anti-CD3 (CD3, positive control), irrelevant peptides (Irr.), $LMP2_{426-434}$ peptide (LMP2). (C) Percentage of Ki-67 staining at day 28 among $CD8^+$ T cells from the ctrl vs DDB cultures (n=5 different donors) and (D) co-expression of surface CD107a and intracellular granzyme-b (GzB) as surrogate for degranulation following $LMP2_{426-434}$ exposure. (E) Fluorescence-based cytotoxicity

assay (cell tracer yellow – CTY or cell tracer violet – CTV) showing loss of targets loaded (LMP2+) or not (LMP2-) with the $LMP2_{426-434}$ antigen (numbers indicate percentages of total events) and (F) compiled results at different target:T-cell ratio (from 3 different donors). The significant p-values are indicated on the panels and error bars indicate SEM.

Figure 4



2.7.4 PD-L1/TIM-3 blockade imparts no consistent gene expression signatures to expanded antigen-specific CD8+ T cells

To gain more insight into the biological impacts of double immune checkpoint blockade on antigen-reactive T cells, we submitted sorted day 28 multimer-positive T cells from three donors to paired transcriptome and TCR alpha-beta single-cell RNA sequencing (scRNA-Seq). Gene expression was compared between donors, conditions (no checkpoint blockade vs. DDB) and clonotypes. Global gene expression patterns across the three donors revealed strong donor-specific clustering (Figure 5A). The impact of DDB on gene expression for each donor antigen-specific T cells, was assessed using published human T-cell gene sets17. This enabled a detailed assessment of T-cell activation, proliferation, terminal differentiation, exhaustion and metabolism-associated gene expression as these processes are likely to be impacted by DDB. Overall, when all T cells from each condition were compared, no consistent change of gene expression signature could be identified across all donors (Figure 5B). We next evaluated whether clonotype-specific signatures could be identified. VD(J) sequencing revealed that the LMP2426-434 specific T cells were oligoclonal in all donors and conditions (1-4 clones representing more than 80% of all cells – Figure 5C and Supplementary Table 3). Most dominant clonotypes were shared between experimental conditions (albeit in some instances at different frequencies) and a few clonotypes were shared between donors (e.g. clonotype 1 and 3 common to donors 1 and 2, Supplementary Table 3). We next analyzed the expression of several pathways in the abundant clonotypes, defined as those identified in at least 10 cells and representing at least 1% of the repertoire in both the DDB and control conditions from the same donor. Similar to comparisons involving all clonotypes, no consistent pattern was found across donors when comparing the DDB versus control condition on a per clonotype basis (Figure 5D). Interestingly, our data unveiled divergent gene expression patterns for one clonotype (Clonotype 3) which was shared by Donor 1 and 2. Relative to its counterpart in the control condition, Donor 1 Clonotype 3 in the DDB condition expressed higher levels of genes related to T-cell exhaustion/differentiation and had a lowered expression of genes associated with T-cell proliferation. The same clonotype in the DDB condition from Donor 2 had, on the contrary, an increased expression of the genes associated with T-cell activation without any transcriptional changes related to T-cell dysfunction (T-cell exhaustion, terminal differentiation,

low proliferation). Although obtained from a limited number of cells and donors, these results suggest that donor rather than clonotype related features may determine T-cell outcomes following DDB.

To assess whether clonotype-specific transcriptional signatures may impact their expansion and clonal hierarchy within the cultures, we used bulk mRNA collected from sorted multimer negative and positive CD8+ T cells at day 21 and 28 from the same donors. RNA of suitable quality was obtained for 23 out of a potential of 24 samples (the multimer-positive fraction of the control condition from Donor 3 had to be excluded) and subjected to complementary determining region 3 (CDR3) region sequencing of the T-cell receptor-beta chain (TCRβ). Day 28 multimer-positive T-cell CDR3 sequencing matched very well paired VDJ sequencing of single cells (Supplementary Figure 2), confirmed the oligoclonality of the multimer-positive T cells at both day 21 and 28 and revealed that TCR repertoire diversity in the multimer-negative T cells was not affected by DDB (Figure 6A). Further, the multimer positive and negative fractions had limited overlap (Supplementary Figure 3), suggesting that sorting effectively separated most CD8+ antigen-specific cells from the rest of the T cells in culture. Clonotype hierarchy among multimer-positive T cells at day 21 and day 28 showed no consistent pattern of evolution in DDB relative to the control condition (Figure 6B-C). However, the proportion of certain clonotypes in these fractions (both DDB and control) varied importantly (>20%) between day 21 and 28. This was notably the case for Clonotype 3 from Donor 1 in the DDB condition, which declined markedly from day 21 to day 28, whereas this clonotype's frequency changed only slightly between day 21 to 28 of DDB exposure in Donor 2 (Figure 6B). We then explored whether the clonotypes with altered abundance between day 21 and day 28 in the same donor and condition displayed a specific transcriptional profile at day 28. In the DDB condition, clonotype 3 of Donor 1, which showed a decreased abundance from day 21 to day 28, had a higher expression of genes related to T-cell activation, differentiation and exhaustion relative to other clonotypes from the same donor in the same condition (Figure 6D). Although less striking, we also noticed a weak trend of increased expression of genes related to exhaustion in clonotypes with decreased abundance at day 28 and a rise in the expression of genes related to proliferation in clonotypes with an increased abundance at day 28, irrespective of experimental conditions (Figure 6D). Taken together these data show that DDB has limited impact on the clonal diversity of the expanded T cells overtime. The proportion of the various clonotypes in time can nonetheless fluctuate in both culture conditions and that the gene

expression signatures at day 28 offers possible explanations for such fluctuations. Globally, these results suggest that DDB confers no consistent transcriptional features to expanded antigen-specific CD8+ antigen-specific T cells but may alter activation/dysfunction and cell cycle-related processes in a clonotypes and donor-dependent fashion.

Figure 5

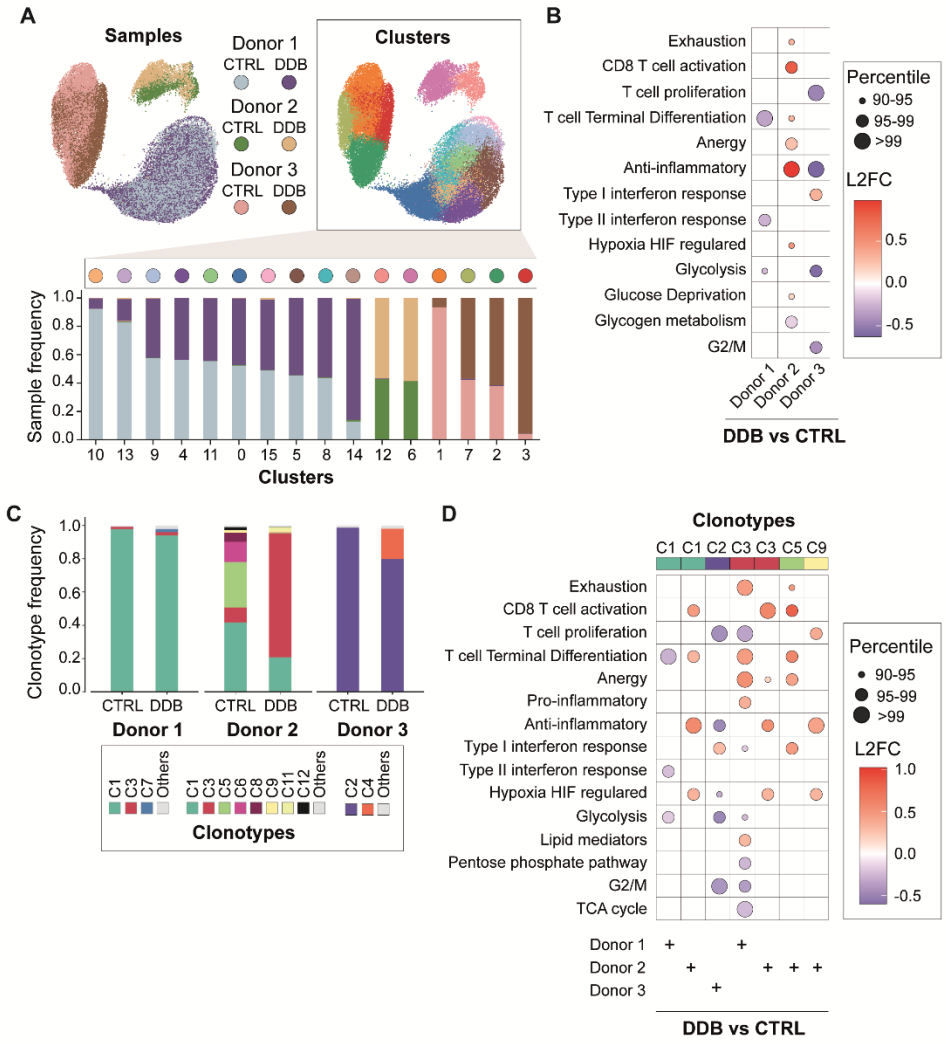


Figure 5. Single-cell RNA-sequencing of antigen-specific T cells after double immune checkpoint inhibition.

(A) T-stochastic neighbor embedding (t-SNE) of normalized single-cell gene expression after dimensionality reduction from control (CTRL) and delayed double blockade (DDB) conditions, color coded by donor and experimental condition on the left and by cluster on the right. The barplot represents the percentage of each sample (colored as above left) in each cluster labeled on the x-

axis and color-coded at the top of the graph. (B) Dot plot representing the change in the expression of genes related to pathways of interest in the DDB condition compared to the CTRL condition. The color of the dots represents the log2 fold-change of the genes in the pathway and the size of the dot is representative of the percentile ranking of the comparison in random gene sets (see Methods). (C) Clonotype frequencies in the CTRL and DDB conditions for each donor. (D) Similar to (B) comparing the cells of selected clonotypes/patients between the two experimental conditions.

Figure 6

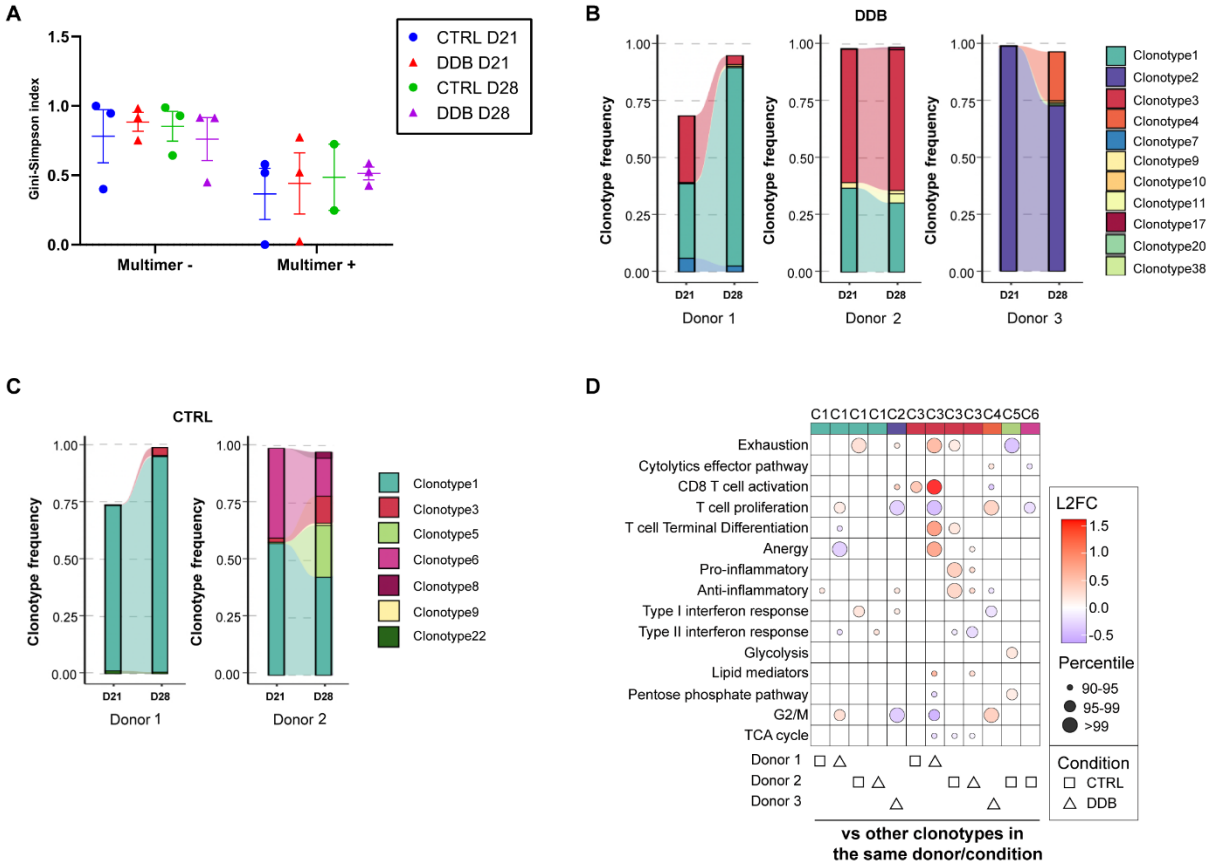


Figure 6. Impact of DDB on clonal diversity and stability in time.

(A) Estimate of TCR repertoire diversity using the Gini-Simpson index among HLA-multimer negative and positive T cells at day 21 and 28. (B) Clonal hierarchy and clonal relatedness among HLA-A0201-LMP2$_{426-434}$ multimer positive T cells between day 21 and 28 using the day 28 repertoire as reference in the DDB (B) or control condition (C) as determined by bulk CDR3 sequencing. The control condition in Donor 3 was not assessed due poor RNA quality at day 28.

(D) Dot plot representing the change in the expression of genes related to pathways of interest when comparing a clonotype of interest in a donor/experimental condition to all the other clonotypes in the same donor/condition. The clonotypes selected are those whose abundance vary by more than 20% between day 21 and 28.

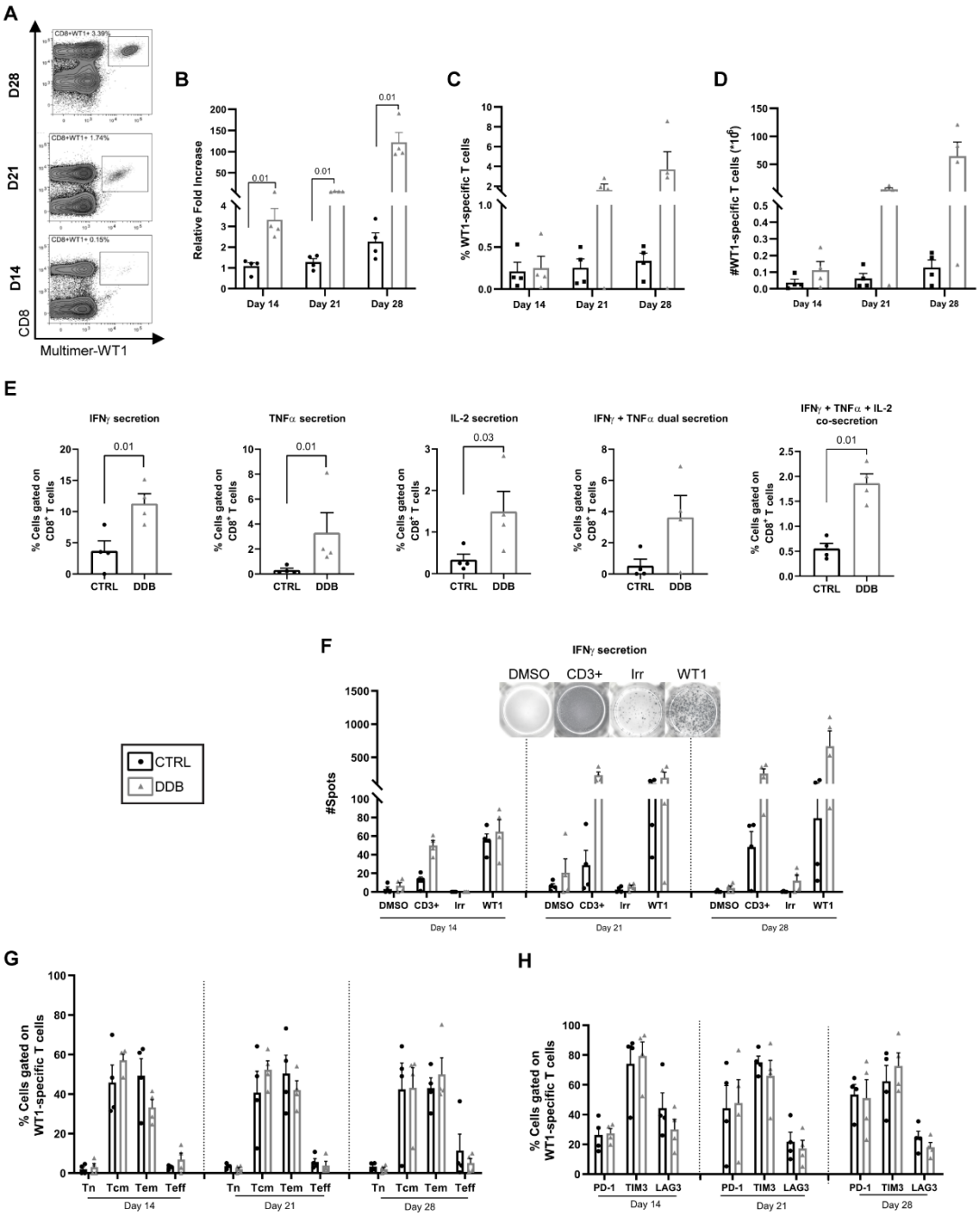
2.7.5 The benefits of dual PD-L1 and TIM-3 blockade extend to TAA-specific T cells

The EBV-derived $LMP2_{426-434}$ antigen stimulates a memory T-cell repertoire in more than 90% of adults(211). The *ex vivo* expansion of naïve T cells is generally considered more challenging for several reasons such as size of the repertoire and amount of stimulation required. We sought to determine the impact of dual PD-L1/TIM-3 blockade on the priming and expansion of naïve T cells. It was previously shown that CD8+ T cells specific against Wilm's tumor 1 (WT1) derived peptides, a clinically relevant tumor-associated antigen (TAA), are phenotypically naïve in healthy individuals(212) but can be expanded from a majority of such donors(213, 214). Volunteer donor T cells were stimulated with an HLA-0201 restricted WT1 peptide ($WT1_{37-45}$) using the same stimulation/expansion conditions for $LMP2_{426-434}$ specific T cells. WT1-specific T cells expanded following a similar pattern as $LMP2_{426-434}$-specific T cells (Figure 7A-C). The expansion, percentages and number of antigen-specific cells as well as their cytokine secretion were improved in the DDB condition relative to cultures without immune checkpoint blockade, again without altering T-cell differentiation patterns or exhaustion marker expression (Figure 7D-H). We conclude that our results obtained with $LMP2_{426-434}$ extend beyond virus-specific memory T-cell expansion and that double PD-L1/TIM-3 immune checkpoint blockade can improve the expansion of functional TAA-specific $CD8^+$ T cells from naïve repertoires.

Figure 7. Improved expansion of functional WT1-specific T cells with DDB. (A) Representative HLA-A0201-$WT1_{37-45}$ multimer staining (WT1) of $CD8^+$ T cells. (B) Cell expansion expressed as fold increase relative to cell input at the beginning of the culture ($15x10^6$) in ctrl (no blocking antibodies) or delayed double anti-PD-L1 and anti-TIM3 blockade (DDB) condition, as well as percentage (C) and absolute numbers (D) of WT1-specific T cells at different time-points in culture. (E) Proportion of intracellular cytokine expression (IFNγ, TNFα, IL-2, and multiple cytokines) following WT1 exposure at day 28 of the culture in $CD8^+$ cells and (F) IFN-γ ELISpot results at days 14, 21, and 28 of culture following exposure to vehicle alone (DMSO),

peptides not used in the culture (irrelevant, Irr) as negative controls, anti-CD3ε (positive control) and the targeted peptide (WT1). (G) T-cell differentiation; naïve (Tn), central memory (Tcm), effector memory (Tem), and effector T cells (Teff) and (H) percentage of immune checkpoint surface expression on WT-1 specific T cells. Four (4) different donors, p-values are indicated on the figures, error bars indicate SEM.

Figure 7



2.8 Discussion

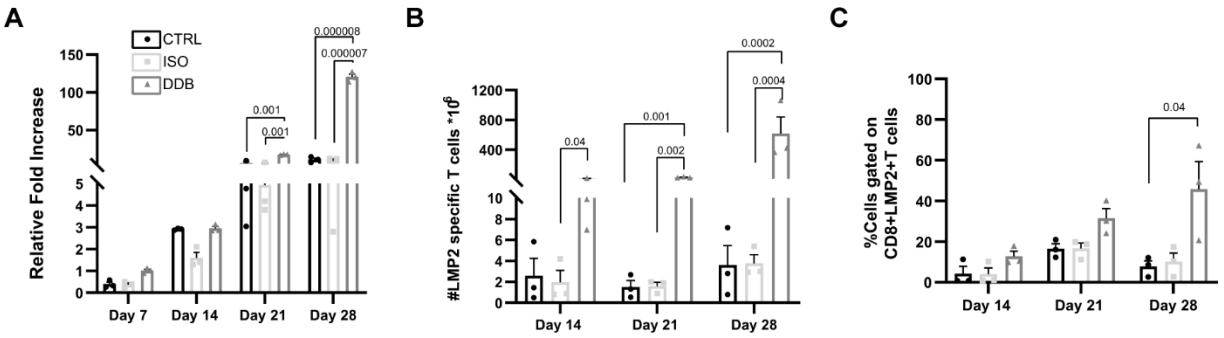
The stimulation and expansion of antigen-specific T cells for adoptive immunotherapy is an attractive strategy to target a wide variety of cancer antigens. However, T-cell expansion is limited by the expression of inhibitory receptors and the development of T-cell dysfunction. We confirmed and extended previous studies revealing that immune checkpoint receptors are expressed on repeatedly activated T cells and that their corresponding ligands can be present on antigen-presenting cells used *ex vivo*. Co-expression of PD-1 and TIM-3 has been used to describe highly exhausted $CD8^+$ T cells, and combined blockade of these receptors in murine models has previously been shown to improve tumor control relative to single blockade(126, 127, 215). We show that antibody-mediated immune checkpoint blockade targeting PD-1 or TIM-3 alone is insufficient to improve $CD8^+$ T-cell expansion while the combination improves T-cell expansion and antigen-specific reactivity. These data confirm the synergistic potential of immune checkpoint blockade and the particular relevance of TIM-3 and PD-1 as inhibitory receptors in $CD8^+$ T cells(127). The reasons for this synergistic effect might be due to the mobilization of different signaling intermediates by the two receptors. While PD-1 relies on the recruitment of SHP phosphatases to mediate its effects (as for several other negative co-stimulatory molecules), TIM-3 uses Bat3 and Fyn for its stimulatory and inhibitory effects, respectively. We reasoned that delaying TIM-3 blockade by one week after the start of the culture even in the presence of TIM-3 ligands on APC would be beneficial, given previous experimental evidence that TIM-3 provides activation signals following a first T-cell stimulation(119, 123). This contrasts with PD-1, which conveys inhibitory signals in both exhausted T cells and early after activation(216), justifying the use of anti-PD-L1 at culture initiation. We realize that several refinements may be required to fully leverage the potential agonistic/antagonistic effects of TIM-3 on T-cell activation and expansion and determine whether anti PD-L1/PD-1 blockade may also be improved by altering the timing of the inhibition. In addition, whether dual PD-L1/TIM-3 blockade improves T-cell expansion and function across multiple types of T-cell manufacturing protocols should be tested, just like further combinations with blockade of additional inhibitory receptors. Nonetheless, our work provides a new strategy for T-cell expansion and offers novel biological insights on the effects of PD-L1/PD-1 axis and TIM-3 blockade in human antigen-specific T-cell clonotypes submitted to multiple antigenic stimulations.

Our data support that sustained dual PD-L1/TIM-3 blockade through several rounds of antigenic stimulations provides ongoing benefits without exacerbating T-cell dysfunction or curbing expansion as inferred by previous studies of using inhibitory receptor gene deletion(208). This was assessed through phenotyping, functional assays, and gene expression in single cells. Our results indicate that antigen-specific $CD8^+$ T cells expanded under combined TIM-3/PD-L1 blockade are functional as well as specific and that their clonal composition is generally stable in time and relative to their counterpart not exposed to immune checkpoint blockade. It has been reported that immune checkpoint blockade *in vivo* can shape the T-cell repertoire(217-219). Thus, from a perspective of T-cell therapy, it was essential to define whether an intervention during *ex vivo* T-cell expansion alters the clonal identity of the cellular product. While we did not observe consistent effects of PD-L1/TIM-3 blockade on T-cell transcriptome or clonotype distribution, altered transcriptional profiles in a clonotype and donor-dependent manner nonetheless suggests that immune checkpoint blockade in adoptive T-cell therapy may require personalization.

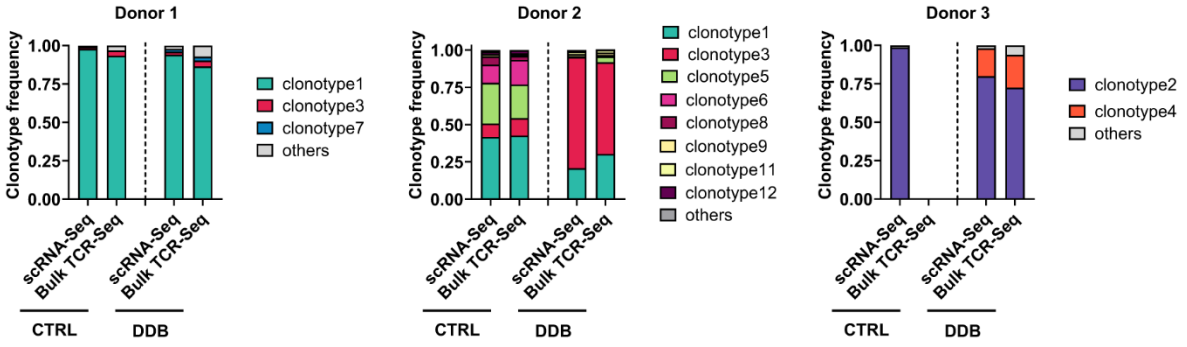
Further work will be required to understand why some clonotypes may be more susceptible to differentiation/exhaustion following immune checkpoint blockade at different time-points (previous activation/proliferation history, differentiation status at the beginning of checkpoint blockade, etc). Nevertheless, our data suggest that even after 28 days in culture, the antigen-specific T cells are highly functional, with a third expressing Tcm markers. This indicates that antigen-specific T cells generated in high numbers after dual immune checkpoint blockade could further expand after transfer and likely respond to further immune checkpoint blockade administered *in vivo*.

We conclude that dual PD-L1/TIM-3 blockade is a readily applicable strategy to improve the expansion of functional antigen-specific $CD8^+$ T cells expansion *ex vivo* for adoptive immunotherapy.

2.9 Supplementary Figures

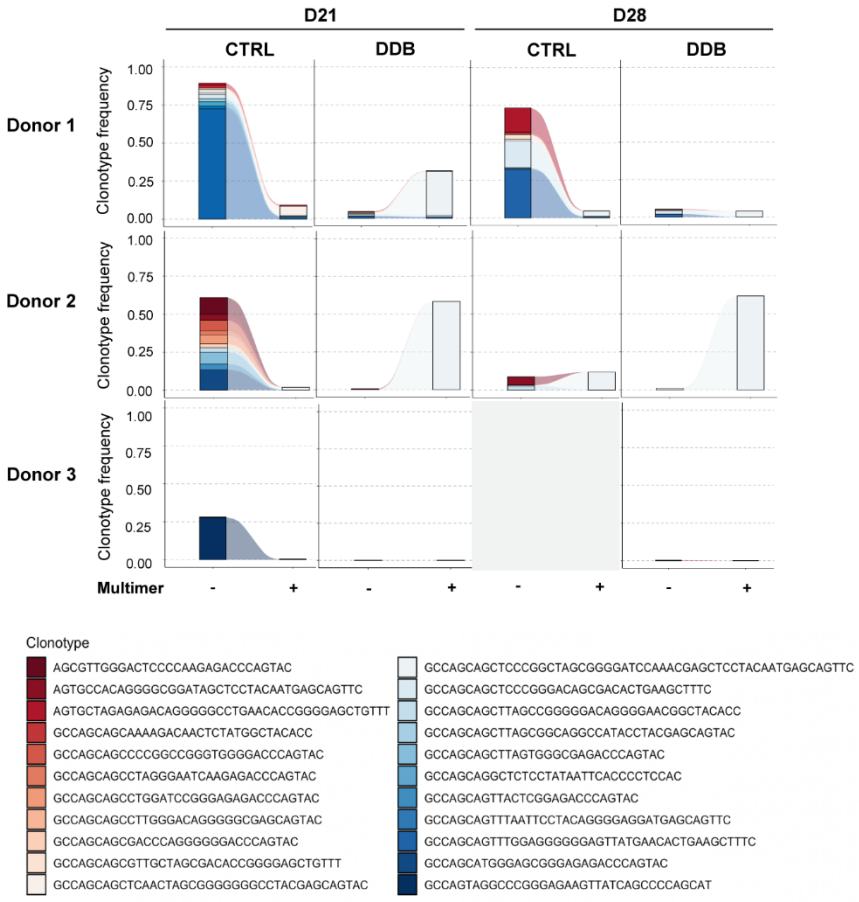


Supplementary Figure S1. Validation of the DDB strategy using isotype control antibodies. (A) Cell expansion relative to input at the beginning of the culture (15x106) in function of time and culture condition; no antibodies (ctrl), isotypes (ISO) according to the Delayed double blockade (DDB) scheme or DDB. Absolute count (B) and percentage (C) of HLA-A0201-LMP2$_{426-434}$ (LMP2) multimer positive T cells in the same conditions and at the same time-points. 3 different donors, significant p-values are indicated, error bars indicate SEM.



Supplementary Figure S2. Correlation between clonotype determination using single-cell RNA sequencing (scRNA-seq) and bulk CDR3 beta-chain TCR sequencing. Comparison of clonotype repertoire among sorted HLA-A0201-LMP2$_{426-434}$ multimer positive T cells at day 28

from the control condition (CTRL) and delayed double blockade (DDB) condition according to scRNA-seq or bulk CDR3 sequencing (bulk TCR-Seq) for each donor. The bulk TCR-Seq from the CTRL condition in Donor 3 was not considered due to poor RNA quality.



Supplementary Figure S3. Limited overlap between HLA-A0201-LMP2$_{426-434}$ multimer positive and negative T cells. Bulk CDR3 beta-chain TCR sequencing data from sorted multimer positive (+) and negative (-) fractions from all donors in both experimental condition (control - CTRL and delayed double blockade – DDB) at the day 21 and 28 time points showing limited clonotype overlap. Clonotypes present in both fractions (identified by beta-chain CDR3 sequences) are represented with none being abundant (>10%) in both fraction simultaneously. Due to poor RNA quality, the multimer-positive fraction of the control condition in Donor 3 could not be analyzed.

2.10 Supplementary Tables

Supplementary Table S1: Single cell filtering results. Initial number of barcodes represents the output from CellRanger v3.0.2 while the number of cells after filtering corresponds to the cells kept for downstream analysis.

Sample	Initial number of barcodes	Number of cells after filtering
CTRL1	20182	12753
CTRL2	8550	1950
CTRL3	27019	9109
DB1	19724	10101
DB2	11107	2665
DB3	30813	8981

Supplementary Table S2: Gene list used for analysis.

TCA cycle	G2-M	Pentose Phosphate Pathway		Lipid mediators		Glycogen Metabolism	Glycogen Metabolism	Glucose Deprivation
ACLY	AURKA	G6PD	ADIPOQ	GH1	FASN	GBE1	GBE1	NRN1
ACO1	BIRC5	G6PDH	TNFRSF9	HGF	IRS2	GYS1	GYS1	FAM129A
ACO2	BUB1	PGLS	AgRP	ICAM1	KLF15	GYS2	GYS2	IL23A
CS	BUB1B	PRPS1	ANGPT1	IGFBP1	PPARG	UGP2	UGP2	SPRY1
DLAT	CCNA2	PRPS1L1	ANGPT2	IGFBP2	PPARGC1A	AGL	AGL	GADD45B
DLD	CCNB1	PRPS2	ANGPTL3	IGFBP3	RETN	PGM1	PGM1	HSPA1A
DLST	CCNB2	RBKS	ANGPTL4	IGFBP4	SIRT3	PGM2	PGM2	HSPA1B
FH	CCNF	RPE	ANGPTL6	IGFBP6	SLC2A4	PGM3	PGM3	GPNMB
IDH1	CDC20	RPIA	TNFSF13B	IGFBP7	SREBF1	PYGL	PYGL	HAVCR2
IDH2	CDC25B	TALDO1	BMP4	IL1b	ACACB	PYGM	PYGM	SOAT2
IDH3A	CDC25C	TKT	CTSD	IL6	AXIN1	GSK3A	GSK3A	ADAMTS6
IDH3B	CDK1		CTSS	IL10	CCND1	GSK3B	GSK3B	
IDH3G	CDKN2D		CCL2	IL11	CDK4	PHKA1	PHKA1	
MDH1	CENPA		CCL5	INS	CEBPB	PHKB	PHKB	
MDH1B	CENPF		RARRES2	LEP	CEBPD	PHKG1	PHKG1	
MDH2	CKS2		CFD	LIF	DKK1	PHKG2	PHKG2	
OGDH	KIF20A		CRP	LCN2	E2F1			
PC	PLK1		CXCL8	CSF1	FABP4			
PCK1	RACGAP1		DPP4	MIF	FASN			
PCK2	TOP2A		ESM1	MPO				
PDHA1			S100A12	GHRL				
PDHB			AHSG	GHSR				
SDHA			FGF1	LIPE				
SDHB			FGF2	LPL				
SDHD			FGF21	AGT				
SUCLA2			FGF23	CEBPA				
SUCLG1			LGALS3	CFD				
SUCLG2			GCG	FABP4				

Glycolysis	Hypoxia-HIF regulated		Type II Interferon Response	Type I Interferon Response	Anti-inflammatory	Pro-inflammatory	Anergy	T- Cell Terminal Differentiation
ALDOA	LAMB1	IL10RA	IFNG	IRF1	TIGIT	IL1A	EGR3	TIGIT
ALDOB	ALDOA	IL15RA	CXCL9	IFIH1	IDO1	IL1B	NR4A3	PDCD1
ALDOC	ADM	ITGA6	CXCL10	IFITM3	LGALS3	TNF	EGR1	CD274
BPGM	BCL2	ITK	STAT1	DDX58	PDCD1	IFNG	NR4A2	CTLA4
ENO1	BCL2L1	JUND	CD274	IFI44L	FOXP3	TBX21	EGR2	LAG3
ENO2	FOS	IGHG4	CDKN1A	IFI6	ENTPD1	CCL3	TNFSF11	HAVCR2
GALM	JUN	MAP3K5	MYC	IFITM2	CD274	CCL4	IRF4	CD244
GCK	SRC	MAP2K1	SMAD7	NAMPT	CSF2	PRF1	GCH1	CD160
GPI	CREBBP	MAP2K2	IRF1	OASL	CTLA4	GZMA	GADD45B	
HK2	CCR6	MIF	HLA-F	RTP4	CXCL12	GZMB	NFATC1	
HK3	CEBPB	NFATC1	HLA-G	TREX1	CXCL5	GZMK	JARID2	
PFKL	ENO1	NFKB2	HLA-A	ADAR	CXCL8	GZMH	SLC29A3	
PGAM2	FAS	NFKBIE	HLA-E	TENT5C	MIF	CD8A	HLF	
PGK1	FASLG	TP53	HLA-C	LY6E	PTGS2	FASLG	ZFP36L1	
PGK2	FKBP4	TGM6	HLA-B	MCOLN2	VEGFA	CCL2	RNF19A	
PGM1	GAPDH	TNF	HLA-DRA	APOBEC3G		CCL20	ADORA2B	
PGM2	SLC2A1	TNFRSF1B	HLA-DRB5	IL15		IL2	NOCT	
PGM3	SLC2A3	TRADD	HLA-DRB1	ISG15		IL6	DUSP6	
PKLR	CSF2	TNFSF10	HLA-DQA1	MX1		IL12a	NDRG1	
TPI1	IFNG	TRAP1	HLA-DQB1	TLR3		IL17a	ADORA2A	
	IFNB1	VEGFA	HLA-DQB1-AS1			IL23a	HSPA1A	
	IL13		HLA-DQA2			PTGS2	PFKP	
	IL1R1		HLA-DQB2			TLR4	FYN	
	IL2RA		HLA-DOB			TNF	CTSE	
	IL4		HLA-DMB				ZNF629	
	IL5		HLA-DMA				F2R	
	IL6R		HLA-DOA				RNF128	
	CXCL8		HLA-DPA1				LAG3	
			HLA-DPB1				RGS16	
			HLA-DPA1				E2F1	
							E2F2	

T-Cell Proliferation	CD8 T-Cell Activation	Cytolytics effector pathway	Exhaustion
UBE2T	CD69	EOMES	TOX
CENPU	CCR7	TBX21	TOX2
CHEK1	CD27	GZMB	EOMES
AURKA	BTLA	PRF1	TBX21
CAPG	CD40LG	FASLG	NR4A2
PCLAF	IL2RA	GZMH	PRDM1
BUB1B	CD3E	GZMA	PDCD1
CCNB2	CD47		HAVCR2
DLC1	EOMES		CD244
CDK1	GNLY		CD160
CCNA2	GZMA		LAG3
SERPINB2	GZMB		CD38
PCNA	PRF1		ENTPD1
TOP2A	IFNG		CD101
STAT5A	CD8A		IFNG
Il2	CD8B		TNF
	FASLG		IL2
	LAMP1		CTLA4
	LAG3		
	CTLA4		
	HLA-DRA		
	TNFRSF4		
	ICOS		
	TNFRSF9		
	TNFRSF18		

Supplementary Table S3: TCR alpha/beta chain sequences of the most abundant clonotypes across donors and conditions.

Clonotype ID	nt_TRA	nt_TRB	aa_TRA	aa_TRB	Donor
clonotype1	TGTGCTGCCCTCATGGATAGCAACTATCAGT TAATCTGG	TGCGCCAGCAGTGATGACGGGATGAACACT GAAGCTTTCTTT	CAALMDSNYQLIW	CASSDDGMNTEAFF	1 and 2
clonotype2	TGTGCTGTGCTCATGGATAGCAACTATCAGT TAATCTGG	TGCGCCAGCAGTGGGGACGGTATGAACACT GAAGCTTTCTTT	CAVLMDSNYQLIW	CASSGDGMNTEAFF	3
clonotype3	TGTGCAATGAGCGCGGAGGAGGACATGCGC TTT	TGTGCCAGCAGCTCCCGGCTAGCGGGGATC CAAACGAGCTCCTACAATGAGCAGTTCTTC	CAMSAEEDMRF	CASSSRLAGIQTSSYNEQFF	1 and 2
clonotype4	TGTGCAATGAGCGCGGAATCAAATTCCGGGT ATGCACTCAACTTC	TGTGCCAGCAGCTTAGGGGGTTATGAGCAG TTCTTC	CAMSAESNSGYALNF	CASSLGGYEQFF	3
clonotype5	TGTGCAGGGGCCGGGGCTGGGAGTTACCAA CTCACTTTC	TGCGCCAGCAGCTTGGAGGGACAGGCGAGC TCCTACGAGCAGTACTTC	CAGAGAGSYQLTF	CASSLEGQASSYEQYF	2
clonotype6	TGTGCTGTGACTAACTTTGGAAATGAGAAATT AACCTTT	TGCGCCAGCAGCCAACTACAGGGATACGAG CAGTACTTC	CAVTNFGNEKLTF	CASSQLQGYEQYF	2
clonotype7	TGTGCAATGAGAGAGCCTCTCACGGGAGGA GGAAACAAACTCACCTTT	TGTGCCAGCAGTGGACTAGCGATCTCCTACG AGCAGTACTTC	CAMREPLTGGGNKLTF	CASSGLAISYEQYF	1

nt: nucleotide; aa: amino acid; TRA: TCR alpha chain; TRB: TCR beta chain.

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Chapter Three

3. CD5 deletion in ex vivo stimulated human antigen-specific T cells does not improve expansion and functionality

Shirin Lak1†, Béatrice Portier1†‡, Valérie Janelle1, Cédric Carli1, Émilie Dufour1, Mathieu Pelletier1, Fabien Touzot2,3,4, Heather J Melichar1,5, Jean-Sébastien Delisle1,5,6

†Equal contribution.

1 Centre de recherche de l'Hôpital Maisonneuve-Rosemont, Montréal, Qc, Canada
2 Centre de recherche du CHU Sainte-Justine, Montréal, Qc, Canada
3 Department of Pediatrics, Université de Montréal, Montréal, Qc Canada
4 Division of Immunology and Rhumatology, CHU Sainte-Justine, Montréal, Qc, Canada
5 Department of Medicine, Université de Montréal, Montréal, Qc, Canada
6 Division of Hematology-Oncology, Hôpital Maisonneuve-Rosemont/CIUSSS-EMTL, Montréal, Qc, Canada

‡ Current address: CHU de Lille, Lille, France

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Correspondence to:
Jean-Sébastien Delisle MD, FRCPC, PhD
Centre de recherche de l'Hôpital Maisonneuve-Rosemont
5415, Boul. de L'Assomption
Montréal, Qc
H1T 2M4
Canada

e-mail : js.delisle@umontreal.ca

3.1 Author's Contribution

SL: Study design, conducting all experiments except sequencing experiments, data analysis and discussion, preparation of all main and supplementary figures (except Figure 1), and writing of the first draft of the manuscript. 45% of the total effort.

BP: Conducting and analysis of experiments to evaluate the sgRNA, preparation of Figure 1

VJ: Assisting in preparation of all figures

CC: Study design and technical assistance in the cell cultures

ED: Trainee, Assistance in the cell cultures

MP: Trainee, Assistance in the cell cultures

FT: Study design, CRISPR/Cas9 protocol design

HJM: Study design

JSD: Study design, data analysis, general discussion, and manuscript writing

3.2 Acknowledgments

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3.3 Abstract

The type-I transmembrane glycoprotein CD5 is expressed by all T cells and is recognized as a negative regulator of T-cell activation. As such, it represents an attractive target to improve adoptive T-cell immunotherapies. However, the role of CD5 in the regulation of human antigen-specific activation, expansion, differentiation, and function remains incompletely understood. Moreover, whether CD5 signal modulation can be harnessed to improve current adoptive immunotherapies using antigen-specific T cells is unclear. By performing clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated CD5 deletion in primary human T cells undergoing ex vivo stimulation and expansion, we found that the absence of CD5 expression has very limited impact on T-cell growth, differentiation and function. This was true for both anti-CD3/CD28 mediated stimulation and antigen-specific T cells expanded using a clinically compliant protocol and serial antigenic stimulations ex vivo. These results suggest that CD5-mediated regulation of TCR signaling does not significantly impact (positively or negatively) the function and fate of ex vivo expanded antigen-specific human T cells and, as such, may not be prioritized as a target to improve human T-cell expansion and function in immunotherapeutic contexts.

Keywords: CD5, immune checkpoint, CRISPR/Cas9, adoptive immunotherapy, T cell

3.4 INTRODUCTION

The cell surface glycoprotein CD5 is expressed on all T-cell subsets(1, 2). The expression intensity of this type-I transmembrane glycoprotein that belongs to the scavenger receptor cysteine-rich (SRCR) family has been shown to correlate with T-cell receptor signal strength. In both mouse and human T cells, CD5 expression correlates with TCR avidity and increases following T-cell activation(3, 4). Mounting evidence support that such modulation of CD5 expression has functional consequences as CD5 can negatively regulate T-cell receptor (TCR) signaling, thereby protecting T cells from over-activation and ultimately from activation-induced cell death(2, 5). CD5 localizes at the immunological synapse, where it serves as a scaffolding molecule for several negative regulators of TCR signaling and co-stimulation downstream of CD28(2, 6). Although the role of CD5 in T-cell biology is still imperfectly understood, several lines of evidence place CD5 as an attractive target for immune checkpoint therapy. Among them, the antibody-mediated blockade of CD5 has led to enhancement of T-cell function and improved outcomes in pre-clinical models of cancer(7). In addition, work performed in CD5-deficient mice and CD5-deficient murine T cells ex vivo have likewise revealed a role for CD5 in the limitation of T-cell activation and anti-tumoral activity(5, 8). More recently, the deletion of CD5 through Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 approaches have been performed to primarily limit fratricide among human T cells bearing a CD5 targeting chimeric antigen receptor (CAR)(9-11). Such anti-CD5 CAR T cells are being developed to treat refractory T-cell neoplasms, and preliminary evidence suggests that CD5-negative CAR T cells might be more effective(11), in line with the notion that the dominant role of CD5 in mature T cells is that of an immune checkpoint. However, whether CD5-deletion can be an adjunctive measure to enhance the expansion and function of human antigen-specific T cells is unknown. We have performed CRISPR/Cas9-mediated deletion of CD5 in human T cells and incorporated CD5-deletion into a clinical compliant protocol to expand antigen-reactive T cells. Our findings indicate that CD5 deletion in human T cells ex vivo does not impact T-cell expansion, differentiation and antigen reactivity. Collectively our data suggest that CD5 is not a priority target to enhance the expansion and function of human antigen-specific T cells, especially in contexts of adoptive immunotherapy.

3.5 RESULTS

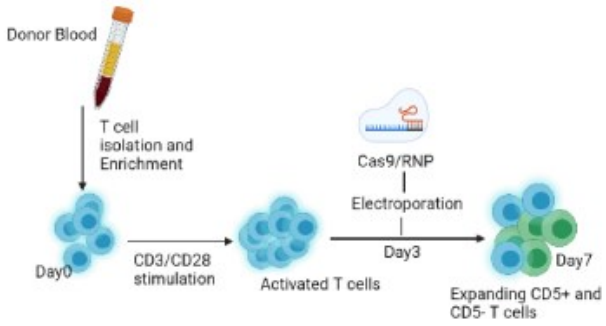
3.5.1 CD5 targeting in human T cells

Four CD5-targeting small guide (sg)RNA were designed for initial assessment of efficacy in CD5-expressing Jurkat cells. Ribonucleotide (RNP) made of the sgRNA and Cas9 were nucleofectedin Jurkat cells and surface CD5 staining was assessed three days later. The sgRNA with highest efficacy (sgRNA#2 targeting CD5 exon 5) was selected for further evaluation (Supplementary figure 1). Then, primary human T cells were stimulated with anti-CD3/CD28 for three days before nucleofection with either Cas9 alone or the sgRNA/cas9 RNP and assessed after an additional three to four days for CD5 surface expression (Figure 1A-B). Despite significant donor-dependent variability, CD5-negative CD8+ and CD4+ T cells were readily generated. Genomic deletion of CD5 on activated T cells was performed with Track indels analysis (TIDE) which confirmed a significant proportion of insertion-deletion mutations (indels) from the predicted site of edition (Figure 1C-D).

Figure 1. CD5 deletion in primary human T cells. (A) Schematic representation of T-cell stimulation/editing process using anti-CD3/CD28. (B) One representative dot plot and compiled data for CD8 and CD4 T cells (n=4) following nucleofection with Cas9 alone (negative control) or CD5-sgRNA/Cas9 ribonucleoprotein (RNP). (C) TIDE analysis showing the percentage of insertions or deletions (total indels 60.7%) following editing of primary T cells (dark pink denotes significant rates of indels, black non-significant). (D) Percentages of aberrant sequences downstream of the predicted edition site (nucleotide 339) in the test sample (nucleofection of RNP) versus control (nucleofection of Cas9 alone). Error bars represent standard error to the mean (SEM). (next page)

Figure 1

A



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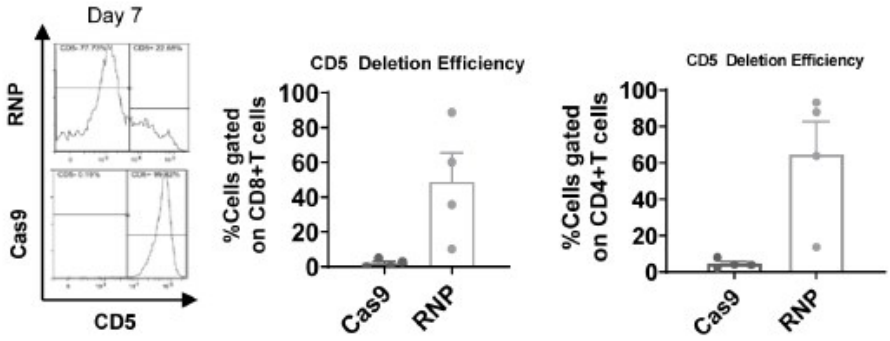

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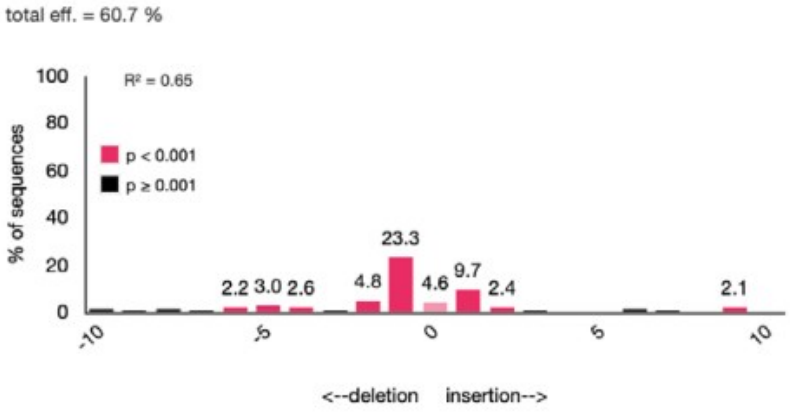

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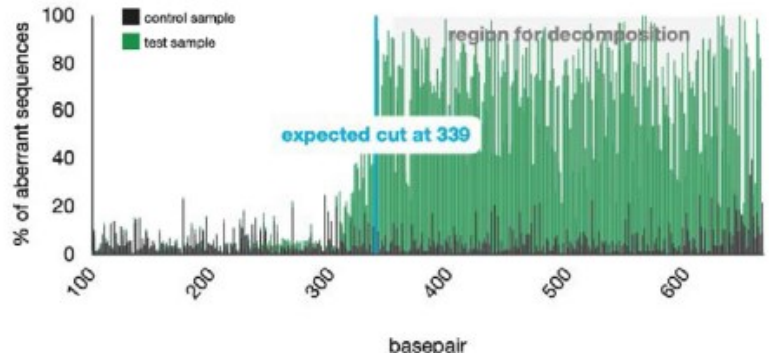
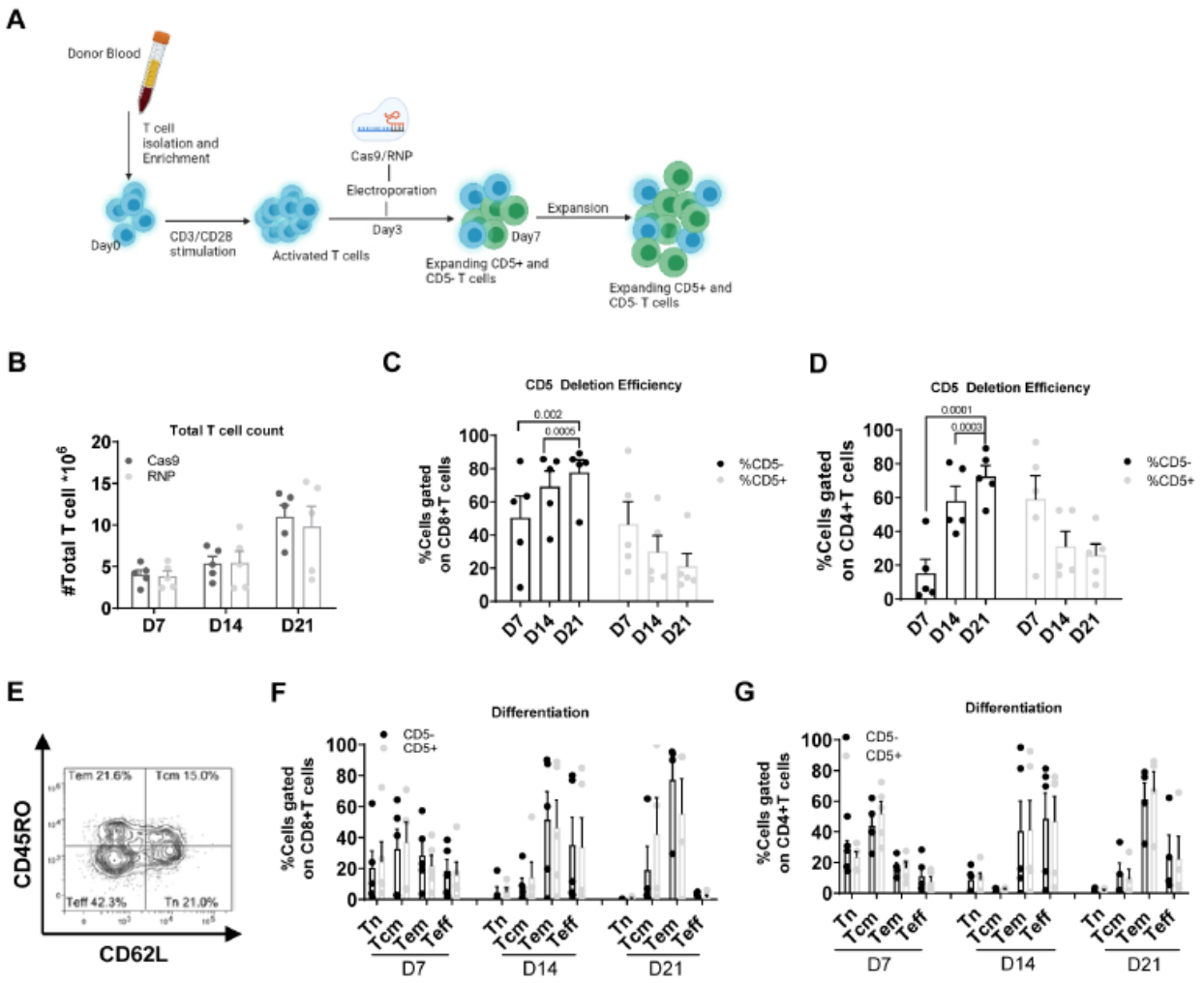


3.5.2 The absence of CD5 expression does not impact human T-cell expansion and differentiation following anti-CD3/CD28 stimulation.

We extended our previous data on T cells stimulated with anti-CD3/CD28 at day 0 and nucleofected with RNP at day 3 by using repeated anti-CD3/CD28 stimulations weekly (Figure 2A). After one (day 7), two (day 14), and three (day 21) stimulations, there was no difference in cell counts between the unedited (nucleofection with Cas9 alone) and the edited (nucleofection with RNP) cultures (Figure 2B). However, when the proportion of CD5-negative and positive T cells was assessed in the edited condition, the percentage of CD5-negative T cells gradually increased from day 7 to day 21 in both CD8 and CD4 T cells, suggesting that CD5-negative T cells may outcompete their CD5-positive counterparts in the same culture (Figure 2C-D). However, this relative increase in the proportion of edited T cells did not come with other phenotypic evidence of increased activation such as increased effector differentiation(12, 13) (Figure 2F-G, gating strategy shown in Supplementary Figure 2). Indeed, the proportion of effector memory (Tem) and effector (Teff) T cells increased with serial stimulations at the expense of "early" memory central memory T cells (Tcm) and naïve T cells in both conditions as predicted from previous studies(14, 15). Similarly, no difference was noted in the percentages of CD5-positive and CD5-negative CD4 or CD8 T cells expressing the common T-cell activation/exhaustion markers PD-1, TIM-3, LAG3, and 2B4 (Supplementary figure 3). Taken together, these results suggest that CD5 has a limited impact on the expansion and phenotypes of ex vivo repeatedly stimulated T cells.

Figure 2. Anti-CD3/CD28 mediated stimulation and expansion of CD5-deleted T cells. (A) Schematic representation of the CD5 editing and repeated anti-CD3/CD28 stimulation protocol. (B) Total cell count harvested from control (unedited T cells nucleofected with Cas9 alone – Cas9) or CD5-edited cultures (RNP) at day 7, 14 and 21 (D7, D14, D21 respectively). (C) Histograms representing the percentage of CD5-positive and CD5-negative CD8+, or (D) CD4+ T cells in the CD5-edited cultures (n=5 different donors/cultures). (E) Representative dot plot showing the distribution of T cells as naïve (Tn), central memory (Tcm), effector memory (Tem) and effector (Teff) T cells based on CD62L and CD45RO expression. (F) CD8+ and (G) CD4+ T cell differentiation in function of time in culture and CD5 expression. (next page)

Figure 2



3.5.3 CD5 deletion does not increase human antigen-specific expansion and reactivity

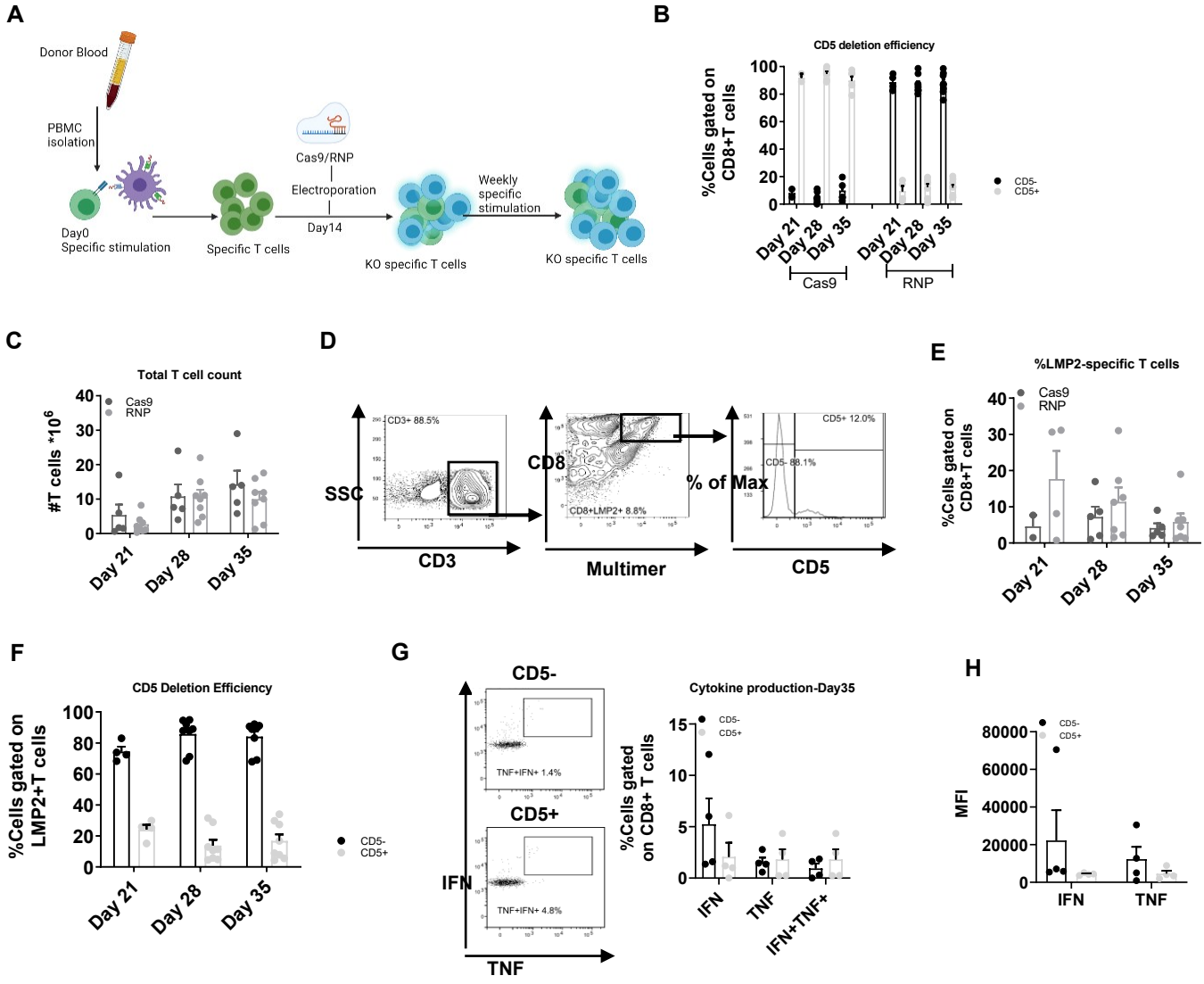
Given that anti-CD3/CD28 is a vigorous form of stimulation, we next sought to determine if CD5 deletion could be leveraged to expand human T cells stimulated in a more physiologic context. To this end, we used a clinically relevant protocol that has previously been shown to expand CD8+ T cells specific for a HLA-A0201 restricted epitope from Latent membrane protein-2 of the Epstein-Barr virus (LMP2426-434) (15, 16). We performed RNP nucleofection (or nucleofection with Cas9 alone as control) after two rounds of stimulation with antigen loaded autologous dendritic cells (day 14 of the culture) (Figure 3A). Following genetic edition at day 14, we re-stimulated the

T cells with antigen loaded dendritic cells weekly for three weeks. Hence, the impact of serial antigenic stimulation on T-cell growth and function could be assessed. When looking at the entire CD8 compartment, CD5 deletion was very effective (>70%) at all time points after nucleofection (days 21, 28 and 35), but this did not result in increased cell counts in the CD5-deleted culture relative to the cultures exposed to nucleofection with Cas9 alone (Figure 3B-C). Similarly, the percentage of HLA-A0201-LMP2426-434 fluorescent multimer-positive CD8+ T cells did not differ between the two conditions (Figure 3D-E). Further analysis of the multimer positive fractions enabled the comparison of CD5-negative and CD5-positive antigen-specific T cells from the same culture (Figure 3F). The expression of CD5 did not impact T-cell differentiation or exhaustion marker expression among multimer positive T cells (Supplementary figure 4) and cytokine production upon antigen re-exposure (Figure 3G-H). In order to establish whether this lack of effect was due to the timing of the CD5-deletion, additional experiments where performed with T cells that had undergone CD5-deletion (or nucleofection with Cas9 alone) prior to the first antigenic stimulation. As for T cells edited at day 14, CD5-deletion at day 0 did not impact antigen-specific yield or antigen-specific cytokine release following two rounds of stimulation with antigen pulsed dendritic cells despite a modest increase in total T-cell expansion (Supplementary figure 5). Taken together, these results show that CD5 editing can be incorporated in clinically relevant protocols to expand antigen-specific T cells but that the presence or absence of CD5 does not alter growth, differentiation, proportions or cytokine secretion of antigen-specific T cells.

Figure 3. Generation and expansion of CD5-deleted antigen-specific human T cells. (A) Schematic representation of gene editing integration into antigen-specific T-cell expansion protocols. (B) CD8+ T-cell CD5 surface expression 1-3 weeks (and the same number of antigenic stimulation) after genetic-editing (RNP) or nucleofection with Cas9 alone (done at day 14 of the culture (n=4-8 independent donors/cultures). (C) Calculated number of total T cells following nucleofection in the same conditions (n=4-8 independent donors/cultures). (D) Approach to study CD5 expression on HLA-A2 0201/LMP2426-434 fluorescent multimer-positive T cells and (E) percentage of multimer-positive T cells in culture in function of time. (F) One-representative intracellular staining including the control negative (stimulated with irrelevant peptide MART1), control positive(stimulated with PMA-Iono), and test condition, and compiled results showing the percentage of CD5-positive and CD5-negative CD8+ T cells in the CD5-edited condition secreting cytokines at the end of the culture following a brief re-exposure to antigen (n=4 independent

donors/cultures). (G) Mean fluorescent intensity (MFI) of intracellular cytokine staining among cytokine secreting cells according to CD5 expression in the CD5-edited condition. Error bars represent SEM.

Figure 3



3.6 DISCUSSION

The function of the CD5 receptor has remained enigmatic for several decades (1, 2), but the sum of available data from both murine and human mature T cells now position this receptor as a likely immune checkpoint. The deletion of CD5, based on CD5-deficient mouse data, represented a very attractive approach to increase the reactivity of ex vivo expanded T cells for adoptive immunotherapy. Our data show limited discernible effects of CD5 deletion on the characteristics of ex vivo stimulated human T cells. One reason for that might be that the ex vivo expansion of antigen-specific T cells requires strong stimulations that may obscure the role of CD5 as a fine regulator of TCR signaling. However, the lack of difference in cytokine secretion after simple peptide re-exposure suggest that CD5-deleted T cells are not more reactive than their unedited counterparts that have comparable differentiation status and expression of other common inhibitory receptors. On that note, future studies should focus on the impact of CD5-deletion on the blockade (or deletion) of other immune checkpoints. One of the proposed mechanisms leading to TCR signalling inhibition downstream of CD5 is the phosphorylation of FYN(17), which in turn can modulate the suppressive effects of TIM-3(18). Also, given that several mechanisms putatively mediating the inhibitory properties of CD5 rely on ubiquitination and degradation of TCR signalling molecules(6), CD5 could provide additive or synergistic inhibitory effects when combined with the blockade of other immune checkpoints that use other mechanisms to suppress TCR signalling (such as the recruitment of phosphatases like SHP-1)(19). The characterization of TCR and co-stimulatory receptor signaling activation in CD5-deleted T cells will be required to design the best co-inhibition strategies.

In conclusion, CD5-deletion reveals that CD5 is not a central regulator of antigen-specific human T cells expansion, differentiation and antigen reactivity. More studies are required to define the molecular effects of CD5 deletion in order to better understand how this immune checkpoint can be leveraged for immunotherapy.

3.7 METHODS

Cell procurement Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque gradient (STEMCELL Technologies) from leukoreduction system chambers provided by Héma-Québec (LRSCs)(20). All donors provided written informed consent, and this study was

approved by the Héma-Québec and Hôpital Maisonneuve-Rosemont Research Ethics Committees. Freshly collected PBMCs were resuspended in freezing media (90% fetal bovine serum and 10% dimethyl sulfoxide (DMSO)), transferred to Mr. Frosty containers (NALGENETM Cryo 1ºC freezing container), and stored in vapor-phase liquid nitrogen for future use.

3.7.1 Design of sgRNA and gene-editing.

CD5 gene has two different transcripts each composed of 11 exons. Exons common to the two transcripts were targeted. We used Integrated DNA Technology (IDT), CHOPCHOP (https://chopchop.cbu.uib.no/) and the Broad Institute GPP sg RNA Design tool platform for sgRNA design and avoided « GCC » and « TT » at the hinge site of the sgRNA and polymeric nucleotides(21-23). The potential off-target sites were evaluated « in-silico » with Cas-OFFinder data base (http://www.rgenome.net/cas-offinder) and IDT tools The scaffold sequence was taken from Ran et al(24). We designed four sgRNA: CD5sgRNA 1, 2, 3, 4 which targeted respectively exon 3, 5, 9 and 6. The sgRNA were synthetized by Genscript. The efficiency of sgRNA-mediated deletion at the DNA level, was assessed using TIDE software (Tracking of Indels By Decomposition)(25, 26) following sequencing (IRIC core genomics platform).

3.7.2 Nucleofection

Transfection of Cas9 or RNP into Jurkat or primary T cells was performed with the Nucleofector 2b Device (LONZA), programed to ensure high efficiency and cell viability. After harvesting/ isolating the cells we combined the cells with the Cas9 (Alt-R® S.p. Cas9 Nuclease-IDT) as control condition or the Cas9+CD5sgRNA (from Genscript) test condition resuspended in the appropriate cell-type specific Nucleofector® solution and transfered to an amaxa certified cuvette from the Human T Cell Nucleofector Kit (Lonza Bioscience (via VWR)). After the nucleofection the cells were washed with warm complete T cell media and were transferred to the culture plate/ GRex.

3.7.3 T-cell stimulation

Primary human CD3+ T cells were enriched by negative immune-magnetic selection from the previously frozen PBMCs using EasySep™ Human T Cell Enrichment Kit (Stemcell Technologies) based on the manufacturer's protocol. Isolated cells were stimulated with monoclonal anti-CD3 (17A2 (RUO)) and anti-CD28 antibodies (37.51 (RUO)) both from BD

Pharmingen. 6-well culture plates were coated with the anti-CD3 antibody by diluting the anti-CD3 antibody at 1 µg/mL in sterile PBS and then incubated the plate at 5% CO2 at 37°C for 1.5 hours. After 1.5 hours, the CD3 solution was aspirated and the wells were washed with 1X PBS two times. T cells were seeded with 2x106 cells per well/ 2ml in complete T cell media. Next, anti-CD28 antibody was added to the media at 1 µg/mL. Antigen-specific T cells were stimulated using a clinically compliant protocol as previously described ((16) and www.clinicaltrials.gov NCT03091933) from 15x106 PBMCs and expanded through multiple weekly stimulations using irradiated (40 Gy) autologous, peptide-loaded monocyte-derived DCs at a 1:10 (DC:PBMC) in a G-Rex6® Well Plate vessel (Wilson Wolf Manufacturing, New Brighton, MN). Our complete T-lymphocyte culture (CTL) media (Advanced RPMI 1640, 10% human serum, 1X L-glutamine(Gibco)) was supplemented with the following cytokines; week 1: IL-21 (30 ng/mL) and IL-12 (10 ng/ mL) (Both from Feldan), week 2: IL-21, IL-2 (100 IU/mL), IL-7 (10 ng/mL) and IL-15 (5 ng/mL) (STEMCELL Technologies), subsequent weeks: IL-2, IL-7 and IL-15. Medium, including cytokines, was refreshed every 3 to 4 days, and specific T cell cultures were restimulated once every week with peptide-loaded monocyte-derived DCs (moDCs). Cell concentration was adjusted to 1:10 ratio each week. All cell cultures were performed in monitored incubators (37°C in 5% CO2 and 5% air humidity). Monocyte isolation and DC differentiation have been generated as previously described(16). Briefly, monocytes were obtained using the adherence method whereby PBMCs were plated in adherent plastic plates (Sarstedt) in media (X-Vivo 15 medium (LONZA) supplemented with 5% human serum, 2 mM L-glutamine and 1mM Sodium Pyruvate (Gibco), 1000 U/ml (100 ng/ml) IL-4 and 800 U/ml (50ng/ml) GM-CSF (Both from STEMCELL technologies)) and incubated in a CO2, 37°C incubator for 7 days. On day 4, media was replaced with fresh media supplemented with IL-4 and GM-CSF. On day 7, DCs were matured with maturation media containing 1000 U/ml (100 ng/ml) IL-4, 800 U/ml (50ng/ml) GM-CSF (Both from STEMCELL technologies), 10 ng/ml TNF-α (STEMCELL technologies), 1 µg/ml PGE2 (SIGMA), 10 ng/ml IL-1β (Stemcell), 100 ng/ml IL-6 (Miltenyi Biotec) and loaded with desired peptide (1 µg/mL LMP2426-434 (CLGGLLTMV) from JPT Peptides. Lastly, DC media was supplemented with IFN-γ 1000U/ml (Feldan) for the last 24 hours of culture.

3.7.4 Cell count and flow cytometry

Cell viability and cell counts were assessed by the countess automated cell counter (Invitrogen) using trypan blue (Invitrogen) at a 1:1 ratio with the cellular suspension in cell counting chamber slides (Invitrogen-C10283). For cell surface staining, harvested cells were washed twice in staining buffer (2% fetal bovine serum) and stained for cell surface markers. Cells were incubated with relevant antibodies for 30 min at 4°C in the dark, washed twice and re-suspended in staining buffer. Flow cytometry was performed using a Fortessa flow cytometer (BD Biosciences), and data were analyzed with Flowlogic Software (inivai). The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in the forward and side scatter.

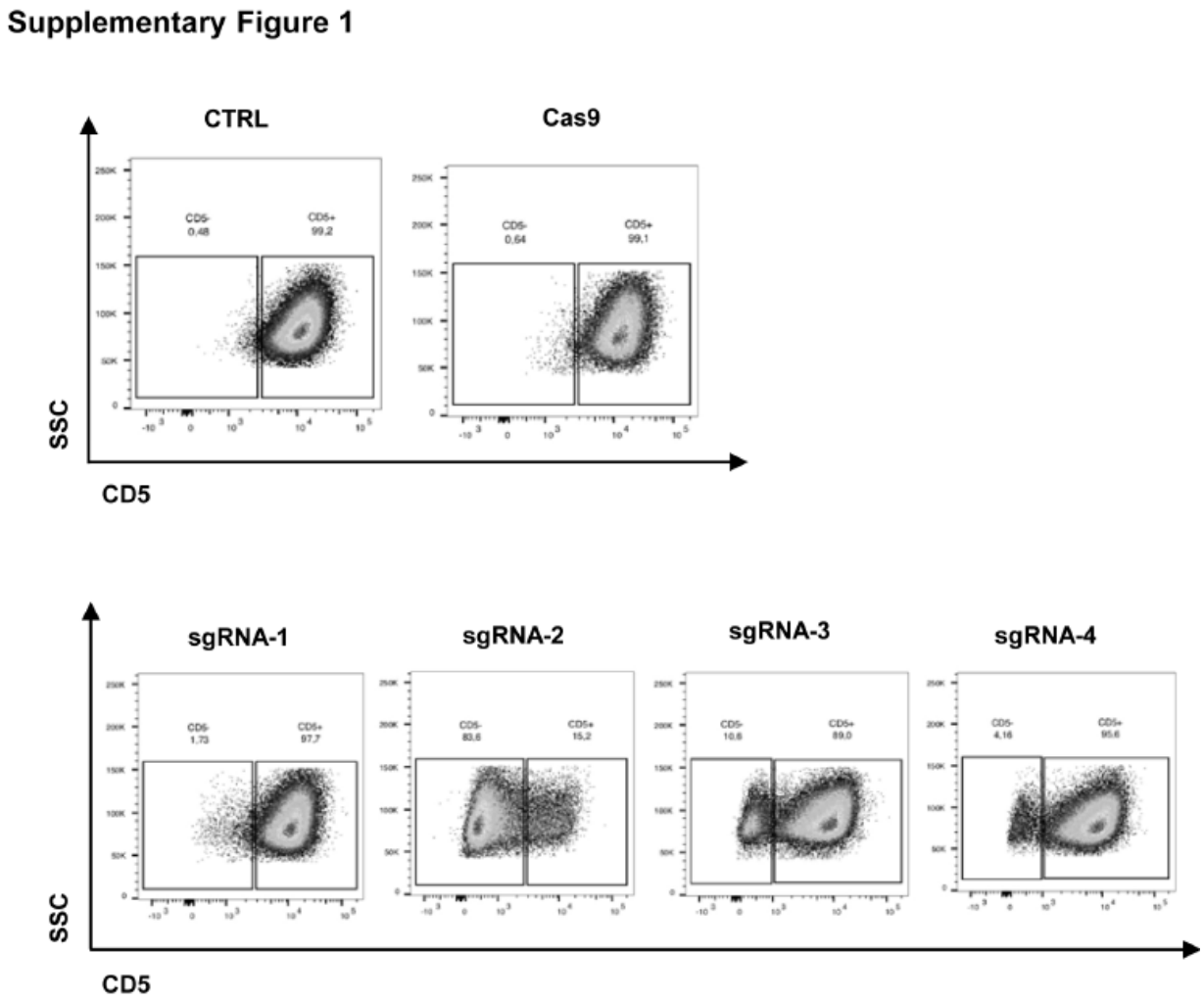
The following anti-human monoclonal (clone name in parenthesis) antibodies (mAb) were used for staining: CD3 (SKY7), CD3 (UCHT1), CD8 (SK1), CD45RO (UCHL1), LAG3 (T47.530), and CD4 (RPA-T4) all from BD Biosciences; CD62L (DREG-56), TIM3 (F38-2E2), PD-1 (EH12.2H7), CD5 (UCHT2) from Biolegend and CD8 (RPA-T8) and CD244 (eBioDM244) from Invitrogen. According to the manufacturer's instructions for intracellular detection of cytokines, cells were permeabilized, fixed and stained using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience). Before the fixation step, up to 106 cells were stimulated to produce cytokines with the indicated peptide (0.5 µg/ml) (test condition) or PMA (50 ng/mL)-ionomycin (500 ng/mL) (Sigma-Aldrich) (positive control) and an irrelevant peptide (HLA-A0201 restricted but not used as a stimulator in the culture-negative control- MART1 in this study) for 4 h at 37°C. Cells are suspended in CTL media plus brefeldin A (Biolegend) to block the secretion of cytokines during the stimulation period. Cells were then harvested and stained for cell-surface markers, including CD3, CD5, CD4, and CD8 in 4 oC for 20 minutes. Next, fixed and permeabilized cells were stained with intracellular cytokine detection antibodies; IFN-γ (4S.B3), and TNF-α (Mab11) all purchased from BD Biosciences) at RT for 20 minutes.

3.7.5 Statistics

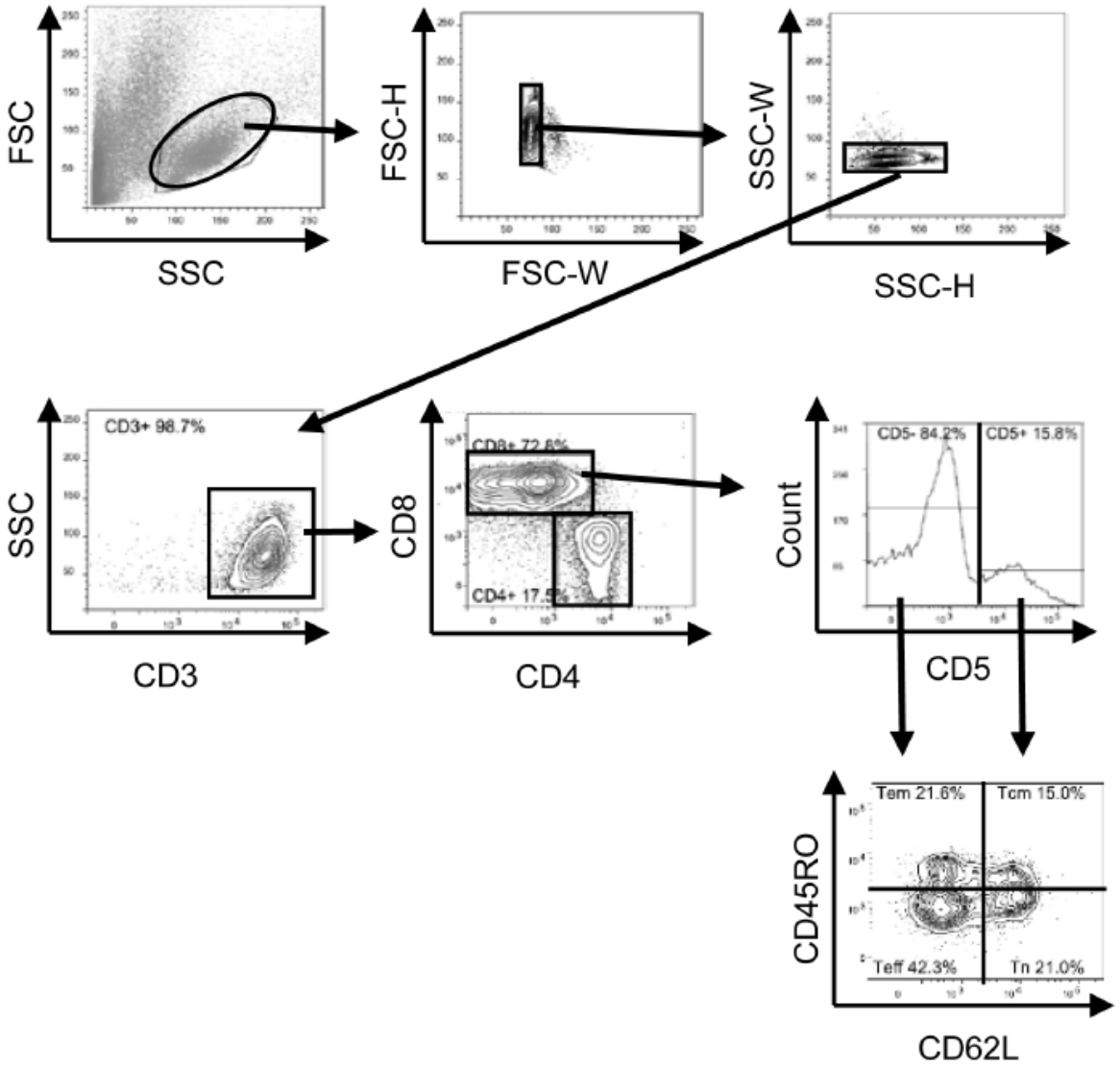
Statistical significance was analyzed with the GraphPad software, version 8.01. Differences in the cell count were calculated by t-test for paired data or the Wilcoxon test for independent groups. p values of 0.05 or less were considered significant.

3.8 Supplementary Figures

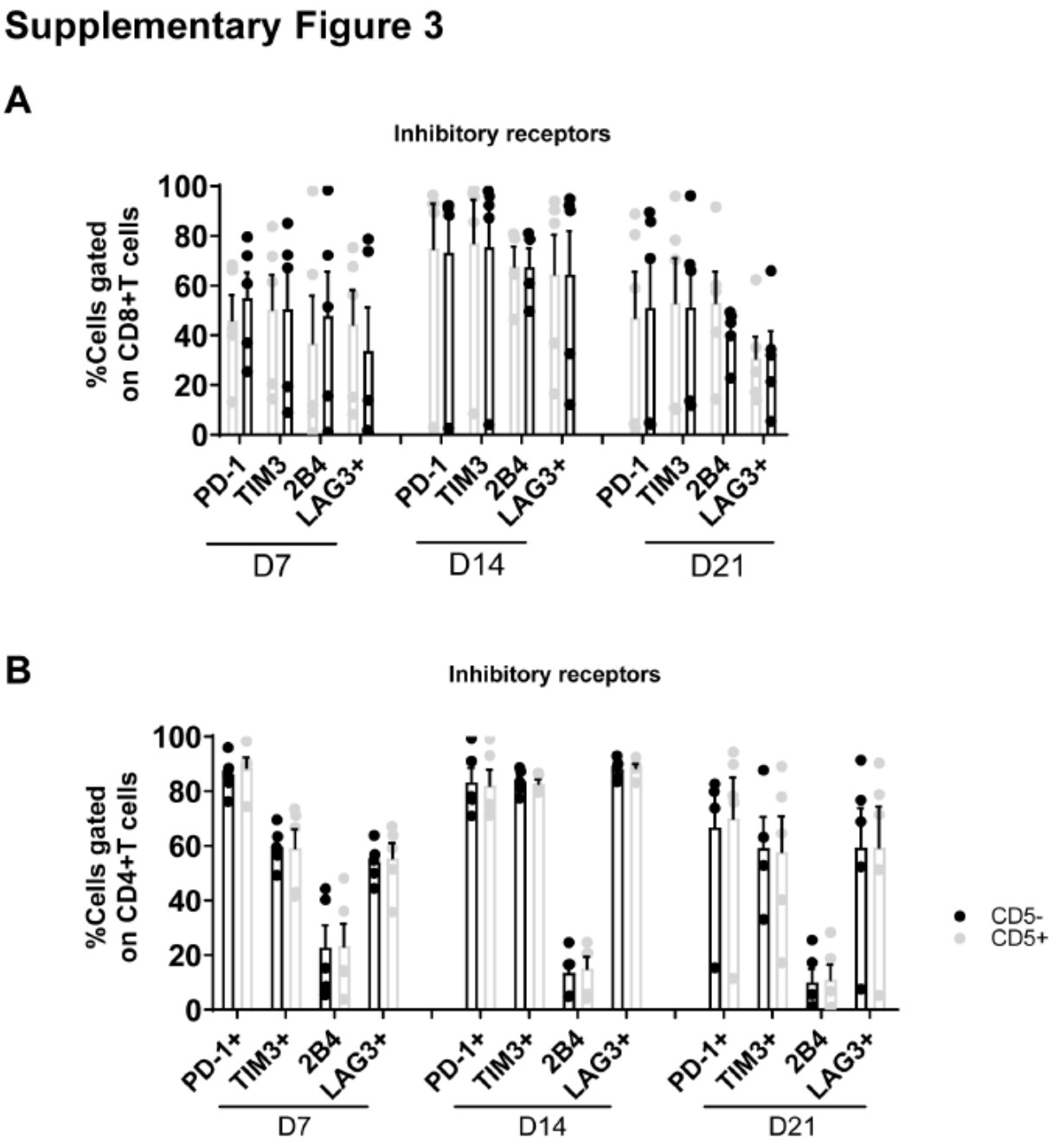
Supplementary figure S1. Identification of most suitable CD5sgRNA in Jurkat cells. Representative staining of CD5 expression on unedited (top) and edited Jurkat cells (bottom). On the top panel, CTRL is for non-nucleofected cells, Cas9 for nucleofection of Cas9 alone and SSC is for (side scatter). High editing efficiency of sgRNA-2 is shown in the bottom panel.



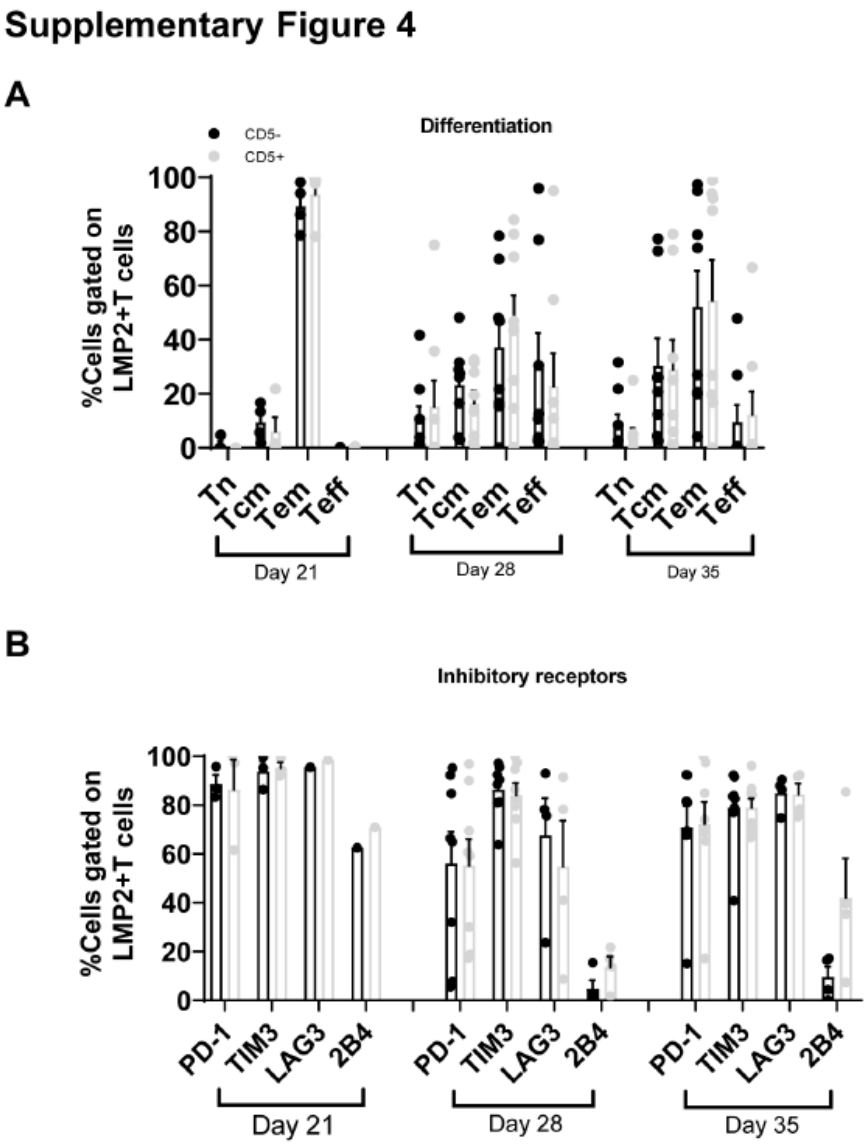
Supplementary figure S2. Gating strategy to assess the phenotype of T cells. Here shown for T-cell differentiation of CD8+ T cells.



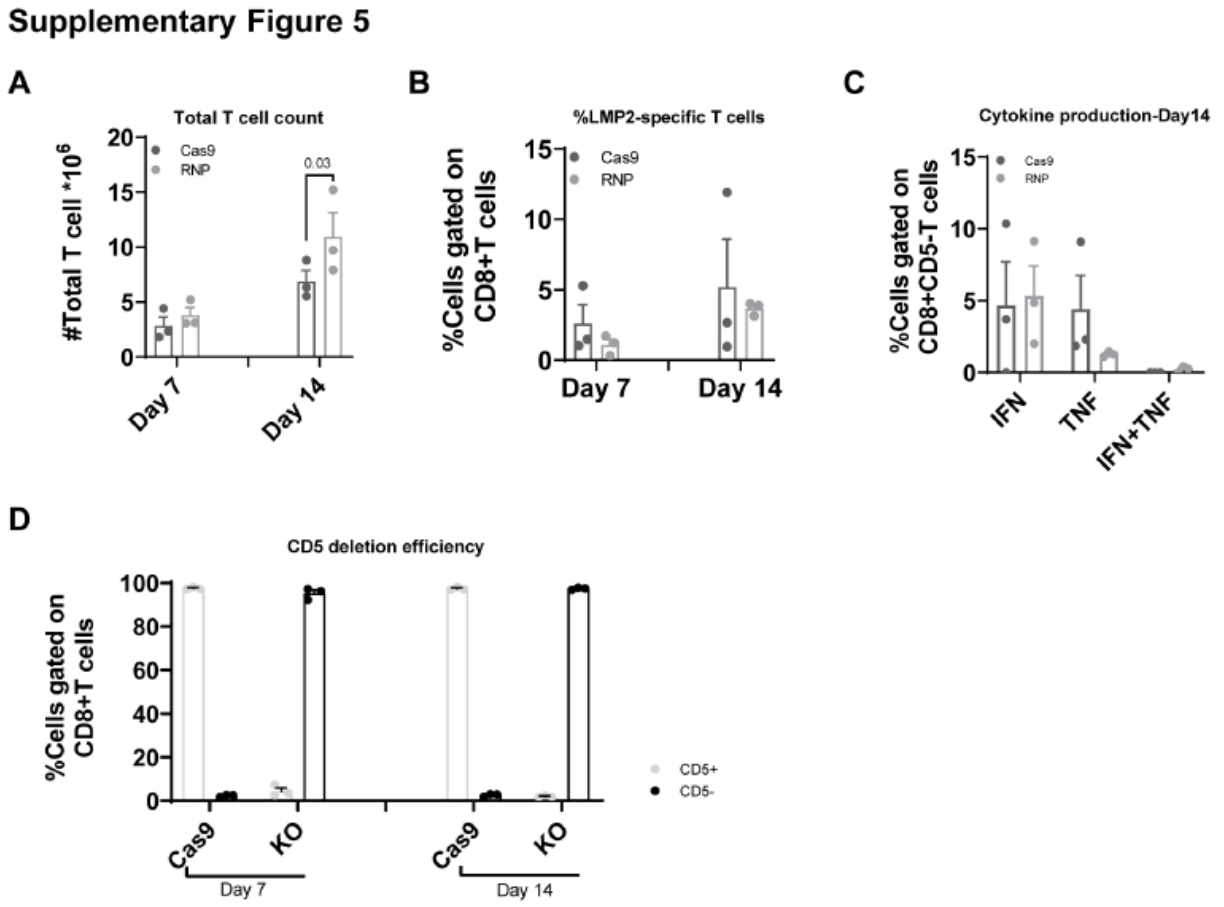
Supplementary figure S3. Inhibitory receptor expression following anti-CD3/CD28 stimulation. (A) Compiled results on CD5-expressing or CD5-negative CD8+ and (B) CD4+ T cells from 4 different donors/cultures at three time-points **(days 7, 14, and 21).**



Supplementary figure S4. T-cell differentiation and inhibitory receptor expression following repeated antigen stimulations. (A) T-cell differentiation based on CD45RO and CD62L expression showing the percentage of naïve (Tn), central memory (Tcm), effector memory (Tem) and effector (Teff) among CD5-positive and negative antigen-specific T cells as assessed by HLA-peptide multimer staining (LMP2+) at three time points following gene-editing and additional weekly antigenic stimulation (one to three weeks after gene-editing at day 14 of culture). Four different donors/cultures. (B) Assessment of inhibitory receptors on the same cells. Error bars are for SEM.



Supplementary figure S5. Antigen-specific T-cell expansion following gene-editing prior to culture initiation. (A) Total T-cell expansion in function of time in unedited (nucleofected with Cas9 alone) or edited T cells (RNP) following one (day 7) or two (day14) antigenic stimulations (p=0.03 for Cas9/RMP comparison at day 14). Three independent donors/cultures (B) Percentage of antigen-specific (LMP2) as assessed by HLA-peptide multimer staining or (C) cytokine producing cells upon peptide re-exposure (n=3 independent cultures/donor). (D) Histogram representing the percentage of CD5 expression in total $CD8^+$ T cells submitted to nucleofection with Cas9 alone (Cas9) or RNP (KO) and showing high gene-editing efficiency in the RNP condition.



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Chapter Four

4. Discussion

4.1 The efficiency of antigen-specific T cell adoptive therapy: Promises and Pitfalls

Immunotherapy, especially checkpoint blockade therapy, has revolutionized cancer treatment. However, a significant portion of patients does not respond to immunotherapy or experience relapse and tumor progress. One likely reason is not all the patients bear tumor-reactive T cells to eliminate tumor cells following the set free by checkpoint blockade. Directing the immune cells to target antigens specifically for adoptive cell therapy is a compelling strategy to treat a broad range of malignancies and propose an appealing alternative to checkpoint blockade or a potential modality to use alongside immune checkpoint blockade. Accordingly, clinical-grade protocols have been developed to produce specific T cells *ex vivo* and transfer them to patients in large numbers. Recent success in targeting the post-transplant viral infections associated with EBV and CMV has proven that specific T cell therapy is efficient and feasible. Early clinical trials demonstrated a 70%-90% protection in patients (193, 220). Increasingly, discovering a multitude of novel antigens, several of which are specific (especially in cancer), offers several opportunities if one can produce high-quality T cells from the natural repertoire.

Although the adoptive cell therapy with specific T cells has shown benefit in many studies, it is a labor-intensive method, and the protocols need to be improved. Additionally, since the therapy outcomes are correlated with the function and survival of the T cells, they can be further improved if the number of the cells, their functionality, and *in vivo* persistence could be augmented. The current protocol to produce specific T cells for adoptive cell therapy is via multiple specific stimulations and long co-cultures. Repeated stimulations can activate cells to go through vigorous proliferation and reduce expansion time while keeping the cell density. However, as discussed in chapter one, persistent long-term stimulation leads to the expression of several inhibitory receptors and T cell dysfunction. **As a result, the optimal strategy that promotes a sufficient number of long-lasting and functional specific T cells is still missing and needs to be further defined.**

Another major problem is the low efficiency of developing specific T cells from naïve repertoire. The successful clinical response achieved with virus-specific T cell transfer is partially due to already existing memory cells. Because most people have already encountered EBV and CMV, the circulating memory repertoire enhances the efficiency of *ex vivo* expansion. As mentioned in chapter one, naïve T cells specific to most tumors or microbial antigens usually have low frequencies in peripheral blood. Optimized antigen presentation and co-signaling are critical to prime T cells from naïve repertoire (discussed in chapter one). However, the negative regulation modulated by inhibitory receptors affects the T cell priming, especially in repetitive stimulation settings, and resembles the priming in chronic infection(221).

Consequently, considering the role of inhibitory receptors in exhaustion and priming T cells, blocking the checkpoints during the *ex vivo* expansion may lead to improved culture conditions to generate functional, long-lasting specific T cells.

Accordingly, utilizing a previously developed protocol(222), we aimed to generate clinical-scale virus-specific T cells expansion through repetitive stimulation of PBMCs with antigen-pulsed monocyte-derived dendritic cells in three to four weeks. We focused on expanding the circulating memory T cell repertoire targeting EBV envelope protein LMP2, a highly immunogenic antigen associated with the latent viral cycle(223). $LMP2_{426-434}$ is an HLA-A0201 restricted antigen, and since most people are latently infected with EBV, we anticipated a high efficiency of our protocol on the stimulation and expansion.

Our results highlighted that despite T cell growth and expansion after each stimulation, LMP2-specific T cell numbers and percentages stagnated or, in fact, decreased slightly but constantly beyond day 14 (chapter 2 fig1.A-C). **We also demonstrated that the poor development and expansion of antigen-specific T cells were associated with gradual differentiation from being mostly central memory cells to effector memory and effector cells at the end of the culture.** Additionally, these terminally differentiated cells expressed high levels of various inhibitory receptors, including PD-1, TIM3, LAG3, and 2B4 (chapter2, fig1.D, E), extending our previous results(222). Among these, high and persistent expression of PD-1 is indicated as the marker of the T cell exhaustion state. The association of expression of multiple inhibitory receptors with the exhaustion of specific T cells has been identified in chronic viral infections and cancers(101).

Accordingly, the presence of exhaustion markers, PD-1, and TIM3 on *ex vivo* expanded specific T cells, and the declined proliferation and expansion suggest that the T cells are pushed toward some level of exhaustion prior to adoptive transfer. T cell exhaustion can result in inadequate response to therapy, so preventing it will be essential to improve the treatment.

As repeatedly stated, it is critical to notice and emphasize that the inhibitory receptors are essential for immune response homeostasis, and their diverse function in exhaustion is due to their elevated and sustained expression. However, it is vital to characterize specific T cells in terms of inhibitory receptor expression; even if they do not lead to exhaustion, they will suppress and decline T cell activation and function.

The acquisition of exhaustion-like phenotype by *ex vivo* expanded specific T cells is likely due to constant antigen exposure and prolonged culture, leading to upregulation of several inhibitory receptors. To confirm the negative regulation, we tested our antigen-presenting cells (antigen-pulsed monocyte-derived dendritic cells). We showed Ceacam-1, Gal9, and PD-L1 expression, the main ligands of TIM3 and PD-1 by DCs (Chapter2, Fig1.G), providing a rationale to target the PD-L1/PD-1 and TIM3.

Our observations and results suggest that the *ex vivo* expanded virus-specific T cells can undergo an exhaustion process similar to CTLs during chronic infection or cancer. As a result, one can expect that they may also react to checkpoint therapy the same way the exhausted T cells do.

4.2 Using immune checkpoint therapy to boost the immune response in ACT

There are several available strategies to improve adoptive T cell products during the expansion and before the infusion to the patient. Here we submit a rapid and straightforward method to eliminate or lessen the possibility of exhaustion via blocking the inhibitory receptors. One advantage of this approach is evaluating several combinatorial strategies and assessing the T cells' characteristics before the infusion.

Checkpoint blockade therapy has been associated with improved anti-tumor immune response and enhanced tumor regression in several types of cancer. Anti-PD-1/PD-L1 and anti-CTLA-4

therapies have been approved by the FDA and are a part of the cancer therapy regimes for over a decade. To date, there are several new IRs in clinical studies to get FDA approval, like TIM3 and LAG3 blocking antibodies. Accordingly, we first sought to recognize the best inhibitory receptor among the expressed ones to target for immune checkpoint blockade. Our results show that most of the exhausted specific T cells express TIM3 and PD-1, the primary inhibitory receptors involved in exhaustion. On average, 50% of the LMP2-specific T cells express both TIM3 and PD-1 at day 21(chapter2, Fig1. F). Accordingly, co-expression of TIM3 and PD-1 has been observed on terminally differentiated and dysfunctional T cells in several mouse tumor models(224-226). For instance, the expression of both TIM3 and PD-1 inhibitory receptors is positively correlated with the severe exhaustion of TILs in melanoma(227). However, exhausted T cells are a heterogenous pool of cells. They present dynamic differentiation state, inhibitory receptor expression, function, and finally, their response to immunotherapy interventions differs. In our study, the cells expressing both TIM3 and PD-1 inhibitory receptors are still viable at the time of the harvest (day 28), they are not severely exhausted (as assessed by functional studies), and we expected that at least a fraction of them would react to ongoing checkpoint blockade.

As a result, we chose to study the impact of blocking TIM3 and/or PD-L1 (the primary ligand of PD-1) via blocking monoclonal antibodies starting from day zero of the culture. PD-L1 blocking was chosen for first being cost-effective compared to PD-1 blocking antibodies while showing the same results (228). And secondly, recent studies suggest some possible extra advantages of blocking PD-L1instead of PD-1(229). Targeting PD-L1 may lead to inhibiting additional PD-ligand interactions, such as PD-L1/CD80 interaction, which has been shown to inhibit T cell responses(229-232). However, the extent of the proposed additional mode of action was not evaluated in this study.

Surprisingly, anti-PD-L1 or anti-TIM3 treatment alone did not show any significant change in the numbers of either CD8+ or antigen-specific CD8+ T cells compared to the untreated control (Chapter2 Fig2. A-C). One explanation would be the non-redundant function of inhibitory receptors in the negative regulation of TCR signaling. Several inhibitory receptors are expressed by activated or exhausted T cells, so, likely, blocking just one IR may not be sufficient to disrupt the negative regulation of TCR activating signals. The synergistic function of inhibitory receptors in exhaustion and immune response has been previously documented in chronic infections and

cancer(233-235). Accordingly, TILs isolated from most of the patients resistance to PD-1/PD-L1 checkpoint blockade therapy demonstrate TIM3 upregulation(236, 237), supporting the possible efficacy of combination checkpoint blockade. It also can partially explain why there was no significant change following PD-L1 or TIM3 single blocking.

These data and ours suggest multiple layers of immune regulations mediated by several inhibitory receptors. As a result, while many of the checkpoint inhibitors can be effective as monotherapy, the lack of response in some patients and relapses has shifted the efforts to overcome the challenge by blocking several IRs (182, 238).

Many previously mentioned inhibitory receptors co-express with PD-1 on exhausted T cells and are mechanistically related (discussed in chapter one). It was then demonstrated that their dual blockade could efficiently reverse tumor growth in preclinical studies and led to the synergistic reversal of exhaustion in mouse models. Patients receiving more than one checkpoint therapy exhibited enhanced immune response resulting in superior cancer regression and overall survival. The combination of anti-PD-1 and anti-CTLA-4 blocking agents has been studied in melanoma in several clinical trials(238, 239). Patients who received combination therapy showed significantly lower rates of mortality and improved tumor regression. Similar results were obtained in renal cell carcinoma and lung cancer with the same combination of blocking antibodies(175).

Notably, antibodies directed to diverse inhibitory receptors are in different stages of clinical studies to treat different types of cancers. However, each inhibitory receptor serves to downregulate particular T-cell functions. Not all the checkpoint combinations efficiently recover T cells' exhaustion state, which could be due to the inharmoniousness of the targeted inhibitory receptors. Therefore, understanding the ligand/receptor interaction, the cells that express these IRs, and the underlying mechanisms are essential to target the best receptor(s) and design checkpoint therapies for the desired outcome.

T cell growth and specific T cell expansion significantly enhanced in PD-L1/TIM3 dual blocked condition compared to the control (no blocking antibody) (chapter2, fig2. A-C). We also confirmed the results using isotype antibodies (chapter2 supplementary fig1. A-C). Most importantly, enhanced proliferation and expansion in blocked conditions did not associate with terminal differentiation or severe exhaustion despite what is expected (chapter2, Fig2. D, E). However, we noticed a significant decrease in the percentage and expansion of antigen-specific T cells on day

14 compared to the control condition. These cells expressed vigorous growth at day 21 and significantly expanded. As a result, the decline could be due to the early blockade of inhibitory receptors. As mentioned in chapter 1, inhibitory receptors have a crucial function in T cell activation, and their early blockade could lead to hyperactivation of T cells. These cells could go through AICD and apoptosis. Since we had significant generation and expansion of specific T cells at the time of the harvest, which was our main aim, we did not evaluate these cells further.

Indeed, preclinical studies support the positive outcome of blocking both TIM3/PD-1 and reinvigoration of tumor-specific T cells over a single blockade of either(240, 241). Blocking both TIM3 and PD-1 or PD-L1 in melanoma patients was evaluated in some clinical studies, and the results from early clinical trials were encouraging(242). Interestingly, the same study also showed that blocking both TIM3 and PD-L1 limits tumor growth more than either monotherapy in advanced solid tumors. Anti-PD-L1 therapy has shown successful tumor regression and low toxicity in several different types of cancer. A more recent mouse study reported enhanced efficiency of oncolytic virotherapy in refractory lung cancer in combination with anti-TIM3 and anti-PD-1 therapy compared to monotherapy(243).

Collectively, our initial results confirm the possibility of checkpoint blockade during *ex vivo* expansion without compromising the specific T-cell generation. Secondly, it highlights the significant impact of blocking TIM3 and PD-L1 on T-cell growth and specific T-cell generation improvement.

Exhausted T cells also have altered proliferation capacity and lose cytokine secretion and killing potential stepwise but constantly. The severity of exhaustion is reflected in their function. Higher antigen load and co-expression of several inhibitory receptors are correlated with the lower functional capability. The pattern and number of expressed inhibitor receptors are also associated with different levels of T cell exhaustion. For instance, cells expressing both PD-1 and TIM3 are severely exhausted than PD-1+TIGIT+ T cells(244). As a result, the complex of co-expressed receptors can affect the outcome of checkpoint therapy to improve T cells' function and release an immune response. Several studies have documented the possibility of reversing the loss of function in exhausted T cells through checkpoint blockade. A comparison of TILs before and after checkpoint therapy revealed a shift of dysfunctional T cells to active functional tumor-specific T cells(245). In mouse models, simultaneous *in vivo* blockades of TIM3 and PD-1 have been

associated with restoring antiviral immunity and virus control. Notably, they reported, double blockade of TIM3 and PD-1 enhanced cytokine secretion and killing potential of virus-specific CD8+ T cells pointedly compared to the blocking of either of pathways alone(246).

Consequently, as discussed, co-expression of multiple inhibitory receptors leads to severe exhaustion; thus, one may hypothesize that blocking multiple inhibitory receptors may also synergically boost specific T cells' function.

CD8+Tcells are believed to be the majority of the immune cells aiming at the malignant cells in the tumor microenvironment of several cancers such as lung, renal, and colorectal (247-249). Additionally, since most previous studies demonstrate the effect of checkpoint blockade mainly on CD8+ T cells, we accordingly focused on this T cell subpopulation and did not evaluate or track the CD4+ T cell changes. However, one must keep in mind that PD-1 and TIM-3 co-expression is particularly relevant for CD8+ cells and that other immune checkpoints (such as LAG3, for instance) may be better targets to improve CD4+ T-cell expansion and function(177, 250).

In agreement with previous studies, we observed significant improvement in the proportion of functional T cells following blocking both TIM3 and PD-L1 (chapter2, fig4). The improved specific functionality of the CD8+ T cells was demonstrated through enhanced secretion of cytokines, including IFN-γ, TNF-α, and IL-2, in response to specific secondary stimulation. Also, the proliferation was measured by Ki-67 expression, and a comparison between the control and dual checkpoint blockade showed a significant increase in Tim3/PD-L1 blocked condition (chapter2, fig4. A-C). Therefore, the effect of checkpoint blockade is also reflected in the function of antigen-specific $CD8^+$ T cells; they expand more and are specifically directed to the expansion of specific T cells and not the polyclonal nonspecific T cells existing in the culture.

Ultimately, for the first time, our study verified that combined blocking of PD-L1 and TIM3 is an effective modulation to generate significantly more specific T cells via *ex vivo* expansion for adoptive therapy.

In line with a few previous studies, the co-expression of PD-1 and TIM3 on specific T cells and their synergistic impact on their exhaustion suggests intracellular crosstalk(251). The exact cellular mechanism associated with this synergy impact is poorly understood, partially due to the

controversial rule of TIM3 as a co-signaling receptor. PD-L1 and TIM3 are expressed by specific T cells, but TIM3 lacks the conventional signaling motifs (ITIM/ITSM) and utilizes Fyn/Bat3 tyrosine kinases to dampen TCR signals. Bat3 limits the access of Fyn to its binding site and functions as the gatekeeper of TIM3 downstream signaling. The TIM3 regulatory role is associated with T cell dysfunction in cancer and chronic viral infection(121). Additionally, the TIM3 cytoplasmic tail binds to several SH-2 domain-containing proteins such as PI3K and LCK tyrosine kinase(119). Enhanced TIM3 downstream signaling promotes mTOR1 activity indirectly(121). On the other hand, PI3K/Akt/mTOR signaling pathway is known to be inhibited by PD-1(252, 253), suggesting an essential node in CD8+T cell exhaustion/effector function.

Taken together, our results suggest a partially unknown synergy between TIM3 and PD-1/PD-L1 downstream signaling, which can have significant implications on developing checkpoint blocking antibodies and combinatorial therapeutic strategies.

4.3 The importance of inhibitory receptor physiology in specific T cell differentiation

Several studies have documented different expression profiles of co-inhibitory receptors during acute or chronic viral infections, in the tumor microenvironment, and in various stages of exhaustion. The distribution of inhibitory receptors differs on T cell subpopulations, and it is associated with their functional profile and response to checkpoint therapy. Hence, beyond the immediate use for the ACT, exploring the hierarchy of inhibitory receptors could reveal the exhaustion process in *ex vivo* expanded antigen-specific T cells and their potential as a checkpoint therapy target *in vivo*. The data from mouse models also have shown a hierarchy of function and expression for IRs(227). IRs engage and affect a different part of the TCR complex, signaling, and their expression profile(254). They may interfere directly with TCR like PD-1 or MHC molecules, as revealed in LAG3 and TIGIT(255). Several inhibitory receptors also target different members of TCR downstream signaling cascades, including TIM3, CTLA-4, and 2B4(32, 90).

Accordingly, the optimal timing to block each IR is essential in targeting multiple checkpoint blockades to achieve maximum therapeutic benefits. For instance, PD-1 is expressed shortly following the TCR activation, and it is a part of the immunological synapse; therefore, its primary

function is to control T cell activation and prevent hyperactivation. Preclinical studies showed enhanced apoptosis and anergy of antigen-specific T cells following the PD-1 blockade before antigen priming(256). In contrast, CTLA-4 will have a prevalent effect on the lymph node during T-cell priming (175, 204, 257). Thus, our immune checkpoint blockade protocol targeted the PD-L1/PD-1 interaction in both activated (non-exhausted) T cells at the beginning of the culture as well as gradually differentiated and dysfunctional T cells. The resulting increased in T-cell expansion might have been secondary to enhanced early activation or/and reinvigoration of exhausted T cells. Dissecting the effects of PD-1 blockade at the different stages of the expansion protocol may help tailor the best timing for PD-1 blockade.

Little data is available in the literature regarding the effect of timing in checkpoint therapy, and in this notion, TIM3 has been the most controversial inhibitory receptor. Depending on the immune response context (acute or chronic) and the specific cell type, TIM3 can have both co-inhibitory and co-stimulatory functions. As discussed before, TIM3 can promote T cell activation and function during an acute immune response. TIM3+ T cells have been reported to be the most responsive in active tuberculosis and *Listeria monocytogenes* infection(120, 125). Studies on tumor-reactive T cells also support the positive effect of transient TIM3 expression in cellular activation(258). Notably, such positive co-stimulatory results have been recorded while TIM3 is expressed transiently and seems independent of the ligands, including Gal-9 and HMGB1(259). Accordingly, in settings of persistent antigen expression such as chronic viral infection and tumor microenvironment, TIM3 co-expression with other inhibitory receptors is associated with exhaustion or T cell dysfunction(123).

To address the discrepancies mentioned above, we changed our *ex vivo* expansion protocol and checkpoint blockade timepoints. Instead of starting the blockade from day zero of the culture, we delayed the TIM3 blocking for one week and added the antibody at day 7. Of note, day zero was chosen by literature review and considering the fact that TIM3 and PD-1/PD-L1 are expressed highly during the first week of our co-culture (chapter2, fig1).

We showed that delaying TIM3 blocking for one week is associated with an even more increase in T cell expansion and antigen-specific T cell generation without inducing further exhaustion (chapter2, fig2). Additionally, the cells from delayed double blocked conditions not only show

enhanced cytokine production and proliferation but we also observed increased killing potential. Enhanced cytotoxicity was confirmed via *in vitro* cytotoxicity and granzyme secretion assays. We concluded that increased expansion, proliferation, cytokine production, and killing ability of the generated specific T cell are mediated by blocking PD-L1 and TIM3 during the *ex vivo* expansion. This did not come at the expense of increased terminal effector differentiation or exhaustion marker expression. Accordingly, we could expect better clinical outcomes based on the number and quality of T cells manufactured from our protocol.

Notably, some studies suggest a shift in the effector and memory T cell balance toward developing more short-lived effector T cell because of the TIM3 stimulatory signals(259). Using a mouse model, they show enhanced TCR and T cell activation signaling in TIM3 expressing models compared to TIM3 knocked out mice. However, TIM3 signaling led to exhaustion in the long run by restricting the long-lived memory cell development and impaired the CD8+T cell response to restimulation. Nevertheless, in the current study, the double blocked/ delayed TIM3 inhibition was not correlated with further T cell differentiation or any significant change in early memory or effector T cells.

Our results suggest a more complex role for TIM3 during specific T cell expansion and may confirm the co-stimulatory impact of short-term TIM3 expression. However, the exact underlying mechanism still needs to be characterized. We also propose more targeted checkpoint blockade while considering their downstream signaling to prevent possible induced resistance and benefiting from more efficient synergy.

4.4 Checkpoint blockade efficiency during the *ex vivo* expansion is not limited to memory repertoire or viral antigens

As discussed previously, priming naïve T cells requires professional antigen-presenting cells expressing co-stimulatory signals. Upon successful TCR stimulation, specific T cells start to proliferate and differentiate into effector and memory cells. In our method, peptide-pulsed dendritic cells utilized as APCs express CD80/CD86, the primary receptors providing co-

stimulatory signals in naïve T cell priming. However, our previous data indicate that even with the antigen-pulsed dendritic cells, the expansion of antigen-specific T cells from naïve repertoire is challenging and unpredictable(260). We evaluated the feasibility of generation and expansion of T cells specific to Wilms tumor antigen 1 (WT1) peptide epitope with checkpoint blockade. WT1 is a tumor suppressor gene, but its overexpression has also been found in various types of leukemia and solid tumors(261, 262). As for LMP2-specific T cells, blocking TIM3/PD-L1 increased T-cell yield and antigen-reactive WT1-specific T cells relative to the control condition. This may results from optimized antigen-presenting signals at priming. As some previous studies suggest, checkpoint blockade may improve the co-stimulatory signaling receptor expression and signal(56). PD-1/PD-L1 axis targets CD28-mediated co-signaling pathways, a vital part of IS, and priming naïve T cells. CD28+T cells have been detected as the most proliferating and functional cells in patients receiving anti-PD-1 therapies(263, 264).

The results presented here illustrate the importance of co-signaling in immune response and show how checkpoint blockade can facilitate generating antigen-specific T cells for adoptive therapy. Additionally, we present the importance of culture conditions and the positive impact of checkpoint blockade in specific T cell generation targeting different types of antigens. We showed that our method is feasible to generate functional specific T cells from a naïve repertoire targeting tumor associate antigen. We specifically described the impact of PD-L1 and TIM3 blockade to improve T cell *ex vivo* expansion protocol targeting viral and TAA antigen**s.** Taken together, we were able to generate high numbers of specific T cells that elicit a more robust immune response for adoptive T cell transfer through our suggested method.

4.5 Other inhibitory receptors to consider as a candidate for combinatorial therapies

In search of another target for checkpoint blockade during *ex vivo* expansion, we pursued to evaluate the impact of CD5 deletion in specific T cell generation. CD5 has been suggested to have a dual role, including the negative regulator of T cell activation and a T cell priming mediator as a component of the immune synapse(265). CD5 is highly expressed by regulatory lymphocytes such as Tregs and Bregs, so it has been primarily studied as a negative regulator of T cell activation

and a checkpoint. It is also a part of the immune synapse and is physically associated with the TCR complex(266). However, studies of CD5 deficient mice show its antigen-dependent and independent recruitment to the IS (267). As a result, its role in IS stability is unlikely and probably acts only as a TCR signal regulator(268). Notably, CD5 extracellular domain has been proven to be unessential for its inhibitory signaling as a result, blocking mAbs is expected to have modest influence(268, 269). Accordingly, deleting but not blocking CD5 in tumor mouse models led to enhanced TCR signaling and T cell-mediated anti-tumor immunity(270). Consequently, we decided to delete the CD5 protein using the CRISPR/Cas9 system.

Targeted deletion of inhibitory receptors via CRISPR/Cas9 in antigen-specific T cells is challenging. It may affect T cell survival due to technical hurdles, and the complete elimination of negative regulatory signals may cause T cell hyperactivity. For instance, PD-1 permanent deletion in effector T cells is associated with hyperactivity and severe immune response-related side effects(216). Probably due to its vital role as T cell function regulator during immune response homeostasis. We tried several CRISPR/Cas9 protocols to target CD5 and, after multiple experiments, successfully deleted CD5 in activated and specific T cells (chapter 3, fig1,2).

Although a modest increase in the expansion of CD5 deleted T cells, there was no significant change in the specific T cell generation or growth (chapter 3, fig2, 3). One reason could be that CD5 is not a direct negative modulator of TCR and mainly plays a scaffolding role, facilitating CBL-CBLB localization to the plasma membrane(271, 272). Hence, it could negatively modulate TCR signaling by controlling the CBL-CBLB ubiquitination and not directly affecting T cell function or response.

Next, we assessed the significance of CD5 absence in antigen-specific T cell cytokine secretion and did not see any pattern among the donors. There are still some more experimental gaps in our study, such as more severe functional assessment of the active T cells at different time points from the culture and in vivo experiments.

Finally, based on our current results, we do not recommend CD5 as a target for checkpoint blockade. However, there might be some benefit in combining the CD5 deletion with other checkpoint blockades.

4.6 Immune repertoire changes under therapy and its associations with clinical outcome

Given the degree to which checkpoint blockade influences the function and phenotype of *ex vivo* expanded T cells, we sought to unveil the biological underpinnings of such effect. Transcriptional profile and clonal diversity were performed considering previous data linking immune checkpoint blockade (administered to patients) with changes in clonality(273). Thorough characterization of the T cells following *ex vivo* checkpoint blockade would provide valuable insight to improve our understanding of the exhaustion state. We used scRNA-Seq to be able to link transcriptome with each clone expanded in control vs. experimental condition. Additionally, the changes of TCR repertoire within separate heterogeneous T cell populations before and after checkpoint blockade may reflect clonotype-dependent effects of immune checkpoint blockade.

Next-generation deep sequencing of complementarity-determining region 3 (CDR3) regions in the rearranged T-cell receptor b (TCRβ) chain was performed using the bulk mRNA samples of sorted LMP2-specific T cells at day 21 28. For each CDR3 sequence identified in a pool, the frequency within each individual at two different time points was subsequently calculated. It revealed that the specific T cells are highly oligoclonal, and a few abundant clones are shared between donors and conditions (Chapter 2, fig 6. A). Analysis of the traceable clones demonstrated that some clones disappeared or showed up with no consistent pattern across donors and conditions and were highly donor-dependent (chapter 2, fig 6. B, C).

Notably, observed clonal expansions and losses did not show dramatic repertoire changes, and we showed the persistence of most of the specific clones after TIM3 and PD-L1 blockade. These findings are consistent with previous studies that the diversity and clonality of T cells are markedly patient and tumor-type dependent(273).

To elucidate the blockade effect on transcriptome and T cell clonality at the single-cell level and identify the gene signature associated with active/expanded clones, we performed single-cell together with VDJ sequencing. After successfully clustering the specific T cells to different donors and conditions, it first confirmed the CDR3-TCR sequencing, and we could get a more robust estimate of clonal abundance. Then we investigated the TCR repertoire of specific T cells between the control, and TIM3/PD-L1 blocked conditions to evaluate if the hyperexpanded clonotypes are associated with any specific gene signature. T-cell activation, proliferation, terminal

differentiation, exhaustion, and metabolism-associated gene expression were assessed associated with T cell repertoire. We highlighted evidence of increased exhaustion in double blocked conditions compared to control in some clonotypes of some donors (chapter 2, fig 5. A-D). This can partially explain the disappearance of some clones; they probably were severely exhausted and failed to expand (or died) between the day 21 and 28. Reciprocally, some clones with a proliferation gene expression signature at day 28 saw their relative proportion increase from day 21 to day 28. Although the relationship to immune checkpoint blockade could not be established, our study is the first to map *ex vivo* expanded human antigen-specific clonotypes in the function of their gene expression signature. Examination of the gene signatures showed considerable donor to donor variability without notable differentially expressed gene programs between different donors and clones, suggesting a strong influence of yet unidentified donor-related factors and/or stochastic events occurring during culture. This argues for better characterization of donors (age, sex, previous infections, etc.).

In addition, through the expansion, specific T cells show a dynamic process of proliferation and differentiation; thus, sequencing specific cells in different time points would have improved the predictive potential of our analysis. However, it was not possible to sort enough specific T cells for all the conditions and time points in our case.

Unspecific broadening of T cell repertoire following the checkpoint blockade could enhance immune side effects and be associated with increased risk of relapse and shorter disease-free survival(274, 275). Despite the promising outcome of adoptive cell therapy, many treatments elicit severe on-target, off-tumor side effects (discussed in chapter one), making the pretransfer evaluations necessary. TCR sequencing allows for tracking the dynamic changes of specific T cell clones during *ex vivo* expansion and highlights the changes that may lead to altered immune response following the transfer.

Utilizing sequencing across the CDR3 region of the TRBV gene in either bulk populations of sorted specific T cells or at the single-cell level, we compared the double blocked condition with control. Regardless of modest changes in some clones, we did not detect any significant change in clonal diversity following the checkpoint blockade. Additionally, gene expression analysis confirmed our previous experiments regarding expanding highly active clones in double-blocked conditions. As mentioned, there were some limits to our approach; however, the data support the

safety of *ex vivo* expanded products combined with checkpoint blockade and highlight the challenge of selecting the proper regime and the importance of personalized immunotherapies.
This concept of a tailored approach to patient treatment based on the molecular analysis of tumor genes and proteins has been validated with highly efficacious kinase inhibitors that target specific subgroups of tumors.
Our results also hinted at donor-dependent features (scRNA sequencing data) and the personalized nature of adoptive immunotherapy treatments. While such variability from donor to donot adds a certain level of uncertainty and may unsettle regulatory agencies, it may not be different to current immunotherapy approaches that leads to vastly different outcomes, such as immune checkpoint blockade(276). Nonetheless, the deep analysis of the adoptive cell therapy product and characterization of the cells before the transfer could advance cancer immunotherapy and improve the efficiency of the therapy.

4.7 Heterogeneity and fate choice: T cell exhaustion in ex vivo expanded antigen-specific T cells

CD8 T cell differentiation is a tightly regulated process generating effector and memory T cells over the course of acute infections. In cancer and chronic infection, this differentiation program is disrupted due to the persistence antigen exposure, and antigen-specific CD8 T cells differentiate to a hyporesponsive state generally referred to as T cell exhaustion. As discussed in detail in chapter one, virus-specific exhausted T cells are a heterogeneous population and consist of at least two subpopulations: a progenitor CD8 T cell subset that proliferates and gives rise to a terminally differentiated progeny. Terminally differentiated T cells do not replicate and display high expression of multiple inhibitory receptors. Notably, the progenitor subset responds to checkpoint blockade and is the prime target of immunotherapy. However, due to the progressive nature of the exhaustion, ultimately, cells will reach the severe state of dysfunction/exhaustion, which is highly resistant to therapeutic reprogramming. In our study, the LMP2-specific T cells responded efficiently to the PD-L1 and TIM3 checkpoint blockade, which hints at their early exhaustion state. As a result, we anticipated further differentiation or the progressive upregulation of inhibitory receptors. However, their exhaustion phenotype did not progress, and the cells were viable and highly functional at day 28. Also, the single-cell transcriptome of the LMP2-specific T cells

comparing all clonotypes or dominant clonotypes across donors and conditions showed no consistent pattern or significant changes in any of the pathways. However, based on our bulk TCR sequencing results, some clones disappeared from day 21 to day 28, which had a higher expression of genes related to T-cell activation, differentiation, and exhaustion relative to other clonotypes from the same donor in the same condition (chapter2, Fig6D).

These results all point to the fact that we probably are not reinvigorating the exhausted T cells or changing their fate in our method. In fact, the severely exhausted fraction of the cells go through apoptosis and disappear, and the significant positive response we show is due to the expansion of the early state exhausted specific T cells. In other words, checkpoint blockade during the ex vivo expansion of the specific T cells preserves the early differentiated T cells and improves their growth and development. We did not evaluate this hypothesis further, but one could anticipate that the cell growth and specific T cell expansion will continue till all the progenitors are exhausted. Accordingly, the cells will keep responding to the checkpoint blockade till the state of complete dysfunction.

Taken together, estimating distinct differentiation state dynamics, fate choices, and functional states of the ex vivo expanded cell before the transfer have the utmost importance. Delineating the regulatory mechanisms defining distinct T cell states and determining the requirements for therapeutic reprogramming of these states will provide needed insights for the design of effective immunotherapies for the treatment of cancer and chronic infections

4.8 Conclusions

Antigen-specific T cell adoptive therapy targeting refractory viral infections after HSCT has been proven to provide a potentially curative, pathogen-specific, and non-toxic treatment against the virus(192, 277-279). This approach offers several opportunities for patients resistant to conventional pharmacotherapies or other forms of immunotherapy; and provides an accelerated specific immune response with the minimal off-tissue effect. Adoptive transfer of virus-specific T cells in patients following the HSCT results in high viral clearance (280).

Isolation of the tumor specific-T cells from the tumor-infiltrating cells is another suitable strategy(281). However, the majority of the TILs are polyclonal T cells, and less than 10% of them

can recognize tumor antigens(274, 282). Additionally, the high load and persistent expression of antigens, along with the immunosuppressive nature of the tumor microenvironment, pushes the TILs toward exhaustion and the dysfunctional state (283, 284). They show functional impairment, low anti-tumor reactivity, and early disappearance, making the use of TILs as an adoptive cell therapy challenging(281, 285). Preventing this kind of functional exhaustion will improve the efficiency of ACT products and therapy outcomes. Hence, our approach may also be applied to the expansion of TILs, which are antigen-experienced and often expressing PD-1 already at harvest(281).

Accordingly, in this study, we successfully established and developed an *ex vivo* model to generate sufficient numbers of highly functional specific T cells targeting different types of immunogens for the ACT. **To our best knowledge, for the first time, checkpoint blockade was combined with the *ex vivo* expansion-specific T cell generation to ameliorate the negative regulation mediated by inhibitory receptors.** We observed the improved generation of specific T cells via checkpoint blockade combinations (TIM3+PD-L1 targeting a diverse type of peptide (viral antigen- LMP2 or TAA- WT1).

Our work has revealed important insights into T cell biology based on phenotyping and receptor profiling. We offer new biological insight into how different inhibitory receptors can synergize to improve specific T cell generation and expansion and their function. We show that the outcome of combining checkpoint therapy and specific T cell adoptive therapy could be influenced by at least two factors: the relative timing of checkpoint blockade administration and the physiology of targeting inhibitory receptors. Much remains unknown about the mechanism of action of checkpoints alone or combined. Accordingly, we advise that the best timing and combination of receptors to achieve maximum synergy depends on the physiology of each checkpoint and its part in modulating TCR signaling.

4.9 Limitations and Future direction suggested by our study

Administration of checkpoint blockade during *ex vivo* expansion of antigen-specific T cells could improve T cells' yield and function for adoptive cell therapy. In this study, we highlighted that blocking several inhibitory receptors may not be functionally redundant. In fact, they could act

synergically to improve immune response, specifically if the targeting receptors are expressed by different cell populations/subsets or utilized other signaling motifs. However, as we highlighted, timing and the proper combinations of checkpoint blockade are vital factors affecting the efficiency of adoptive immunotherapy products.

Although our results contributed to a better understanding of specific T cell therapy combined with checkpoint therapy, this study has several limitations. Our study's short-term *ex vivo* nature makes it hard to follow up the long-term effects on survival, expansion, and function associated with other immune response-related factors like TME. Checkpoint blockade resulted in the proliferative growth of specific T cells, but these cells keep differentiating and will display altered phenotype and function at later times or after infusion to the patient. This raises the question about the necessity of maintaining the checkpoint therapy after adoptive transfer. In this notion, and considering that our ex vivo expanded antigen-specific T cells still express several inhibitory receptors such as PD-1, TIM3, and LAG3 at the time of harvest, one could expect their positive response to checkpoint blocking after the transfer.

We concentrated our study mainly on the function and phenotype of specific T cells influenced by checkpoint blockade and did not evaluate the exact mechanism of action of each blocking antibody. Given the number of potential ligands of TIM3, the aftereffects of blocking their interaction might be different. Literature review suggests a difference between the contribution of each ligand in TIM3 signaling, and depending on which TIM3-ligand interaction is blocked, the clinical outcome might change. In the current study, our anti-TIM3 blocking antibody was reported to target the Gal9-TIM3 interaction mainly(286, 287), but we did not get the chance to address other possible scenarios specifically. Considering that our antigen-presenting DCs were expressing other TIM3 ligands, too, it might be imperative to determine which TIM3-ligand interaction is dominant and can optimally modulate our specific T cell product. Of note, blocking multiple TIM3-ligand pathways may improve the T cell yield but knowing the fact that TIM3 ligands are not exclusive, complete blockade may cause unknown adverse consequences(288, 289).

Although we attempted to address several questions regarding the mechanism of the highlighted synergy between TIM3 and PD-L1 blockade through single-cell transcriptome, the low number of donors obscured potential differences. Further experiments are needed to disclose the contribution

of actively transcribed genes to very stable transcripts to the specific T cell expansion following checkpoint blockade. Another challenge that our study faced was the sampling bias. We had no way to evaluate if the clonal input was the same between control and blocked conditions, and again, due to the low number of specific T cells at some time points, only a snapshot of the TCR repertoire was taken. Currently, several new combinatorial immunotherapy strategies and agents are being developed, and as reflected by our results, it would be essential to verify their synergy through mechanisms of action. A deeper understanding of the underlying mechanisms may also lead to identifying new targets to facilitate the stratification of responsive patients.

The mechanism underlying TIM3/PD-L1 blockade's synergy remains to be investigated precisely; it will be essential to identify and specify which TIM3-ligand interaction is responsible for the potential immunological response *in vivo*. We suggest a thorough evaluation of the phosphorylation state of the proteins involved in downstream signaling pathways of TIM3 and PD-1 in specific T cells under checkpoint blockade. Using selective inhibitors of various adaptor proteins and enzymes such as PI3K and Akt would help determine the crosstalk between TCR signaling and TIM3/PD-1.

Additionally, as mentioned in chapter one, CD5 is a scavenger receptor. Since the scavenger receptors function as a complex network of receptors and ligands, therapeutic intervention is far more complicated than the more common inhibitory receptors such as PD-1 and TIM3. As a result, we advise using CD5 for therapeutic purposes after considering all these possible associations with other ligands and receptors. This being said, our most negative results may be dependent on the methods we used. We integrated CD5 deletion in *ex vivo* cultures in order to eventually incorporate this modification in cell therapy protocols involving antigen-specific T cells. Our results are at odds with previous murine studies done in vivo with CD5 knock-out T cells showing increased anti-tumor activity or human CD5 deleted CAR T cells showing increased activation profile and killing activity(290). The reasons for these discrepancies are unclear but may depend on several factors including the type of stimulation used or CRISPR-Cas9 off-target effects that we are currently investigating.

To finish, optimizing culture conditions or T cell genetic modifications are widely used to avoid a decline in the number or function of *ex vivo* expanded specific T cells, and preconditioning T cells is still developing. Another relatively successful therapeutic intervention includes the modulation

of co-stimulatory molecules to increase the efficiency of cancer immunotherapy. Several co-stimulatory molecules have been approved by preclinical and mouse model studies(291, 292). Co-stimulatory deficient CD8+ T cells display weak anti-tumor activity and the inability to survive and generate memory T cells. Furthermore, overexpression of the co-stimulatory molecules enhances the specific immune response and positively correlates with tumor regression(293). Our study supports the idea of manipulating co-signaling receptors for more robust specific T cell generation. For further improvement, modulating co-stimulatory receptors such as 4-1BB and OX40 along with checkpoint blockade should be considered(292). OX40 has also been shown to reduce the levels of T-cell exhaustion in the glioma microenvironment(294).

Our work integrates immune checkpoint modulation for the optimization of ACT based on antigen-specific T cells. As such, we show that the two most effective forms of cancer treatment can be used together, at least to generate better T-cell products. A reasonable objective would be to use these enhanced T-cell products in clinical studies that investigate the combination of immune checkpoint blockade with ACT.

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