Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse intitulée:

Transcriptional regulation of effector CD8+ T cell differentiation and molecular dysfunction during HIV-1 infection

Régulation transcriptionnelle de la différenciation et de la dysfonction des lymphocytes T CD8+ effecteurs au cours de l'infection par le virus de l'immunodéficience humaine de type 1

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Résumé

Les cellules T CD8+ jouent un rôle primordial dans le contrôle des infections virales en limitant la dissémination des cellules infectées. Lors de l'infection chronique par le virus HIV, les cellules T CD8+ HIV-spécifiques ne se différencient pas en cellules effectrices fonctionnelles capables de tuer les cellules infectées par le virus ; ces cellules ne sont plus capables de proliférer ou de produire l' IL-2. Ces cellules expriment PD-1 et l'engagement de PD-1, par son ligand, aboutit a plusieurs de ces déficits fonctionnels des cellules T . Le rôle de PD-1 dans la régulation d'évènements transcriptionnels contrôlant la différentiation et l'obtention des fonction effectrices des cellules T CD8+ reste à démontrer.

Id2 joue un rôle central dans la différenciation des cellules T CD8+ effectrices. Nous avons émis l'hypothèse que le défaut de maturation observé chez les cellules T CD8+ PD-1 high HIV-spécifiques (CD8+PD-1hi) au cours de l'infection chronique par le virus HIV pouvait être lié à la diminution d'expression du régulateur Id2. Nous avons ainsi démontré que l'engagement de PD-1 contribuait à une diminution d'expression de Id2 et de ses cibles transcriptionnelles. La surexpression de Id2 de ces cellules a permis de restaurer l'expression de marqueurs tels que Granzyme B et Bcl-2 et diminuir l'expression du marqueur de maturation de CD27.

La famille des cytokines à chaine γ joue un rôle clef dans la survie et l'homéostasie des cellules T. Dans ce travail, nous avons démontré que l'IL-15 était unique grâce à ses capacités de stimulation de l'expression d'Id2 et ses propriétés favorisant la survie ainsi que la différenciation des cellules T CD8+ effectrices. l'IL-15 induit la prolifération de toutes les populations de cellules T mémoires provenant de donneurs sains. L'addition de cette

cytokine aux sous-populations cellulaires Ttm et Tem a permis leur différenciation en cellules effectrices capables de produire Granzyme B alors que la stimulation par l'IL-15 des cellules Tcm ne favorise pas leur différenciation. Un test de cytotoxicitié par cytométrie en flux nous a permis de confirmer que la stimulation de cellules T CD8+ HIV spécifiques par l'IL-15 favorisait l'expression de Id2 et restaurait les fonctions cytotoxiques des cellules T CD8+ HIV spécifiques.

En conclusion, nous avons pour la première fois dans cette thèse défini les mécanismes moléculaires impliqués dans la modulation de l'expression du régulateur transcriptionnel Id2 par l'IL-15. Nous avons également révélé comment l'engagement de PD-1 conduisait a une altération de l'expression et de la fonction d'Id2 et favorisait la diminution des fonctions effectrices des cellules T CD8-HIV spécifiques. Une perspective de traitement avec des agents tels que l'IL-15 ou le bloquage de PD-1, en combinaison avec les traitements conventionnels, pourrait contribuer à une meilleure stimulation des réponses immunes favorisant ainsi la réactivation des cellules T CD8+ et permettant la destruction de cellules T CD4+ infectées de manière latente.

Mots-clés : HIV, CD8 CTL, PD-1, Id2, IL-15, différenciation des cellules effectrices, cytotoxicité

Abstract

CD8+ T cells play a fundamental role in controlling viral replication and dissemination by killing virus-infected cells. However during chronic HIV infection HIV-specific CD8+ T cells fail to differentiate to functional cytotoxic effector cells and develop functional defects such as loss of IL-2 secretion, decreased proliferation and express high levels of PD-1. Persistent expression of PD-1 and triggering by its ligand results in immune dysfunction; it is not known how PD-1 signaling influences transcriptional events involved in T cell differentiation and effector function.

We found that the transcriptional regulator Id2 was downregulated in PD-1hi HIV-specific CD8+ T cells when compared to PD-1low CMV-specific CD8+ T cells from the same HIV-infected donors. Since Id2 has been shown to play a central role during differentiation of effector CD8+T cells, we hypothesized that skewed maturation of the PD-1hi HIV-specific CD8+ T during chronic HIV infection could result from decreased levels of Id2. We found that signals transduction pathways downstream of PD-1 ligation inhibited the expression of Id2; transfection of PD-1hi effector cells from HIV infected individuals with a Tat-Id2 construct could reverse an apoptotic fate associated with the exhausted phenotype. Finally, overexpression of Id2 restored expression of Granzyme-B and Bcl-2 and led to a decreased expression of the T cell maturation marker CD27.

Although the extrinsic signals and costimulation needed to activate cell proliferation and effector function are well known, signal-transduction pathways that regulate differentiation of memory cells to effector cells are beginning to be understood. The γ–chain family of cytokines is essential for the survival and homeostasis of T cells; they have pleiotropic

effects on the differentiation of effector and memory virus-specific CD8+ T cells. IL-15 was unique among γ-chain cytokines in upregulating the expression of Id2 and promoting the survival and differentiation of effector memory CD8+ T cells.

IL-15 induced proliferation of all memory subsets from healthy subjects but only induced differentiation, Granzyme-B production, and cytotoxic effector function in CD8+ Ttm and Tem cells. Stimulation of Tcm with IL-15 failed to induce their differentiation; this was associated with their decreased *ex vivo* levels of IL-15Rα when compared to Tem and Ttm subsets. Finally, we developed a single cell flow-cytometry cytotoxicity assay, and found that stimulation of CD8+T cells from HIV chronically infected subjects with peptide plus IL-15 induced the differentiation of tetramer+ CD8+ Ttm cells and restored Id2 expression and their cytotoxic activity .

Overall, we illustrate in this thesis, for the first time, the molecular mechanisms of effector T cell differentiation mediated by IL-15 and its downstream transcriptional regulator Id2; we reveal how PD-1 engagement leads to alteration of the Id2 pathway leading to decreased effector function of the HIV-specific CD8+ T cells. Immunotherapy with agents such as IL-15 or PD-1 blocking antibody that increase levels of Id2 expression , in combination with HAART, should trigger the functional re-activation of HIV-specific CD8+ T cells and the killing of latently HIV-infected CD4+ T cells.

Keywords : HIV, CD8 CTL, PD-1, Id2; IL-15, effector cell differentiation, cytotoxicity

TABLE OF CONTENTS

CONTENTS

LIST OF FIGURES

CHAPTER 3

CHAPTER 4

CHAPTER 5

ABBREVIATIONS

APC: Antigen Presenting Cell

AIDS: Acquired Immunodeficiency Syndrome

Bcl-2: B-cell lymphoma 2

Bcl-6: B-cell lymphoma 6

Blimp-1: B lymphocyte-induced maturation protein 1

CBA: Cytometric Bead Array

CCR5: Chemokine (C-C motif) Receptor 5

CCR7: Chemokine (C-C motif) Receptor 7

CD3ζ: Cluster of Differentiation 3ζ

CD4: Cluster of Differentiation 4

CD8: Cluster of Differentiation 8

CD27: Cluster of Differentiation 27

CD28: Cluster of Differentiation 28

CD38: Cluster of Differentiation 38

CD45RA: Cluster of Differentiation 45RA

CD57: Cluster of Differentiation 57

CD70: Cluster of Differentiation 70

CD80: Cluster of Differentiation 80

CD86: Cluster of Differentiation 86

CD107a: Cluster of Differentiation 107a

CD127: Cluster of Differentiation 127

CD160: Cluster of Differentiation 160

CDK: Cycline Dependent Kinases

CHI: Chronically progressing subjects

CMV: Cytomegalovirus

CTL: Cytotoxic T Lymphocyte

DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-

EOMES: Eomesodermin

EC: Elite Controller

Env: Enveloppe

FASL: Fas Ligand

Gag: Group Antigen

GALT: Gut-Associated Lymphoid Tissue

GrzB: Granzyme B

gp120: Glycoprotein 120

HAART: Highly Active Antiretroviral Therapy

HIV: Human Immunodeficiency Virus

HLH: Helix-Loop-Helix

ID: Inhibitory of DNA binding

IFN : Interferon

IL-2 : Interleukin 2

IL-15: Interleukin 15

IL-7: Interleukin 7

IS: Immunological Synapse

KLRG-1: Killer cell Lectin-like Receptor G1

LCMV: Lymphocytic Choriomeningitis Virus

LM: Listeria Monocytogenes

MHC: Major Histocompatibility Complex

MPEC: Memory Precursor Effector Cells

mRNA: messenger Ribonucleic acid

Nef: Negative factor

NK : Natural Killer Cell

PAMPs : Pathogen-Associated Molecular Patterns

PBMC : peripheral blood mononuclear cell

PCR: Polymerase Chain Reaction

PD-1: Programmed cell death 1

PD-L1: Programmed cell death Ligand 1

PD-L2: Programmed cell death Ligand 2

pDC: plasmacytoid dendritic cell

Pol: Polymerase

pRb: retinoblastoma

siRNA: small interfering RNA

SLEC: Short-Lived Effector Cells

ST: successfully HAART treated

STAT-3: signal transducer and activator of transcription 3

STAT-4: signal transducer and activator of transcription 4

STAT-5: signal transducer and activator of transcription 5

ST: Successfully Treated

T-bet: T-box transcription factor

TCR: T cell receptor

Tcm: T central memory

Tem: T effector memory

Temra: T terminally differentiated effector

Ttm: T transitional memory

Tscm: T stem cell-like memory cells

TNF: Tumor Necrosis Factor

TRAF: Tumor necrosis factor receptor associated protein

VV: Vaccinia Virus

ZAP-70ζ-associated protein-70

Ai mie genitori, Alfonso e Cristina,
per avermi sostenuto
in questo faticoso percorso della mia vita
e avermi fatto sentire sempre
il loro amore e la loro vicinanza
nonostante un oceano di mezzo.
Senza il loro supporto questo dottorato
non sarebbe stato possibile.

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CHAPTER 1

INTRODUCTION

1. Immunological Memory

Immunological memory is defined as the ability of the immune system to respond against a previously encountered pathogen. This characteristic was described for the first time by Thucidydes in the fifth century B.C., indeed he noticed that people who survived plague were not infected a second time. The property of the immunological memory are based on the fact that both antibody and T cell responses which mediate the adaptive immune response, are more efficient in responding the second time to the same antigen than in the first encounter. Immunological memory is the basis for generating protective immunity against many pathogens and represents the hallmark for vaccine strategies. While it is clear that B cell production of antibody is critical for the protective features of many vaccines, long-lived T cell immunity is also a critical component for good vaccine.

1.1 Generation of memory CD8+ T cells

Once a mature naïve T cell population exits the thymus after positive and negative selection, it circulates in the blood and in the periphery while expressing a wide repertoire of diverse T cell receptors (TCRs) that can recognize pathogens and induce efficacious immune responses. Naïve T cells are maintained by the signals provided by the continuous but transient interaction of their TCR with self–peptide major histocompatibility complexes (MHC) on antigen-presenting cells (APCs). Upon infection dendritic cells migrate to the lymphoid organs and present antigen to naïve T cells. This interaction is responsible for the formation of immunological synapses (ISs) between T cells and APC leading to more stable interactions. A naïve T cell activated by antigen stimulation undergoes proliferation, cytokine production, and differentiates into effector cells. Several studies have shown that the immune response requires three signals: 1) antigenic stimulation upon engagement of the TCR and CD8 as a coreceptor, 2) costimulatory signals, such as CD28, and 3) inflammatory cytokines such as IL-12 and IFNs (Curtsinger, Schmidt et al. 1999; Curtsinger, Johnson et al. 2003; Curtsinger, Lins et al. 2003; Kolumam, Thomas et al. 2005). The T cell-APC interactions lead to clonal expansion and effector and memory formation. The few naïve T cells specific for the cognate antigen are able to divide more

than 15 times (up to 50.000 fold expansion) in response to lymphocytic choriomeningitis virus (LCMV) and Listeria monocytogenes (LM), leading to their differentiation into effector cytotoxic T lymphocytes (CTLs) that will kill infected cells (effector phase). Functional CTLs exert their cytotoxic activity through the secretion of effector molecules, such as granzymes and perforin, as well as the secretion of cytokines such as interferon-γ (IFN) and tumor necrosis factor (TNF). CD4+ T cells may also differentiate into T helper 1 (Th1) cells that secrete IFNγ, TNF and interleukin-2 (IL-2). These events regulate the antiviral immune response and mediate direct killing of virus infected cells. Once the antigen has been cleared, most of the effector CTL (90%-95%) die by apoptosis (contraction phase) while the remaining cells (5-10%) establish a pool of the long-lived population of memory cells. It was originally proposed that the few cells that survive the contraction phase were randomly selected. However, recent studies have shown that some cells have more memory potential when compared to others. Studies in mice have helped in the discovery of transcription factors (described later) and cell surface markers that can predict which cells differentiate into effector function and those that instead generate the memory T cell population for life-long persistence. Among these markers, increased levels of IL-7 receptor subunit α (IL-7Rα or CD127), CD27, and B cell lymphoma 2 (BCL-2), as well as decreased expression of killer cell lectin-like receptor G1 (KLRG1) have been shown to preferentially induce the generation of long lived memory CD8+ T cells (Schluns, Kieper et al. 2000; Kaech, Tan et al. 2003; Joshi, Cui et al. 2007; Kurtulus, Tripathi et al. 2011; Dunkle, Dzhagalov et al. 2013). While adoptive transfer experiments using TCR transgenic cells have shown that $KLRG^{hi}$ $CD127^{lo}$ cells slowly decrease in number after transfer in comparison to the long lived $KLRG^{lo}$ $CD127^{hi}$ cells that were maintained over time (Joshi, Cui et al. 2007; Sarkar, Kalia et al. 2008). Thus $KLRG^{hi}$ $CD127^{lo}$ cells have been referred as “short-lived effector cells” (SLECs), while $KLRLG^{lo}$ $CD127^{hi}$ cells as” memory precursor effector cells” (MPECs). Memory cells that are generated after the contraction phase have the ability to control secondary exposure to antigen by the rapid acquisition of effector function, increased frequency and localization to peripheral sites of infection.

1.2 Central memory and Effector memory T cell subsets:

Memory T cells are heterogeneous and different subsets have been identified among CD4+ and CD8+ T cell population. The main two subsets are central memory and effector memory cells (Tcm and Tem, respectively). These two populations are distinguished based on their homing capabilities and effector functions. Effector cells home preferentially to the periphery; indeed they lack the expression of lymph-node homing markers such as CD62L and CCR7. In contrast central memory cells are found preferentially in the lymph-node as they are CD62L+ and CCR7+. Tcm share similar properties with naïve T cell, but do not express CD45RA on their cell surface and can rapidly differentiate into effector cells upon re-exposure to the same antigen. They have high proliferative potential and secrete high levels of IL-2 (Sallusto, Lenig et al. 1999); Tcm are more efficient at reconstituting the memory T cell pool and mediating protective immunity compared to Tem (Wu, Kirman et al. 2002; Wherry, Teichgraber et al. 2003; Castiglioni, Gerloni et al. 2004; Bouneaud, Garcia et al. 2005; Klebanoff, Gattinoni et al. 2005). Tem have lower proliferative capabilities and IL-2 production, however, they can rapidly exert cytotoxic and effector functions (IFN-γ secretion). Many other transitory subsets have been identified in both CD4+ and CD8+ and within the Tem other subsets that can be distinguished based on the expression of CD27 and CD28 (Romero, Zippelius et al. 2007). In humans, there is another effector memory CD8+ T subset, the terminally differentiated effector cells (Temra), that are positive for CD45RA expression but they do not express CCR7. Recently, a human memory T cell subset with stem cell–like properties has been described (Tscm)(Gattinoni, Lugli et al. 2011). These cells share many of the characteristics of naïve T cells cell ($CD45RO^-$, $CCR7^+$, $CD45RA^+$, $CD62L^+$, $CD27^+$, $CD28^+$ and IL-$7R\alpha^+$), however, they also express CD95, IL-2Rβ, CXCR3, and LFA-1, typical markers of memory T cells (Gattinoni, Lugli et al. 2011). Therefore, Tscm are a long-lived human memory T cell population with increased capacity for self-renewal and increased ability to generate central memory and effector memory T cells. In summary, Tem provides immediate protection against second exposure to a previously

encountered pathogen and provide rapid protection at entry sites of infection, whereas Tcm remain principally in the lymphoid tissue where they can rapidly proliferate and differentiate into effector cells to resupply the effector T cells at peripheral sites.

1.3 Models of memory T cells generation

Because of the heterogeneity among the effector and memory CD8+ T cell population, different models of T cell differentiation have been proposed during infection.

Separate-precursor model: this model proposes that when naïve T cells exit the thymus, these cells are differentially pre-selected to become effector or memory CD8+ T cells upon activation. However, evidence suggests that this model is unlikely. More sophisticated studies using adopting transfer of single CD8+ T cells have shown that naïve T cells can differentiate in both effector and several memory CD8+ T cell subsets (Stemberger, Huster et al. 2007; Gerlach, van Heijst et al. 2010).

Linear differentiation model: this model suggests that naïve T cells that respond to antigenic stimulation can give rise to effector cells. Once the antigen has been cleared, effector cells can either die by apoptosis, become senescent terminally differentiated T cells, or differentiate into memory CD8+ T cell subsets (Wherry, Teichgraber et al. 2003).

Bifurcative differentiation model (or asymmetric cell division): this model implies that early after antigen stimulation, within the first cell division, one T cell precursor can give rise to two daughter cells: the proximal daughter cell (closer to the antigen-presenting cell (APC)) differentiates into effector cells that will die by apoptosis once the antigen has been cleared. The distal daughter cell (further from the APC) gives rise to Tcm, Tem and Ttm (Chang, Palanivel et al. 2007) .

*Self-renewing effector mode*l: this model proposes that Tcm and effector T cells, generated from naïve T cells, have self- renewal abilities. Tcm are found in the lymph nodes and undergo homeostatic proliferation and can differentiate into Tem. Tem can give rise to terminally differentiated effector cells but cannot self-renew (Ahmed, Bevan et al. 2009).

1.4 Role of cytokines in the homeostasis and differentiation of effector CD8+ T cells

Memory CD8+ T cells that are generated after an acute infection are maintained in a cytokine-dependent manner. Several studies have shown the role of γ-chain cytokines (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) in regulating survival and homeostatic proliferation of memory CD8+ T cells. Among them IL-2, IL-15 and IL-7 have been shown to regulate different stages of memory differentiation and maintenance. These cytokines have some overlapping functions, but at the same time, regulate different points of the immune response. These overlapping functions are due to the fact that these cytokines share a common cytokine-receptor subunit and signaling pathways. All the receptors of the gamma chain cytokines utilize the gamma chain (γc) subunit, which signals through JAK3. Moreover, IL-2 and IL-15 receptors share a common β chain which signals through Janus kinases 1 (JAK1). IL-15Rα may also mediate signaling through tumor necrosis factor receptor associated factor 2 (TRAF2) binding motif. IL-2 binds to IL-2Rα alone with low affinity while IL-7 binds IL7R, which is composed of two subunits: the common γchain and the unique α-chain that confers cytokine specificity. Memory cells are known to be maintained by homeostatic proliferation which leads to high division rate when compared to naïve T cells (Tough and Sprent 1994) and the continual division is mediated by IL-15 while inhibited by IL-2 (Ku, Murakami et al. 2000). Different groups have shown that IL-7 and IL-15 are the major cytokines involved in the maintenance and homeostatic proliferation of memory CD8+ T cells (Schluns, Kieper et al. 2000; Schluns, Williams et al. 2002; Mueller, Petrovas et al. 2005; Purton, Tan et al. 2007). In particular, IL-15 seems to mediate T cell proliferation while IL-7 is implicated in providing survival. Other researchers have also revealed that overexpression of IL-15, through IL-15 transgenic mice, increases the total number of memory CD8+ T cells (Marks-Konczalik, Dubois et al. 2000; Fehniger, Suzuki et al. 2001). IL-7 seems to regulate the differentiation of effector CD8+ T cells that express high levels of CD127 (IL-7Rα) into long lived memory cells (Kaech, Tan et al. 2003). Indeed IL-7 is more important than IL-15 in supporting memory cell formation from recently activated CD8+ T cells. Other groups have shown reduced maintenance of antigen-specific CD8+ T cells in IL-15-deficient mice (Becker, Wherry et al. 2002; Schluns, Williams et al. 2002). Rubinstein et al. demonstrated that IL-15 and IL-2 induced

the preferential accumulation of short-lived effector/memory (KLRG1hiCD127lo) CD8+ T cells while stimulation with IL-7 mediates increased numbers of the long lived memory precursors KLRG1loCD127hi cells (Rubinstein, Lind et al. 2008). As cytokines that signal through the γc receptors, IL-15 and IL-7 support survival of memory CD8+ T cells at the molecular level by increasing the levels of anti-apoptotic molecules such as Bcl-2. Antigen specific CD8+ T cells have high levels of Bcl-2 while naïve T cells express lower Bcl-2. In addition, CD8+ T cells express higher levels of Bcl-2 than the CD4+ memory cells (Homann, Teyton et al. 2001). The signaling pathway triggered by the γc cytokines signal through signal transducer and activator of transcription 5 (STAT5) and are inhibited by suppressor of cytokine signaling-1 (SOCS-1).

1.5 Transcription factors that control effector and memory CD8+ T cell differentiation

Several transcription factors that regulate effector and memory CD8+ T cell development have been identified. These transcriptional regulators have been shown to act in pairs: when the expression of one of the two molecule increases, they induce either memory or effector differentiation. Among these pairs of transcriptional regulators, t-bet and eomesodermin, Id2 and Id3, Bcl-6 and Blimp-1 have been shown to function in coupled to regulate these properties. In particular, cells that express increased levels of T-bet, B lymphocyte-induced maturation protein (Blimp-1), inhibition of DNA binding 2 (Id2) and signal transducer and activator of transcription 4 (Stat4) during an immune response, induces the differentiation of effector CD8+ T cells in short lived effector cells. These molecules contrast the expression of other transcriptional factors, Eomesodermin (Eomes), B cell lymphoma 6 (Bcl-6), Id3 and Stat3 that, when upregulated, induce the differentiation of effector cells into long-lived memory CD8+ T cells for life-long persistence (reviewed in (Kaech and Cui 2012)).

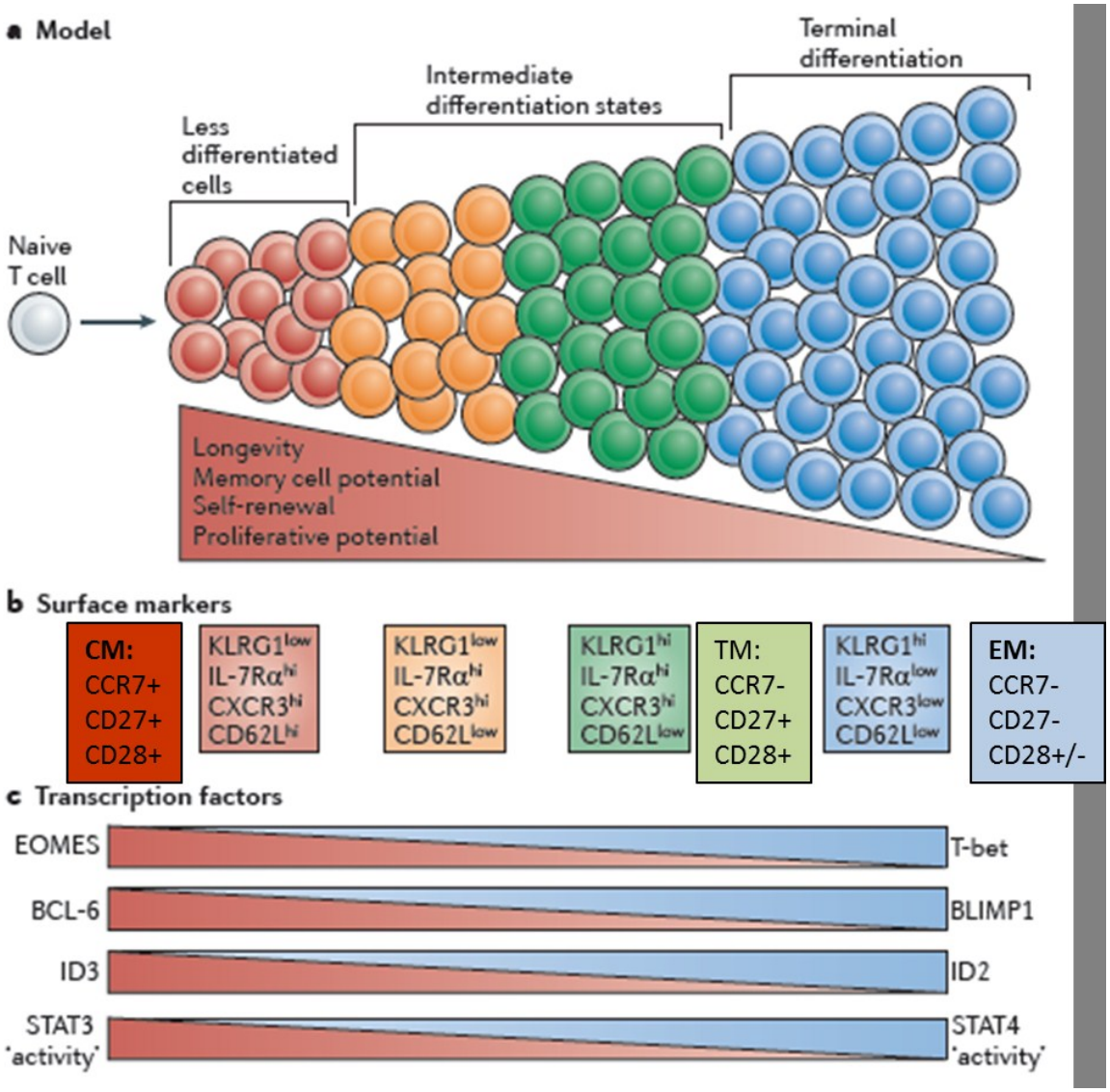

Figure 1: Different stages of CD8+ T cell differentiation:

Memory CD8+ T cells are heterogeneous and can be distinguished based on cell surface markers or the expression of different sets of transcriptional factors: central memory are less differentiated and express CD27, CCR7, and CD28 expression on their cell surface while showing greater self-renewal and proliferative capabilities. At the transcriptional level, Tcm express high levels of BCL-6, Id3, STAT3 and eomes. As cells differentiate in effector cells, they lose expression of CCR7, CD27, and CD28 on their cell surface and

express high levels of transcriptional factors T-bet, Id2, Blimp-1 and STAT4 that regulate their effector functions. Adapted from (Kaech and Cui 2012)

1.5.1 T-bet and eomesodermin

T-bet and eomesodermin are two T box transcription factors that play important roles in promoting effector and memory CD8+ T cell differentiation. Their role in mediating effector CD8+ T cell functions is confirmed by studies where they show that the two transcription factors are required for the expression of INFγ, granzyme B, perforine, CXCR3 and CXCR4 (Intlekofer, Takemoto et al. 2007; Joshi, Cui et al. 2007; Banerjee, Gordon et al. 2010; Pipkin, Sacks et al. 2010). Expression of T-bet is upregulated in the effector phase of the immune response by antigen presentation and by IL-12 and mammalian target of rapamycin (mTOR) activity (Takemoto, Intlekofer et al. 2006; Joshi, Cui et al. 2007; Rao, Li et al. 2010; Joshi, Cui et al. 2011).When memory CD8+ T cell are generated, T-bet expression decreases whereas eomes is induced by RUNX-3 and IL-2 (Banerjee, Gordon et al. 2010; Joshi, Cui et al. 2011).

1.5.2 Blimp-1 and Bcl-6

Blimp-1 and Bcl-6 are another pair of transcription factors involved in B and T cells differentiation. Blimp-1 expression is high on effector CD8+ T cells and its levels are increased by IL-2, IL-12 and IL-21 cytokines (Gong and Malek 2007; Kwon, Thierry-Mieg et al. 2009; Kalia, Sarkar et al. 2010; Pipkin, Sacks et al. 2010). In these cells Blimp-1 regulate effector functions such as T cell trafficking to sites of inflammation and INFγ and Granzyme B expression (Kallies, Xin et al. 2009; Rutishauser, Martins et al. 2009). As memory CD8+ T cells form Blimp-1 expression levels decrease and Bcl-6 levels increase (Cui, Liu et al. 2011). In mice, IL7Rαhi KLRGlo memory precursors express high levels of Bcl-6 and its levels are maintained and increased in antigen-specific memory cells after LCMV infection, and this coincides with decreased levels of Blimp-1 (Gong and Malek 2007). Other studies have shown that Bcl-6 is important for the formation of Tcm and its

expression may be mediated by IL-10 and IL-21 (Ichii, Sakamoto et al. 2002; Gong and Malek 2007; Ichii, Sakamoto et al. 2007).

2. Id proteins

Inhibitor of DNA binding or differentiation (Id) proteins belong to a family of helix-loop-helix (bHLH) transcription factors. There are two main families of the HLH proteins: E-proteins and Id proteins. Id proteins were named due to their ability to inhibit DNA binding (Benezra, Davis et al. 1990). In mammals, four known members of Id proteins exist; Id,1 Id2, Id3 and Id4, all encoded by four different genes (Benezra, Davis et al. 1990; Deed, Jasiok et al. 1994).

2.1 The properties of Id proteins

Id proteins and E proteins both have a highly conserved helix-loop-helix (HLH) domain that consist of two amphipathic α-helices separated by a loop of variable sequence and length (Pesce and Benezra 1993). The HLH domain is required for homo- and hetero-dimerizzation of these proteins. Next to the HLH domain, E proteins contain a basic amino acid domain which is fundamental for their binding to DNA within the canonical "E-box" recognition sequence, CANNTG (Figure 2) (Barone, Pepperkok et al. 1994). However, Id proteins do not possess the basic domain, thus they are not able to bind DNA and act by inhibiting the transcriptional regulation of bHLH factors, in particular, the E-proteins (Figure 3) (Lasorella, Uo et al. 2001) (Zebedee and Hara 2001; O'Toole, Inoue et al. 2003)

Id proteins, through the inhibition of E-protein transcriptional activity, control various critical cells function, including cell cycle, differentiation, apoptosis, cell senescence, tumorigenesis and neoplastic transformation (Barone, Pepperkok et al. 1994) (Andres-Barquin, Hernandez et al. 2000).

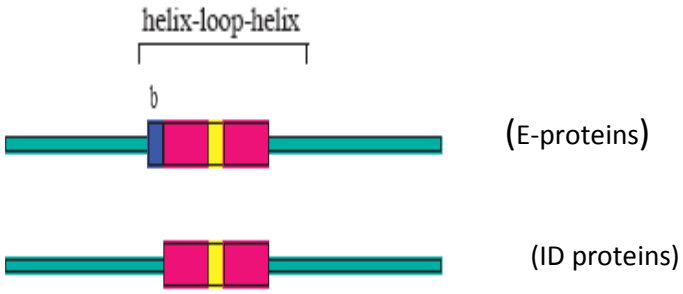

Figure 2: Schematic structure of E-proteins and Id- proteins

The basic DNA binding region (b) that bound the HLH region is shown. (Adapted from (Norton 2000)).

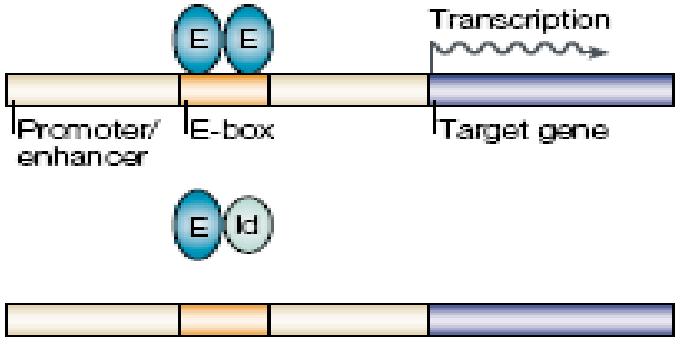

Figure 3: Id and E-protein interaction

E-proteins form heterodimers that bind DNA and induce transcription of genes involved in apoptosis and blockage of cell cycle progression. When Id proteins bind to E-proteins, they form a complex that cannot bind to DNA and thus inhibits E-proteins transcriptional activity (adapted from (Engel and Murre 2001)).

2.2 Id proteins in cell cycle control

The role of Id proteins in regulating cell cycle has been shown in mice and cell line models. These studies have shown that Id proteins are implicated in the G1/S transition of the cell cycle. Lasorella et al. have demonstrated that resting and undifferentiated cells have lower levels of Id proteins and its expression increases when cells enter the cell cycle and proliferate, suggesting that Id proteins may play a role in regulating cell growth (Lasorella, Uo et al. 2001). Mechanistic studies via the use of small interfering RNA have shown that the silencing of Id proteins results in cell cycle arrest (Barone, Pepperkok et al. 1994). Two main pathways have been shown to correlate Id proteins to cell cycle control. The first one is by inhibiting the binding of E-protein transcription factors: following mitogenic signals, increased levels of Id may sequester E-proteins from forming heterodimers and prevent them from binding the DNA. The Id-E protein interaction results in a consequent inhibition of E-protein target gene expression such as the cyclin-dependent kinase (CDK) inhibitors, (i.e. p21CIP1 and p27KIP1) (Trabosh, Divito et al. 2009). Moreover, Ids can inhibit the expression of p16INK4a promoter through an E-protein-dependent mechanism, while Id2 inhibits p15INK4b (Alani, Young et al. 2001). Id proteins have been shown to preferentially target the ubiquitously expressed E-proteins (E12, E47, E2-2, and HEB), (Benezra, Davis et al. 1990; Norton and Atherton 1998). In addition to E proteins, Ets transcription factors and the tumor suppressor proteins of the Retinoblastoma (pRb) family are also targets of Id proteins (Iavarone, Garg et al. 1994; Biggs, Zhang et al. 1995; Lasorella, Iavarone et al. 1996). The direct interaction between pRB and Id proteins (Id2 in particular) is thought to potentiate the S phase. This interaction in fact results in the dissociation of pRB from the E2F transcription factor. Once dissociated from pRB, (p107 and p130) E2F is free to bind to DNA and induce the expression of genes involved in cell cycle progression. Free E2F transcriptional complex then activates the expression of genes required for progression through S phase of the cell cycle (Figure 4).

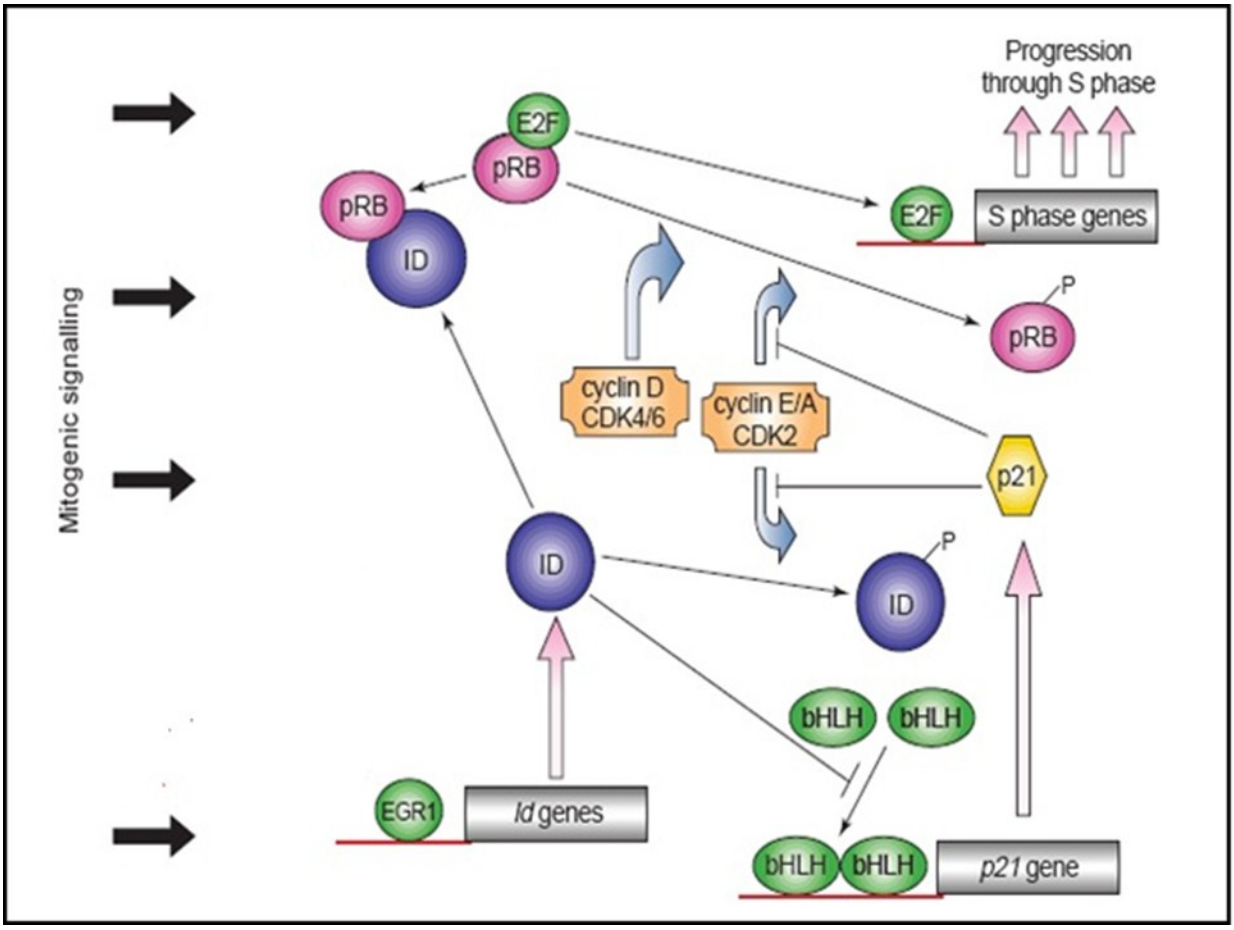

Figure 4: Id proteins in cell cycle control

Schematic representation shows how Id proteins regulate cell cycle progression. Mitogenic signaling pathways, during G1 phase of the cell cycle, promotes events that induce the phosphorylation of the cyclin-dependent kinase (CDKs). CDKs phosphorylate pRB (p107 and p130), leading to release of the transcription factor E2F. Free E2F is able to bind to DNA and thus to induce the expression of genes important for the S phase of the cell cycle. (Norton 2000).

2.3 Ids in Development and Differentiation

Because of the ability of Id proteins in regulating proliferation and differentiation, these proteins regulate many aspects of development. Studies in mice with Id protein knockout have shown that the absence of Id1 and Id3 together leads to altered differentiation and

proliferation of neural cells and are embryonic lethal, whereas the absence of Id2 resulted in decreased proliferation of many cell types and decreased body size was observed in these mice (Lasorella, Uo et al. 2001; Sikder, Devlin et al. 2003). Id1 knockout mice alone have no evident defects whereas Id3 knockout mice exhibit defects in B cell proliferation and humoral immunity. The importance of Id2 in regulating development was further confirmed by a study where the loss of Id2 lead to the absence of Peyer's patches and lymph nodes as well as decreased numbers of epidermal Langerhans cells and natural killer cells (Sikder, Devlin et al. 2003).

2.4 Role Id2 and Id3 in memory CD8+ T cell differentiation

Few studies in mice have recently shown the new role of Id2 and Id3 in regulating the differentiation of effector and memory CD8+ T cell formation. The first study from Cannarile et al in 2006 had shown that after antigen stimulation, Id2 expression was increased in effector cells and its expression remained high in the memory compartment (Cannarile, Lind et al. 2006). CD8+ T cells from Id2 knockout mice infected with *Listeria monocytogenes* were able to respond to the antigen, to proliferate, and differentiate into effector cells but were more susceptible to spontaneous apoptosis compared to wild-type cells, resulting in a decreased ability to clear *Listeria* after infection (Cannarile, Lind et al. 2006). CD8+ T cells in absence of Id2 have decreased levels of the pro-survival Bcl-2 molecule and increased levels of the proapoptotic Bim and Ctla-4, suggesting that Id2 was mediating the survival of effector cells that undergo contraction during the immune response. Moreover, the few cells that survived in the absence of Id2 expression expressed a different phenotype, maintaining CD27 and CD127 expression and exhibiting a more central memory phenotype in comparison to wild-type cells that had instead differentiated into effector memory CD8+ T cells (Cannarile, Lind et al. 2006).

Another more recent study in mice showed that the ratio between Id2 and Id3 expression levels are important for determining which cells become long-lived memory cells and those that become short-lived effector cells after an immune response (Yang, Best et al. 2011). Indeed, high levels of Id3 preferentially lead to the differentiation of long-lived memory

cells while low levels of Id3 promote short-lived effector cell differentiation. Contrary to Id2 expression that is upregulated early after antigen stimulation, Id3 expression levels were decreased in effector cells and its expression became high when memory cells were generated. Id3 levels were found to be modulated right before the change in KLRG-1 and CD127 expression on the cell surface. Therefore, Id3 expression could be an indicator for the identification of memory precursors ($CD127^{hi}KLRG\text{-}1^{lo}$). Id3 deficient mice were not able to accumulate $CD127^{hi}$ memory cells. Moreover, Id2 knockout cells were lacking $KLRG\text{-}1^{hi}$ short lived effector cells. This study suggest that Id2 and Id3 regulate the differentiation of effector and memory cells differently and that their expression could be used to predict memory precursors.

3. CD8+ T cell exhaustion

During an acute infection, naïve T cells are stimulated by antigen to undergo clonal expansion, differentiate into effector T cells that secrete cytokines and exert cytolytic activity. Once the infection is resolved, a small subset of these functional effector T cells survive the contraction phase and further differentiate into highly polyfunctional memory T cells. Memory cells have the capacity to rapidly respond to previously encountered antigens, which include the ability to reacquire the homing receptors to secondary lymphoid tissues, high proliferative capabilities, and the ability to self-renew by undergoing homeostatic proliferation driven by IL-7 and IL-15 (Becker, Wherry et al. 2002; Goldrath, Sivakumar et al. 2002). These properties allow memory T cells to confer protective immunity. However, during a chronic viral infection, T cells lose these characteristics because of the continued exposure to the antigen, a functional impairment known as exhaustion. T cell exhaustion was described for the first time almost 10 years ago in murine LCMV models where researchers showed that during chronic infection, antigen-specific T cells were dysfunctional and were not able to kill virally infected cells (Barber, Wherry et al. 2006). During exhaustion, CD8+ T cells undergo a progressive loss of their effector functions. The events that regulate this loss are influenced by the level and persistence of antigen stimulation and by CD4+ T cell help. If the viremia is low and CD4+ T cell

numbers are not lost, CD8+ T cells are still functional, as during some latent infections. Typically, when viral infection persists, IL-2 production, proliferation, and ex vivo killing are the first effector functions to be lost during chronic infection (exhaustion I) (Wherry, Blattman et al. 2003). This stage is followed by partial exhaustion II when TNF-α production begins to be impaired. Reduction in INF-γ production following antigen stimulation follows, a stage of exhaustion that is the result of high viral load and low CD4+ T cell numbers (Wherry, Blattman et al. 2003). Finally, the continuous presentation of the antigen to antigen-specific CD8+ T cells leads to death by apoptosis. After these first studies in mice, it became evident that humans had similar kinds of CD8+ T cell exhaustion. For example during human immunodeficiency virus (HIV), hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, virus-specific CD8+ T cells appear to lack *ex vivo* effector functions.

3.1 HIV infection

Human immunodeficiency virus (HIV) is a lentivirus, a member of the retrovirus family, and if untreated, leads to acquired immunodeficiency syndrome (AIDS). HIV is an enveloped viral particle that contains two copies of positive single-stranded RNA that code for nine genes: *gag, pol, env, tat, rev, nef, vif, vpr* and *vpu*. HIV infects CD4+ T cells, macrophages and dendritic cells. HIV infection leads to a gradual loss of CD4+ T cells through direct or indirect mechanisms: killing of CD4+ T cells directly mediated by the virus, indirect killing of infected cells by cytotoxic CD8+ T cells, killing of CD4+ T cells mediated by abortive HIV infection and/or increased rates of apoptosis of uninfected cells (Banda, Bernier et al. 1992; Groux, Torpier et al. 1992; Cottrez, Manca et al. 1997; Herbein, Van Lint et al. 1998; Badley, Pilon et al. 2000; Esser, Graham et al. 2001; McCune 2001; Alimonti, Ball et al. 2003; Doitsh, Cavrois et al. 2010). When the numbers of CD4+ T cells become very low, the immune system is compromised and the disease is characterized by susceptibility to infection with organisms that are not typically pathogenic (McCune 2001; Mattapallil, Douek et al. 2005).

3.1.1 Clinical Course of HIV infection

HIV infection is characterized by a peak in plasma viremia, usually reaching more than a million RNA copies/ml, and occurring 3-4 weeks after HIV entry. This period is characterized by non-specific flu-like symptoms and coincides with decreased numbers of circulating CD4+ T cells (McMichael, Borrow et al. 2010). The partial viral control correlates temporally with an HIV-specific CD8+ T cell response which peaks just prior to the decline in viremia and is important in maintaining the viral load at stable levels (Koup, Safrit et al. 1994). This essential role of CD8+ T cells in controlling HIV disease progression has been confirmed in non-human primate models (Matano, Shibata et al. 1998; Jin, Bauer et al. 1999; Schmitz, Kuroda et al. 1999). Indeed, it has been shown that depletion of CD8+ T cells leads to a rapid increase in viremia while upon reappearance of CD8+ T cells, viremia decreases during acute and chronic SIV (Jin, Bauer et al. 1999; Schmitz, Kuroda et al. 1999). Chronic HIV infection, also known as the latent phase, lasts an average of 10 years and begins when the viral load decreases and CD4 T cell counts are stabilized. Throughout this phase, CD4+ T cells are gradually depleted from the circulation (McMichael, Borrow et al. 2010). When CD4+ T cells numbers decrease below 200 cells/μl, the immune system is susceptible to opportunistic infections and tumors and this immune dysfunction leads to AIDS. The rate of this depletion varies from one individual to another, for reasons that remain to be fully elucidated (Sun, Williams et al. 2004). The depletion of CD4+ T cells is thought to contribute to the inability of CD8+ T cells to mount effective responses and control viremia during chronic infection.

3.1.2 Role of CD8+ T cells in HIV infection:

CD8+ T cells play an important role during HIV infection and are essential in controlling viral replication and for the killing of virally infected cells. Their ability in controlling viremia is mediated by the secretion of soluble factors (CAF, MIP-1α, MIP-1β, RANTES) and by the release of perforin and Granzyme A and B that mediate direct lysis of infected cells. HIV-specific CD8+ T cells from patients in the acute phase of HIV infection are still able to control viremia and exert strong cytotoxic activity. Indeed, the development of

tetramer stainings have provided the data showing that the frequency of antigen-specific CD8+ T cells can reach up to 18% during acute infection (Gea-Banacloche, Migueles et al. 2000; Addo, Yu et al. 2002). As the infection progresses, the CD4:CD8 T cell ratio is inverted as a consequence of CD4+ T cells loss (Chun, Justement et al. 2002). During the late stages of infection, the frequency of CD8+ T cells begins to decline (Margolick, Munoz et al. 1995). Furthermore, during chronic HIV infection, the capability of CD8+ T cells to exert a cytolytic response diminishes and usually, in the absence of treatment, the disease progresses to AIDS (Klein, van Baalen et al. 1995). How CD8+ T cells are lost during HIV infection is still under debate and different mechanisms have been proposed (Reviewed in: (Gougeon 2003)). HIV specific CD8+ T cells have been shown to be more sensitive to Fas/CD95-induced apoptosis when compared to cytomegalovirus (CMV)-specific CD8+ T cells (Mueller, De Rosa et al. 2001). One possible mechanism of CD8+ T cell apoptosis was shown to be the effect mediated by HIV infected macrophages on bystander cells (Cottrez, Manca et al. 1997; Herbein, Van Lint et al. 1998; Badley, Pilon et al. 2000; Vlahakis, Algeciras-Schimnich et al. 2001). In contrast, other groups have shown that increased apoptosis of antigen-specific CD8+ T cells was not dependent on Fas but these cells were more sensitive to TNF receptor 2 and caspase mediated apoptosis leading to decreased levels of the pro-survival Bcl-2 molecule (Derby, Snyder et al. 2001). Other groups have shown that apoptosis of antigen-specific and total CD8+ T cells can occur *in vitro* by interaction between gp120 and CXCR4 that is expressed on the cell surface of CD8+ T cells. However, other mechanisms of CD8+ T cell depletion as a consequence of T cell exhaustion have been more recently described during HIV infection.

3.2 T cell exhaustion during HIV infection

It has been shown that negative regulatory pathways play an important role in T cell exhaustion. Among them, three different pathways are the principal mediators of exhaustion: cell surface inhibitory receptors (such as PD-1), cytokines (such as IL-10) and immunoregulatory cells (such as Treg cells). Inhibitory receptors have the important function of limiting T cell activation by reducing downstream signaling of the T-cell

receptor (TCR). Thus, a robust immune response is mediated by a perfect balance between positive and negative signals. However, the persistant expression of negative regulatory receptors during chronic infection leads to an impaired ability to respond to infections, further leading to exhaustion.

3.2.1 Programmed death 1 (PD1)

3.2.1.1 Structure

Programmed death 1 (PD-1) and its ligands, PD-L1 and PD-L2, mediate inhibitory signals that control the balance between T cell activation, tolerance and autoimmunity (Nishimura, Nose et al. 1999; Nishimura, Okazaki et al. 2001). PD-1 is a 288 amino acid type I transmembrane protein consisting of one immunoglobulin (Ig) superfamily domain and an intracellular domain containing an immunorecepor tyrosine-based inhibitory motif (ITIM) as well as an immunoreceptor tyrosine-based switch motif (ITSM). PD-1 expression is very low or undetectable on resting cells and its expression is induced transiently on activated T cells, B cells, natural killer T cells, activated monocytes, and dendritic cells (DCs). PD-1 binds to two ligands, both members of the B7 family, called PD-L1 (programmed death ligand-1 or B7-H1) and PD-L2 (B7-DC) (Chen 2004; Greenwald, Freeman et al. 2005). PD-L1 is constitutively expressed on many cell types whereas PD-L2 is induced on antigen-presenting cells during inflammation. PD-1 binding to its ligands (PD-L1 and PD-L2) induces a cascade of events that inhibit TCR signaling. This is a mechanism to control autoimmunity and to prevent constant T cell activation, proliferation, cytokine production, particularly IFN-γ, TNF-α, IL-2, and cytolytic function. The mechanisms that PD-1 uses to dampen TCR signaling are mediated, in part, by the inhibition of ZAP70 phosphorylation and the association of the TCR with CD3ζ. Moreover, engagement of PD-1 inhibits glucose metabolism mediated by CD3-CD28 and also inhibits downstream signaling of Akt by inhibiting CD28 mediated activation of phosphatidylinositol 3-kinase. The role of PD-1 in regulating peripheral tolerance to self-antigen is mediated by its function in the development of regulatory T cells and in inhibiting self-reactive T cells. The role of PD-1

in regulating immune tolerance has been described with PD-1 knockout mice that develop autoimmune disease (Nishimura, Nose et al. 1999; Nishimura, Okazaki et al. 2001). Other studies have demonstrated the treatment with blocking PD-1 antibodies as a therapeutic intervention for immunotherapy to cancer.

3.2.1.2 Role of PD-1 in CD8+ T cell exhaustion

The signaling pathways that are mediated by PD-1 are still not well understood, however, extensive studies have been conducted in the role of PD-1 for mediating T cell exhaustion during immune response. The first studies that were done in order to understand PD-1 function during chronic viral infection have been conducted in a lymphocytic choriomeningitis virus (LCMV) mouse model (Barber, Wherry et al. 2006). Barber et al. were the first to describe that PD-1 expression regulates CD8+ T cell exhaustion in mice infected with LCMV clone 13 (a LCMV variant that generates chronic viral infection). PD-1 expression levels were high on virus-specific CD8+ T cells and blocking the PD-1: PD-L1 interaction during chronic viral infection rescues T cell function while decreasing viral load, suggesting that the inhibition of PD-1 crosslinking may be used as a potential application in the treatment of chronic infection. These first studies were conducted in mice, however, the role of PD-1 in regulating exhaustion of CD8+ T cells was then confirmed in humans during chronic viral infections, such as HIV (Petrovas, Casazza et al. 2006; Trautmann, Janbazian et al. 2006; Zhang, Zhang et al. 2007) and in SIV infected Rhesus macaques (Petrovas, Price et al. 2007), (Velu, Titanji et al. 2009). Different groups have shown that PD-1 expression levels from chronically HIV infected donors are upregulated in particular on HIV-specific CD8+ T cells and its levels were positively correlated with viremia and negatively with CD4+ T cell number. Moreover, PD-1hi cells show a decreased capacity to proliferate and to produce cytokines. Longitudinal studies have shown that the use of highly active antiretroviral therapy (HAART) was able to decrease PD-1 levels on HIV-specific CD8+ T cells, suggesting that the continuous antigen presentation to these cells and viremia are responsible for the increased PD-1 expression

(Day, Kaufmann et al. 2006; Trautmann, Janbazian et al. 2006). In support of this, other groups have shown that SIV-specific CD8+ T cells for a specific epitope that had undergone mutational escape had decreased PD-1 expression on their cell surface. (Petrovas, Price et al. 2007; Salisch, Kaufmann et al. 2010). Other groups have shown that dominant clonotypes within the HIV-specific CD8+ T cell pool with increased levels of PD-1 failed to survive in culture and produced lower levels of cytokine when compared to subdominant clonotypes (Conrad, Ramalingam et al. 2011). The role of PD-1 in regulating apoptosis has also been shown by Petrovas et al. in 2006 where they showed that PD-1 expression levels during HIV-infection correlated with increased susceptibility to spontaneous and CD95/Fas-induced apoptosis and is associated with decreased Bcl-2 expression levels (Petrovas, Casazza et al. 2006). HIV-specific CD8+ T cells have been shown to be more susceptible to apoptosis when compared to CMV-specific CD8+ T cells from the same HIV-infected patients, possibly due to the higher levels of PD-1 on their cells surface. In fact, PD-1hi populations were found to be more susceptible to apoptosis than PD-1 negative CD8+ T cells, independent of antigen specificity. Moreover, increased PD-1 expression on CD8+ T cells from HIV-infected donors correlates with a pro-apoptotic phenotype with lower levels of the pro-survival Bcl-2 molecule, decreased levels of CD127, and higher levels of CD95/Fas surface receptor when compared to the PD-1lo compartment. PD-1hi cells also showed increased binding of MitoTracker suggesting that modification within the mitochondria of these cells may be responsible for increased susceptibility to apoptosis (Petrovas, Mueller et al. 2007).

3.3 HIV Impairment of CD8+ T cell maturation

As discussed earlier, during an acute infection, naïve T cells after antigen stimulation give rise to effector and memory T cells. These populations are distinguished based on their specific phenotype. Naïve T cells express CD45RA, costimulatory molecules, and homing receptors. As cells differentiate into effector cells (CD45RA+ CD28-CD27-CCR7-), Tem (CD45RA-CCR7-CD28-CD27-) and Tcm (CD45RA-CD28+CCR7+CD27+), certain markers, such as CCR7 for Tem and CD27 for Tcm, are gained or lost. Appay at al. showed

that during the chronic phase of HIV infection, antigen-specific cells that were generated expressed a distinguishable differentiation phenotype (based on CD28 and CD27) when compared to Epstein-Barr Virus (EBV) and HCV-specific CD8+ T cells (Appay, Dunbar et al. 2002). Other groups have shown that HIV-specific CD8+ T cells expressed lower levels of perforin on their cell surface and these cells were not able to exert cytotoxic functions in relation to CMV and EBV-specific cells in HIV-infected patients that instead are granzyme A/perforin+ CD45RA+ CD27- (Strengell, Matikainen et al. 2003; Zhang, Shankar et al. 2003). Moreover, different groups demonstrated that HIV-specific CD8+ T cells have lower CD28 expression and are CD27+, suggesting a decreased effector phenotype (Ogg, Kostense et al. 1999; Roos, van Lier et al. 2000). CD28 and CD27 are costimulatory receptors. CD28 mediates TCR activation upon TCR triggering while CD27 facilitates the generation of antigen-specific CD8+ T cells. CD28+ cells have longer telomere lengths and therefore, have increased proliferative potential in contrast to CD28- cells. As a result, decreased levels of CD28 on HIV-specific CD8+ T cells are associated with diminished proliferation (Speiser, Migliaccio et al. 2001). CD27 negative cells that are associated with an effector phenotype express more Granzyme A and perforin when compared to cells that express CD27 on their cell surface (van Baarle, Kostense et al. 2002). Therefore, HIV-specific CD8+ T cells that express high levels of CD27 and have lower proliferative capabilities and decreased perforin and cytotoxic activity. Moreover, HIV-specific memory CD8+ T cells were also found to have a pre-terminally differentiated phenotype (CD45RA-CCR7-), when compared to CMV-specific cells that instead expressed a terminally differentiated (CD45RA+CCR7-) phenotype (Champagne, Ogg et al. 2001; Ellefsen, Harari et al. 2002). In summary, during the chronic phase of HIV infection, antigen-specific CD8+ T cells should have an effector/effector memory phenotype, however these cells express a pre-terminally differentiated phenotype. The skewed maturation of these cells is responsible for decreased effector functions and this leads to viral escape from immune containment.

3.4 Highly Active antiretroviral Therapy

Highly active antiretroviral therapy (HAART) is the treatment for HIV infected individuals. HAART is able to decrease the rates of viral replication thus delaying the progression to AIDS. HAART is a combination therapy of three different drugs: two nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PI), two NRTIs and non-nucleoside reverse transcriptase inhibitors (NNRTI) or other such combinations. HAART therapy has been shown to efficiently decrease viremia and increase CD4+ T cell numbers in the majority of cases. Upon initiation with HAART, the plasma viral load undergoes two exponential phases of decay (Perelson, Essunger et al. 1997). The first phase is characterized by a rapid decline of the viral load as result of the loss of productively infected CD4+ T cells, which are mainly activated CD4+ T cells. The second phase is less rapid and consists of the loss of infected macrophages and dendritic cells, which are more resistant to virus–induced cytopathic effects than CD4+ T cells. The majority of the patients treated with HAART have a reduction in viremia that drops below 25 copies of viral RNA per ml of plasma (the detection limit of sensitive assay) by week 20 and remains low to undetectable except for occasional increases in viral load called viral “blips” (Perelson, Essunger et al. 1997; Posevitz, Arndt et al. 2008). Following HAART, the recovery of CD4+ T cell numbers undergoes two phases. First, there is a rapid increase in the CD4+ T cells that mostly have a memory phenotype, which happens during the first weeks from the treatment (Autran, Carcelain et al. 1997). This phase is followed by an increase in the naïve CD4 + T cell numbers (Autran, Carcelain et al. 1997).

HYPOTHESIS AND AIMS:

We have previously shown that during chronic HIV infection, there is an impaired distribution of the memory CD8+ T cell subsets that is associated with high levels of PD-1. The majority of CD8+ T cells are arrested in a transitional memory (Ttm) phenotype, are not able to differentiate to effector/effector memory (Tem) cells and lack cytotoxic functions. Functional and phenotypical defects are not restored even under HAART treatment. PD-1 limits T cell activation by attenuating T cell receptor signaling, however, it is not known whether PD-1 also acts by downregulating genes important for differentiation and T cell functions.

In the first part of my thesis (Chapter 2) I used a system biology approach as an unbiased methodology in order to identify pathways downstream of PD-1 that are associated with CD8+ T cell dysfunction and T cell exhaustion. I analyzed global gene expression profile of highly purified PD-1 high HIV-specific CD8+ T cells when compared to PD-1 low CMV-specific CD8+ T cells from the same chronic HIV-infected individuals and found that HIV-specific CD8+ T cells express low levels of the transcriptional modulator Id2. Due to the fundamental role of Id2 in mediating effector CD8+ T cell differentiation we decided to further investigate whether defects in Id2 expression could be responsible for the impaired maturation of the PD-1 high cells during chronic HIV infection. We hypothesized that:

- PD-1 crosslinking inhibits T cell function by interfering with the expression of Id2, leading to decreased maturation of CD8+ T cells and exhaustion.

In the second part of my thesis (Chapter 5) I wanted to identify upstream signals that can upregulate Id2 expression and that could restore CD8+ differentiation during HIV-infection. Id2 and the common γ-chain cytokine IL-15 play similar roles in mediating survival and differentiation of effector CD8+ T cells; both Id2 or IL-15 knock-out mouse lack cells with an effector/Tem phenotype. Moreover, Id2 has two potential STAT5 binding sites at its promoter and IL-15 mediates survival of effector cells by inducing phosphorylation and transcriptional activity of STAT5. Finally, both Id2 and IL-15

upregulate the pro-survival Bcl-2 and downregulate the proapoptotic Bim molecules. Thus I focused on defining the mechanisms of action of IL-15 in promoting CD8+ T cell differentiation and defining the role of Id2 in this outcome. We hypothesized that:

a) Id2 upregulation by IL-15 is responsible for human effector CD8+ T cell differentiation;
b) treatment with IL-15 will rescue Id2 expression and differentiation of HIV-specific CD8+ T cells and effector function.

In order to assess the impact of IL-15 on effector function of HIV-specific CD8+ T cells, I optimized a cell-based flow cytometry assay (Chapter 4) that measures the cytolytic activity at the single cell level.

A better understanding of the molecular events involved in the generation of effector cell mediated by IL-15 is critical for the development of effective strategies for HIV treatment that aim at the restoration of effector and cytotoxic ability of antigen-specific CD8+ T cells.

CHAPTER 2

LOSS OF ID2 DURING CHRONIC HIV INFECTION IS MEDIATED BY PD-1 TRIGGERING AND IS RESPONSIBLE FOR THE EXHAUSTION OF HIV-SPECIFIC CD8+ T CELLS

Loss of ID2 during chronic HIV infection is mediated by PD-1 triggering and is responsible for the exhaustion of HIV-specific CD8+ T cells

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KEYWORDS: HIV, PD-1, PD-L1, HIV-specific CD8+ T cells, cell survival

ABSTRACT

Background: During chronic HIV infection virus-specific $CD8^+$ T cells develop functional defects such as loss of IL-2 secretion, decreased proliferation and self–renewal capability and high level of expression of co-inhibitory molecules, most notably PD-1. We have shown that PD-1hi cells accumulate at the Ttm/Tem memory subsets and do not progress to fully differentiated effector cells. Little is known on the mechanisms downstream of PD-1 signaling that influence transcriptional/epigenetic events involved in T cell differentiation and effector function.

Results: Transcriptional profiling allowed us to show that the transcriptional modulator Id2, which is important for proliferation and survival of many cell types, was downregulated during HIV infection in cells expressing high levels of PD-1. We hypothesized that PD-1 might be inhibiting T cell differentiation to effector cells by interfering with the expression of Id2. We found that HIV-specific $CD8^+$ T cells from viremic donors showed decreased levels of Id2 mRNA when compared to CMV-specific CD8+ T cells from the same HIV-infected individual. Id2 levels were most profoundly decreased in the PD-1 high population compared to PD-1 negative counterparts. We further showed that engagement of PD-1 inhibited expression of Id2 mRNA and protein in $CD8^+$ T cells from healthy uninfected subjects that resulted in decreased proliferation and increased apoptosis. Transfection of PD-1hi effector cells from HIV infected individuals with a Tat-Id2 construct that localizes in the nucleus can reverse the apoptotic defect and restore effector function of these cells associated with the exhausted phenotype.

Conclusion: This study provides a molecular mechanism for PD-1/PD-L1 interaction in downregulation of Id2 expression and decreased differentiation of CD8 effector cells in chronic infection and strongly supports the potential therapeutic benefit of anti-PD-1 blocking antibody in restoring immune function in individuals with HIV infection and other chronic viral diseases.

INTRODUCTION

CD8+ T cells play a fundamental role in chronic viral infections; they are essential for controlling viral replication and for killing of virally infected cells. During chronic HIV infection HIV-specific CD8+ T cells are continuously exposed to the antigen and are found to be functionally impaired, as measured by reduced proliferative abilities, decreased capacity to produce cytokines and effector molecules leading to exhaustion (Greenwald, Freeman et al. 2005; Barber, Wherry et al. 2006). Others and we have shown that HIV-specific CD8+ T cells during chronic HIV infection upregulate the levels of the programmed death 1 (PD-1) receptor (Day, Kaufmann et al. 2006; Trautmann, Janbazian et al. 2006; Zhang, Zhang et al. 2007). PD-1 expression levels strongly correlate both with viral load and with the reduced capacity to produce cytokines and to proliferate. Cytomegalovirus (CMV)–specific CD8+ T cells from the same donors express decreased levels of PD-1 on their cell surface and their cytokine production is not affected (Trautmann, Janbazian et al. 2006). HIV-specific CD8+ T-cells have been shown to express a pre-terminally differentiated phenotype (CD45RA-CCR7- CD27+), when compared to CMV-specific cells that instead express a terminally differentiated effector phenotype (CD45RA+/-CCR7-CD27-) suggesting that HIV-specific CD8+ T cells may not complete their differentiation during the immune response (Champagne, Ogg et al. 2001; Appay, Dunbar et al. 2002; Ellefsen, Harari et al. 2002). PD-1 crosslinking results in attenuation of the signaling pathways downstream of TCR, but it can also induce the expression of genes that are specifically modulated upon its engagement by the ligand leading to decreased T cell function (Quigley, Pereyra et al. 2010; Patsoukis, Sari et al. 2012). However, it is not known whether PD-1 crossliniking also inhibits genes that are involved in CD8+ T cell differentiation during infection. Extensive studies have recently shown that transcription factors are important in regulating the memory versus effector differentiation of CD8+ T cell subsets during an immune response. Among them T-bet and eomes, Blimp-1 and Bcl-6 and the inhibitors of DNA binding (Id2 and Id3) have been shown to act as pairs of antagonistic transcriptional regulators that play critical roles in this process (Intlekofer, Takemoto et al. 2007; Joshi, Cui et al. 2007; Banerjee, Gordon et al. 2010; Pipkin, Sacks et

al. 2010) (Cannarile, Lind et al. 2006; Gong and Malek 2007; Kwon, Thierry-Mieg et al. 2009; Kalia, Sarkar et al. 2010; Pipkin, Sacks et al. 2010; Cui, Liu et al. 2011; Yang, Best et al. 2011) .

Id proteins together with the E-proteins belong to the family of the helix-loop helix (HLH) family of transcriptional regulators (Barone, Pepperkok et al. 1994; Iavarone, Garg et al. 1994; Lasorella, Uo et al. 2001) (Andres-Barquin, Hernandez et al. 2000). Id proteins are not able to bind to DNA because they lack a basic DNA binding domain and instead they function by inhibiting E-protein transcriptional activity leading to increased survival, cell cycle and differentiation of many cell types (Norton 2000; Engel and Murre 2001). Recently it has been shown that Id proteins regulate T cell differentiation of mature CD8+ T cells (Cannarile, Lind et al. 2006; Yang, Best et al. 2011; Masson, Minnich et al. 2013). Knockout studies in mouse models demonstrated that, in the absence of Id2 ,CD8+ effector cells were more susceptible to apoptosis when compared to wild type cells, and these cells consistently showed decreased levels of the pro-survival *Bcl-2* and increased levels of the pro-apoptotic *Bim* mRNA (Cannarile, Lind et al. 2006). This study was confirmed by others where they show that Id2 is required for the formation of short-lived effector memory CD8+ T cells after infection, while Id3 expression is required for the generation of long lived memory cells (Yang, Best et al. 2011).

Here we used a systems biology approach to identify genes that are modulated in exhausted cells from chronically HIV infected individuals in order to assess if the downregulation of genes involved in the generation of effector CD8+ T cell could explain the decreased differentiated phenotype and effector functions of PD-1hi antigen-specific CD8+ T cells. Transcriptional profiling allowed us to show that Id2 was downregulated in HIV-specific CD8+ T cells when compared to CMV-specific CD8+ T cells from the same subjects . We hypothesized that inhibitory receptors such as PD-1 act by inhibiting T cell effector functions not only by reducing TCR signaling, but also by decreasing the expression of genes that control differentiation of antigen specific CD8+ T cells into terminally differentiated effector cells.

RESULTS

1. Ex vivo Id2 levels are greatly reduced in HIV-specific CD8+ T cells

We compared the transcriptional profiles of sorted HIV-specific and CMV-specific CD8+ T cells from HIV viremic individuals using BioConductor Linear models (LIMMA). Extracted RNA from these populations was analyzed for global gene expression in order to identify a unique signature for exhausted HIV-specific CD8+ T cells (Fig.1). We found that HIV-specific and CMV-specific CD8+ T cell populations exhibited differential expression (upregulated or downregulated) of 307 genes. Among them 149 mRNAs were downregulated in HIV-specific CD8+ T cells and 158 were upregulated when compared to CMV-specific CD8+ T cells. By microarray analysis we found that genes upregulated in HIV-specific CD8+ T cells were enriched for those of inhibitory receptors such as PD-1 and CD160. Surface expression of PD-1 also showed increased levels of PD-1 in the HIV-specific CD8+ T cells as previously reported (Supplementary Figure 1D) (Trautmann, Janbazian et al. 2006). HIV-specific CD8+ T cells have a poorly differentiated effector phenotype indeed we found that they expressed higher levels of CD27 and decreased expression of all killer cell lectin-like receptors (KLRs); the latter molecules regulate effector memory differentiation (Sarkar, Kalia et al. 2008) . At the cell surface levels when we stained HIV-specific CD8+ T cells for CD45RA, CCR7 and CD27 we found that more than 60% of the cells expressed a transitional memory (Ttm) phenotype (63% ± 7) and only 20% of them had an effector memory (Tem) phenotype (29% ± 4), while a small fraction of these cells differentiated into TEMRA suggesting that these cells were blocked at the Ttm stage and could not progress to fully differentiated effector cells (Supplementary Fig.3). By gene array analysis we found that Id2, which inhibits E protein transcription factors and is associated with survival and differentiation of effector CD8+ T cells in mice (Cannarile, Lind et al. 2006; Yang, Best et al. 2011; Masson, Minnich et al. 2013), was downregulated during HIV infection in HIV-specific CD8+ T cells when compared to CMV-specific CD8+ T cells from the same HIV infected individuals (Fig.1). The array data were validated by Real Time PCR and we found that for all the epitopes examined (Table I) not only Id2 levels were decreased in the HIV-specific CD8+ T cells when compared to

CMV-specific CD8+ T cells (0.8± 0.1, 4.9± 0.7, P<0.01), but also the prosurvival Id2 target molecule , Bcl-2, (1.1± 0.5, 3.2 ±0.2 , P<0.01) while we found increased levels of the pro-apoptotic Bim molecule (22.1±2, 1.7± 1.8, P<0.01) (Supplementary Fig.1A, B,C). Studies *in vivo* have shown that members of the Bcl-2 family play an important role in regulating the contraction phase and that loss of Bcl-2 is associated with a decreased number of effector CD8+ T cells suggesting that loss of both Id2 and Bcl-2 in the HIV-specific CD8+ T cells may be responsible for the decreased survival of these cells especially of those with an effector phenotype (Hildeman, Zhu et al. 2002; Pellegrini, Belz et al. 2003; Wojciechowski, Jordan et al. 2006). Id2 has been shown to bind to E-protein transcription factors, thus inhibiting E-protein target genes that are involved in cell cycle arrest and apoptosis (Norton 2000; Cannarile, Lind et al. 2006). Therefore, we examined the global gene expression profile of genes downstream of E-protein transcription factors (supplementary Fig.2). We identified sets of genes upregulated in HIV-specific CD8+ T cells that regulate apoptosis (increased levels of Caspase 9 and Bid molecules, decreased Bcl-2 expression) and genes associated with blockage of the cell cycle (upregulation of the cyclin-dependent kinase inhibitors p21CIP1, and p15). Of note, p21CIP1 and p15 are upregulated by PD-1 crosslinking most probably as a result of the downregulation of Id2 expression (Kowanetz, Valcourt et al. 2004; Schlegel, Eichhoff et al. 2009; Patsoukis, Sari et al. 2012).

Taken together, these results show that PD-1 high HIV-specific CD8+ T cells from viremic patients do not differentiate to terminal effector cells ; this is associated with a distinct gene expression profile when compared to CMV-specific cells from the same patients. Moreover, genes involved in survival of effector CD8+ T cells leading to cell differentiation, such as Id2 were decreased in the HIV-specific CD8+ T cells when compared to CMV-specific CD8+ T cell subset.

2. Effector memory and transitional memory subsets from chronically infected HIV+ individuals have decreased levels of Id2 when compared to healthy donors.

Other groups have already shown that PD-1 expression levels are increased not only in HIV-specific CD8+ T cells, but also correlates with cellular differentiation (Petrovas,

Chaon et al. 2009; Yamamoto, Price et al. 2011). To study the impact of PD-1 on Id2 expression and CD8+ T cell differentiation, we further investigated whether the defect of Id2 expression during HIV-1 infection was specific to antigen-specific CD8+ T cells or whether it was a general defect of effector cells. We sorted memory CD8+ T cell subsets (gating strategy on Supplem. Fig. 4) and we compared Id2 expression from chronically HIV infected and uninfected individuals in the different subsets. Comparison of gene expression of each subset shows that in healthy individuals Id2 was expressed at higher levels in effector and transitional memory CD8+ T cells when compared to Tcm (Fig.2 A,B,C). However during chronic HIV infection Tem and Ttm failed to upregulate Id2 expression levels that we found to be significantly lower in viremic patients when compared to healthy individuals (Fig. 2 A,B). No significant differences were found in the Tcm compartment between the two groups of subjects (Fig 2C). PD-1 levels gated on naïve CD8+ T cells were used as a negative control for PD-1 expression. The decreased levels of Id2 in Tem and Ttm from viremic subjects correlated with increased levels of PD-1 in these cells. As previously described, we found that Tem and Ttm expressed higher levels of PD-1 compared to Tcm and that PD-1was increased in all memory subsets from viremic patients when compared to healthy donors (Fig.2 D, E, F respectively).

3. High levels of PD-1 during HIV-infection negatively correlate with Id2 expression

The low levels of Id2 expression in Ttm and Tem, the subsets that express the highest levels of PD-1 during chronic HIV-1 infection, prompted us to further validate our hypothesis of decreased Id2 expression during HIV infection as a consequence of PD-1 expression. Thus CD8+ CD45RA- PD-1 positive and negative cells from 9 HIV viremic patients were highly purified (>95%) as shown in supplementary Figure 5 and Id2 expression was assessed by Real Time PCR. We found that PD-1 hi cells had decreased levels of Id2 mRNA when compared to the PD-1 negative counterpart (0.1± 0.05, 0.9±0.2 respectively, P=0.0045) (Fig.3 A). Moreover, levels of Id2 negatively correlated with PD-1 expression (p=0.0045 spearman r=0.8) (Fig.3B). We assessed the expression levels of Bcl-2 and Bim by Real Time PCR and showed that Bcl-2 expression was also drastically

decreased in the PD-1 positive compartment when compared to PD-1 negative populations (5±1, 25±6 respectively, P<0.03)(Fig.3D) while Bim mRNA levels were increased (17±3, 2±0.9, P=0.007) (Fig.3C). When we measured PD-1 ligand (PD-L1) on monocytes from the same donors we found that there was a significant positive correlation between PD-1 and PD-L1 expression levels (p=0.01, r=0.7)(Fig.3D). Interestingly, Id2 expression levels were not decreased in successfully HAART treated patients (ST) or in HIV infected patients that controlled infection without treatment (elite controllers) (data not shown). Of note, CD8+ T cells from elite controllers and ST patients express lower levels of PD-1 on their cell surface (Day, Kaufmann et al. 2006; Trautmann, Janbazian et al. 2006). Collectively, these data show that during chronic HIV infection the levels of Id2 and its target molecule Bcl-2 are decreased in the PD-1 high population when compared to PD-1 negative cells in patients that express high levels of PD-L1 suggesting that PD-1 modulates of their expression levels.

4. PD-1 crosslinking decrease Id2 mRNA and protein levels

The inverse correlation between PD-1 and Id2 levels prompted us to further investigate whether the crosslinking of PD-1 by its ligand, PD-L1, resulted in the downregulation of Id2 expression levels. Primary human CD8+ T cells were cultured with anti-CD3-CD28-isotype beads or PD-L1-anti-CD3-CD28 beads for 24 to 48h and we assessed Id2 mRNA levels by Real Time PCR or protein levels by Western Blot. Incubation of CD8+ T cells with PD-L1 anti-CD3-CD28 beads for 24h resulted in downregulation of Id2 gene expression (Fig.4A) when compared to the Isotype beads control (3±0.5, 0.1 ±0.07, P=0.008). ID2 protein levels were also found to be drastically reduced after 48h of incubation with PD-L1 CD3-CD28 when compared to ISO-CD3-CD28 beads (Fig.4 D,E) (25± 5, 100 ±0, P=0.004). Incubation with PDL1-CD3-CD28 beads resulted in a significantly decreased production of IL-2 as measured by ELISA when compared to cells incubated with ISO-CD3-CD28 beads (Fig.4C) (358±74, 7±2 P=0.009). Triggering of PD-1 resulted in increased apoptosis most probably as a consequence of decreased levels of Bcl-2 expression (Fig. 4B)(ISO 1.7±0.2 PD-L1 0.08±0.03, P=0.013).

We investigated the signaling pathways that were modulated during HIV-infection after PD-1 crosslonking. We found that many of the signaling pathways downstream of Id2 were downregulated in the PD-1 positive cells after PD-1 crosslinking when compared to the PD-1 negative compartment. We found decreases in the cyclins and cell cycle regulation, and in G1/S checkpoint regulation. Increased TNFR-1 and TNFR-2 signaling was observed within the PD-1 positive cells and more interestingly there was a downregulation of IL-15 and Jak/STAT signaling involved in effector CD8+ T cell differentiation (Fig. 4F). These data suggest that PD-1 ligation in CD3-CD28 stimulated cells induces both decreased T cell function such as IL-2 production and a specific transcriptional program in primary human cells that leads to a loss of Id2 expression.

5. Ectopic Id2 expression rescues the survival of PD-1 hi effector memory CD8+ T cells

Finally, we tested whether overexpression of Id2 would improve the function of HIV-specific T cells. To rescue Id2 expression we cloned Id2 cDNA into a tat vector, purified the protein and labeled this recombinant protein with FITC in order to track transduced cells by flow cytometry (Supplementary Fig.6A) (Becker-Hapak, McAllister et al. 2001). Tat peptide allows the protein to enter the cells by micropinocytosis (Wadia, Stan et al. 2004). To track the cellular localization of the protein we extracted nuclear and cytosolic fraction and assessed ID2 protein expression by Western Blot analysis (Supplementary Fig.6B). We found that 1 h post transduction by Id2-tat all the protein was localized within the nucleus of CD8+ T cells probably due to Id2 nuclear localization signals (Kurooka and Yokota 2005). This system allowed us to overexpress Id2 in CD8+ T cells purified from chronically HIV infected donors; transduced cells and controls were then recultured in the presence of autologous PBMCs depleted of CD8+ T cells, to allow PD-1 triggering by its ligand that is highly expressed on antigen presenting cells (APC). Overexpression of Id2 resulted in a significant decrease in apoptosis of PD-1 hi effector CD8+ T cells as measured by Annexin V, staining when compared to control tat transfected cells (22% ± 9%, 72% ± 7%, respectively P=0.014) (Fig.5A,B). CD8+ T cells that were transduced with the two proteins were sorted after 12h for Tem ID2-FITC

positive and Tat-FITC positive cells to assess whether ID2 overexpression was able to restore CD8+ T cell effector function at the molecular level. We found that Ttm and Tem transfected cells with ID2 had increased levels of the effector molecule Granzyme B, and decreased levels of the inhibitor of cell cycle p21CIP1 (Fig.5C,D). Moreover, ID2 expression also rescued cells from their apoptotic phenotype as measured by increased levels of Bcl-2, Myc and decreased Bim expression; they also expressed a more differentiated phenotype as measured by decreased levels of CD27 (Fig.5 E,F,G,H). Therefore, enforced ID2 expression increases survival of exhausted PD-1 hi effector T cells and leads to their differentiation into effector cells.

DISCUSSION

During an immune response, naïve T cells are primed by antigen to undergo clonal expansion and to differentiate into antigen-specific effector cells that secrete cytokines and exert cytolitic activity. Once the antigen is cleared, most of the effector cells (90%-95%) die by apoptosis (contraction phase) while the remaining cells (5-10%) establish the pool of long-lived population of memory cells. Under normal conditions, several transcription factors have been shown to regulate effector and memory CD8+ T cell development. Among them Id2 plays a critical role in the generation of effector CD8+ T cells during an immune response and its expression levels are maintained in the memory compartment both in mice and humans in the most differentiated Ttm and Tem populations (Cannarile, Lind et al. 2006) (Chapter 4). Effector CD8+ T cells from mice lacking Id2 are not able to survive and this results in decreased antigen clearance (Cannarile, Lind et al. 2006). We (chapter 4) and others have also shown that after antigen priming, activated virus specific CD8+ T cells receive signals that induce Id2 expression (Cannarile, Lind et al. 2006). The increased levels of Id2 promote differentiation and survival of effector cells which are important to exert cytotoxic function, proliferation and cytokine production. Antigen-specific CD8+ T cells that are able to limit infection, as during CMV infection, have a terminally differentiated phenotype, they lack the expression of CD27, CCR7 and CD28 molecules, and have a

better ability to produce granzyme B (Appay, Dunbar et al. 2002; Jaspan, Gaumer et al. 2003; Trautmann, Janbazian et al. 2006). Thus, transcriptional regulation is required for memory differentiation to confer protective immunity. During chronic infection, such as HIV, the continued exposure to the antigen leads to a functional impairment of T cells known as exhaustion (Barber, Wherry et al. 2006). It has been already shown that the functional exhaustion of CD8+ T cell is affected also at the transcriptional level and this might reflect their altered phenotypic and effector characteristics. PD-1 plays an important role in T cell exhaustion, and its engagement by PD-L1 has been shown to affect both genes downstream of TCR as well as specific gene expression signature that reflects the transcriptional consequences of PD-1 receptor ligation (Quigley, Pereyra et al. 2010).

In this study, we show that PD-1 hi HIV-specific CD8+ T cells from chronic HIV infected individuals are blocked in a transitional memory phenotype and this is associated with a downregulation in Id2 expression levels when compared to CMV-specific CD8+ T cells from the same HIV-infected donor. Moreover not only was Id2 downregulated in the HIV-specific CD8+ T cells, but there was a modulation of its target genes with decreased levels of the pro-survival Bcl-2 molecule and increased levels of the pro-apoptotic Bim. Members of the Bcl-2 family have been shown to regulate survival of effector cells during the contraction phase of an immune response. Indeed overexpression of Bcl-2 mediated survival of antigen specific cells that survive the contraction phase (Hildeman, Zhu et al. 2002; Pellegrini, Belz et al. 2003; Wojciechowski, Jordan et al. 2006). These findings suggest that the decreased frequency of cells that have an effector phenotype within the HIV-specific CD8+ T cells may be due to decreased levels of genes involved in the survival and differentiation of effector cells.

Here we have also compared Id2 expression levels in memory CD8+ T cell subsets from chronic HIV-infected subjects and healthy individuals. Under normal conditions Id2 expression levels are upregulated on transitional memory and effector memory CD8+ T cells and lower Id2 expression levels are found in the central memory compartment. However, when we compared the gene expression profiles of CD8+ T cell memory

subsets from chronic to healthy individuals we found that only in Tem and Ttm populations from HIV infected individuals was Id2 expression reduced, and these two memory subsets are those that express the highest PD-1 levels. No significant differences in Id2 expression were found in the Tcm compartment between the two groups of subjects probably because Tcm express lower levels of PD-1 on their cell surface. Indeed, when we compared Id2 expression from total memory PD-1 positive and PD-1 negative populations Id2 expression levels were found to be drastically decreased in the cells with high PD-1 levels and we also found a negative inverse correlation between Id2 expression levels and PD-1 MFI. PD-1 expression during HIV infection has been associated with an apoptotic phenotype as measured by decreased levels of the prosurvival molecules Bcl-2 and CD127 (IL-7 receptor α) and increased binding of MitoTracker, a marker that measures mitochondrial mass (Petrovas, Chaon et al. 2009). Here we show that the apoptotic phenotype of the PD-1 high cells is mediated by its engagement by PD-L1. During chronic HIV infection, the continuous antigenic stimulation is responsible of the increased levels of both PD-L1 on monocytes and PD-1 on pre-terminally differentiated cells (Trautmann, Janbazian et al. 2006; Meier, Bagchi et al. 2008). Our results show that PD-1 crosslinking on CD8+ T cells from healthy individuals with anti-CD3-CD28-PD-L1 beads results in the downregulation of both Id2 protein and mRNA expression levels which leads to decreased survival abilities (measured as Bcl-2 levels) and decreased proliferation (measured by IL-2 production).

Finally we have shown that restoration of Id2 expression with a tat fusion protein rescues survival of PD-1 hi effector CD8+ T cells from chronic HIV-infected individuals measured by Annexin V levels and that at the molecular level Id2 overexpression restores Bcl-2 upregulation and decreases Bim. Id2 overexpression leads to increased Granzyme B expression and decreased levels of the maturation marker CD27, showing that these cells are able to exert cytotoxic function and to differentiate into effector cells.

In conclusion, this study identifies a new molecular pathway that is important for CD8+ T cell differentiation that is negatively regulated during chronic HIV infection and is downstream of PD-1. Our studies do not identify the mechanism by which PD-1 ligation

induces downregulation of Id2, or whether this happens by direct or indirect pathways. However, our results suggest further investigations into how Id2 regulates T cell differentiation of antigen-specific cells and how we may reverse the decreased Id2 levels observed in patients with chronic phase HIV infection.

METHODS

Study population

Study population included 9 chronically progressing subjects (CHI) infected for more than 6 months based on CD4 T cell counts under 500/mm3 or declining CD4 T cell counts. Informed consent was obtained and research conformed to ethical guidelines established by the ethics committee of Centre Hospitalier de l'Université de Montreal.

Peptides and Tetramers

Soluble pMHC monomers were generated as previously described (Altman, Moss et al. 1996). The peptides and tetramers used to analyze the CMV and HIV-specific CD8+ T cell response were: NLVPMVATV (A*02 CMV), TPRVTGGGAM (B*07 CMV), FLGKIWPSYK (A*02 Gag), ILKEPVHGV (A*02 Pol), SLYNTVATL (A*02 Gag), RLRPGGKKK (A*03 Gag), RPGGKKKYKL (B*07 Gag), TPGPGVRYPL (B*07 NEF), GEIYKRWII (B*08 Gag), FLKEKGGL(B*08 NEF), RLRPGGKKK (A*03 POL)

Phenotypic analysis

PBMCs were resuspended in PBS containing 2% FCS and stained with Tetramer-PE at 0.3μg per 10^6 cells. The following cocktail was used for phenotyping CD8+ T cells: CD3-Alexa 700 (BD), CD8 PB (BD), CD45RA H7 (BD), CCR7 Pe-Cy7 (BD), CD27 Qdot655 (Invitrogen) and PD-1 APC (eBioscience). Dying cells were eliminated with a LIVE/DEAD viability dye (Invitrogen). We acquired a minimum of $1x10^6$ events for all cytometry-related experiments using a BD LSRII flow cytometer and analyzed with DIVA software v5.0 (BD).

Real Time PCR

Primers and probes were obtained from IDT and Roche respectively. Multiple 100 cell samples from healthy or HIV infected individuals where sorted by FACS ARIA (BD) into a 96 well plate gating on the population of interest. We used the Cells Direct One-Step qRT-PCR Kit for reverse transcription and preamplification (18 cycles) of the RNA targets of interest without a separate RNA purification step. qPCR was performed on a Roche Light Cycler 480 and analysis was performed according to the ΔΔct method.

Gene array analysis of HIV vs CMV specific CD8+ T cells

Sorted HIV and CMV- specific CD8+ T cells were processed as previously described (Peretz, He et al. 2012). Analysis of the genome array output data was conducted using the R statistical language (R Development Core Team) and various software packages from Bioconductor, an open source project for the analysis and comprehension of high-throughput genomic data (Gentleman et al., 2004). First, arrays displaying unusually low median intensity, low variability, or low correlation relative to the bulk of the arrays were discarded from the rest of the analysis. Quantile normalization was applied, followed by a log2 transformation. The LIMMA package (Bioconductor; Smyth, 2004) was used to fit a linear model to each probe and to perform a (moderated) Student's *t* test on differences of interest. Here, samples were stratified into HIV and CMV specific groups. The contrast was designed and fit into a liner model. The results were displayed in individual heatmaps of genes differentially expressed between the different groups (HIV and CMV). Gene expression within each heatmap is represented as gene-wise standerized expression (Z-score), with $|FC|>1.3$ and p-value <0.05 and FDR $<5\%$ chosen as the significant levels unless specified otherwise in the title or legend of the heatmap. The expected proportions of false positives (FDR) were estimated from the unadjusted p-value using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

Pathway analysis of PD-1 high and PD-1 negative cells after PD-1 crossliniking

Highly purified CD8+CD45RA- PD-1 high and PD-1 negative cells (based on Suppl. FIg.5) from chronic HIV infected individuals were stimulated for 3h or 12h with PD-L1-anti-CD3-CD28 beads and samples were processed as previously described (Peretz, He et al. 2012). Illumina Probe IDs were imported into the Ingenuity software and mapped to the Gene Symbol from Ingenuity database. Genes that had adjusted p-value < 0.05 at each comparison and Fold change > 1.3 or < -1.3 and associated with a canonical pathway in Ingenuity's Knowledge Base were used for pathway analysis. Heat map colors represent the ratio of regulated genes / pathway after stimulation with anti-CD3-CD28-PD-L1 beads (pathways not over-represented are dark blue). Pathways were regrouped according to some key immune or biological pathways. The over-representation test was performed using Fisher Exact Test. Statistical significance achieved at $p < 0.05$

Construction and Purification of Tat-Id2 Protein

We cloned the Id2 cDNA into pTATv2.2 vector and expressed it in BL-21 (DE3) star E. coli (Novagen). The expression of the protein was done using 1 mM isopropyl thiogalactoside. « After 5 h of induction at 37 °C and an additional overnight culture at 25 °C, cells were harvested and sonicated in 6 M guanidine buffer containing 10 mM Tris·HCl and 100 mM NaH2PO4 (pH 6.3). Lysates were clarified by centrifugation and loaded (10 mL/500 mL culture) on HisTrap chelating columns (Qiagen). Bound proteins were washed with 6 M urea buffer (pH 6.0), and the target proteins were eluted with 6 M urea buffer (pH 4.5). The eluted proteins were desalted using PD-10 Sephadex G-25 columns (GE Healthcare) with PBS (10% glycerol), and aliquots were stored at −80 °C. The purity of proteins was characterized by limulus amoebocyte lysate (LAL) (Lonza) and Picogreen assays (Invitrogen). The endotoxin level was 27.0 EU/mg, and the bacterial DNA level was 8.6 ng/mg of protein in four independent preparations » (Michelle Becker-Hapak et al. 2001).

Preparation of antibody-coupled beads

Combinations of anti-CD3 (clone 2C11), PD-L1-hIgG1 or Iso-hIgG1 were covalently attached to Dynabeads M450 glycidyl ether beads following the manufacturer's directions (Invitrogen). We loaded same ammount of proteins during preparation by keeping constant the molar ratios of the proteins using hamster IgG or hIgG1-Fc antibodies as filler, as appropriate (Broeren, Gray et al. 2000; Riley, Mao et al. 2002). We used 1 μg of anti-CD3 (20% of total protein) and 4 μg of PD-L1 (80%) or control Ig were used per 10^7 beads.

Statistical analysis

We used GraphPad Prism 5.0c (GraphPad software, San Diego, CA) for statistical analysis. Statistical analysis of group differences was examined using the nonparametric Mann–Whitney U test or unpaired two-tailed Student's t tests. *$P < 0.05$ and **$P < 0.01$ were considered to be significant.

FIGURE LEGEND

Table I: Clinical characteristics of study population

Figure 1: HIV-specific CD8+ T cells represent a distinct subset with a unique transcriptional profile: PBMCs were stained with Live/dead cell viability dye, anti-CD3, anti CD8 and sorted for HIV-specific and CMV-specific CD8+ T cells using MHCI/peptide tetramer staining. Heat map illustrating the differentially expressed genes between HIV and CMV-specific CD8+ T cells is shown.

Figure 2: Effector memory and transitional memory subsets from chronically infected HIV+ individuals have decreased levels of Id2 when compared to healthy donors. PBMCs were stained with antibody against CD3, CD8, CD45RA, CCR7 and CD27, and memory CD8+ T cell subsets were sorted from Healthy donors and HIV+ chronically infected patients. a) Id2 mRNA levels on memory CD8+ T cell subsets was assessed by Real Time PCR and normalized by GAPDH. b) PD1 MFI on EM, TM and CM subsets from the two groups of subjects.

Figure 3: Id2 expression is downregulated in exhausted PD1+ population cells during HIV chronic infection: PBMCs were stained with anti-CD3, anti-CD8, anti-CD45RA, and memory CD8+ T cells were sorted by gating on PD-1 positive and PD-1 negative populations form HIV chronic infected patients. Relative expression of a) Id2 d), Bcl-2 e) and Bim mRNA levels measured by Real Time. b) MFI of PD1 expression on sorted cells in relation to Id2 mRNA levels and in relation to c) PD-L1 MFI on CD14+ cells.

Figure 4: PD-1 crosslinking leads to decreased Id2 mRNA and protein levels and a consequent decrease in T cell function: Primary human CD8+ T cells were cultured with CD3-CD28-ISO or PDL1 beads. a) Id2 and b) Bcl-2 expression measured by Real Time PCR at 24h. c) Expression of IL-2 cytokine was measured by ELISA d)Total lysates of equal amount of cells were subjected to immunoblotting as indicated. Id2 protein and actin blots were performed in parallel as loading controls. e) Densitometric quantification was

performed using ImageQuant software. F) Heat map showing statistically significant canonical pathways (Ingenuity PathwayAnalysis Software) commonly regulated at 3h and 12h when comparing PD-1 high versus PD-1 low to baseline. Columns represent high versus low comparisons at each time point. Rows represent significantly regulated canonical pathways.

Figure 5: Ectopic Id2 expression rescues the survival of PD-1 hi effector memory CD8+ T cells: We cloned the Id2 cDNA into the pTATv2.2 vector, purified the recombinant tat-tagged ID2 protein and labeled it with FITC. Primary human CD8+ T cells from chronic HIV infected donors were transduced with 3ug of the ID2-tat-FITC construct or tat-FITC alone and cocultured with CD8 depleted PBMCs. a) Gating strategy of Tem cell plotted for Annexin V after transfection with ID2-tat or tat alone. b) Histograms represent Annexin V levels in 5 chronic HIV-infected individuals after tranfection. 12h post transduction with tat or ID2-tat construct 100 CD8+ Tem and Ttm cells were sorted and expression of c) GranzymeB, d) p21, e) CD27, f) Bcl-2, g) Bim and h) Myc was assessed by Real Time PCR. Expression was measured by Fold change.

Supplementary Figure 1: Ex vivo Id2 levels are greatly reduced in HIV-specific CD8+ T cells. Sorted HIV-specific and CMV-specific CD8+ T cells were analyzed for the mRNA expression of a) Id2 b), Bcl-2 c) and Bim, while d) MFI of PD-1 was measured by flow cytometry.

Supplementary Figure 2: HIV-specific CD8+ T cells represent a distinct subset with a unique transcriptional profile: Heat map illustrating the differentially expressed genes with the Id2-Id3 filter between HIV and CMV-specific CD8+ T cells.

Supplementary Figure 3: HIV-specific CD8+ T cells have a Ttm phenotype. PBMCs were stained for HIV-tetramers; HIV-specific CD8+ T cells were plotted based on CD45RA, CCR7, CD27, expression for Tem (CD45RA-CCR7-CD27-), Ttm (CD45RA-CCR7-CD27+), Tcm (CD45RA-CCR7+CD27+) and naïve (CD45RA+CCR7+CD27+).

Supplementary Figure 4: Gating strategy for memory CD8+ T cell subsets. PBMCs were plotted for CD3+, CD8+ cells viable cells and CD45RA negative cells to sort memory

subsets. Memory subsets were sorted based on CCR7, CD27 expression for Tem (CD45RA-CCR7-CD27-), Ttm (CD45RA-CCR7-CD27+), Tcm (CD45RA-CCR7+CD27+).

Supplementary Figure 5: Gating strategy for sorted PD-1 high and PD-1 negative cells. Total PBMCs were stained with antibodies against CD3, CD8, CD45RA, and PD-1. Dead cells were excluded by LIVE/DEAD staining. CD3+, CD8+ cells were plotted for CD45RA. CD45RA negative cells were plotted for PD-1 and 100 positive and negative cells were sorted by FACS ARIA (BD).

Supplementary Figure 6: ID2-TAT construct localizes into the nucleus of CD8+ T cells: a) Schematic representation of the ID2-tat construct. b) Nuclear and cytosolic fractions were extracted 1h post transduction and ID2 expression was measured by Western blot. 1mM of the ID2-tat was loaded directly on the gels of the WB and was used as positive control.

Supplementary Figure 7: Id2 expression levels are decreased in PD-1 hi population independently of other negative regulators. PBMCs were sorted for double positive (CD160+, PD-1+), double negative (CD160-, PD-1-) and single positive (CD160-, PD-1+) memory (CD45RA -) CD8 + T cells from 9 chronic HIV infected individuals. A) Id2 expression was assessed by Real Time PCR; B) PD-1 MFI on the different subsets.

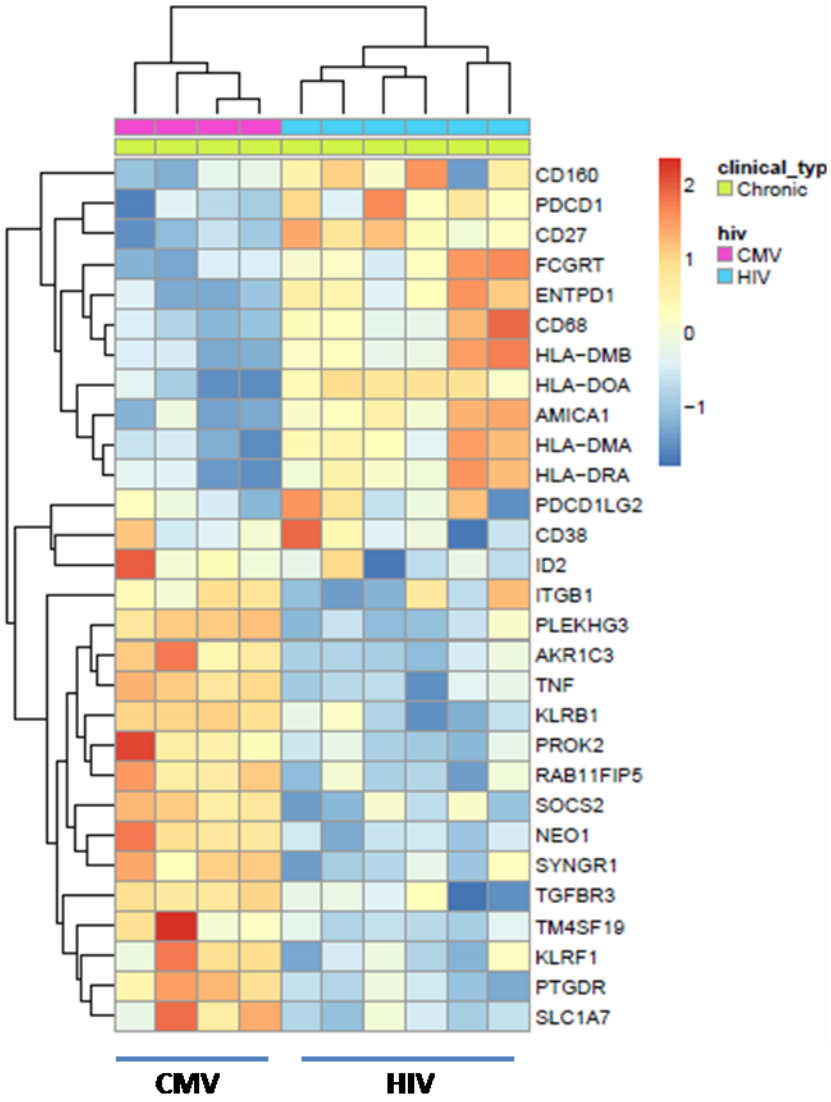
Contributions

A.N. performed all the experiments, analyzed-interpreted data and prepared graphics. F.A.P.supervised the project, designed experiments, participated in performing experiments. H.T and F.D. and J.A. helped in analysis and preparation of graphics. Z.H. prepared plasmids for positive controls. R.B. provided with the samples. K.G. and C.S. analyzed gene array data. R.P.S. supervised the project.

Table I: Clinical characteristics of study population

Patient ID	Time Point	CD4 *cells/mm3*	CD8 *cells/mm3*	LOG VL	VL	tetramers		
DRPI026	15-Sep-06	450	898	5.24	173497	B7CMV pp65	A3HIVgag kkr	
IDR192	4-Mar-09	368	1320	4.54	34363	B7CMV pp65	B7 HIV nef	B7 HIV SM9
IDR194	25-Feb-09	383	354	4.32	20925	A2 CMVpp65	A2 HIV p15	A2 HIV pol
DRPI041 v15	2005JN29	475	640	4.75	56838	A2 CMVpp66	A2 HIV p15	
NYU008	4-Dec-08	557	1313	4.44	27766	B705 kk10	A3 HIV pol	B8 HIV nef
C401	8-Jun-12	317	470	4.71	51000	A2 CMVpp68	A2 HIV gag p17	
ACT71748	9-Feb-2007	571	1266	3.77	5897	A2 HIV p15	A2NV9 (CMV)	
DRPI40	6-Dec-05	345	511	4.86	71910	A2 HIV p15		

Figure 1


CD160
PDCD1
CD27
FCGRT
ENTPD1
CD68
HLA-DMB
HLA-DOA
AMICA1
HLA-DMA
HLA-DRA
PDCD1LG2
CD38
ID2
ITGB1
PLEKHG3
AKR1C3
TNF
KLRB1
PROK2
RAB11FIP5
SOCS2
NEO1
SYNGR1
TGFBR3
TM4SF19
KLRF1
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Chronic
hiv
CMV
HIV
CMV
HIV

Figure 2

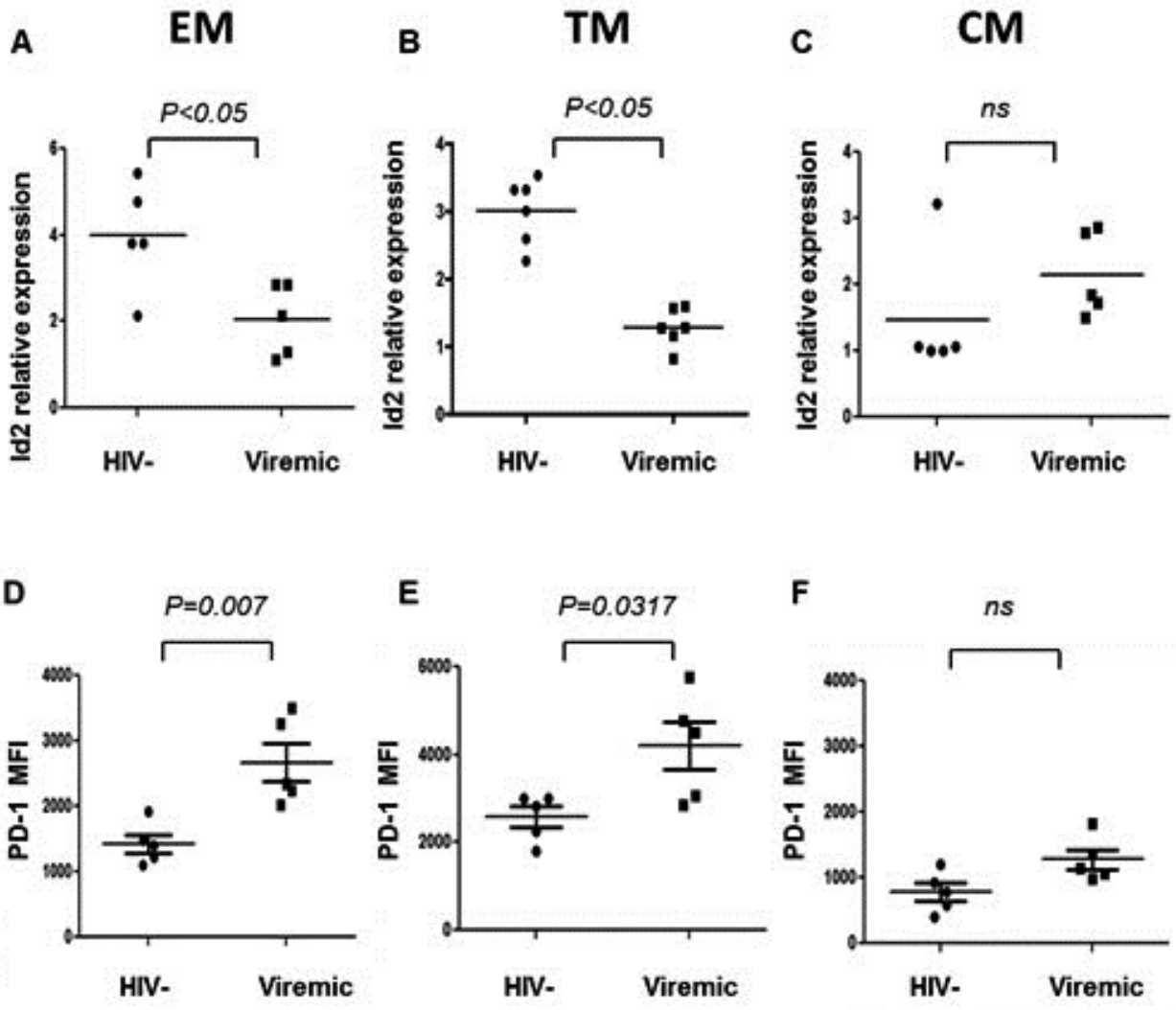

A
EM
P<0.05
Id2 relative expression
HIV-
Viremic
B
TM
P<0.05
Id2 relative expression
HIV-
Viremic
C
CM
ns
Id2 relative expression
HIV-
Viremic
D
P=0.007
PD-1 MFI
HIV-
Viremic
E
P=0.0317
PD-1 MFI
HIV-
Viremic
F
ns
PD-1 MFI
HIV-
Viremic

Figure 3

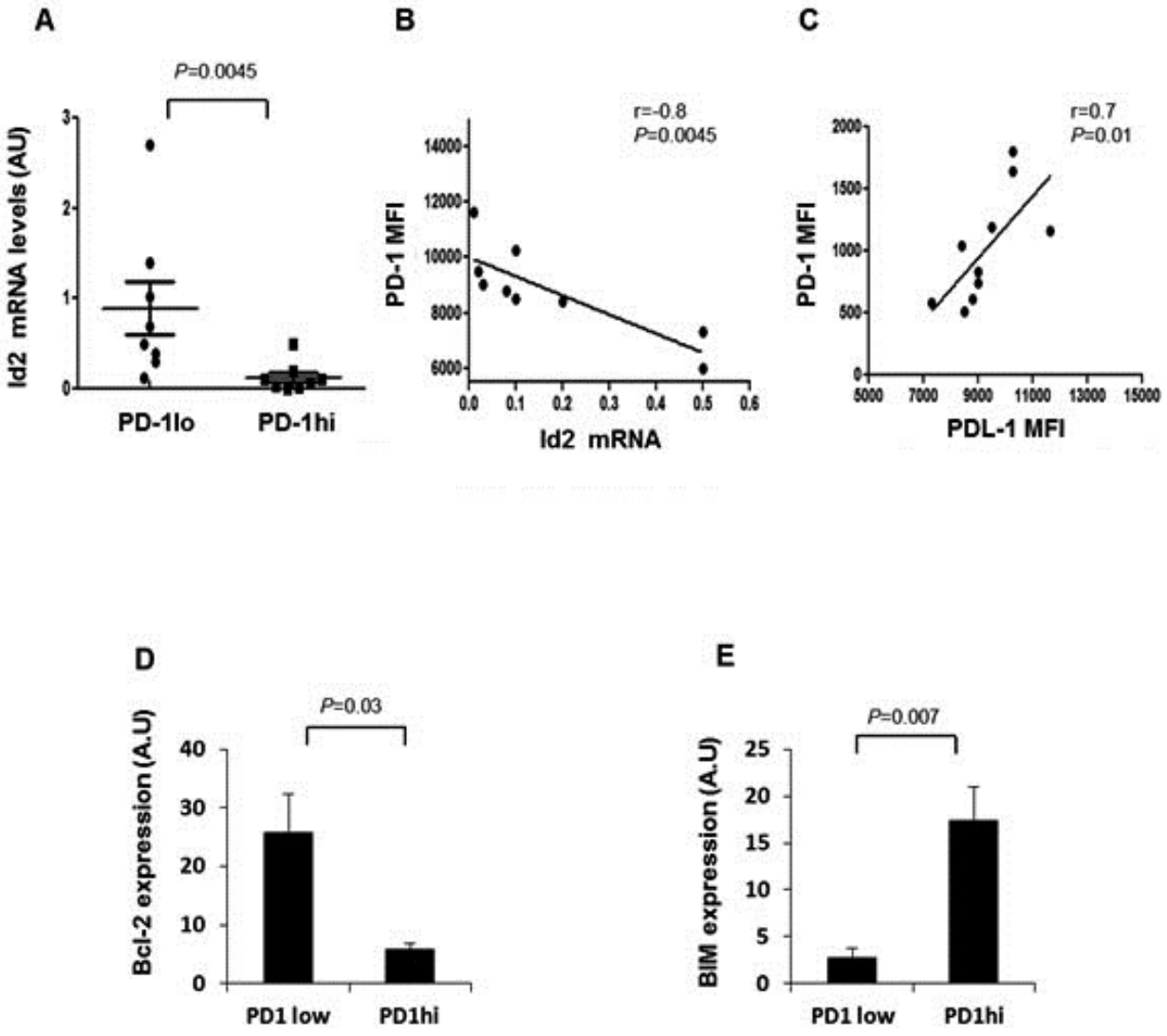
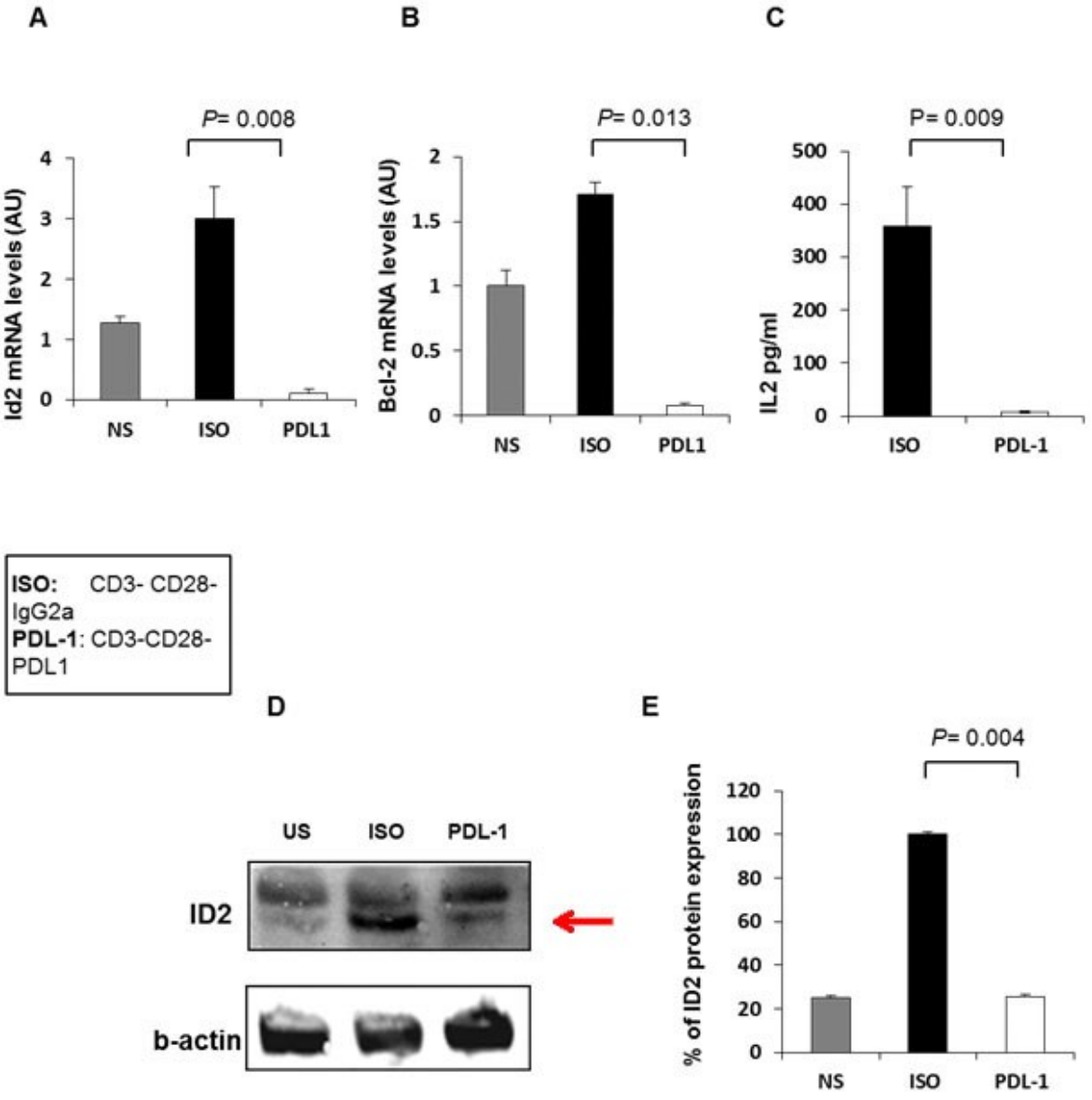

A
P=0.0045
Id2 mRNA levels (AU)
PD-1lo
PD-1hi
B
r=-0.8
P=0.0045
PD-1 MFI
Id2 mRNA
C
r=0.7
P=0.01
PD-1 MFI
PDL-1 MFI
D
P=0.03
Bcl-2 expression (A.U)
PD1 low
PD1hi
E
P=0.007
BIM expression (A.U)
PD1 low
PD1hi

Figure 4


A
B
C
P= 0.008
P= 0.013
P= 0.009
Id2 mRNA levels (AU)
Bcl-2 mRNA levels (AU)
IL2 pg/ml
NS
ISO
PDL1
PDL-1
ISO: CD3- CD28- IgG2a
PDL-1: CD3-CD28- PDL1
D
E
US
ISO
PDL-1
ID2
b-actin
P= 0.004
% of ID2 protein expression

F

Figure 4

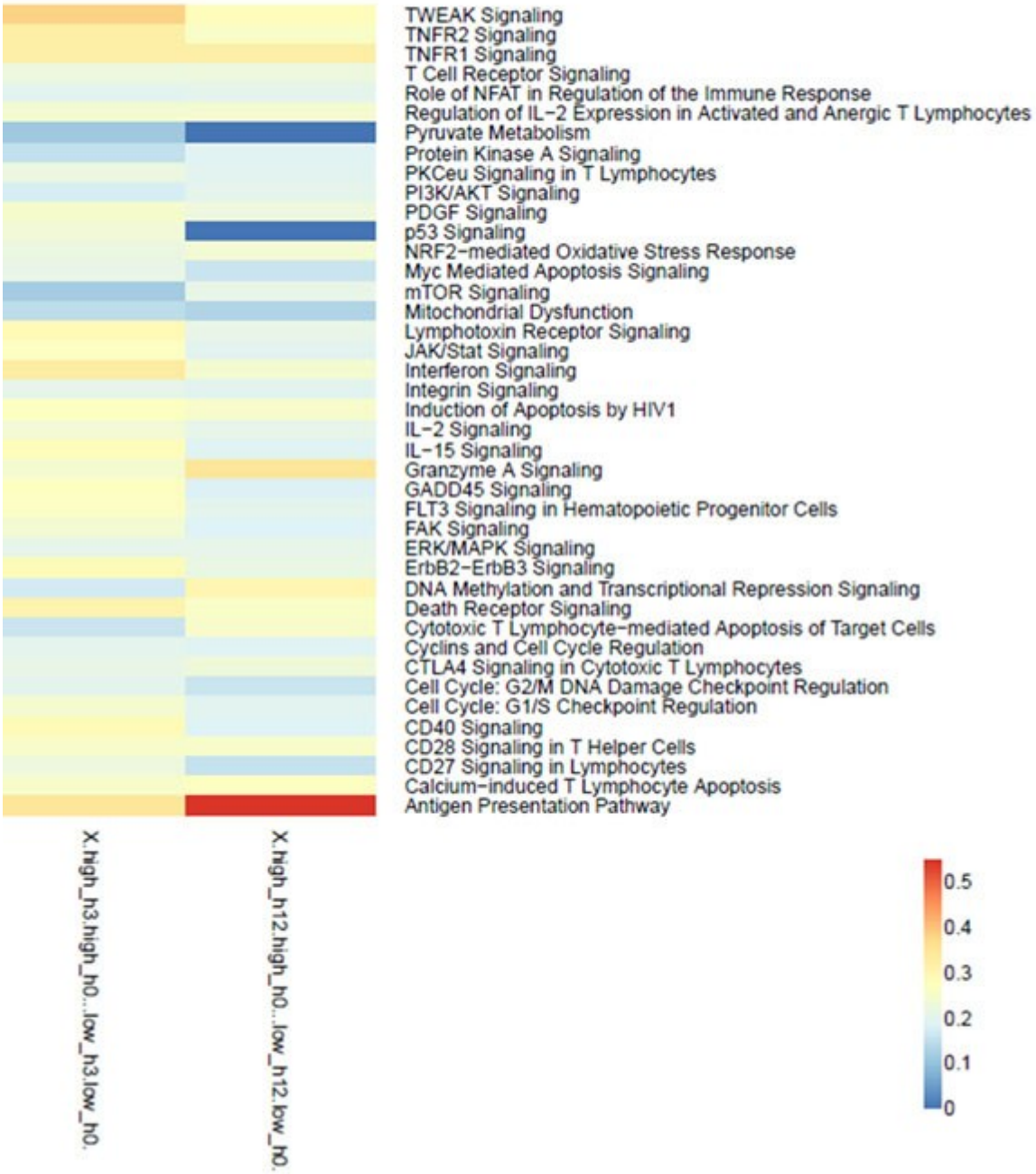

TWEAK Signaling
TNFR2 Signaling
TNFR1 Signaling
T Cell Receptor Signaling
Role of NFAT in Regulation of the Immune Response
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes
Pyruvate Metabolism
Protein Kinase A Signaling
PKCeu Signaling in T Lymphocytes
PI3K/AKT Signaling
PDGF Signaling
p53 Signaling
NRF2-mediated Oxidative Stress Response
Myc Mediated Apoptosis Signaling
mTOR Signaling
Mitochondrial Dysfunction
Lymphotoxin Receptor Signaling
JAK/Stat Signaling
Interferon Signaling
Integrin Signaling
Induction of Apoptosis by HIV1
IL-2 Signaling
IL-15 Signaling
Granzyme A Signaling
GADD45 Signaling
FLT3 Signaling in Hematopoietic Progenitor Cells
FAK Signaling
ERK/MAPK Signaling
ErbB2-ErbB3 Signaling
DNA Methylation and Transcriptional Repression Signaling
Death Receptor Signaling
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells
Cyclins and Cell Cycle Regulation
CTLA4 Signaling in Cytotoxic T Lymphocytes
Cell Cycle: G2/M DNA Damage Checkpoint Regulation
Cell Cycle: G1/S Checkpoint Regulation
CD40 Signaling
CD28 Signaling in T Helper Cells
CD27 Signaling in Lymphocytes
Calcium-induced T Lymphocyte Apoptosis
Antigen Presentation Pathway
X.high_h3.high_h0...low_h3.low_h0.
X.high_h12.high_h0...low_h12.low_h0.
0.5
0.4
0.3
0.2
0.1
0

Figure 5

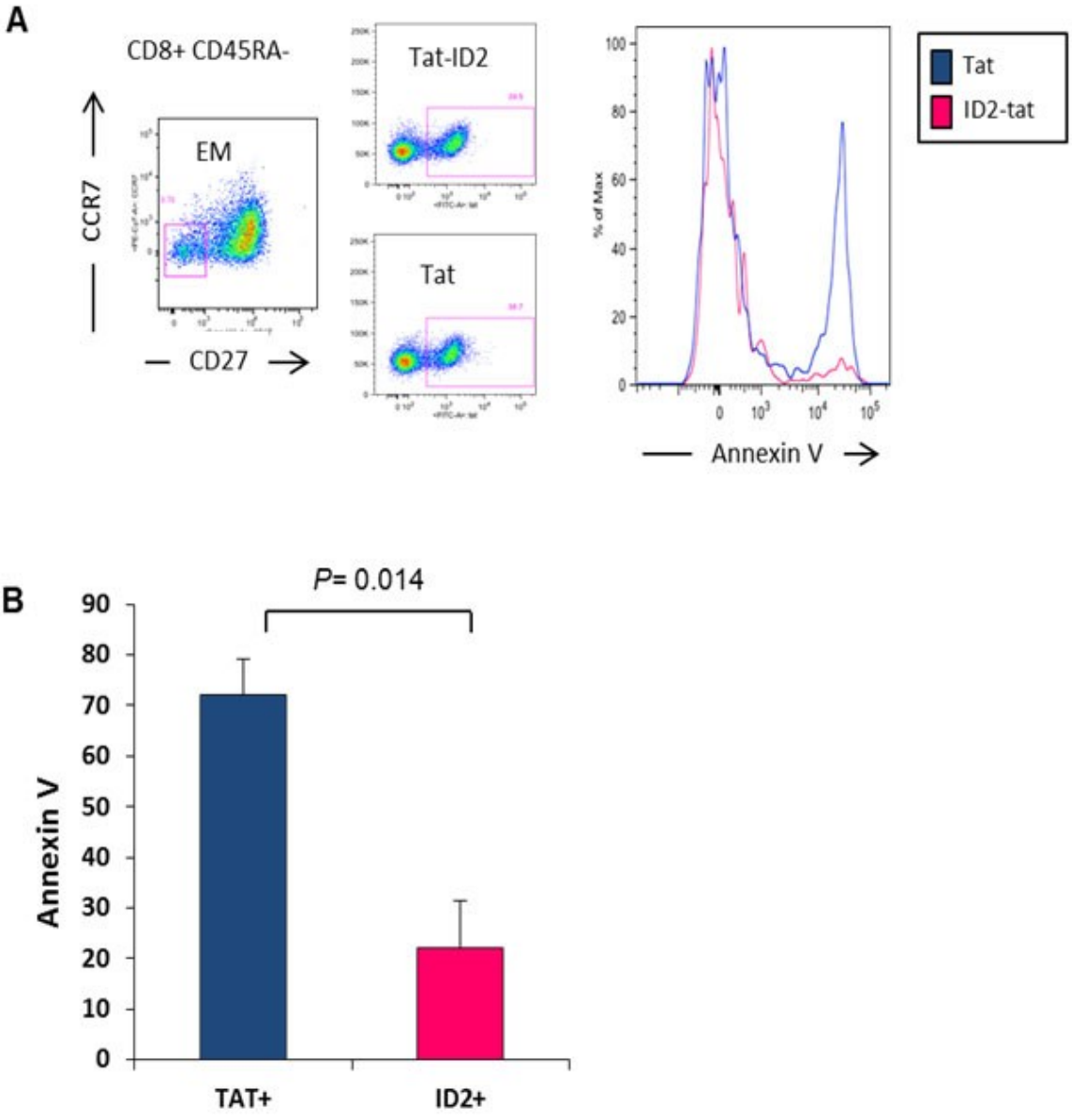
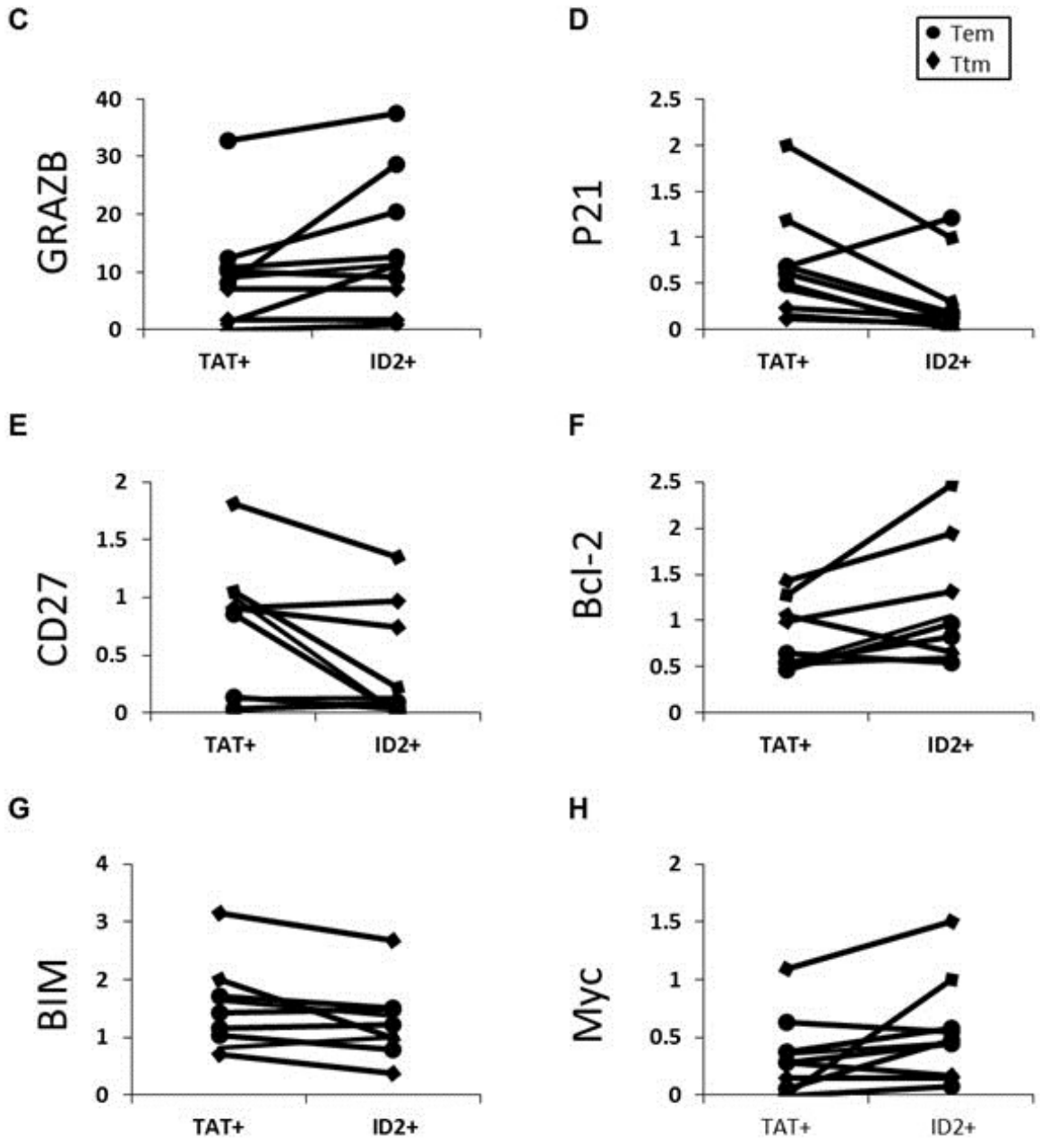
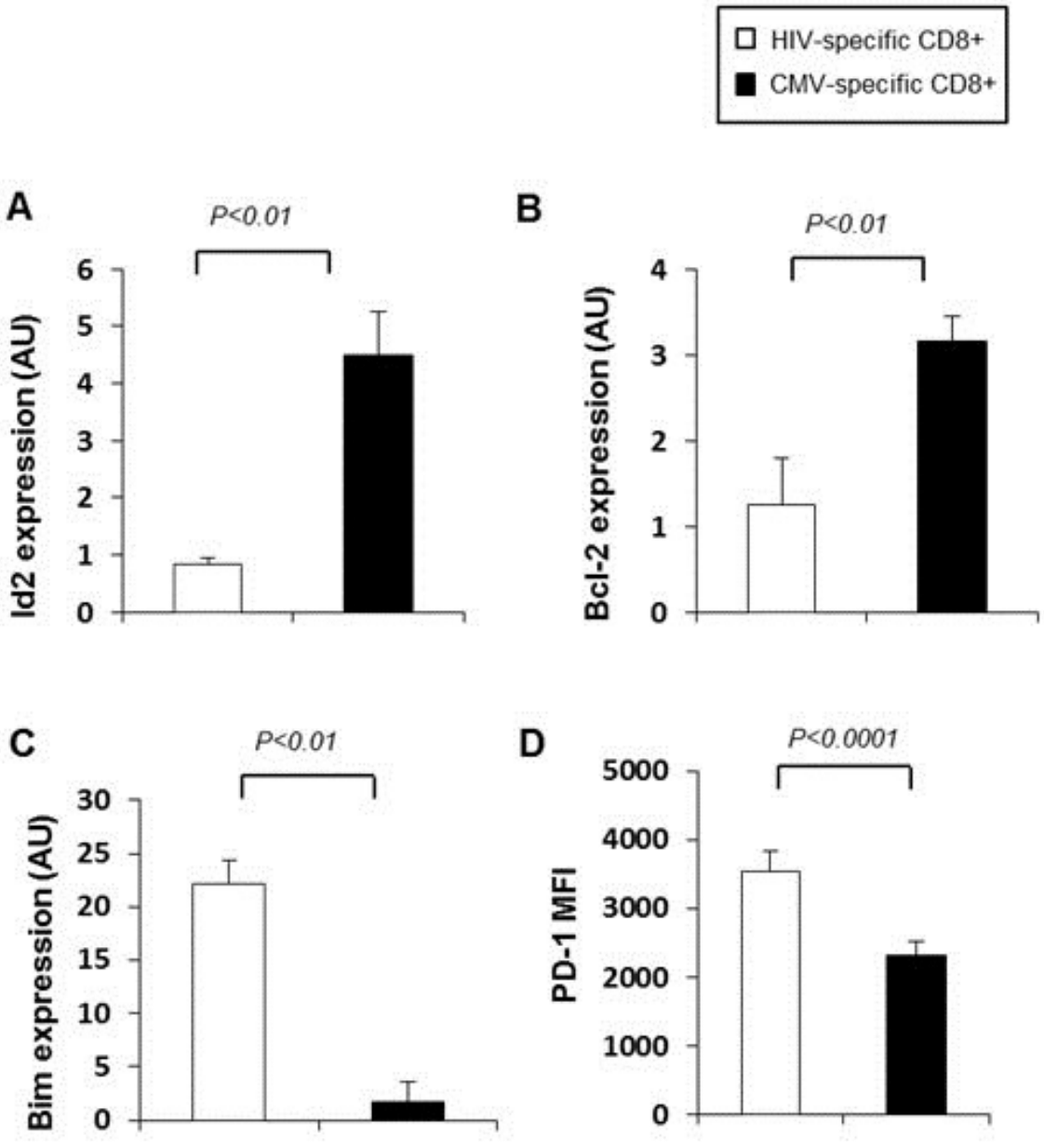
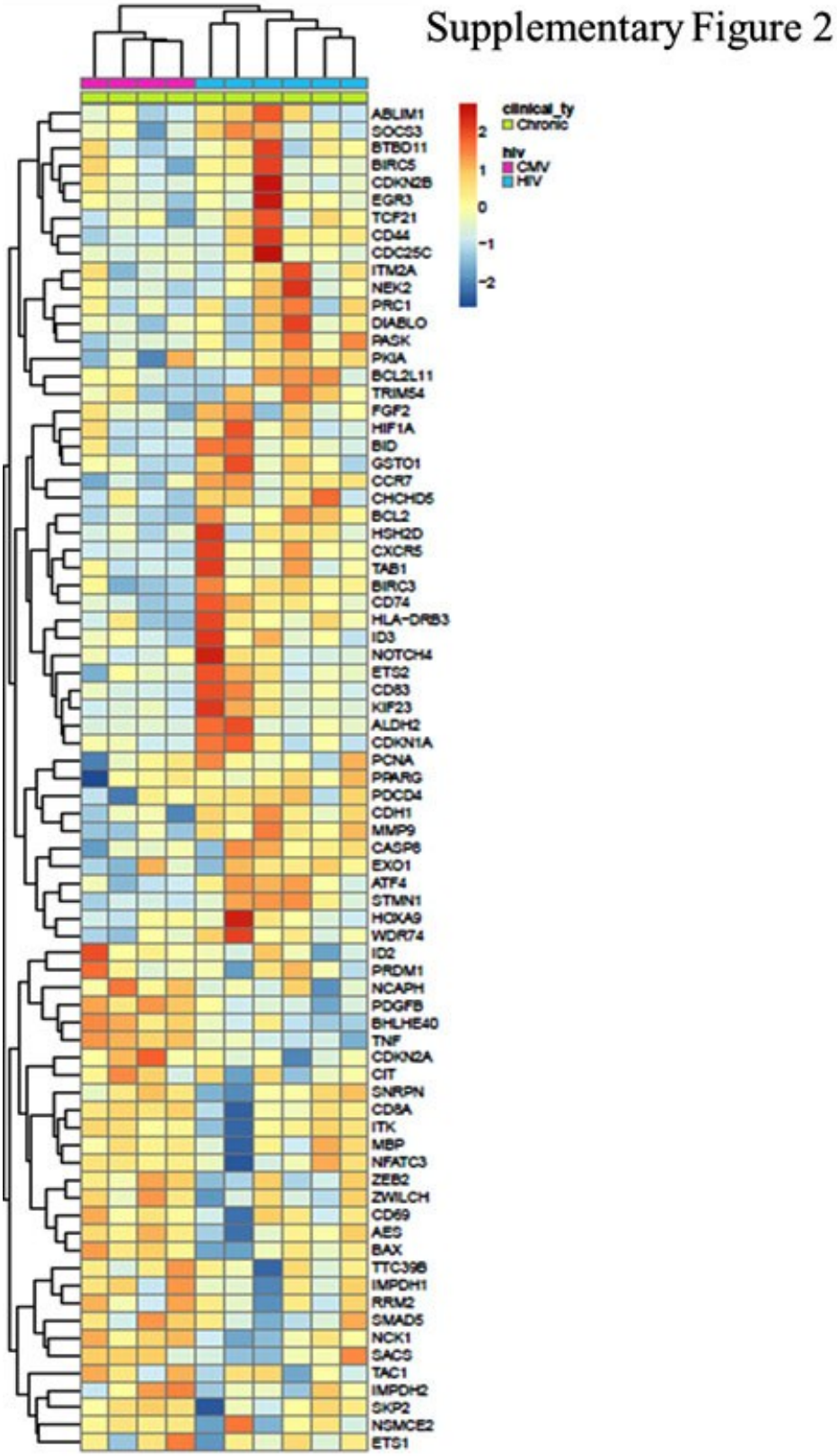

A
CD8+ CD45RA-
EM
CCR7
CD27
Tat-ID2
Tat
% of Max
Annexin V
Tat
ID2-tat
B
P= 0.014
Annexin V
TAT+
ID2+

Figure 5


C
GRAZB
40
30
20
10
0
TAT+
ID2+
D
Tem
Ttm
P21
2.5
2
1.5
1
0.5
0
TAT+
ID2+
E
CD27
2
1.5
1
0.5
0
TAT+
ID2+
F
Bcl-2
2.5
2
1.5
1
0.5
0
TAT+
ID2+
G
BIM
4
3
2
1
0
TAT+
ID2+
H
Myc
2
1.5
1
0.5
0
TAT+
ID2+

Supplementary Figure 1




Supplementary Figure 2
clinical_ty
Chronic
hiv
CMV
HIV
2
1
0
−1
−2
ABLIM1
SOCS3
BTBD11
BIRC5
CDKN2B
EGR3
TCF21
CD44
CDC25C
ITM2A
NEK2
PRC1
DIABLO
PASK
PKIA
BCL2L11
TRIM54
FGF2
HIF1A
BID
GSTO1
CCR7
CHCHD5
BCL2
HSH2D
CXCR5
TAB1
BIRC3
CD74
HLA-DRB3
ID3
NOTCH4
ETS2
CD83
KIF23
ALDH2
CDKN1A
PCNA
PPARG
PDCD4
CDH1
MMP9
CASP8
EXO1
ATF4
STMN1
HOXA9
WDR74
ID2
PRDM1
NCAPH
PDGFB
BHLHE40
TNF
CDKN2A
CIT
SNRPN
CD8A
ITK
MBP
NFATC3
ZEB2
ZWILCH
CD69
AES
BAX
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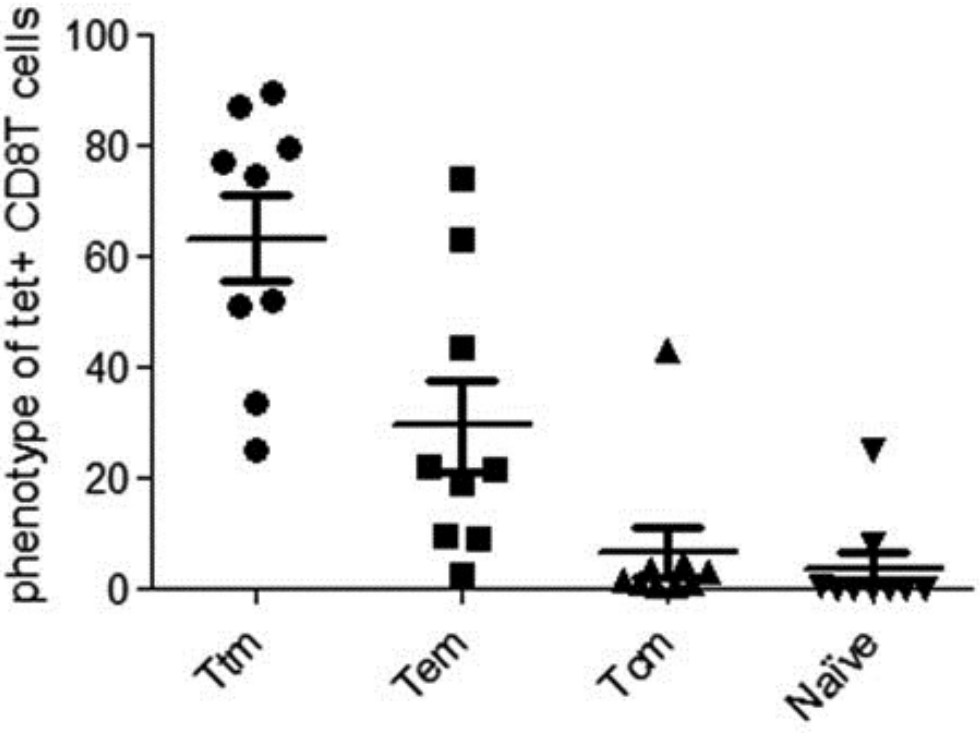
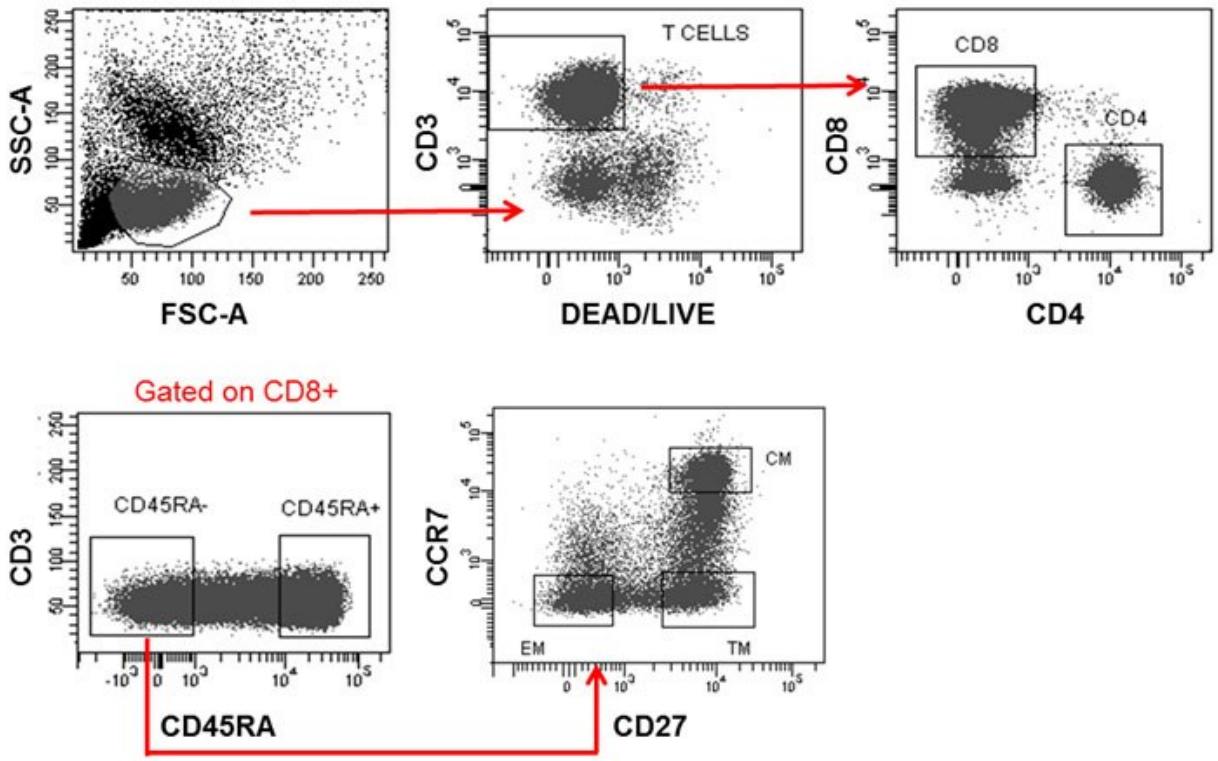
SKP2
NSMCE2
ETS1

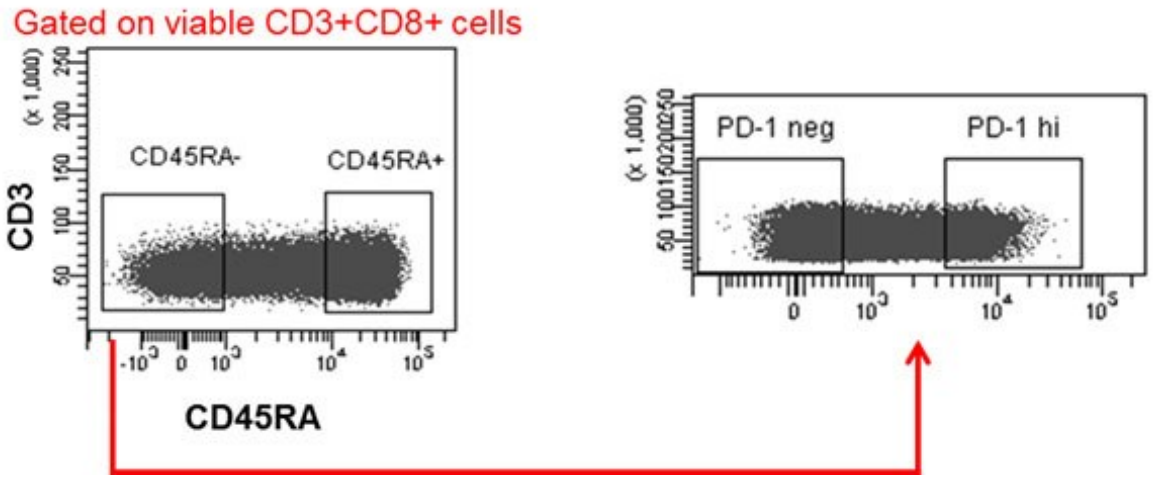
Supplementary Figure 3



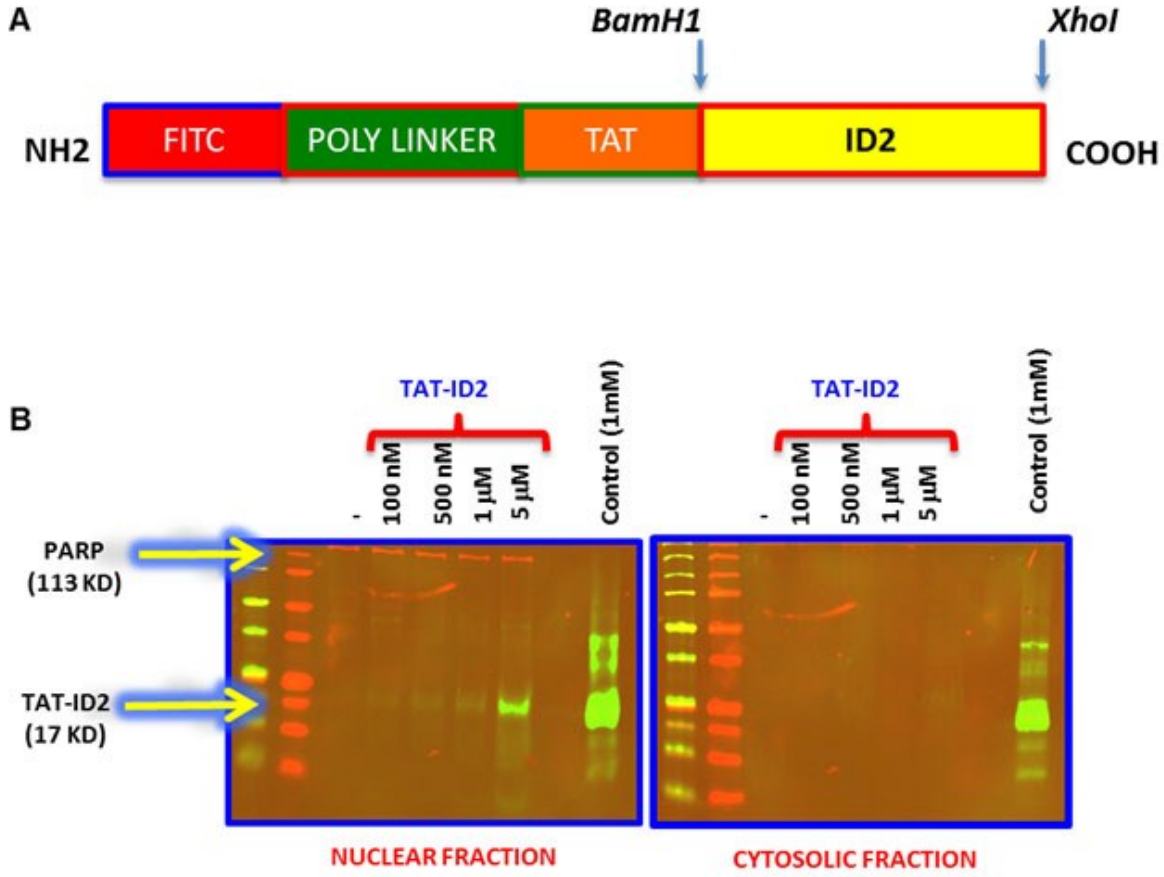
Supplementary Figure 4



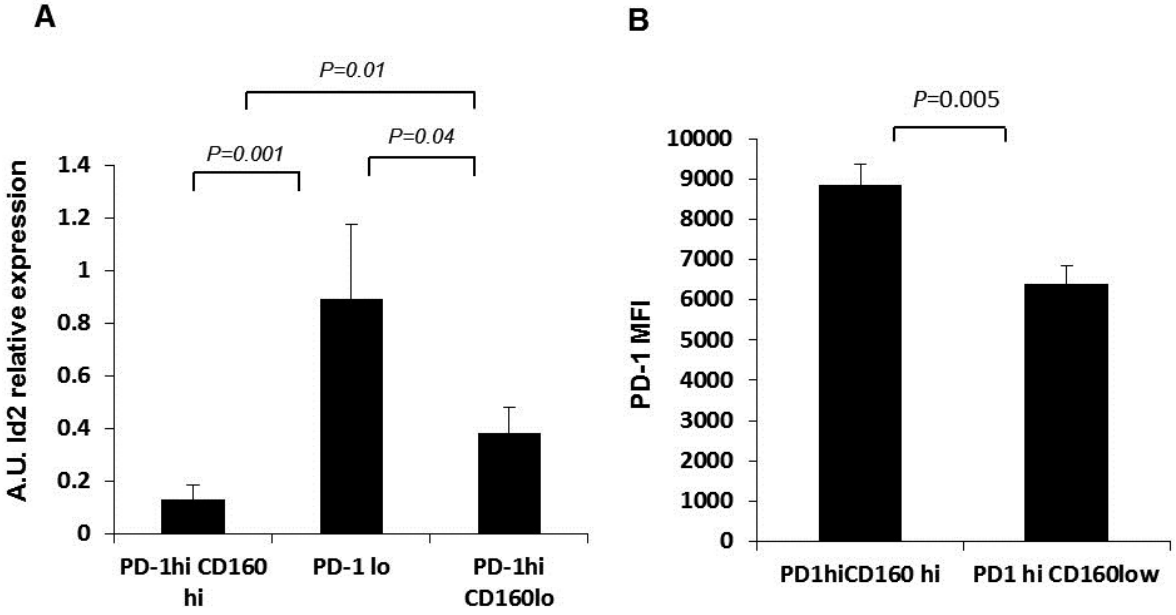
Supplementary Figure 5



Supplementary figure 6



Supplementary Figure 7:



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CHAPTER 3

CELL-BASED FLOW CYTOMETRY ASSAY TO MEASURE CYTOTOXIC ACTIVITY

Cell-Based Flow Cytometry Assay To Measure Cytotoxic Activity

Authors: Alessandra Noto[1], Pearline Ngauv[1], Lydie Trautmann[1]

[1] Vaccine and Gene Therapy Institute Florida, Port Saint Lucie, Florida

KEYWORDS: Cytotoxicity, Effector CD8+ T cells, Tetramers, Target CD4+ T cells, CFSE, Flow cytometry

SHORT ABSTRACT

This protocol describes a sensitive, cell-based cytotoxicity assay. By enumerating the decrease in frequency of live target CD4+ T cells in the presence of an increasing number of effector CD8+ T cells, this assay allows for the direct assessment of cytolytic activity of antigen-specific CD8+ T cells.

LONG ABSTRACT

Cytolytic activity of CD8+ T cells is rarely evaluated. We describe here a new cell-based assay to measure the capacity of antigen-specific CD8+ T cells to kill CD4+ T cells loaded with their cognate peptide. Target CD4+ T cells are divided into two populations, labeled with two different concentrations of CFSE. One population is pulsed with the peptide of interest while the other remains un-pulsed. Pulsed and un-pulsed CD4+ T cells are mixed at an equal ratio and incubated with an increasing number of purified CD8+ T cells. The specific killing of autologous target CD4+ T cells is analyzed by flow cytometry after co-culture with CD8+ T cells containing the antigen-specific effector CD8+ T cells detected by peptide/MHCI tetramer staining. The specific lysis of target CD4+ T cells measured at different effector versus target ratios, allows for the calculation of lytic units, $LU_{30}/10^6$ cells. This simple and straightforward assay allows for the accurate measurement of the intrinsic capacity of CD8+ T cells to kill target CD4+ T cells.

INTRODUCTION

Cytolytic activity is the major function of CD8+ T cells but is still rarely measured as assays used for this purpose are cumbersome and have been difficult to standardize. Accurate measurement of this function is of paramount importance when characterizing effector functions of CD8+ T cells, as no reliable predictors of effective cell-mediated cytotoxicity have been described yet (Appay, Van Lier et al. 2008; Betts and Harari 2008). Here, we propose a new functional assay to measure the cytotoxic activity of antigen-specific CD8+ T cells on target CD4+ T cells. Several approaches have been developed to provide alternatives to the gold standard chromium release assay (Roder, Haliotis et al. 1980). We present here a cell-based assay that reveals the entire process of killing as it measures the death of live target cells. This method was derived from protocols of flow cytometry-based cytotoxic assays *in vivo* in mice (Barber, Wherry et al. 2003; Byers, Kemball et al. 2003) and *in vitro* in humans (Devevre, Romero et al. 2006). In this protocol, the antigen-specific CD8+ T cells contained in the total CD8+ T cell population are used as effector cells and autologous CD4+ T cells are used as target cells. Effector CD8+ T cells of interest are enumerated using MHCI/peptide tetramers (Altman, Moss et al. 1996). Death of target cells is calculated by the ratio between peptide loaded/non-loaded CD4+ T cells. We have previously shown that this method was reproducible, sensitive, specific and did not depend on the number of effector cells within the total CD8+ T cell population (Mbitikon-Kobo, Bonneville et al. 2012). By enumerating both the number of effector and target cells in the co-culture assay, the intrinsic capacity of CD8+ T cells to kill target cells can be calculated and expressed in lytic units (Bryant, Day et al. 1992).

PROCEDURE

1. Preparation of effector CD8+ T cells

1.1. Thaw autologous cryopreserved PBMCs (2-3 vials of $50X10^6$ cells) by transferring the cryovial from liquid nitrogen to a 37°C water bath. If frequency of tetramer positive cells is high (1% or more within total CD8+ T cells), go directly to step 1.7. If frequency is low (less than 1% of total CD8+ T cells), expand cells for 6 days (continue to step 1.2).

1.2. Wash the cells by filling up the tube to 50 ml with complete RPMI (4 mM L-glutamine, and 100 U/ml penicillin and streptomycin, supplemented with 10% FBS). Count PBMCs and resuspend cells at a concentration of $5X10^6$/ml in complete RPMI.

1.3. Add specific peptide (5 µg/ml) and IL-2 (10 ng/ml) to PBMCs.

1.4. Set-up culture in 96 deep well plate; seed 1 ml to each well.

1.5. After 3 days of culture, replace half of the cell culture medium with fresh complete RPMI.

1.6. After 6 days of culture, collect all PBMCs with multichannel pipette and transfer cells in sterile reservoir.

1.7. Count, wash, and resuspend PBMCs at $5X10^7$/ml in the recommended separation buffer in 14 ml round bottom tubes. Add human CD8+ T cell enrichment cocktail at 50 µl/ml cells. Mix and incubate at room temperature for 10 minutes. Add magnetic particles at 150 µl/ml and incubate for 5 minutes.

1.8. Bring the cell suspension up to 7 ml by adding the separation buffer. Proceed to immunomagnetic isolation of untouched CD8+ T cell by placing the tube into the magnet. After 5 minutes, with the tube still in the magnet, pour the cells of interest into a new 15 ml conical tube. The expected purity should be higher than 95%.

1.9. Resuspend CD8+ T cells in 450 µl in complete RPMI.

1.10. Add 225 µl of complete RPMI into 5 screw cap tubes.

1.11. Prepare serial dilutions (from 1:2 to 1:32) by transferring 225 µl of CD8+ to the next tube (*see figure 1 upper panel*).

2. Preparation of target CD4+ T cells

2.1. Thaw autologous cryopreserved PBMCs (1 vial of $50X10^6$ cells) by transferring the cryovial from liquid nitrogen to a 37°C water bath.

2.2. Transfer the cell suspension to a 50 ml conical tube containing 10 ml of RPMI. Wash cells and resuspend PBMCs at $5X10^7$/ml with separation buffer in 14 ml round bottom tubes. Add human CD4+ T cell enrichment cocktail at 50 µl/ml cells. Mix and incubate at room temperature for 10 minutes. Add magnetic particles at 100 µl/ml and incubate for 5 minutes.

2.3. Bring the cell suspension up to 7 ml by adding separation buffer. Proceed to the isolation of CD4+ T cells by placing the tube into the magnet. After 5 minutes, with the tube still within the magnet, pour the desired cells into a new 15 ml conical tube. The expected purity should be higher than 95%.

2.4. Count CD4+ T cells, split them into two 15 ml conical tubes and wash in warm 1X PBS.

2.5. Prepare 0.4 µM CFSE-high working solution in warm PBS.

2.6. Prepare 0.04 µM CFSE-low working solution by diluting 10X CFSE high working solution in warm PBS.

2.7. Resuspend cells at $20X10^6$/ml of PBS (if the number of CD4+ T cells is less than $10X10^6$/ml resuspend cells in 0.5 ml of PBS) and stain half of the CD4+ T cells by adding one equal volume of 0.4 µM CFSE-high working solution (final concentration 0.2 µM). For example, for 1 ml of cells add 1 ml of the CFSE working solution. Repeat for the other half of the CD4+ by adding one equal volume of 0.04 µM CFSE-low working solution (final concentration 0.02 µM). Stain cells for 15 minutes at 37°C in 5% CO_2 atmosphere.

2.8. After the incubation, pellet cells at 1500rpm for 5 minutes and resuspend in 1 ml of warm complete RPMI to quench the labeling reaction.

2.9. Pulse CFSE-low CD4+ T cells by adding the peptide of interest at a final concentration of 5 µg/ml in the complete RPMI and incubate for 45 minutes in 5% CO_2 atmosphere at 37°C.

2.10. Wash CFSE-high and CFSE-low CD4+ T cells in complete RPMI. Repeat for a total of two washes. Resuspend at a concentration of $2X10^5$ cells/ml each in complete RPMI.

2.11. Mix the two target populations at a ratio of 1:1 (CFSE-High:CFSE-Low) (*see figure 1 lower panel*).

3. Co-culture

3.1. From each dilution of CD8+ T cells, seed 100 µl in duplicate to round-bottom 96-well plate.

3.2. Add the mixed target CD4+ T cells (100 µl) to each dilution of effector cells for a final volume of 200 µl.

3.3. To measure the basal apoptosis, seed 3 wells with target cells alone.

3.4. Incubate co-culture for 6 hours in 5% CO_2 atmosphere at 37^oC.

4. Flow cytometry staining and acquisition

4.1. Transfer cells to a V-bottom 96 well plate and stain with PE-conjugated pMHC tetramers for 15 minutes in 5% CO_2 atmosphere at 37^oC.

4.2. Wash in 1X PBS-2% FBS and stain cells with Far Red LIVE/DEAD fixable dead cell , αCD3-Alexa700, αCD8-PeCp, αCD4-Brilliant Violet 650 for 30 minutes at 4°C, protected from light.

4.3. Wash cells once with 1X PBS-2% FBS and resuspend in 100 µl with 2% formaldehyde in 1X PBS.

4.4. Acquire cells on flow cytometer.

REPRESENTATIVE RESULTS

The schematic in Figure 1 summarizes the assay. Purified CD8+ T cells (effector cells) were resuspended in 450 µl of complete RPMI and serial dilutions were performed by adding 225 µl of CD8+ T cells to the next tube containing 225 µl of media (upper panel). Purified CD4+ T cells (target cells) were counted, split into two tubes and stained with two different concentrations of CFSE (high and low) as described in the protocol. CFSE-low CD4+ T cells were pulsed with the peptide of interest and CFSE-high CD4+ T cells remained unloaded as a control. CFSE-high and CSFE-low CD4+ T cells were resuspended at $2X10^5$/ml and mixed at a ratio of 1:1 (lower panel). The mixed CD4+ T cells were seeded at 100 µl in each well. Three wells containing the mixed CD4+ T cells were used for triplicate of CD4+ T cells alone (wells in red). 100 µl of each CD8+ T cell dilution was added in duplicate to the remaining wells containing the mixed CD4+ T cells (wells in green). The killing of target cells was assessed by flow cytometry after 6 hours of co-culture.

After incubation, cells were analyzed and gated as in Figure 2. The ratio of viable CFSE-high versus CFSE-low target cells, un-pulsed or peptide pulsed was analyzed from a small FSC-A/SSC-A gate. Live cells were gated on LIVE/DEAD negative. CD4+ T cells were plotted on CFSE in order to analyze the ratio CFSE-low versus CFSE-high cells. From a larger FSC-A/SSC-A gate to measure the total number of effector and target cells, either dead or alive, tetramer+ CD8+ T cells and CFSE-low CD4+ T cells were plotted in order to analyze the ratio of effector versus target cells. The total number of effector CD8+ T cells was determined by gating on CD8+ and tetramer+ cells. The total number of target cells was determined by gating on CD4+ T cells CFSE-low and LIVE/DEAD positive and negative cells.

The results presented in Figure 3 show that the ratio of CFSE-low versus CFSE-high cells (target ratio) was 1:1 when CD4+ target cells were cultured in absence of effector cells. However, the ratio of CFSE-low versus CFSE-high cells was drastically decreased when CFSE-low cells pulsed with peptide were incubated with the total CD8+ T cell population

containing antigen-specific CD8+ effector T cells. As the ratio decreased, the CFSE-low CD4+ pulsed target cells became LIVE/DEAD positive. The dilution of the effector cells (right panel) resulted in restoration of the CFSE ratio and decreased the number of LIVE/DEAD positive peptide-pulsed CFSE-low CD4+ target T cells.

For each well, the ratio between pulsed and un-pulsed live CD4+ target T cells (CFSE-low/CFSE-high) in the presence of different dilutions of effector CD8+ T cells was measured by calculating the ratio between antigen-pulsed CFSE-low CD4+ T cells and un-pulsed CFSE-high CD4+ T cells after 6 hours of culture with effector CD8+ T cells. The percentage of specific lysis for each well was calculated as follows: % specific lysis = 100 – ((CFSE low/CFSE high) in the presence of effector cells / median between the three wells containing target cells alone in the absence of effector cells) x100. To determine the intrinsic cytolytic capacity of antigen-specific CD8+ T cells and compare results between donors, the cytolytic activity was expressed in lytic units as previously reported (Mbitikon-Kobo, Bonneville et al. 2012). The effector versus target ratio (E/T) was measured by calculating the ratio between total tetramer+ CD8+ T cells and total CFSE-low CD4+ T cells for each well. The percentage of specific lysis was plotted in function of the E/T ratio in a log scale as illustrated in Figure 4. A linear regression was calculated from the plot and the equation of the trendline was used to calculate the lytic units $LU_{30}/10^6$ cells (i.e.: the number of CD8+ effectors T cells required to kill 30% of 10^6 target CD4+ T cells).

DISCUSSION

The assay described here allows for the quantification of the principal function of CD8+ T cells: the cytolytic activity. The accurate measurement of this function is of paramount importance when characterizing effector functions of CD8+ T cells, as previous studies reported discordance between this function and the cytokine secretion of antigen-specific CD8+ T cells (Varadarajan, Julg et al. 2011; Trautmann, Mbitikon-Kobo et al. 2012). Due to the short co-culture incubation time (6 hours) and the use of autologous target cells, there is low rate of non-specific killing of target cells. This assay is therefore highly specific and reliable. This assay can also be easily modified to analyze different cell subsets. Effector CD8+ T cells can be replaced by other cell types, such as CD4+ T cells as it has been recently described in the *in vivo* killing assay (Hildemann, Eberlein et al. 2013). Different target cells can also be used to replace CD4+ T cells, as we previously described the use of autologous B cells as target cells (Mbitikon-Kobo, Bonneville et al. 2012). This protocol provides an accurate method to quantify the cytolytic activity of antigen-specific CD8+ T cells. As this method is versatile and easy to perform, measuring the killing capacity of effector cells might be performed more often in the quantification of CD8+ T cell functions.

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Contributions

A.N. performed all the experiments, analyzed-interpreted data and prepared graphics. P.N. participated in performing experiments. L.T. supervised the project.

FIGURE LEGEND

Figure 1. Schematic representation of the cytotoxic assay co-culture. Purified CD8+ T cells were resuspended in 450 µl of complete RPMI and 5 serial dilutions were performed. Purified CD4+ T cells were counted, split into two tubes and stained with two concentrations of CFSE (0.02 µM CFSE-low and 0.2 µM CFSE-high). Only CFSE low CD4+ T cells were pulsed with 5 µg of specific peptide. After 45 minutes, both CFSE low and high CD4+ T cells were washed twice, resuspended at $2X10^5$/ml and mixed at a ratio of 1:1. 100 µl of mixed CD4+ were seeded in a 96 well plate in the presence or absence of 100 µl of each CD8+ T cell dilution for 6 hours.

Figure 2. Gating strategy of the cytotoxic assay. From a smaller FSC-A/SSC-A gate, the ratio of CFSE-low versus CFSE-high cells was analyzed within the CD4+ LIVE/DEAD negative cells. From a larger FSC-A/SSC-A gate, effector cells were analyzed by gating on CD8+ T cells and tetramer+ cells. The number of target cells was analyzed by gating on CD4+ T cells, CFSE-low and LIVE/DEAD positive and negative cells.

Figure 3. Representative results of FACS staining. CFSE-low/CFSE-high ratio (left panels), target cells (middle panels) and effector cells (right panels) in the different co-culture conditions.

Figure 4. Linear regression of specific lysis in function of the effector/target ratio. The equation of the trendline is used to calculate the lytic units $LU_{30}/10^6$ cells.

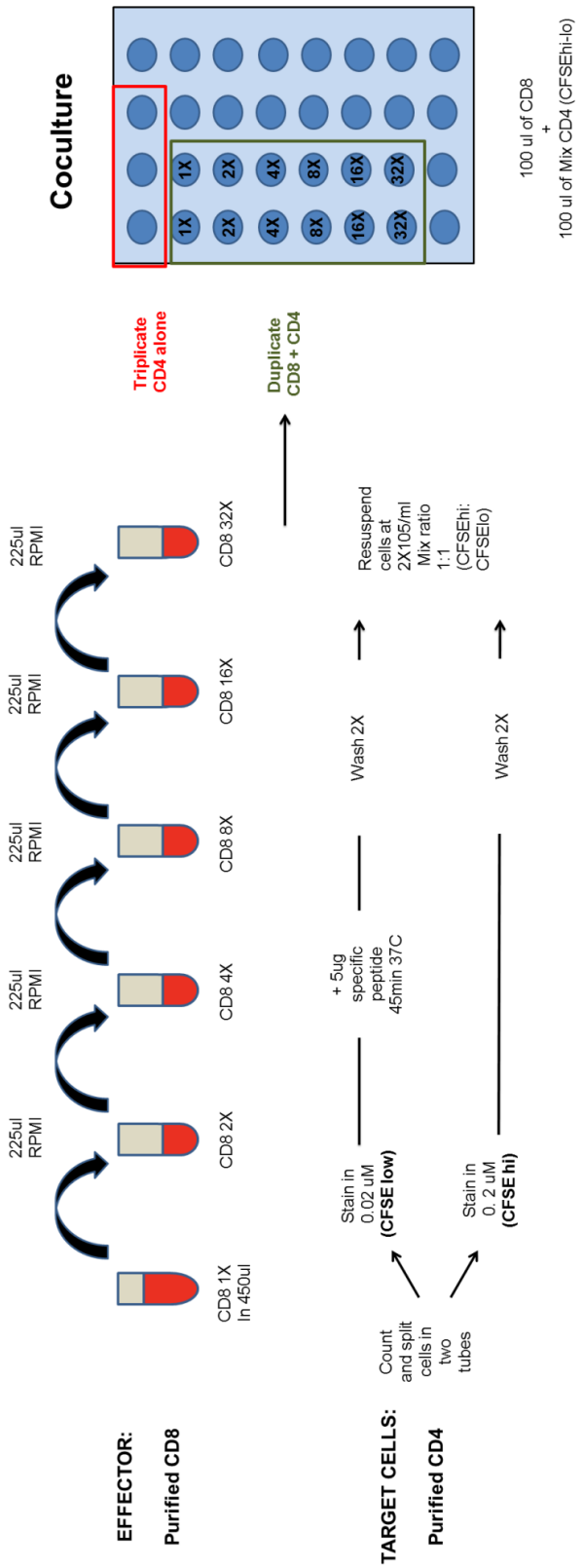

Coculture
1X
2X
4X
8X
16X
32X
100 ul of CD8
+
100 ul of Mix CD4 (CFSEhi-lo)
Triplicate
CD4 alone
Duplicate
CD8 + CD4
EFFECTOR:
Purified CD8
CD8 1X
In 450ul
225ul
RPMI
CD8 2X
CD8 4X
CD8 8X
CD8 16X
CD8 32X
TARGET CELLS:
Purified CD4
Count
and split
cells in
two
tubes
Stain in
0.02 uM
(CFSE low)
Stain in
0. 2 uM
(CFSE hi)
+ 5ug
specific
peptide
45min 37C
Wash 2X
Wash 2X
Resuspend
cells at
2X105/ml
Mix ratio
1:1
(CFSEhi:
CFSElo)

Figure 1

Figure 2

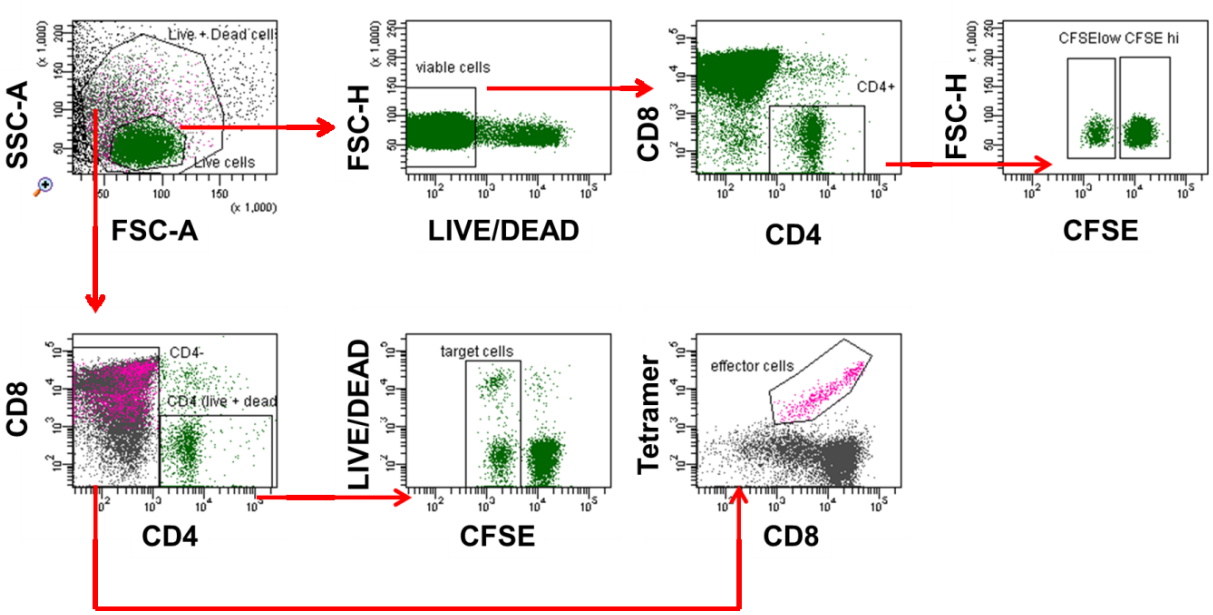

SSC-A
Live + Dead cells
Live cells
FSC-A
FSC-H
viable cells
LIVE/DEAD
CD8
CD4+
CD4
FSC-H
CFSElow CFSE hi
CFSE
CD8
CD4-
CD4 (live + dead
CD4
LIVE/DEAD
target cells
CFSE
Tetramer
effector cells
CD8

Figure 3

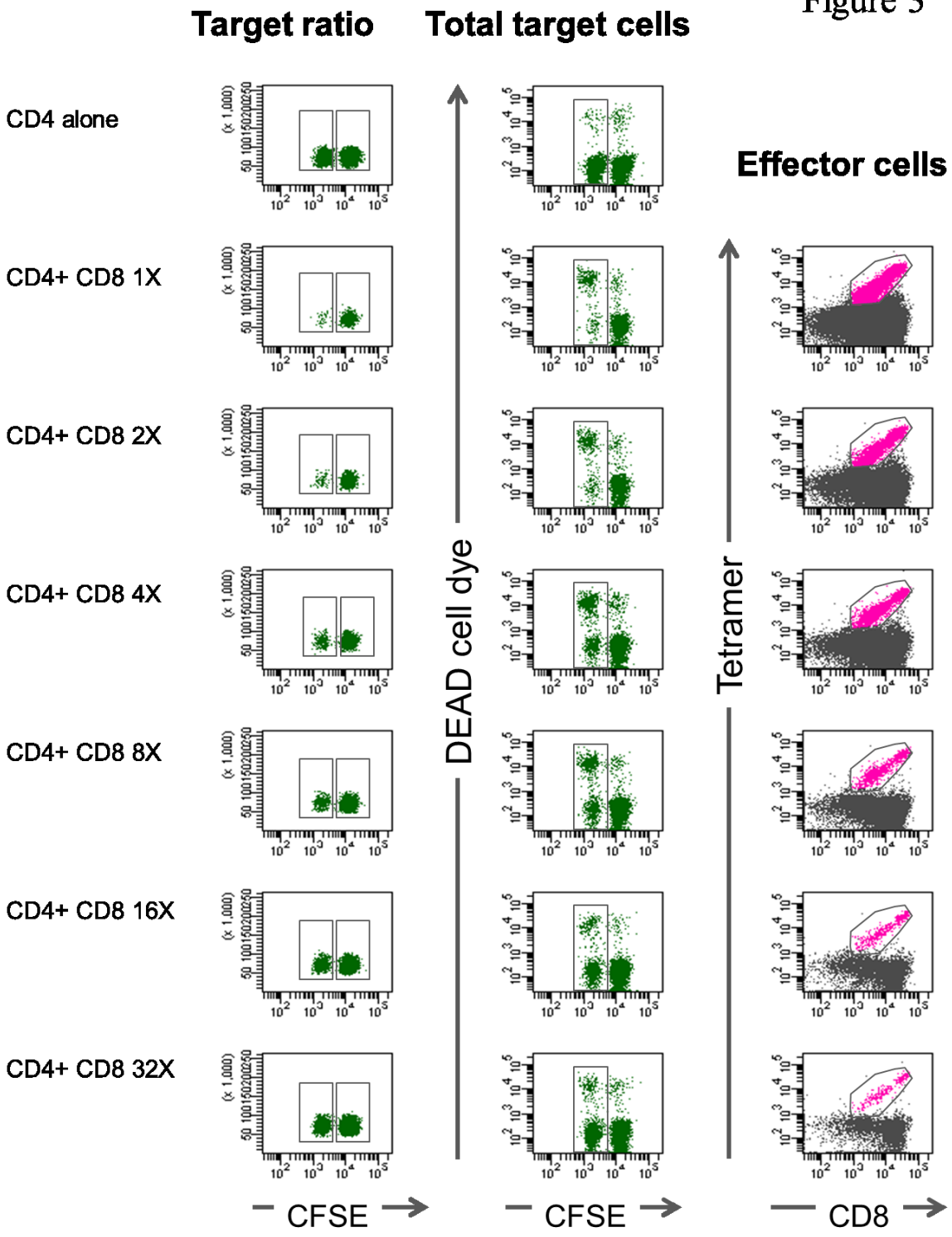
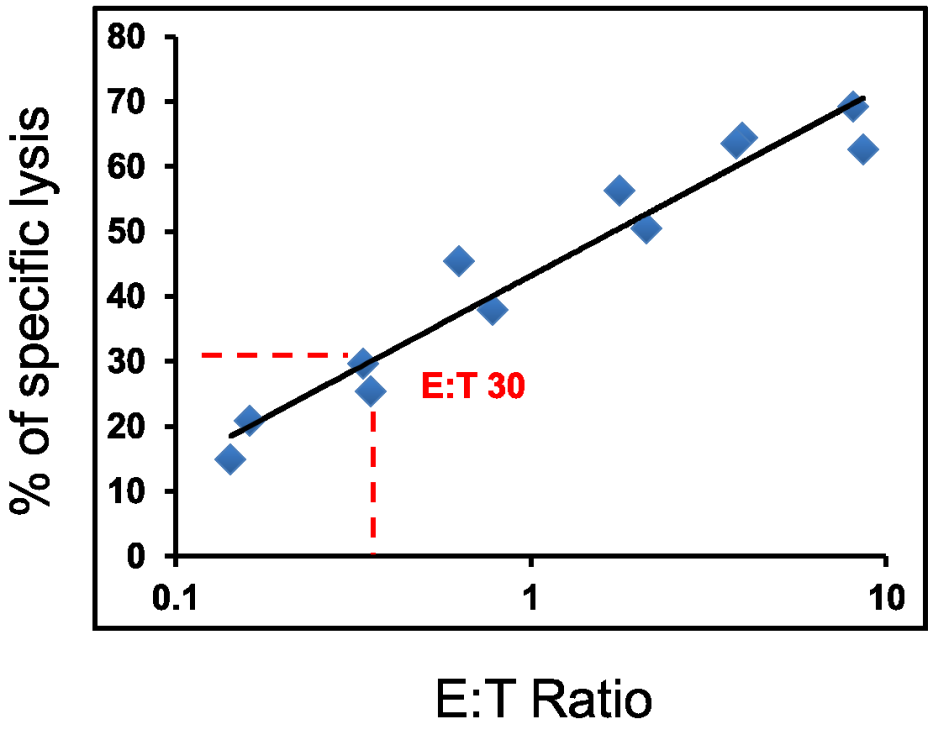

Target ratio
Total target cells
Effector cells
CD4 alone
CD4+ CD8 1X
CD4+ CD8 2X
CD4+ CD8 4X
CD4+ CD8 8X
CD4+ CD8 16X
CD4+ CD8 32X
DEAD cell dye
Tetramer
CFSE
CFSE
CD8

Figure 4



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CHAPTER 4

IL-15 RESCUES TCR SIGNALING IN ANERGIC HIV-SPECIFIC CD8+ T CELLS AND DRIVES DIFFERENTIATION AND EFFECTOR FUNCTION BY UPREGULATING ID2

IL-15 rescues TCR signaling in anergic HIV-specific CD8+ T cells and drives differentiation and effector function by upregulating ID2

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KEYWORDS: Homeostasis, differentiation, Id2, Effector CD8+ T cells, IL-15, cytotoxicity

ABSTRACT

During chronic HIV infection HIV-specific CD8+ T cells develop functional defects that are not restored under HAART. CTL are blocked in a transitional memory phenotype, fail to produce effector cytokines, have decreased proliferative capabilities and are not able to exert cytotoxic function. The transcriptional profile of total memory CD8+ T cells showed downregulation of regulators of differentiation Id2 and Id3 in chronic HIV infected subjects (Chapter 2). Among common γ-chain cytokines, we found that IL-15 was most effective in the upregulation of Id2 expression, proliferation, and survival of human effector memory CD8+ T cells. Human CD8+ memory cell subsets response to exogenous IL-15 did not require trans-presentation and inhibition of Id2 by siRNA abrogated the ability of IL-15 to support proliferation and survival. We found that IL-15 induced proliferation of all memory subsets from healthy subjects but only induced differentiation, GzmB production, and cytotoxic effector function in CD8+ Ttm and Tem cells. Using a single cell flow cytometry cytotoxicity assay of primary antigen-specific CD8+ T cells, we found that stimulation of HIV-specific CD8+ cells with peptide plus IL-15 induced Id2 upregulation, differentiation of tetramer+ Ttm cells and restored their cytotoxic activity. Results from this study provide a strong rationale for the use of IL-15 in therapeutic vaccines and "Cure" interventions in patients receiving HAART to enhance CD8+ CTL killing of HIV-infected cells and prevent replenishment of latent reservoirs by reactivated virus.

INTRODUCTION

During infection, naïve CD8+ T cells are primed by antigen to differentiate into cytotoxic, cytokine secreting effector T cells (Williams and Bevan 2007). In lymphoid tissues, antigen-specific T cells undergo clonal expansion and upregulate molecules important for eliminating the pathogen, including both cytokines and effector molecules that mediate cytotoxicity. Once a foreign antigen has been cleared a small subset of antigen specific effector cells survive the contraction phase and differentiate into memory cells that persist for a prolonged period. After exposure to the same pathogen, memory T cells have the capacity to rapidly respond with enhanced proliferation, cytotoxicity and cytokine production, thereby providing life-long protection.

Memory cells can be found in different stages of differentiation which can be distinguished by the expression of several surface markers (CCR7+, CD27+, CD28+). Among memory CD8+ T cells subsets central memory T cells (Tcm) have the greater memory cell potential, longevity and proliferative potential when compared to effector memory T cells (Tem) and terminal effector (Temra) cells which lose these properties and instead exert immediate effector functions. CD8+ effector cell development is regulated at the transcriptional level: the relative expression of T-bet, Blimp-1, inhibitor of DNA binding 2 (Id2) and STAT-4 during infection determines the population of CD8+ T cells that acquire a KLRG-1^{1hi} IL-$7R^{lo}$ terminally differentiated effector phenotype (Nguyen, Salazar-Mather et al. 2002; Cannarile, Lind et al. 2006; Intlekofer, Takemoto et al. 2007; Joshi, Cui et al. 2007; Banerjee, Gordon et al. 2010; Yang, Best et al. 2011), (Knell, Best et al. 2013). In contrast, expression of genes associated with self-renewal: TCF7, Eomesodermin (Eomes), BCL-6, Id3 and STAT3, prevent the terminal differentiation of effector T cells and maintain the memory cell phenotype (Zhou, Yu et al. 2010), (Ichii, Sakamoto et al. 2002; Banerjee, Gordon et al. 2010; Cui, Liu et al. 2011; Siegel, Heimall et al. 2011; Yang, Best et al. 2011).

Id2 belongs to the family of the helix-loop-helix (HLH) proteins that plays a key role in the differentiation of a number of cell lineages. It has been shown that Id proteins regulate T cell differentiation of mature CD8+ T cells (Cannarile, Lind et al. 2006; Yang, Best et al.

2011; Masson, Minnich et al. 2013). Knockout studies in mouse models demonstrated that, in the absence of Id2, CD8+ effector cells were more susceptible to apoptosis when compared to wild type cells, and these cells consistently showed decreased levels of the pro-survival *Bcl-2* and increased levels of the pro-apoptotic *Bim* mRNA (Cannarile, Lind et al. 2006). This study was confirmed by others where they show that Id2 is required for the formation of short-lived effector memory CD8+ T cells after infection, while Id3 expression is required for the generation of long lived memory cells (Yang, Best et al. 2011). Id2 is a TCF-1/LEF target gene and inhibits the interaction of pRb and E2F for cell cycle progression induced by Myc (Zebedee and Hara 2001; D'Cruz, Lind et al. 2012). Id2 was also recently shown to inhibit E2A activation of TCF-1 and Eomes expression that instead is required for CD8+ Tcm memory cell differentiation and longevity (Masson, Minnich et al. 2013). Thus, the Id2/E2F axis plays a major role in regulating cellular proliferation, and the check-point for CD8+ Tem and Tcm cell differentiation.

Homeostatic cytokines IL-7 and IL-15 that converge on STAT5A/B signaling pathways have essential roles in CD8+ T cell differentiation and long term survival and memory (Oh, Perera et al. 2004; Sato, Patel et al. 2007; Sandau, Kohlmeier et al. 2010; Tripathi, Kurtulus et al. 2010; Yu, Tawab-Amiri et al. 2011; Van Belle, Dooms et al. 2012). IL-15 down-regulates proapoptotic Bax, an intermediate in TRAIL-mediated apoptosis, and increases anti-apoptotic Bcl-2 and Bcl-XL in CD8+ T cells (Becker, Wherry et al. 2002; Burkett, Koka et al. 2004; Surh and Sprent 2008). Although both IL-2 and IL-15 induce identical signal-transduction pathways and proliferative responses in T cells and NK cells, IL-2 favors maintenance of peripheral regulatory T cells and participates in activation-induced cell death while IL-15 preferentially stimulates expansion of CD8+ and NK, and NKT cells (Ring, Lin et al. 2012).

In this study we have identified a key role for IL-15 in upregulating Id2 expression and in driving T cell proliferation and differentiation of CD8+ Tem and Temra while maintaining the homeostasis of Tcm. Furthermore, we show that IL-15 induces the differentiation of tetramer+ CD8+ Ttm cells and can enhance the killing efficiency of HIV-specific CD8+ CTL *ex vivo*.

RESULTS

1. Upregulation of ID2 and BCL-2 in resting memory CD8+ T cells by TCR triggering is necessary for proliferation

Id2 expression increases upon antigen encounter in murine model and its expression is maintained into the memory pool (Cannarile, Lind et al. 2006). Consistent with results from murine models, resting human memory CD8+ T cells *ex vivo* expressed weak to undetectable protein levels of ID2 (Suppl. Fig. 1A). Interestingly we found increased levels of Id2 in Temra, Tem and Ttm subsets *ex vivo* (Fig.1A) which are the cells with a more activated phenotype. Activation of purified resting memory CD8+ T cells with anti-CD3/CD28 led to upregulation of ID2 in 94% of stimulated cells by day 2 ($p=0.0006$) and peak protein levels at day 3 after activation (Suppl. Fig. 1B, C, D). The induction of ID2 coincided with the upregulation of the anti-apoptotic protein BCL-2 (Suppl. Fig. 1C) and was confirmed in proliferating cells as we found ID2 to be significantly up-regulated within anti-CD3-CD28 stimulated proliferating (CFSE lo) memory CD8+ T cells (100% ± 0 $P<0.0001$) (Fig.1 B,C). BCL-2 was also found to be upregulated in these proliferating CD8+ T memory T cells (100% ± 0, $P<0.0001$) while levels of proapoptotic BIM molecule were inversely related to BCL-2 (Fig. 1B,C) . Conversely we also found increasing levels of apoptosis in non-proliferating cells expressing BIM by day 3 of culture compared to the proliferating population that expressed high levels of BCL-2 (Suppl. Fig. 2 A,B).

To confirm that ID2 upregulation was essential for proliferative expansion and cell survival following TCR activation, we used a small interfering RNA (siRNA) specific for Id2 to knock down its expression. Id2 siRNA led to a significant decrease of ID2 protein and of its target BCL-2 (70-80% for each protein, ID2, $p=0.0028$, and BCL-2, $p=0.0012$)(Fig. 1D) when compared to a degenerate control siRNA . Levels of total ERK were not changed upon transfection with either siRNA. Id2-specific knock down by siRNA led to a 1.5 fold increase in spontaneous apoptosis at 3 days post transfection when compared to cells transfected with the degenerate siRNA control (Fig. 1F)($P<0.01$). Significant differences in proliferation were also observed in the absence of Id2 with less than 10% of cells were CFSE low at day 3 after stimulation (Fig 1E, $n=5$)($P<0.001$).

Moreover, Id2 knockout led to a decrease in the frequency of cells with an effector memory phenotype (Fig 1G, n=5)($P<0.01$). To our knowledge, this is the first demonstration that Id2 is an immediate early response gene following TCR activation that directly links the upregulation of Bcl-2, and the downregulation of Bim to the survival and differentiation of the proliferating memory cell population.

2. IL-15 synergizes with anti-CD3/CD28 to upregulate ID2 expression and drive effector cell differentiation of activated CD8+ memory cells

Homeostatic survival and differentiation of Ag-specific CD8+ T cell memory generation is temporally regulated by cytokines. IL-2, IL- 15 and IL-7 signal through high affinity receptor complexes that share the common gamma-chain to activate JAK3 / STAT5A/B and PI3K/AKT/mTOR pathways necessary for long term survival and maintenance (Surh and Sprent 2008). We determined the impact of these cytokines on the levels of ID2; IL-15 upregulated levels of ID2 significantly (Fig 2B) ($P<0.001$). IL-15 also synergized with TCR to upregulate ID2 levels (Fig 2B) ($P<0.001$). IL-2 was the only other cytokine that upregulated ID2 in synergy with TCR (Fig 2A, and Suppl.Fig 3A). Other γ-c receptor cytokines did not have an impact on ID2 expression. The differences observed in the capacity of γ-c receptor cytokines to increase levels of ID2 were not due to lack of specific cytokine receptor expression (Suppl. Fig 3B). Although IL-15Rα expression was undetectable on memory subsets ex vivo by flow cytometry, we found high levels of IL-15Rα mRNA by RT-PCR in Tem compared to Tcm (~ 2 fold, $p= 0.006$) (Suppl. Figure 3C). Levels of IL-15Rα were also higher in Ttm than Tcm (Suppl. Figure 3C).

3. Transcriptional control in CD8+ Tcm cells maintains homeostasis in response to IL-15

To better understand the mechanisms of how transcriptional programs are modulated in memory CD8+ T cell subsets in response to IL-15 we performed multiplexed PCR on the different memory subsets 12h after IL-15 stimulation (Fig 2C). Confirming our earlier results, Id2, and Bcl-2 expression were upregulated in all memory subsets as well as cMyc

after stimulation, with Id2 showing the highest increase in the Tem and Ttm compartments. IL-15 significantly decreased the levels of mRNA expression of Foxo1, and Foxo3a target genes Gadda45, and Bim in Tem and Ttm while p21 (a cell cycle inhibitor) was most significantly reduced in Tem. Interestingly, IL-15 had little effect on the expression of Foxo1 or Foxo3a target genes expression or the negative regulatory molecule Ctla-4 in Tcm cells compared to Tem and Ttm. We also observed a slight decrease in Tbet and Blimp-1 expression in Tem cells compared to Tcm and Ttm at 12h whereas Eomes was downregulated in all subsets at this time point. These results might reflect the temporal relationship between proliferation and differentiation as Id2 levels were just beginning to rise at 12h and peaked at 2 days after IL-15 stimulation (Supplementary Fig. 1B,D).

4. IL-15 induces proliferation and upregulates CD45RA expression on all memory CD8+ T cell subsets but induces differentiation in only Ttm and Tem subsets.

IL-15 is important for maintaining antigen-specific memory CD8+ T cells and effector memory cells. In the absence of IL-15, memory CD8+ T cells express variable levels of IL-7Rα, high levels of CD27, low levels of Granzyme B and effector cytokines, and fail to upregulate BCL-2 and undergo secondary expansion (Sandau, Kohlmeier et al. 2010). Since we found the expression of Id2 was highest in CD8+ Tem cells *ex vivo* compared to Tcm and correlated with Bcl-2 levels, proliferation, and survival of CD8+ memory T cells following anti-CD3/CD28 plus IL-15, we wanted to determine if different memory CD8+ T cell subsets respond to IL-15 as a homeostatic survival signal or could be induced to differentiate by cytokine signals independent of TCR signal. To address this question we cultured highly purified (>95%) CD8+ Tcm, Tem and Ttm cell subsets in the presence or absence of IL-15, with and without low dose anti-CD3/CD28 stimulation. We measured proliferation by Ki67 intracellular staining, survival by annexin V staining, and the capacity of each memory subset to differentiate in response to IL-15 by measuring the frequency of cells expressing CD45RA, CCR7, and CD27 at day 6 of culture. Not unexpected, we found that IL-15 was necessary to maintain the survival of each CD8+ memory T cell subset in unstimulated culture as shown by the low levels of Annexin V + cells when sorted subsets

were exposed to IL-15 (Fig 3A). Significantly greater proliferation was seen in Tem cells compared to Ttm and Tcm (Tcm, 20% ± 6.2% P=0.02; Ttm 35.48% ± 9.1% P=0.02; and Tem, 48.2%± 8.1% P=0.007) when compared to unstimulated cells that did not express any Ki67+ cell (Fig 3B). IL-15 induced the highest rates of proliferation in Tem and Ttm when compared to Tcm compartment consistent with levels of IL-15Rα mRNA expression in the different subsets confirming the direct activation of human CD8+ memory cell subsets by exogenous IL-15. Addition of IL-15 to low dose anti-CD3/CD28 also led to increased numbers of proliferating cells (Tcm 29.96% ± 9.1% P=0.02; Ttm 39.7%± 9.2% P=0.02; Tem 53.28% ± 6% P= 0.009). There was no significant differences in frequencies of proliferations whether IL-15 was added to the cultures in the presence or the absence of TCR triggering. We also looked at the expression of CD45RA as a marker for terminal differentiation of CD8+ effector cells. In memory CD8+ cell cultures stimulated with IL-15 only, approximately half of the proliferating cell population in each memory cell subset upregulated the expression of CD45RA (Tcm 14.6%± 5% P=0.005; Ttm, 18.3% ± 6% P=0.002; Tem 20% ± 5% P=0.004) (Fig. 3C). In contrast, IL-15 modestly increased the percentage of CD45RA positive cells after anti-CD3/CD28 stimulation yet this was significantly less than that induced by IL-15 alone.

To determine whether IL-15 induced differentiation of Tcm we measured percentages of Ttm or Tem generated in cultures 6 day after stimulation with IL-15 or anti-CD3/CD28 plus IL-15 (Fig. 4). IL-15 alone did not induce significant differentiation of Tcm (Fig. 4A) indicating that Tcm cells induced to proliferate by IL-15 retained their Tcm phenotype. In contrast, we observed an inverse relationship between the percentages of cells that lost the Ttm CCR7- CD27+ phenotype and gained the CCR7- CD27- Tem phenotype (~40%) when stimulated with IL-15 ; low dose anti-CD3/CD28 stimulation plus IL-15 effectively induced more than 50% of Ttm cells to differentiate to Tem cells (Fig. 4B) and this was significantly different than TCR stimulation alone(52% ± 8 vs 11% ± 4, P=0.003). Although IL-15 could induce the differentiation of $CD27^+$ Ttm to $CD27^-$ Tem and into Temra (CD45RA+,CD27-,CCR7-)(~5% of total Tm), 10% of the cells that acquired

CD45RA expression retained the $CCR7^{-}$ $CD27^{+}$ Ttm phenotype (Fig. 4B). Tem required IL-15 to differentiate to terminally differentiated effector cells (25%) (Fig. 4C).

CD8+ Tcm did not produce Granzyme B in response to IL-15 whereas anti-CD3/CD28 plus IL-15 induced expression in 40% of stimulated cells (Fig. 5A). In contrast, IL-15 alone was sufficient to induce expression of Granzyme B in the majority of Tem and Ttm cells (Ttm 73%±10% vs 17% ±12% p=0.04; Tem 81% ±2% vs 41%±10% p=0.04) and stimulation by anti-CD3/CD28 activation plus IL-15 did not significantly increase the response. Thus, IL-15 is essential for the homeostatic proliferation of Tcm and for the differentiation and effector function of Ttm and Tem cells.

5. IL-15 induces the differentiation and cytolytic function of HIV-specific CD8+ T cells

Our results demonstrating the ability of IL-15 to induce differentiation of Ttm and Tem memory CD8+ T cells subsets and Granzyme B expression suggest that IL-15 cytokine therapy might be a promising approach to enhance CD8+ T cell-mediated killing of HIV infected CD4+ T cells in HAART subjects. We have optimized a flow cytometry based assay, adapted from previous studies in B cells (chaper 3) (Mbitikon-Kobo, Bonneville et al. 2012), that allows us to analyze the cytotoxic capacity of primary antigen-specific CD8+ T cells *ex vivo* at the single cell level. PBMCs from chronic HIV infected patients were stimulated for 6 days with an optimum HLA class I restricted HIV peptide in the presence or in absence of IL-15. At day five we measured activation and differentiation of tetramer positive cells. HIV-specific CD8+ T cells stimulated with peptide plus IL-15 showed increased proliferation and granzyme B expression when compared to peptide stimulation only (Ki67 14%±10 vs 5%±2 $P<0.01$, GrzB 57%±3 vs 25%±6 $P<0.01$) (Fig 5B,C). Moreover, based upon expression of CCR7, CD27 and CD45RA, we found that IL-15 effectively induced the differentiation of HIV-specific CD8+ Ttm to a Tem phenotype ($p<0.01$, Fig. 5D and Suppl. Fig 4) and this was associated to Id2 upregulation (Fig.5E). We then tested limiting dilutions of tetramer positive CD8+ effector cells for the ability to kill autologous activated CD4+ T cell targets pulsed with peptide. HIV-1 specific CD8+ T

cells from all donors stimulated with peptide plus IL-15 showed enhanced killing of autologous CD4 target cells. CD8+ CTL killing efficiency increased in HIV –infected subjects between 25 to 1.5 fold change in lytic units for the specific target epitopes (Fig.5F).

DISCUSSION

In this study we sought to better understand mechanisms regulating CD8+ T cell effector cell differentiation and to identify interventions that could restore CD8 immune function in HIV infected individuals. We as well as other groups have shown that chronic HIV infection leads to the loss of CD8+ Tcm memory cells and the accumulation of Ttm and Tem cell subsets that express high levels of negative regulatory molecules associated with an arrest in CD8+ T cell differentiation and a failure to undergo proliferative expansion and exert cytotoxic effector function (Trautmann, Janbazian et al. 2006; Ladell, Hellerstein et al. 2008; Quigley, Pereyra et al. 2010; Trautmann, Mbitikon-Kobo et al. 2012; Hardy, Sieg et al. 2013). While proinflammatory cytokines like IL-12 and IFN-α enhance antigen sensitivity and CD8+ T cell cytotoxic activity during an acute infection, chronic infection and persistent inflammation is associated with immune exhaustion and a failure to develop long-term memory (Intlekofer, Takemoto et al. 2007; Joshi, Cui et al. 2007; Knell, Best et al. 2013) (Joshi, Cui et al. 2011) (Takemoto, Intlekofer et al. 2006; Joshi, Cui et al. 2007; Hand, Cui et al. 2010; Favre, Stoddart et al. 2011; Terawaki, Chikuma et al. 2011; Kaech and Cui 2012). Our transcriptional profiling studies of CD8+ T cells identified the downregulation of Id2 and Id3 expression, which play a central role in regulating Tem and Tcm differentiation, in chronic HIV infection compared to healthy controls (chapter 2). Therefore, we sorted human CD8+ memory T cell subsets from PBMCs from normal donors to determine levels of Id2 expression and responsiveness to IL-15. We found that Id2 levels were expressed at increasingly higher levels in Ttm, Tem and Temra. The high affinity IL-15Rα chain was also expressed at higher levels and were 2 fold higher in CD8+ Tem and Ttm compared to Tcm cells. Surprisingly, total CD8+ T cell memory cells

responded directly to exogenous IL-15 by proliferating equally well as to recombinant IL-15/IL-15Rα protein complex (ALTOR) or IL-15 plus autologous monocytes over the range of doses tested (1ng/ml-100ng/ml). Maximum proliferation was seen at 10ng/ml with the proportion of activated cells expressing $Ki67^+$ (range 65-72); HLA-DR^+ (22-52%); PD-1^+ (67-74%) compared to $Ki67^+$ (range 2-5%) in unstimulated cells (Suppl. Fig. 5). A recent study also showed that human CD8+ T cells are responsive to direct stimulation with exogenous IL-15 (Ota, Takase et al. 2010).

ID2 levels peaked 2 days after stimulation and returned to baseline at day 5 while IL-15Rα expression reached a plateau at day 3. Protein expression levels of BCL-2 increased with the same kinetics in cycling cells while BIM inversely correlated with BCL-2 levels in the responding CSFElow cells. IL-15 induced proliferation was dependent on the upregulation of ID2 expression since knockdown of Id2 with a small interfering RNA (siRNA) abrogated IL-15-induced proliferation and BCL-2 protein expression but did not affect BIM protein expression in non-proliferating cells. These finding confirm other studies that show that ID2 levels correlate with cell cycle and is required for upregulation of BCL-2 expression and survival of the proliferating cell population and the temporal regulation of differentiation of effector phenotypes (Knell, Best et al. 2013). Indeed, we found that *ex vivo* Id2 levels were low in undifferentiated CD8+ naïve and Tcm cells while we found increasing expression of Id2 with differentiation of memory CD8+ T cell subsets and the highest levels in Temra. We found IL-15 induced high levels of ID2 expression independent of TCR signals with peak protein levels occurring 2 days after stimulation. In fact, IL-15 –STAT5 signaling was the primary driver of Id2 expression since induction of ID2 by anti-CD3/CD28 was low in the absence of IL-15 and the combination of a TCR signal with IL-15 resulted in only a modest enhancement of Id2 expression (Fig.2B). Stimulation of memory subsets with IL-15 resulted in increased proliferation, survival and differentiation of Ttm and Tem, while a TCR activation plus IL-15 was required to induce differentiation in the CD8+ Tcm cell population. When we stimulated Tcm with IL-15, even in the presence of TCR, we did not see any significant changes in phenotype or expression of transcription factors associated with T cell differentiation (Fig. 4A). Although

we saw upregulation of Id2, albeit significantly lower than in Ttm and Tem cells, stimulated with IL-15, and higher expression of Bcl-2 and cMyc in Tcm, Foxo1 expression was highest in Tcm and remained unchanged after IL-15 stimulation whereas Foxo-1 mRNA levels decreased 2.6 fold in Ttm and 2.2 fold from already low levels in Tem cells (Fig. 2C). Our findings are consistent with the role of Foxo1 in regulating the expression of IL-7Rα and KLF2 and the expression of CD62L and CCR7 on Tcm (Weinreich, Takada et al. 2009; Takada, Wang et al. 2011). Alternately, Tcm express lower level of IL-15Rα and may have a stricter requirement for trans-presentation of IL-15/IL-15Rα by dendritic cells or require IL-7 signals provided in the BM niche.

Our findings support a model in which cells progressively differentiate in response to specific-antigen from a naïve phenotype to Tcm, Ttm, Tem and finally a terminally differentiated effector phenotype Temra (Gattinoni, Lugli et al. 2011). Most importantly, we found that IL-15 can support the proliferation and differentiation of Ttm and Tem but only proliferation of Tcm cells. The most significantly upregulated genes in CD8+ Tem cells in response to IL-15 stimulation included Id2, Bcl-2, cMyc, GzmB, and Cd25 whereas Bim, Gadda45, Cdkn2a (p21), Ctla4, Cd27 , and Foxo-1 were significantly downregulated. Although T-bet, Eomes and Blimp-1 were also slightly downregulated in Tem in response to IL-15 compared to unstimulated cultures, we believe that the time point (12h after IL-15 stimulation) at which mRNA levels were measured might have been too early to detect a role for these transcription factors in differentiating a population of cells that had entered the proliferative growth phase suggesting that proliferation and differentiation programs may be distinct and are temporally regulated.

Since we showed that IL-15 increased both mRNA and protein expression of granzyme B in Ttm and Tem memory CD8+ T cell subsets we wanted to see if IL-15 could induce differentiation and restore effector function in antigen-specific CD8+ Ttm and Tem cells from HIV-1 chronically infected and HAART treated subjects (Champagne, Ogg et al. 2001; Hess, Altfeld et al. 2004; Harari, Enders et al. 2009). IL-15 production by dendritic cells has been shown to be deficient during HIV-1 infection, but can be restored during HAART (Lucey, Pinto et al. 1997; d'Ettorre, Forcina et al. 2002). In both chronic infected

donors and HAART treated subjects, stimulation of PBMCs with peptide plus IL-15 induced the proliferation of 15% of CD8+ CCR7- CD27+ Ttm cells and differentiation to CD27- Tem cells compared to peptide alone. Stimulation with peptide plus IL-15 also significantly upregulated ID2 expression and enhanced the killing efficiency of tetramer positive CD8+ T cells.

Preclinical studies have demonstrated the ability of IL-15 to enhance survival and recall responses of unhelped CD8+ T cells (Becker, Wherry et al. 2002; Oh, Perera et al. 2008; Aubert, Kamphorst et al. 2011) and augment vaccine elicited by CD8+ CTL responses in murine tumor models (Klebanoff, Finkelstein et al. 2004; Yang, Ji et al. 2012; Zhang, Ju et al. 2012; Vincent, Bessard et al. 2013). Of relevance to HIV-1 immunotherapy, the combination of IL-15 and blocking antibodies against PD-1 and CTLA-4 negative regulatory molecules significantly enhanced CD8+ CTL effector function and prolonged survival of metastatic tumor-bearing animals (Yu, Steel et al. 2010). Preclinical safety and toxicity studies conducted in non-human primates showed that daily infusions of IL-15 were safe and resulted in a 100 fold expansion of CD8+ Tem cells (Sneller, Kopp et al. 2011; Waldmann, Lugli et al. 2011).

Numerous studies suggest that the quality of CD8+ Tem and mucosal homing are critical factors for immune control of HIV infection in gastrointestinal tissue, which is a probable source of persistent virus replication in HAART-treated subjects (Oh, Perera et al. 2004; Stoklasek, Colpitts et al. 2010; Hansen, Ford et al. 2011; Buckheit, Salgado et al. 2012; Snell, Lin et al. 2012; Yamamoto, Johnson et al. 2012; Liu, Hawkins et al. 2013). Our findings that IL-15 can enhance expansion and cytolytic effector functions of CD8+ Tem from HIV infected subjects supports the inclusion of IL-15 in therapeutic vaccines and "Cure" strategies, to enhance killing of virus infected cells and prevent reestablishment of the latent reservoir. IL-15 can stimulate virus replication in HIV infected CD4+ T and we have recently shown that IL-15 is much more effective at reactivating virus replication in latently infected CD4+ T cells than IL-7 (Vandergeeten, DaFonseca et al. 2011; Vandergeeten, Fromentin et al. 2013). Thus IL-15 cytokine therapy might serve a dual role

in reactivating latent reservoirs and enhancing CD8+ CTL mediated killing of virus infected cells.

METHODS

Phenotypic analysis

Memory CD8+ T cells subsets were sorted by flow cytometry from whole PBMCs isolated from healthy individuals based on CD45RA, CD27, and CCR7 expression. Naïve cells (N) were characterized as CD45RA+, CD27+, and CCR7+; Central memory (Tcm) gated as CD45RA-, CD27+, CCR7+; transitional memory (Ttm) were characterized as CD45RA-, CD27+, CCR7- , effector memory Tem were identified by the lack of expression of all three markers, (CD45RA-, CD27-, CCR7-); and terminally differentiated effector cells are CD45RA+, CD27-, CCR7-terminally differentiated (CD45RA+CD27- CCR7-) and memory CD8+ T cells (CD45RA- CD27+/- CCR7+/-). PBMCs were resuspended in PBS containing 2% FCS and stained with Tetramer-PE at 0.3μg per 10^6 cells. The following cocktail was used for phenotyping CD8+ T cells: CD3-Alexa 700 (BD), CD8+ PB (BD), CD45RA H7 (BD), CCR7 PE-CF594 (BD), CD27 Qdot655 (Invitrogen), HLA-DR PerCP (Biolegend) and PD-1 APC (eBioscience), Annexin V APC (BD) in Annexine V buffer 10X. We used 0.01% of saponin buffer for the intracellular staining of KI67 FITC (BD) or Granzyme B (BD). Dying cells were eliminated with LIVE/DEAD viability dye (Invitrogen). We acquired a minimum of $1x10^6$ events for all cytometry-related experiments using a BD LSRII flow cytometer and analyzed with DIVA software v5.0 (BD) or sorted by FACS ARIA (BD). Cytokine receptors: IL-2Ra (CD25) (BD), IL-15Ra (CD215)(eBioscience), IL-7R (CD127) (BD), IL21R (CD360)(BD), were measured on anti-CD3/C28 and/or IL-15 stimulated memory CD8+ T cells.

CD8+ T cell Activation

Memory CD8+ T cells were purified by magnetic beads separation (Stem cells; purity>95%) and labeled them with CFSE, then activated or not with 0.05ug/ml of anti-CD3 and 1ug/ml of anti CD28. Kinetics of ID2 (Santacruz) , Bcl-2 (cell signaling) and BIM

(cell signaling) was assessed by Western Blot. Quantification of protein expression was performed by densitometric reading of bands using ImageQuant software and normalized to expression of housekeeping genes β-Actin or rRNA28s.

Real Time PCR

Primers and probes were obtained from IDT and Roche respectively. Multiple 100 cells samples from 9 healthy individuals where sorted by FACS ARIA (BD) in a 96 well plate gating on the population of interest. We used the Cells Direct One-Step qRT-PCR Kit for retrotranscription and preamplification (18 cycles) of the RNA targets of interest without a separate RNA purification step. qPCR were performed on a Roche Light Cycler 480 and analysis was performed according to the ΔΔct method. Gene expression was normalized by Gapdh housekeeping gene.

Transfection and small interfering RNA assay

Purified memory CD8+ T cells from PBMCs by negative selection (stem cell for CD8+ enrichment and milteny for enrichment of the CD45RA negative cells) were preactivated with 0.05ug/ml of antibodies specific for CD3 and 1ug of CD28 for 24h. Cells were then electroporated $3x10^6$ cells for condition using Nucleofactor II technology according to the manufacturer's protocol (amaxa Biosystem). Id2-specific siRNA and negative control siRNA were obtained from Invitrogen (ID2 Validated Stealth DuoPak) and were used to transfected 5ug of siRNA for each condition. Cells were reactivated for 3 days and stained with CD3 A700 (BD), CD8+ PB (BD), CD45RA H7 (BD), CCR7 PeCy7 (BD), CD27 (QDot655)(Invitrogen) , Annexin V (APC)(BD). Half million of cells were kept to measure protein levels by Western Blotting.

Peptides and Tetramers

Soluble pMHC monomers were generated as previously described (Montreal, Canada) (Altman, Moss et al. 1996). The peptides and tetramers used to analyze the HIV-specific CD8+ T cell response were: 2 donors for FLKEKGGL (B*08 NEF), and two donors for

KRWIILGLNK (B*27 gag kk10), SPAIFQSSM (HLA-B*0702 pol) and ISPRTLNAW (HLA-B-5701).

Cytolytic assay

PBMCs from Chronic HIV infected patients were stimulated for 6 day with the specific HIV peptide (1ug) and in presence or in absence of 10ng of IL-15 (R&D). At day 6 PBMCs were enriched for CD8+ T cells by magnetic negative selection (stem cell) and cocultured with target CD4+ T cells in different dilutions as previously described (chapter 3). Purified autologous CD4+ target cells were pre-activated overnight with 0.1ug of anti CD3-CD28 antibodies. The day after, target cells were counted, spited in two tubes and labeled with two different concentration of CFSE (0.02uM and 0.2uM). After the staining only the CFSE low cells were pulsed with the specific HIV peptide (1ug) for 45 min at 37Co. Cells were washed twice to remove the extra peptide and then we mixed the two populations of CD4 T cells in a 1:1 ratio. The cytolytic activity of the HIV-specific CD8+ T cells was expressed in lytic units as previously described (Mbitikon-Kobo, Bonneville et al. 2012).

Statistical analysis

In all experiments, the nonparametric Mann-Whitney or student paired t tests (for $n<5$) were performed for comparisons between populations using Graphpad Prism 5.0 software. P values of less than 0.05 were considered significant.

FIGURE LEGEND

Figure 1: Id2 is upregulated on Effector cells and on activated memory CD8+ T cells. Id2 shut-down leads to significant decrease of Bcl-2 proteins and decrease survival and proliferation of Tem: CD8+ T cell subsets were sorted from total PBMCs obtained from healthy volunteers for naïve (CD8+CD45RA+CCR7+CD27+), Tcm (CD8+CD45RA-CCR7+CD27+), Ttm (CD8+CD45RA-CCR7+CD27-), Tem (CD8+CD45RA+CCR7-CD27-), and Temra (CD8+CD45RA+CCR7-CD27-) subsets and **A)** Id2 mRNA levels were assessed by RT-PCR. Relative expression levels are plotted as fold increase normalized to GAPDH expression. (CM=Tcm; TM=Ttm; EM=Tem; EMRA=Temra) **B)** Memory CD8+ T cells were activated with anti CD3-CD28 for 3 days and highly purified by sorting on viable proliferative CFSE low and resting CFSE high populations. ID2, BCL-2, BIM and actin expression were assessed by Western Blot. (**C)** Histograms showing relative changes in protein expression in CSFE high versus CSFE low cells (N = 5) by densitometric quantification of the Western Blot bands. Purified memory CD8+ T cells were transfected with Id2 or negative control siRNAs and ID2 and its target protein BCL-2 were assessed by Western Blot. **D)** WB bands and densitometric analysis was performed using ImageQuant software (N = 5). Involvement of ID2 in activated and transfected memory CD8+ T-cell populations on different cell properties: **F)** on spontaneous apoptosis and survival (N = 5), **E)** on proliferative capacity (N = 5), and **G)** frequency of effector cells.

Figure 2: The γ-chain cytokines IL-2 and IL-15 positively modulate ID2 protein expression: Purified memory CD8+ T cells were treated or not with 10ng/ml of IL-2, IL-15, IL-7, IL-21 and with 0.05 ug/ml of anti-CD3 and 1 ug/ml of anti-CD28. After 2 days in culture, ID2 expression was assessed using Western Blot assays. **A)** Densitometric quantification of the bands using ImageQuant software (N=5). **B)** Id2 mRNA levels of total memory CD8+ T cells was assessed at 12h in unstimulated cells or after activation in the presence or absence of IL-15 (10ng/ml) and normalized by 28S housekeeping gene. **C)** Gene expression profile of indicated genes was assessed on

sorted memory CD8+ T cell subsets by Real time PCR normalized to GAPDH housekeeping gene was assessed at 12h after IL-15 stimulation (n=3).

Figure 3: IL-15 increases survival, proliferation and CD45RA expression from all memory CD8+ T cell subsets: A) Percentage of apoptosis was assessed by annexin V labeling (n = 5). **B**) Percentage of proliferation was assessed by Ki67 staining. **C**) Differentiation was assessed by the percentage of cells that re-expressed CD45RA.

Figure 4: IL-15 induces differentiation of memory CD8+ T cell subsets: Sorted CM **A**), TM **B**) and EM **C**) cells from healthy subjects were treated in the presence or absence of IL-15 and presence or not of anti CD3/CD28 antibodies and phenotype of the cells was measured by CD27, CCR7 and CD45RA expression after 6 days of culture by SPICE analysis.

Figure 5: IL-15 induces Granzyme production of Ttm and Tem cells and restores effector function of HIV-specific CD8+ T cells: Sorted CM, TM and EM cells from healthy subjects were treated in the presence or absence of IL-15 and of anti-CD3/CD28 antibodies. **A)** Granzyme B production was assessed after 6 days of culture. PBMCs from HIV+ patients were stimulated or not in presence of specific HIV-peptide and IL-15 (10ng). After 6 days of culture with 1ug of HIV specific peptide in presence or not of IL-15 **B)** Granzyme B and **C)** Ki67+ cells were assessed on tetramer+ cells. **D**) The Tem/Ttm ratio on tetramer + cells was measured by CD27 and CCR7 expression. **E**) after 12h from stimulation 100 tetramer + cells were sorted and ID2 expression was assessed by Real Time PCR. **F)** Cytolytic activity was measured by flow cytometry on tetramer + cells: after 6 days CD8 + T cells were purified and co-cultured at limiting dilution with activated CD4+ targets that were first labeled with high and low concentrations of CFSE and then pulsed with the HIV-specific peptide (CFSE low). Lytic Unit is calculated from the linear regression of specific lysis in the function of the effector/target ratio.

Supplementary Figure 1: Upregulation of ID2 and BCL-2 in memory CD8+ T cells by TCR triggering. A) Kinetics of ID2 expression was assessed using Western Blot

assays in resting memory CD8+ T cells. The level of actin expression was measured in parallel as a loading control (N=5). **B**) Memory CD8+ T cells were activated for 1 to 27 hours with 0.05 µg⁄mL of anti-CD3 and 1 µg⁄mL of anti-CD28. *28S* and *id2* mRNA levels were assessed using real time PCR (N = 5). **C**) Kinetics of ID2 (left) and Bcl-2 (right) expression were assessed after activation with anti CD3/CD28. **D**) Densitometric quantifications of the ID2 bands using ImageQuant software (N = 5).

Supplementary Figure 2: Activation of resting memory CD8 T cell with anti-CD3/CD28 induced significant proliferation and decreased cells undergoing apoptosis during *in vitro* culture. Memory CD8+ T cells were purified using AutoMACS (92.8 % of purity), then activated or not for 1 to 5 days with 0.05 µg⁄mL of anti-CD3 and 1 µg⁄mL of anti-CD28. Kinetics of % of CFSE low **A**) and apoptotic **B**) memory CD8+ T cells were thereafter assessed (N ≥ 5).

Supplementary Figure 3: The γ-chain cytokines IL-2 and IL-15 positively modulate ID2 protein expression: purified memory CD8+ T cells where treated or not with 10ng/ml of IL-2, IL-15, IL-7, IL-21 and with 0,05 ug/ml of anti-CD3 and 1 ug/ml of anti-CD28. **A)** After 2 days in culture ID2 expression was assessed using Western Blot assays. The level of actin expression was measured in parallel as a loading control. **B)** Cytokine receptor levels were assessed by flow cytometry on memory CD8+ T cells after activation with anti CD3-CD28 antibodies **C)** IL-15Rα receptor levels were assessed by flow cytometry on memory CD8+ T cells.

Supplementary Figure 4: IL-15 increases differentiation of HIV-specific CD8+ T cells: PBMCs from HIV+ chronic infected individuals were stimulated or not with 1ug/ml of HIV-specific peptide and with or without 10ng/ml of IL-15. Phenotype of tetramer positive cells at day 6 after stimulation of PBMCs by flow cytometry.

Supplementary Figure 5: CD8+ T cells do not require transpresentation of IL-15: Purified CD8+ T cells were stimulated or not with different doses of IL-15 or ALTOR or in the presence of IL-15 plus monocytes and anti-CD3-CD28 stimulation was used as a

positive control. After 6 days of culture **A**) PD-1 **B**) Ki67 and **C**) HLA-DR expression was assessed by flow cytometry.

Contributions

A.N. performed all the experiments, analyzed-interpreted data and prepared graphics. F.A.P.supervised the project, designed experiments, participated in performing experiments. H.T and F.D. and J.Z. helped in analysis and preparation of graphics. Z.H. prepared plasmids for positive controls.R.B. provided with the samples. J.A. corrected the manuscript. R.P.S. supervised the project.

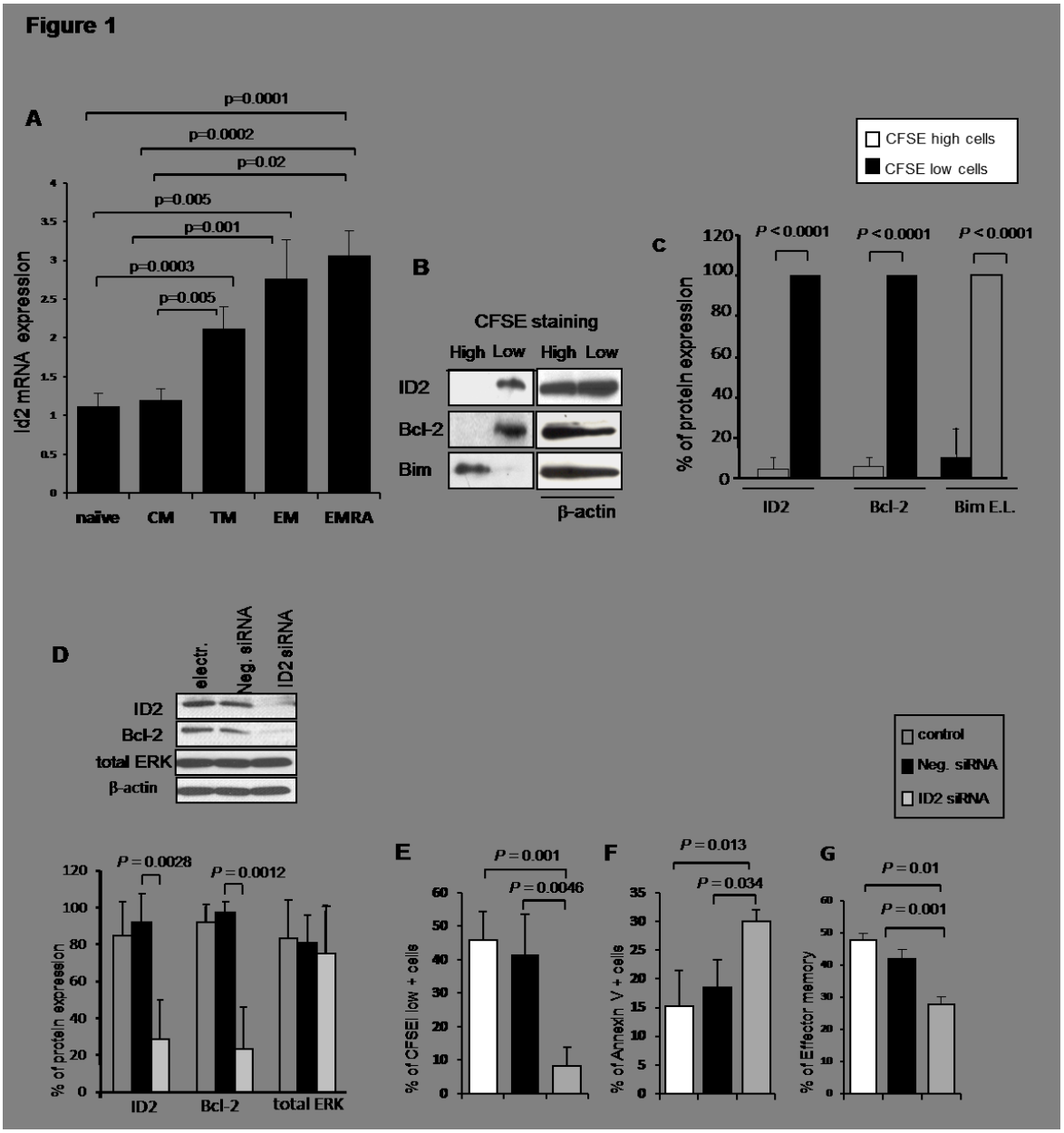


Figure 1
A
p=0.0001
p=0.0002
p=0.02
p=0.005
p=0.001
p=0.0003
p=0.005
Id2 mRNA expression
naive
CM
TM
EM
EMRA
B
CFSE staining
High Low High Low
ID2
Bcl-2
Bim
β-actin
C
CFSE high cells
CFSE low cells
P < 0.0001
P < 0.0001
P < 0.0001
% of protein expression
ID2
Bcl-2
Bim E.L.
D
electr.
Neg. siRNA
ID2 siRNA
ID2
Bcl-2
total ERK
β-actin
control
Neg. siRNA
ID2 siRNA
P = 0.0028
P = 0.0012
% of protein expression
ID2
Bcl-2
total ERK
E
P = 0.001
P = 0.0046
% of CFSEI low + cells
F
P = 0.013
P = 0.034
% of Annexin V + cells
G
P = 0.01
P = 0.001
% of Effector memory

Figure 2

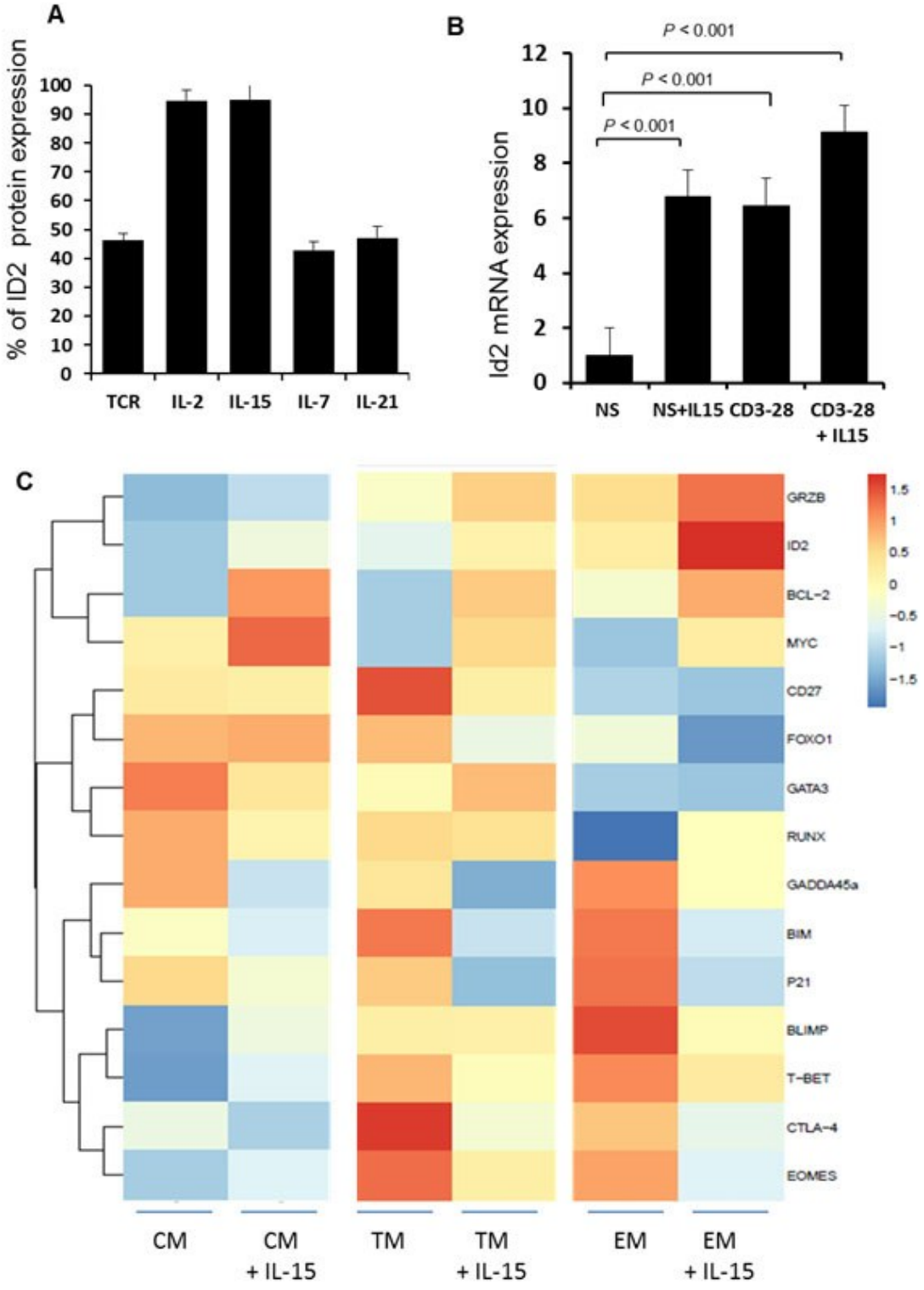

A
% of ID2 protein expression
100
90
80
70
60
50
40
30
20
10
0
TCR
IL-2
IL-15
IL-7
IL-21
B
Id2 mRNA expression
P < 0.001
P < 0.001
P < 0.001
12
10
8
6
4
2
0
NS
NS+IL15
CD3-28
CD3-28 + IL15
C
GRZB
ID2
BCL−2
MYC
CD27
FOXO1
GATA3
RUNX
GADDA45a
BIM
P21
BLIMP
T−BET
CTLA−4
EOMES
1.5
1
0.5
0
−0.5
−1
−1.5
CM
CM + IL-15
TM
TM + IL-15
EM
EM + IL-15

Figure 3

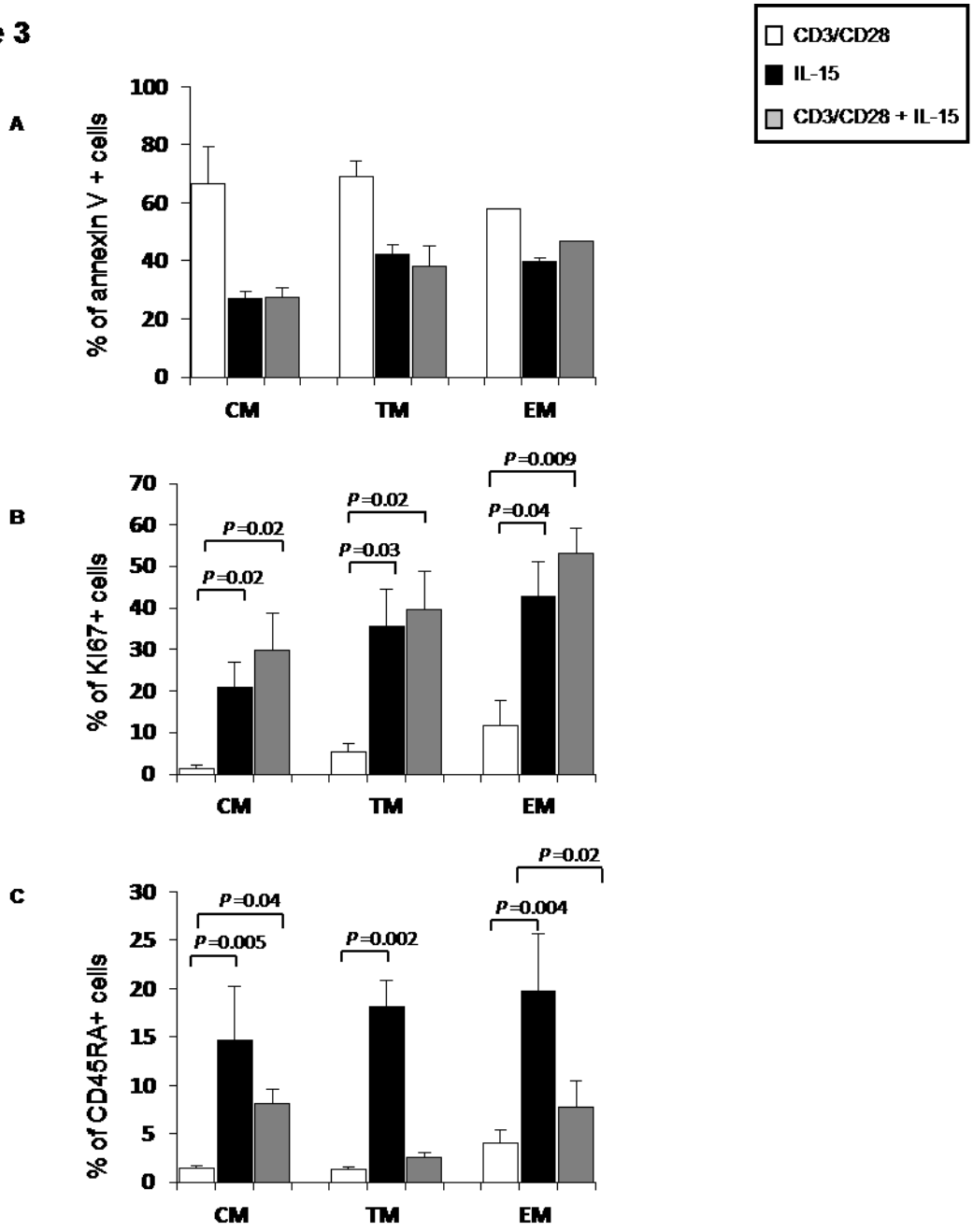

CD3/CD28
IL-15
CD3/CD28 + IL-15
A
% of annexin V + cells
100
80
60
40
20
0
CM
TM
EM
B
% of KI67+ cells
70
60
50
40
30
20
10
0
P=0.02
P=0.02
P=0.02
P=0.03
P=0.009
P=0.04
CM
TM
EM
C
% of CD45RA+ cells
30
25
20
15
10
5
0
P=0.04
P=0.005
P=0.002
P=0.02
P=0.004
CM
TM
EM

Figure 4

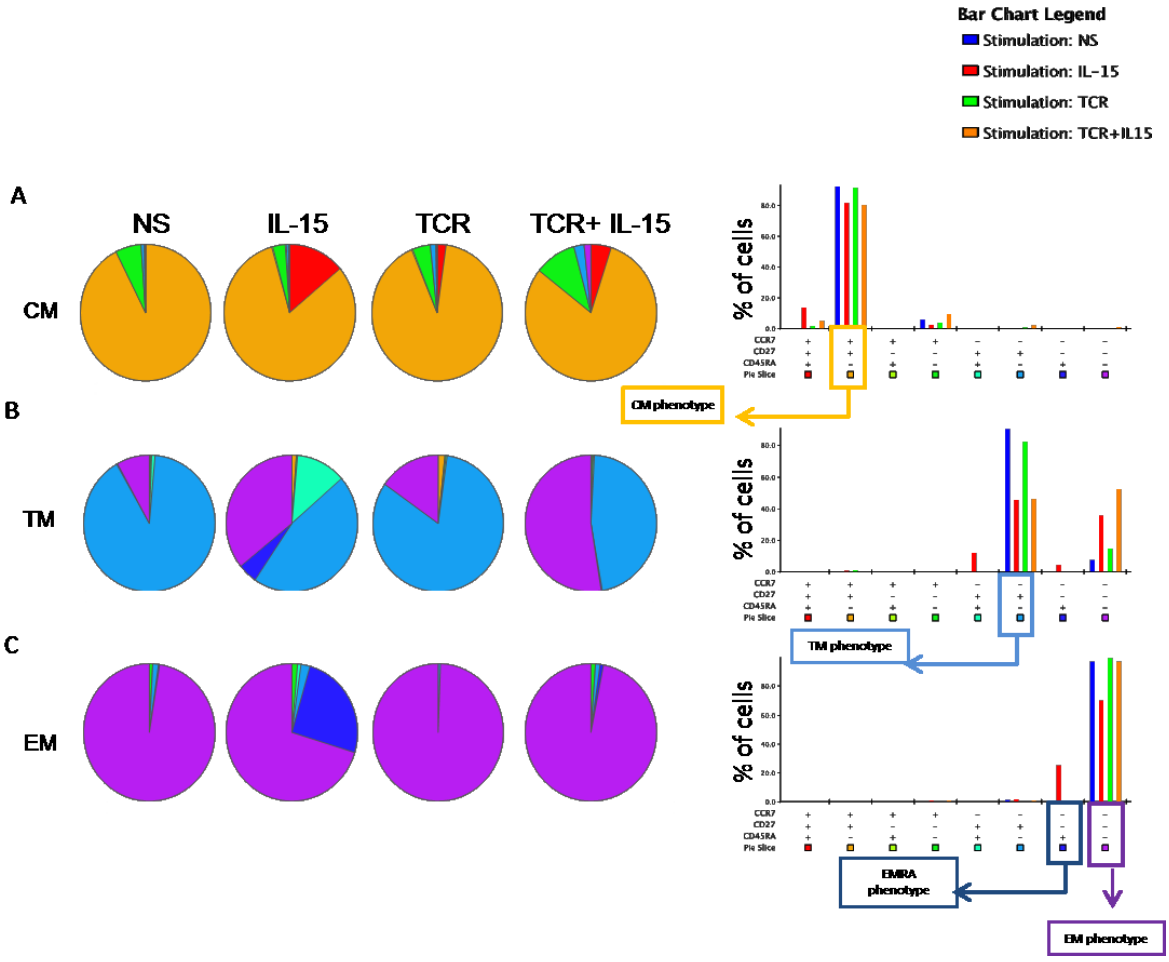
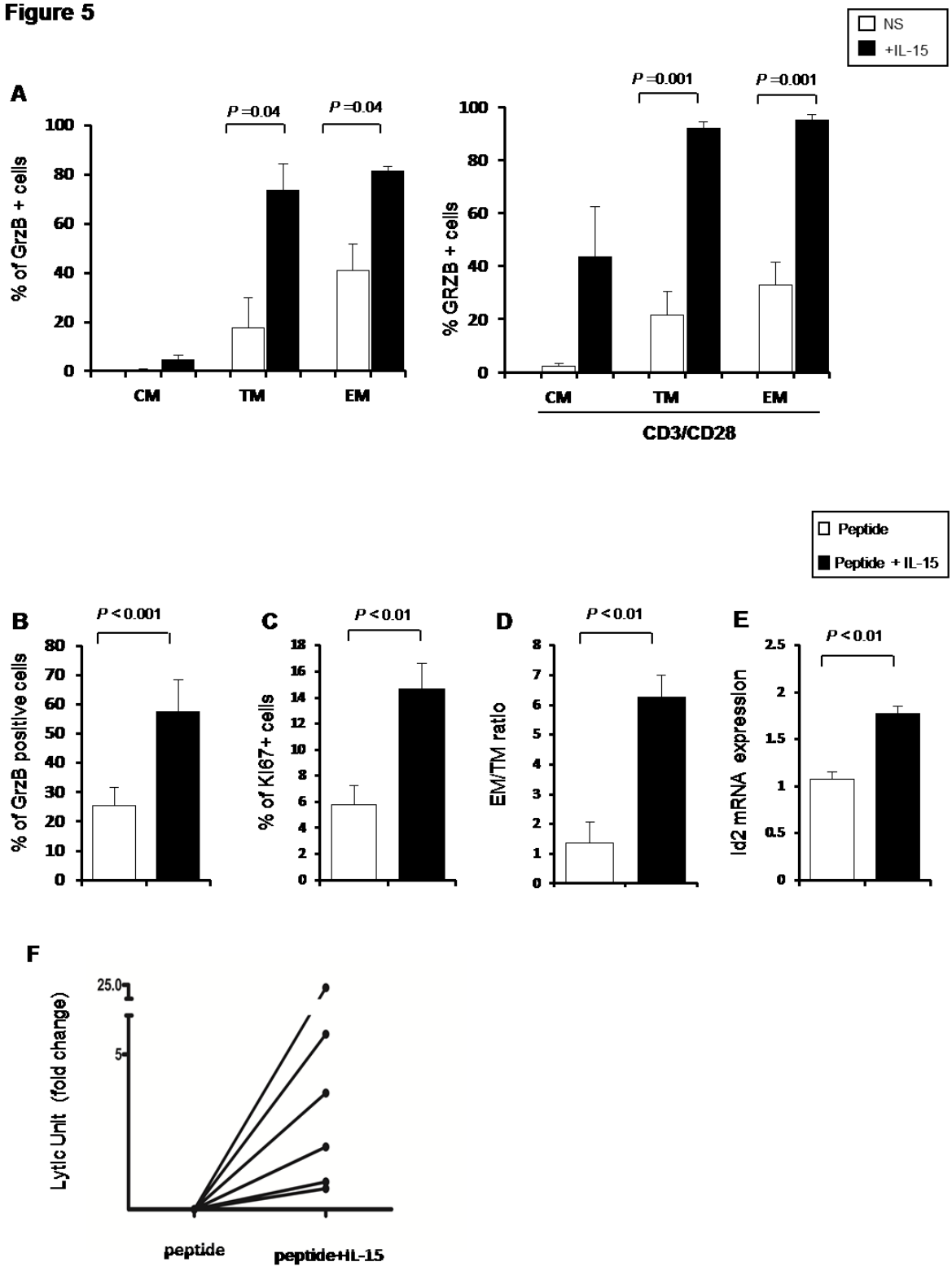
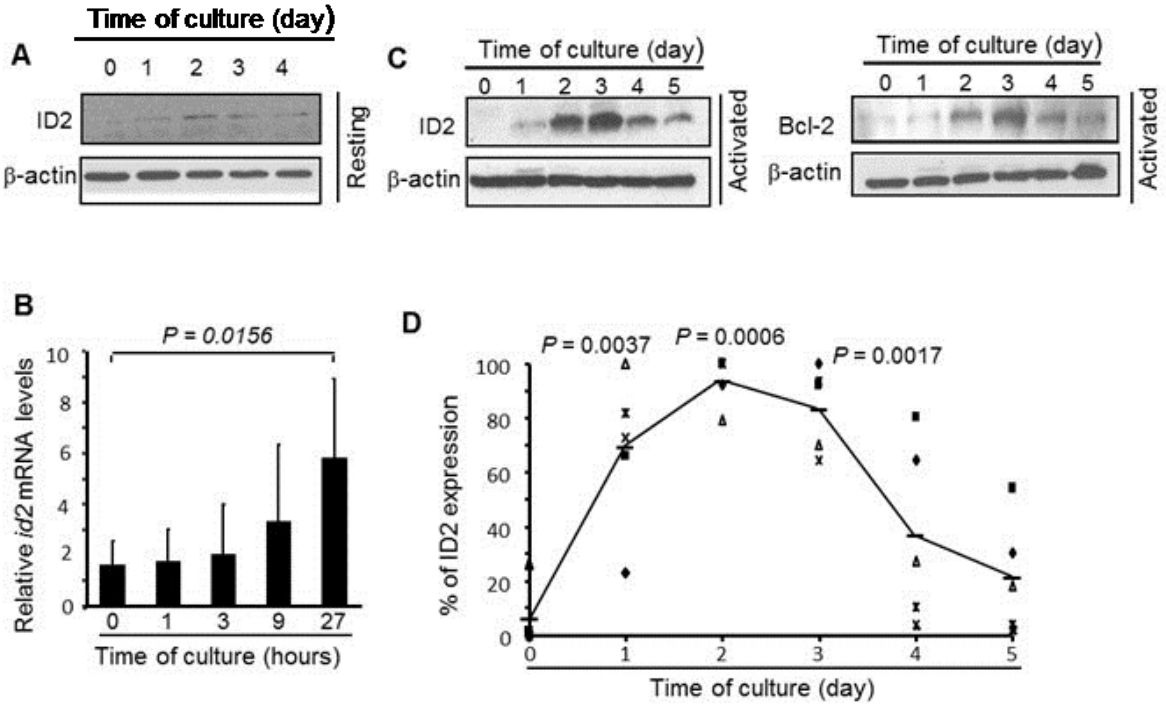

Bar Chart Legend
Stimulation: NS
Stimulation: IL-15
Stimulation: TCR
Stimulation: TCR+IL15
A
NS
IL-15
TCR
TCR+ IL-15
CM
B
TM
C
EM
% of cells
CCR7
CD27
CD45RA
Pie Slice
CM phenotype
TM phenotype
EMRA phenotype
EM phenotype

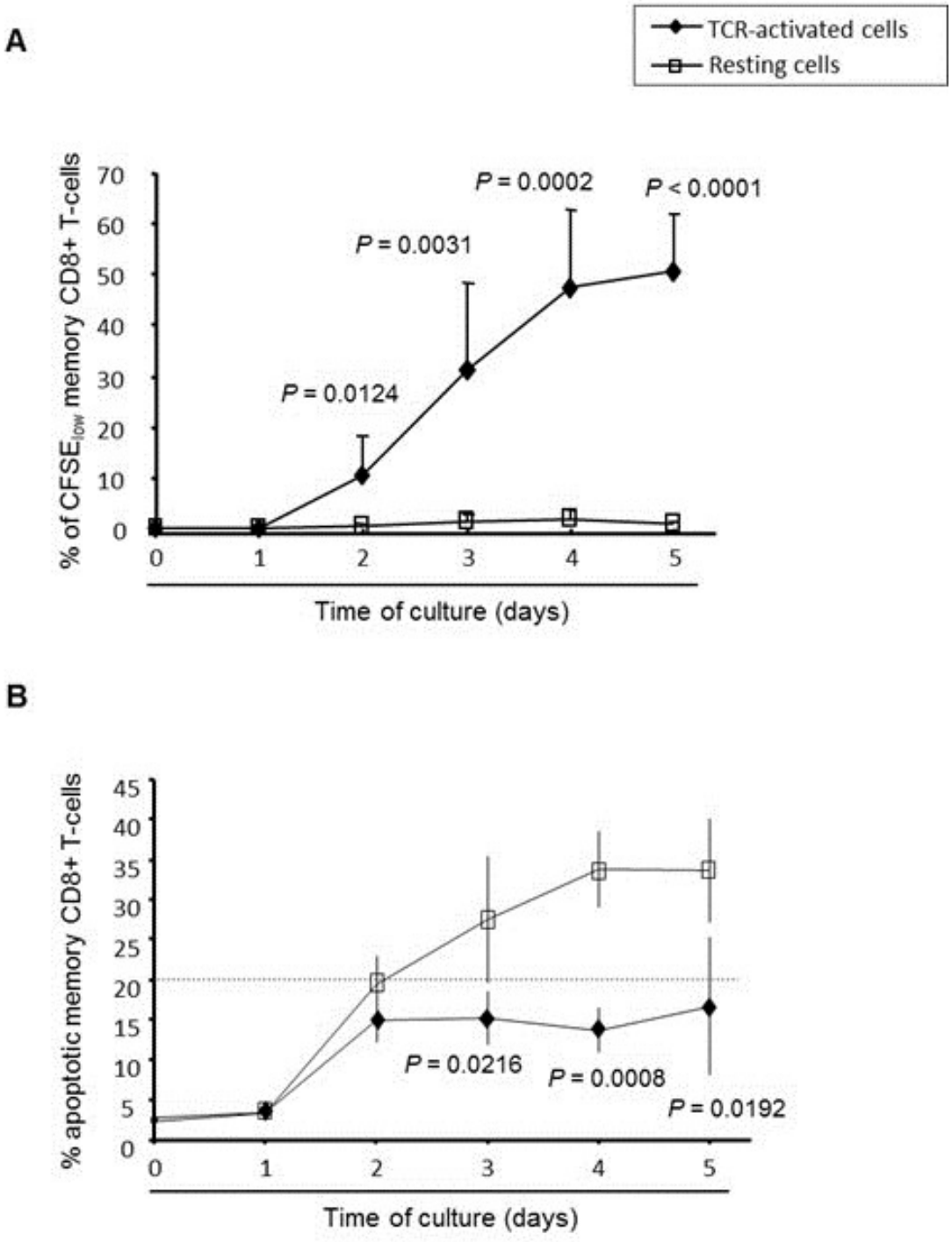
Figure 5


NS
+IL-15
A
% of GrzB + cells
P =0.04
P =0.04
CM
TM
EM
% GRZB + cells
P =0.001
P =0.001
CM
TM
EM
CD3/CD28
Peptide
Peptide + IL-15
B
% of GrzB positive cells
P < 0.001
C
% of KI67+ cells
P < 0.01
D
EM/TM ratio
P < 0.01
E
Id2 mRNA expression
P < 0.01
F
Lytic Unit (fold change)
25.0
5
peptide
peptide+IL-15

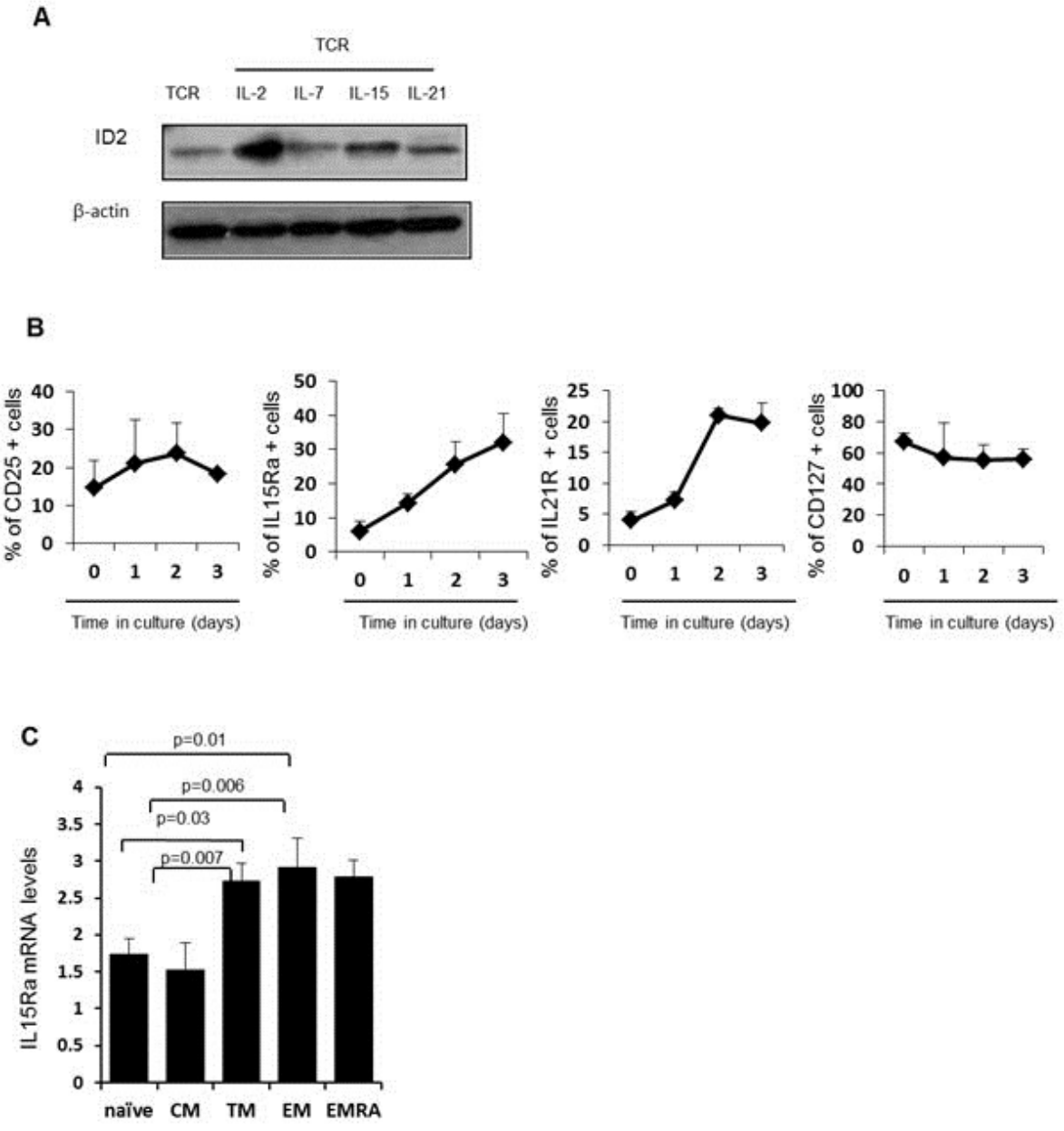
Supplementary Figure 1



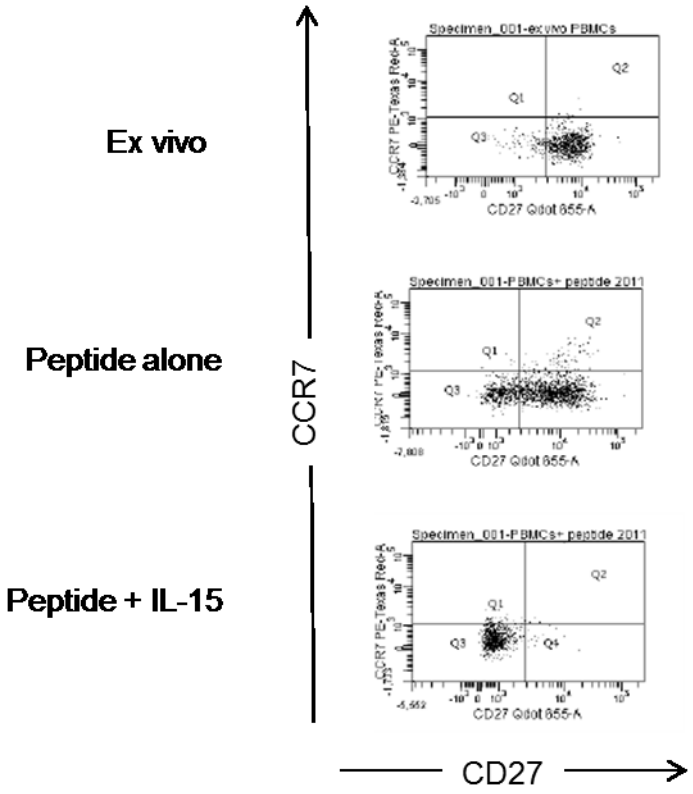
Supplementary Figure 2



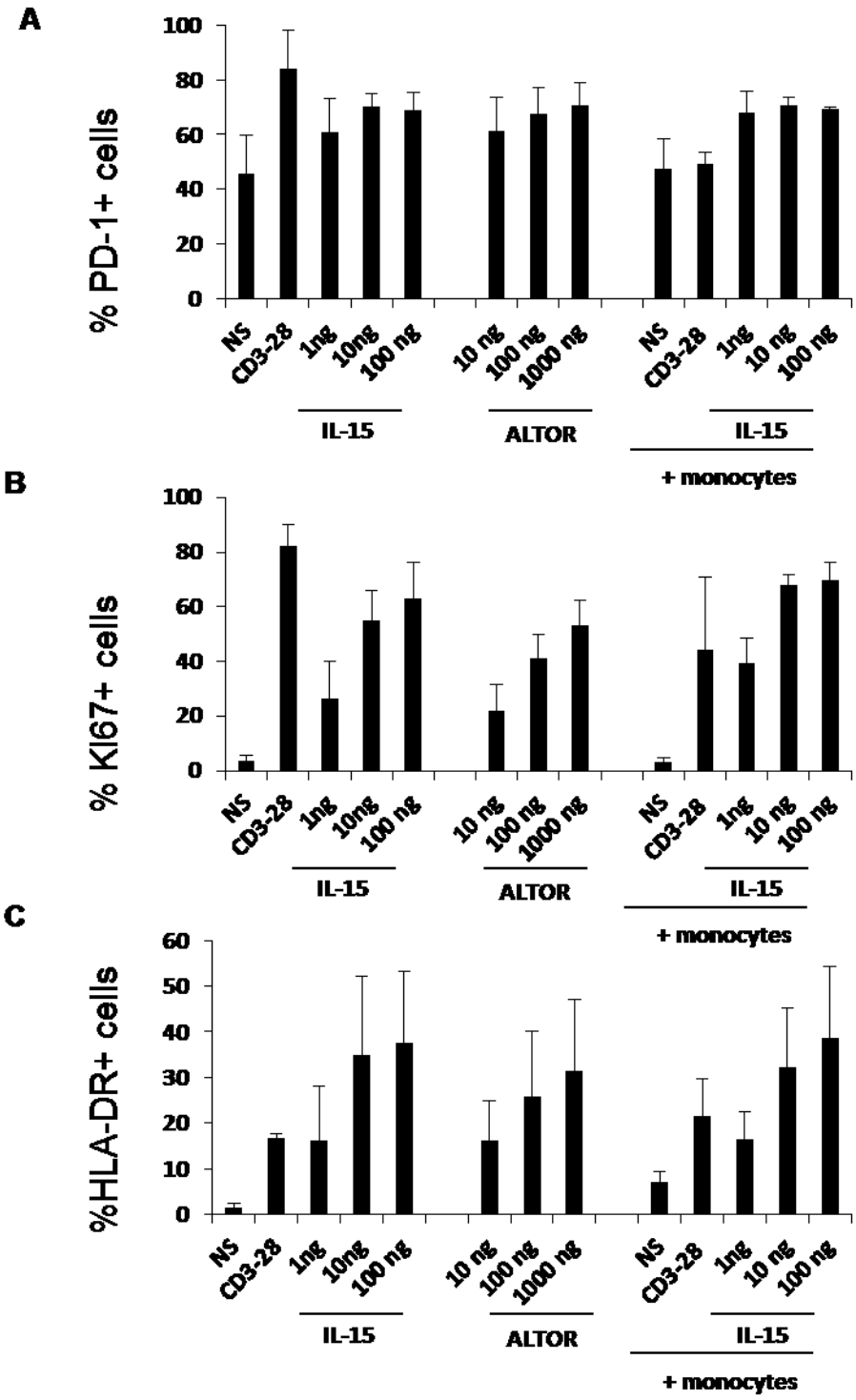
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



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CHAPTER 5

DISCUSSION

1. Summary of findings

CD8+ T cells play a fundamental role in chronic viral infections; they are essential for controlling viral replication and for killing of virally infected cells. However during chronic HIV infection HIV-specific CD8+ T cells express a poorly differentiated effector phenotype that reflects their decreased cytotoxic function; HIV-specific CD8+ T cells from untreated patients develop functional defects such as loss of IL-2 secretion, decreased proliferation and self–renewal capability and express high level of coinhibitory molecules, most notably PD-1. This dysfunction is only partially restored under HAART treatment. Extensive studies have shown how triggering of PD-1 by its ligand regulates immune dysfunction; we have demonstrated that PD-1 colocalizes with TCR in the immunological synapse leading to the inhibition of T cell receptor signaling and activation. It is not well understood how PD-1 signaling influences transcriptional/epigenetic events involved in T cell differentiation and effector function.

In this study we used a system biology approach in order to identify molecules that are modulated during HIV-infection and that are responsible for the decreased effector functions of HIV-specific CD8+ T cells. By gene array analysis we found that the transcriptional modulator Id2, which is important for proliferation and survival of many cell types, was downregulated during HIV infection. We hypothesized that PD-1 might be inhibiting T cell differentiation and perhaps survival by interfering with the expression of Id2. We found that HIV-specific CD8+ T cells from viremic donors had decreased levels of Id2 mRNA when compared to CMV-specific CD8+ T cells from the same HIV-infected individual. Id2 levels were most profoundly decreased in the PD-1 high population compared to PD-1 negative by RT-PCR. We further showed that signals transmitted downstream of PD-1 ligation inhibited expression of Id2 mRNA and protein in CD8+ T cells from healthy uninfected subjects that resulted in decreased proliferation and increased apoptosis. We also found that transfection of PD-1 high effector CD8+ T cells from HIV infected individuals with a Tat-Id2-FITC fusion protein construct that localizes in the nucleus of transduced cells could reverse apoptosis of CD8 effector memory cells. Overexpression of Id2 restored expression of Granzyme B, Bcl-2, and Myc and led to the

decreased expression of the T cell maturation marker CD27. Our study shows that the triggering of PD-1 by PD-1L leads to the downregulation of Id2 expression and to the decreased differentiation of CD8+ effector cells in chronic infection. Understanding the molecular mechanisms of antigen-specific CD8+ T cell exhaustion during HIV infection is important in order to identify interventions that can reverse the effects of PD-1 and other negative coregulatory molecules on CD8 cytotoxic effector function.

The gamma chain cytokines are the main cytokines involved in the maintenance and generation of virus-specific effector CD8+ T cells and their activity is probably mediated by the modulation of a set of antagonistic transcription factors involved in these processes. Of note IL-15 similarly to Id2 promotes survival and differentiation of effector CD8+ T cell. IL-15 has recently been considered as a candidate cytokine for immune therapy to HIV. A better understanding of the molecular events involved in the generation of effector cell mediated by IL-15 signaling is critical for the development of effective strategies for HIV treatment that aim at the restoration of effector and cytotoxic ability of antigen-specific CD8+ T cells. In the second chapter of the thesis I have focused on defining the mechanisms of action of IL-15 in promoting CD8+ T cell differentiation and defining the role of Id2 in this outcome.

Here we show that in healthy individuals the convergence between TCR and IL-15 induces differentiation of transitional memory (Ttm) CD8+ T cells into effector memory (Tem) and terminally differentiated effector cells (Temra) as measured by CD45RA, CD27 and CCR7 expression. The differentiation mediated by IL-15 was supported not only by changes in the phenotype of the cells but also by modifications at the molecular level. Indeed IL-15 stimulation leads to upregulation of Id2 levels and to a cascade of modulation of gene expression downstream of Id2 that result in increased proliferation, survival and differentiation of Ttm and Tem that express high levels of Granzyme B. Stimulation of central memory CD8+ T cells (Tcm) with IL-15 failed to induce their differentiation into effector cells ; this was associated with decreased *ex vivo* levels of IL-15Rα when compared to Tem and Ttm subsets that in contrast to Tcm expressed higher levels of the IL-15 receptor.

We show that during HIV-infection, HIV specific CD8+ T cells have a Ttm phenotype (CD45RA-CCR7-CD27+) and stimulation of these cells with their cognate peptide failed to induce their differentiation into functional Tem or Temra cells (CD45RA+/- CCR7-CD27-) even under HAART. To determine if IL-15 can restore HIV-specific CD8+ T cell function we optimized a cell-based flow cytometry assay that measures the cytotoxic activity of antigen-specific CD8+ T cells. This assay allows the direct assessment of cytolytic activity of antigen-specific CD8+ T cells by enumerating the frequency of apoptotic target cells in the presence of increasing numbers of effector cells. Killing of autologous target CD4+ T cells is analyzed by flow cytometry after co-culture with antigen-specific effector CD8+ T cells detected by peptide/MHCI tetramer staining. This assay allowed us to show that stimulation of HIV-specific CD8+ T cells in combination with IL-15 resulted in their increased cytolytic activity that was accompanied by restoration of the effector phenotype and Id2 expression.

Overall, the data presented in this thesis illustrate for the first time the molecular mechanisms of effector differentiation mediated by IL-15 and its downstream transcriptional regulator Id2. Here we reveal how PD-1 engagement leads to alteration of the Id2 pathway leading to decreased effector function of the HIV-specific CD8+ T cells. Elucidating the signaling downstream of PD-1 crosslinking and its effect on Id2 downregulation may reveal targets for therapeutic intervention. Immunotherapy with agents such as IL-15 treatment or PD-1 blocking antibody that increase Id2 expression levels, in combination with antiretroviral therapy may provide a novel way to restore and enhance the immune response that triggers the functional re-activation of HIV-specific CD8+ T cells and the killing of latently HIV infected CD4+ T cells.

2. Role of transcription factors in the generation of effector cells:

In past years, many studies have focused on understanding the mechanisms of effector CD8+ T cell generation and the roles of transcriptional factors in mediating this process. Murine models have identified sets of transcription regulators and programs that have been shown to have opposite effects on the balance between maintenance of memory T cells and

effector T cell differentiation. While the expression of several of these factors leads to an increase in the frequency of memory T cells others lead to effector differentiation. The best categorized of these inverse TF relationships are: T-bet and Eomesodermin, Id2 and Id3, Bcl-6 and Blimp-1 which have been described in details in Chapter 1 (section 1.5.1 and 1.5.2).

While the use of transgenic and knockout mice for the study of T cell differentiation and function represents good models to better understand these processes many differences at the transcriptional levels have been found between immune cell types of humans and mice despite 80% homology with human genome (Shay, Jojic et al. 2013).

Therefore, little is known about the role of these transcriptional modulators in regulating CD8+ T cell effector differentiation in humans.

This thesis is aimed at investigating the role of Id2 in the differentiation of CD8+ memory cells in human subjects. Id2, together with Id3, regulates memory generation in mouse studies: Id2 and Id3 were shown to be expressed at different stages of the immune response and mediate either generation of long-lived memory CD8+ T cells or short-lived effector cells respectively (Cannarile, Lind et al. 2006; Ji, Pos et al. 2011; Yang, Best et al. 2011). Cannarile et al. have shown that antigen-specific CD8+ T cells from Id2 knockout mice are capable of antigen-mediated activation and proliferation; however, they exhibited a diminished effector response after infection and a delay in the clearance of pathogen (Cannarile, Lind et al. 2006). They also showed that the absence of adequate immune responses was due to the enhanced susceptibility of CD8+ T cells to undergo apoptosis in Id2 knockout mice when compared to CD8+ T cells from wild type animals. All together, mouse studies suggest that Id2 plays an important role in mediating survival of effector CD8+ T cells early during the immune response. Here in our human study we show that Id2 is important for both the proliferation and survival of memory CD8+ T cells; indeed, shut down of Id2 expression using specific siRNA resulted in a complete block of proliferation as measured by CFSE dilution and increased annexin V labeling (Chapter 4, Fig 1 E,F) . This was confirmed at the protein level as we found Id2 to be highly expressed on sorted CFSE low proliferating cells when compared to the CFSE high resting

counterpart (Chapter 4, Fig 1 B,C). Our results suggest that the expression of Id2 allows antigen-specific cells to survive and undergo differentiation during an immune response. We also show that ex vivo Id2 expression levels were very low in naïve and Tcm populations and were significantly increased in the more differentiated Ttm, Tem and terminally differentiated Temra and shut- down of Id2 resulted in decreased frequency of effector cells (Chapter 4 Fig. 1 A,G). In human studies, it is difficult to track antigen specific CD8+ T cell responses and assess the kinetics of Id2 modulation upon infection. However, in vitro culture of memory CD8+ T cell subsets with IL-15 and anti-CD3/CD28 antibodies allowed us to detect upregulation of Id2 in each subset and this correlated with enhanced differentiation into effector cells. Moreover STAT5 and STAT4 binding sites have been found within the Id2 promoter suggesting that inflammatory signals driven by IL-15 promote effector T cell differentiation (Yang, Best et al. 2011).

In contrast to Id2, Id3 was downregulated upon activation of antigen specific cells and was re-expressed later in the memory pool. In agreement with mouse models, our results show that contrary to Id2, Id3 is highly expressed by naïve and Tcm populations but at low levels in the more differentiated Tem, Tm and Temra (data not shown). These findings suggest that the ratios of Id2-Id3 control the signals inducing effector versus memory cell fate differentiation.

3. Regulation of apoptosis by Id proteins during the immune response

Programmed cell death is the major mechanism that regulates the contraction phase during the immune response. Two main pathways have been shown to trigger cell apoptosis: the extrinsic pathway, mediated by activation of pro-apoptotic receptors on the cell surface (mainly TNF-R family), and the intrinsic or mitochondrial pathway, regulated mostly by the Bcl-2 family.

Our data clearly demonstrates that Bcl-2 is a target of the transcriptional modulator Id2. Indeed, we show that absence of Id2 mRNA expression in sorted memory CD8+ T cells rendered cells incapable of upregulating Bcl-2 following antigen stimulation (Chapter 4 Fig.1D). In the absence of both Id2 and Bcl-2, memory CD8 + T cells were unable to

proliferate and showed increased levels of apoptosis. Moreover, induction of Id2 within the proliferating CFSE low memory CD8+ T cells coincided with up-regulation of Bcl-2; while levels of the pro-apoptotic Bim molecule were inversely related to Id2 and Bcl-2 (Chapter 4, Fig 1 B,C). Bim levels were upregulated in memory cells following transfection with specific Id2 siRNA (data not shown). However, this increased Bim expression was not statistically significant, most likely due to the fact that Bim expression was assessed in total memory cells instead of CFSE low cells (cells that express the highest levels of Id2). Alternatively, cells expressing high levels of Bim could be lost by apoptosis, making Bim expression difficult to detect in our assay.

This is in agreement with recent studies that have shown that members of the Bcl-2 family play an important role in regulating the contraction phase during an immune response (Hildeman, Zhu et al. 2002; Pellegrini, Belz et al. 2003; Wojciechowski, Jordan et al. 2006). Indeed overexpression of the pro-survival molecule Bcl-2 or deletion of the pro-apoptotic Bim molecules prevented activation-induced cell death of effector cells induced by antigen (Hildeman, Zhu et al. 2002). However, no effect in preventing deletion was assessed in absence of Fas and TNF-R signaling (Hildeman, Zhu et al. 2002).

Bim expression levels are regulated by the pro-apoptotic transcription factor Foxo3a which is involved in the maintenance of memory CD8+ T cells (van Grevenynghe, Procopio et al. 2008; Sullivan, Kim et al. 2012). During an immune response, Foxo3a deficient cells express lower levels of annexin V and survive the contraction phase when compared to wild-type cells (Tzelepis, Joseph et al. 2013). Both Id2 and Foxo3a regulate Bim expression, thus it is probable that these two transcriptional regulators act together in controlling the survival status of effector cells during an immune response.

Bim and Bcl-2 are known to be induced by the family of the gamma chain cytokines, such as IL-2, IL-15, IL-7, and IL-4 (Nakajima, Liu et al. 1997; Schluns, Kieper et al. 2000; Wojciechowski, Jordan et al. 2006), and Bcl-2 in particular seems to be crucial in maintaining survival following stimulation with these cytokines. As discussed earlier (Chapter 1, section 1.4) IL-7 and IL-15 are the major cytokines involved in the homeostatic proliferation of memory cells. Rubinstein et al. showed in mice that IL-15 induced the

preferential accumulation of short-lived effector/memory KLRG1hi CD127lo CD8+ T cells, whereas stimulation with IL-7 favored accumulation of long lived KLRG1lo CD127hi memory precursors cells (Rubinstein, Lind et al. 2008). STAT5 is activated by the receptors of both IL-7 and IL-15 cytokines and has been implicated in the generation of memory cells. Tripathi et al. have also shown that loss of STAT-5 results in the loss of both effector subsets (KLRG1hi CD127low and KLRG1lo CD127hi CD8+ T cells) (Tripathi, Kurtulus et al. 2010). Moreover, STAT5 is necessary for the expression of Bcl-2 in effector CD8+ T cells. Id2 has two putative STAT5 binding domains within its promoter (Yang, Best et al. 2011). Thus, our results as well as others suggest a role for IL-15 induction in the survival of effector cells in which phosphorylation of STAT5 results in increased expression of Id2 and its target molecule Bcl-2.

4. Role of cytokines in the generation of effector cells:

Our data and recent findings reported by Cieri et al. help resolve the debate of how memory cells are generated in humans. Cieri et al. have shown that sorted naïve T cells, when stimulated with low doses of IL-7 and IL-15 in combination with beads coated with anti-CD3 and anti-CD28 antibodies, were able to differentiate into CD45RA+CD62+CCR7+CD95+ T cells. This T cell subset is phenotypically similar to the recently described memory stem T cell (Tscm) population, a memory subset with stem cell–like properties (Gattinoni, Lugli et al. 2011). Our experiments (Chapter 4 Suppl. Fig. 5) support the linear differentiation model in which Tcm give rise to Ttm and Ttm to Tem. Sorted memory CD8+ T cell subsets were stimulated with or without anti-CD3 and anti CD28 antibodies as well as IL-15. We found that IL-15 alone induced differentiation of Ttm into the more differentiated Tem and Temra subsets, while Tem only gave rise to terminally differentiated Temra, as measured by CD45RA, CCR7 and CD27 expression. Interestingly, Tcm did not differentiate into effector cells following 6 days of IL-15 and TCR stimulation; indeed, they did not downregulate CD27 and CCR7 from their cell surface. This is in agreement with recent findings by Cieri et al. where it was shown that a large proportion of Tcm cellsretained the Tcm phenotype even after 16 to 30 days of TCR

and IL-7 and IL-15 stimulation. This could be due to the fact that Tcm are known to encompass self-renewal capabilities and require more time to give rise to effector cells. Another explanation could be that Tcm cells require the combination of TCR stimulation plus both IL-7 and IL-15. Indeed Tcm express higher levels of IL-7Rα (CD127) (Sarkar, Kalia et al. 2008) (data not shown) and lower levels of IL-15Rα (Chapter 4 Suppl. Fig.3C) when compared to Ttm and Tem, suggesting that they may respond better to IL-7 than to IL-15 stimulation. We have shown that differentiation of T cells is mediated not only by changes in the phenotype of the cells, but also by changes at the molecular level, whereby IL-15 stimulation modulates Id2 target molecules only at the Ttm to Tem and Temra transition (Chapter 4 Fig.2). Cieri et al. also showed that the transition of naïve T cells into memory T cells was associated with a decrease in genes that encode the WNT-beta-catenin signaling transducers T cell factor 7 (TCF7) and lymphoid enhancer-binding factor 1 (LEF1), multiple members of the Kruppel-like factor (KLF) family, Forkhead box P1 (FOXP1) and Id3. Cells with an effector phenotype showed increased expression of Id2, eomesodermin, T-bet, Blimp-1 and zinc finger E-box binding homeobox 2 (ZEB2). These findings further confirm our data whereby increased Id2 expression and modulation of its target genes led to differentiation of the memory CD8+ T cells into effector cells. Our results and the findings from Cieri et. al. suggest that CD8+ T cell differentiation is mediated by the progressive expression of multiple transcriptional regulators rather than being mediated by a selective expression of subset-specific transcriptional factors. Indeed some factors seem to control maintenance of naïve T cells (such as Id3), and a shift in the relative expression of other transcription factors (such as Id2) induce differentiation. Recent studies have shown that some small non-coding RNA molecules microRNAs (miRNAs) are differentially expressed in T cell subsets (Wu, Neilson et al. 2007; Salaun, Yamamoto et al. 2011). Among the miRNAs that have been studied in mature CD8+ T cells, miR-29 is highly expressed in naïve CD8+ T cells and suppresses the expression of T-bet, eomes and IFNγ (Ma, Xu et al. 2011; Steiner, Thomas et al. 2011), while miR-155 expression is increased in effector cells (O'Connell, Kahn et al. 2010). Thus, it would be of interest to gain a better understanding of differential regulation of miRNAs profiles that are associated

with effector or naïve T cell gene signatures and how they are regulated by Id2 expression responsible for T cell differentiation.

5. Transcriptional profiles and pathways associated with progressive immune dysfunction:

Exhausted virus-specific CD8+ T cells have been shown to express increased amounts of multiple inhibitory receptors including PD-1 that contribute to impaired T cell function leading to exhaustion (Yamamoto, Price et al. 2011; Peretz, Cameron et al. 2012). We and other groups have used a systems biology approach in order to investigate new molecules or pathways that are modulated in exhausted HIV-specific CD8+ T cells during chronic HIV infection.

Systems biology is an interdisciplinary and emerging approach that studies "*the interactions between all parts of biological systems, and how these interactions give rise to the function and behavior of that system*" (Peretz, Cameron et al. 2012). System biology finds relationships between independent data and creates networks thus providing the opportunity to discover and elucidate new aspects of the immune response to HIV infection. Genomics, transcriptomics, metabolomics, proteomics and high-throughput techniques are merged together in order to investigate systems through an interactive cycle of experimental analysis, modeling and validation.

Gene array analysis identified a number of molecules that were differentially modulated in HIV-specific CD8+ T cells when compared to CMV-specific CD8+ T cells from the same chronic HIV-infected individuals (Chapter 2 Fig.1). Among differentially expressed genes we found that genes upregulated in HIV-specific CD8+ T cells where enriched for those of inhibitory receptors, most notably PD-1. Here we also showed that HIV-specific CD8+ T cells express lower level of Id2 and increased levels of CD27 suggesting a decreased effector differentiation status of these cells when compared to CMV-specific CD8+ T cells. We thought that PD-1 expression could be responsible for the decreased Id2 mRNA levels in the antigen specific CD8+ T cells.

Indeed our findings have shown that PD1 ligation in CD3-CD28 stimulated cells induces both decreased T cell function, such as IL-2 production, but also a specific transcriptional

program in primary human cells that leads to a loss of Id2 expression (Chapter 2 Fig.4). This is in agreement with Quigley et al. in which they used a system biology approach in order to identify transcription factors that were differentially expressed following PD-1 crosslinking in chronic HIV infected patients when compared to elite controllers (Quigley, Pereyra et al. 2010) . They found three genes to be significantly modulated by PD-1: basic leucine zipper transcription factor ATF-like (BAFT), signal transducer and activator of transcription-1 (STAT1), and interferon regulatory factor-9 (IRF9). Increased BAFT expression, upon PD-1 crosslinking, results in decreased T cell proliferation and cytokine production (Quigley, Pereyra et al. 2010). Our study confirmed these results showing that PD-1 signaling not only inhibits proximal TCR signaling, thus regulating proliferation and T cell activation, but it also induces or inhibits genes that are specifically downstream of PD-1.

Duraiswamy et al. analyzed PD-1hi cells from HIV infected donors as compared to PD-1hi cells from healthy individuals (Duraiswamy, Ibegbu et al. 2011). These studies have shown that PD-1hi cells from healthy individuals express an activated phenotype and are not exhausted. The genes modulated in the PD-1hi cells from HIV-infected individuals were similar to those we found to be significantly modulated in the HIV-specific CD8+ T cells in our study. CD28 was found to be downregulated in PD-1 hi cells from HIV infected individuals while CD27 upregulated. The same group also showed that CD127 (IL7Rα), known to be downregulated on effector cells upon antigen stimulation, was increased in PD-1 hi cells from chronic HIV infected individuals, suggesting that PD-1 expression correlates with decreased differentiation of CD8+ T cells (Duraiswamy, Ibegbu et al. 2011). Thus, in agreement with our findings, PD-1 positive cells from chronic HIV-infected individuals express a less differentiated phenotype probably as a consequence of its continuous engagement of the ligand (PD-L1).

Many groups have shown that the skewed differentiation of HIV-specific CD8+ T cells from chronic HIV infected patients is associated with the expression of different negative regulators, such as PD-1 and CD160 and 2B4. The most exhausted cells express simultaneously multiple negative regulatory receptors on their cell surface and their expression positively correlates with viral load and decreased cytokine production

(Yamamoto, Price et al. 2011; Peretz, He et al. 2012). Blocking the interaction of CD160 and HVEM was able to enhance and rescue CMV and HIV-specific CD8+T cell proliferation and cytokine production. Peretz et al. from our laboratory did transcriptional analysis on CD8+ T cell subsets from HIV infected patients that were sorted based on PD-1 and/or CD160 expression (Peretz, He et al. 2012). These studies represent good approaches that help in defining which genes are involved in T cell activation from those that are instead responsible for CD8+ T cell exhaustion. They compared gene expression from each sorted subset and found that the double positive (PD-1+ CD160+) population and single PD-1+ subsets clustered differently in gene signatures (Peretz, He et al. 2012). Based on this finding we also asked whether the defect in Id2 levels are specific to the PD1 positive cells or is it a general defect of exhausted CD8+ T cells? In Chapter 2 Suppl. Figure 7 we assessed Id2 levels in double positive (CD160+, PD-1+), double negative (CD160-, PD-1-) and single positive (CD160-, PD-1+) sorted memory CD8 + T cells from 9 chronic HIV infected individuals. Id2 levels were decreased in the memory PD-1hi populations independently of CD160 expression in the chronic HIV infected patients, suggesting that the defect in Id2 expression during HIV infection is a direct consequence of PD-1 crosslinking and not of other negative regulators.

Thus, a systems biology approach has helped in the identification of molecules, such as Id2 and BATF, that are important in regulating T cell function and that are significantly modulated during chronic HIV infection. Validation of gene array analysis through functional experiments has helped in revealing a cellular mechanism that explains the dysfunction of CD8 T cells during chronic HIV infection.

6. Restoration of HIV-specific function by blocking PD-1/PD-L1 pathway

Despite the efficacy of HAART in decreasing viral load and restoring CD4+ T cell numbers in 70- 90% of patients, it has been shown that low-level residual HIV replication persists despite therapy. Moreover, HAART has been shown to reduce mortality rates; however, it requires life-long treatment and cannot eliminate cells that are latently infected with HIV. Thus, HAART alone cannot completely eradicate HIV. This is mainly due to the fact that HIV persists as an integrated provirus (latent HIV reservoir) that does not

reactivate under normal circumstances. Furthermore, ongoing HIV replication is present in tissues that are difficult to reach by the drugs, such as lymph nodes, gut and central nervous system. Moreover, antigen specific CD8+ T that are functionally impaired in chronic HIV infected non treated patients, are not totally restored under HAART (Kalams, Goulder et al. 1999; Migueles, Osborne et al. 2008). Therefore, it is important to find new potential therapeutic candidates to use in combination with HAART that will be able to boost CD8+ T cell effector functions and also eradicate HIV.

The results presented in this thesis show that the PD-1/PD-L1 interaction results in decreased proliferation and survival of memory CD8+ T cells as well as decreased levels of Id2 mRNA and protein expression (Chapter 2 Fig.4). Our data shows that PD-1 is highly expressed in antigen-specific CD8+ T cells from chronic HIV infected individuals and positively correlates with PD-L1 expression in monocytes (Chapter 2 Suppl. Fig 1D Fig.3C). This is in agreement with other groups that have shown increased PD-L1 levels during chronic HIV infection in monocytes (Boasso, Hardy et al. 2008). We show that the continuous interaction between PD-1 and its ligand resulted in downregulation of Id2 that led to decreased effector T cell function (Chapter 2 Fig.4). We also show that effector function could be restored by the overexpression of an Id2 construct in *ex vivo* primary CD8+ T cells from HIV infected subjects (Chapter 2 Fig.5). Therefore our findings suggest that Id2 downregulation induced by PD-1 triggering during HIV infection is a novel mechanism by which upregulation of PD-1 leads to defective survival and function of CD8+ T cell effectors.

The combination of systems biology, phenotypic and functional profiling suggests that PD-1 is an ideal target for therapeutic interventions aimed at restoring CD8+ T cell function in HIV infection. Different groups have already shown that PD-1 blockade in various diseases such as HIV, hepatitis B, and hepatitis C is able to restore T cell proliferation, cytokine production and thus effector function (Day, Kaufmann et al. 2006; Petrovas, Casazza et al. 2006; Trautmann, Janbazian et al. 2006; Boni, Fisicaro et al. 2007; Radziewicz, Ibegbu et al. 2007; Nakamoto, Kaplan et al. 2008; Velu, Titanji et al. 2009). While it has been suggested that blocking PD-L1 has a better capacity to restore T cell function than targeting PD-1 itself

(Forler, Kocher et al. 2003; Trautmann, Janbazian et al. 2006), in both cases blocking this interaction resulted in increased HIV-specific CD8+ T cell proliferation (Rosignoli, Lim et al. 2009). Recent *in vivo* studies have been conducted in the rhesus macaque SIV infection model (Velu, Titanji et al. 2009; Titanji, Velu et al. 2010). Velu et al. showed that PD-1 blockade resulted in increased frequencies of SIV-specific CD8+ T cells, increased cytotoxic function and decreased viral load (Velu, Titanji et al. 2009).

Thus our findings are in agreement with previous studies that provide a strong rationale for initiating human clinical trials targeting PD-1 with blocking antibodies in HIV-infected patients. Elucidating the signaling cascade responsible for PD-1 crosslinking in CD8+ T cells and its effects on Id2 downregulation may reveal further specific targets for therapeutic intervention. Immunotherapies such as cytokine treatment or PD-1 blocking antibodies that increase Id2 expression, in combination with antiretroviral therapy may provide a novel way to restore and enhance the immune response. Blocking PD-1 might not only increase differentiation and proliferation of exhausted antigen specific CD8+ T cells that are blocked in a terminally differentiated phenotype but could also reactivate the viral reservoir from latently infected CD4+ T cells (unpublished data from the Chomont Lab). A recent study on HAART treated subjects showed that *ex vivo* stimulation of HIV- specific CD8+ T cells with Gag and IL-2 facilitates elimination of latent viral reservoirs after virus reactivation (Shan, Deng et al. 2012). This study shows that stimulation of CD8+ T cells is able to induce effector function, proliferation, and differentiation. Thus, combination therapy that combines HAART with blocking antibodies for PD-1 signaling and cytokines such IL-15 and IL-7 that enhances differentiation of HIV-specific CD8+ Ttm and Tem into functional effector cells might prove to be more potent in increasing CD8+ T cell killing of latently infected cells and to prevent reactivated virus from reinfecting new CD4+ T cells.

7. Therapeutic potential of IL-15 in restoration of immune function:

7.1 In HIV therapy

Gamma chain cytokines (IL-2, IL-7, IL-15 and IL-21) that play a fundamental role in the regulation of T cell function have been investigated for their ability to restore immune

function in HIV therapy. During HIV infection, production of some of these cytokines, such as IL-2 and IL-15, is downregulated, while IL-7 levels are increased as a consequence of lymphopenia. Extensive phase I and II studies have been done in the late 1990s with IL-2 as a candidate cytokine for treatment of patients with HIV infection. These studies demonstrated that this cytokine increases the frequency of naïve and central memory CD4 T cells, while it had no significant effect on CD8+ T numbers (Kovacs, Baseler et al. 1995; Kovacs, Vogel et al. 1996; Sereti, Imamichi et al. 2005). However, phase III clinical trials demonstrated that IL-2 increased CD25 and FOXOp3 expression (and thus regulatory T cells), which was associated with increased risk of opportunistic diseases (Sereti, Imamichi et al. 2005; Abrams, Levy et al. 2009; Weiss, Letimier et al. 2010). Similar to IL-2, IL-15 signals through the IL-2Rβ (CD122) and γ chain (CD132) receptors and plays an essential role in T cell survival (Wu, Xue et al. 2008).

Our data as well as others demonstrate that stimulation of cells with IL-15 enhances memory CD8+ T cell function in HIV-infected subjects and promotes their survival (Chapter 4 Fig.3), (Waldmann and Tagaya 1999; Marks-Konczalik, Dubois et al. 2000) (Mueller, Bojczuk et al. 2003; Rodriguez, Arulanandam et al. 2007) suggesting a possible role for IL-15 as a vaccine adjuvant or for therapeutic application. We also show that stimulation of memory CD8+ T cells subsets with IL-15 upregulates Id2 and its target genes (Chapter 4 Fig.2C), which are involved in increasing survival, proliferation and differentiation of Ttm in Tem and terminally differentiated Temra. We also show that increased differentiation induced by IL-15 relieves HIV-specific CD8+ T cells from their functional and phenotypic block in a transitional memory phenotype (CCR7-, CD27+, CD45RA-) (Chapter 4. Fig.3D). Indeed during HIV infection these cells are unable to differentiate into functional terminally differentiated effector and effector memory cells (CD45RA+/-, CD27-, CCR7-) even under HAART. HIV-specific CD8+ T cells have decreased Granzyme B levels and thus are not capable of efficient killing of virally infected cells (Jaspan, Gaumer et al. 2003). However we show that IL-15 alone or in combination with anti-CD3-CD28 antibodies resulted in increased Granzyme B production from Ttm and Tem subsets (Chapter 4 Fig. 3A). Moreover, we have optimized a flow-cytometry

based assay that allows measurement of the cytolytic activity of antigen specific CD8+ T cells at the single cell level (Chapter 3). With this assay we have also shown that increased Granzyme B expression after IL-15 and specific HIV-peptide stimulation is responsible for the enhanced ability of HIV-specific CD8+ T cells to recognize and kill HIV-pulsed CD4+ target cells (Chapter 4 Fig. 3B). Our group has also shown that IL-15 was a more potent inducer of viral production from latently infected CD4+ T cells than IL-7 (Vandergeeten, DaFonseca et al. 2011; Vandergeeten, Fromentin et al. 2013). Thus our results provide a good rationale for complementing HAART treatment with IL-15 therapy in eradication strategies aimed at boosting HIV-specific CD8+ T cell responses. In support to these findings, *in vivo* studies in SIVmac251 infected macaques have shown that administration of IL-15 increased the frequency of effector CD8+ T cell which was associated with a decrease in the number of infected cells in the lymph nodes (Mueller, Do et al. 2008). Interestingly, administration of IL-15 in untreated animals does not increase viral replication from SIV infected cells (Mueller, Petrovas et al. 2005; Picker, Reed-Inderbitzin et al. 2006), suggesting that combination therapy with IL-15 during acute infection might be a better contribution for purging HIV virus from latently infected cells and inducing their killing from HIV-specific CD8+ T cells.

7.2 Il-15 as vaccines adjuvants

Many therapeutic HIV-1 vaccines have been tested so far for their efficacy, however only subsets of these have reached phaseII/III clinical trials (Barouch and Korber 2010). An adenovirus 5 (Ad5)-based HIV vaccine expressing HIV-1 clade B gag, pol and nef reached the Step and Phambili phase IIb trials, however this study was halted as it was shown that this vaccine had no effect in preventing HIV infection despite induction of HIV-specific CD8+ T cell responses in 80% of the subjects (Buchbinder, Mehrotra et al. 2008; Sekaly 2008; Corey, McElrath et al. 2009). The RV144 clinical trial in Thailand using a canarypox vaccine ALVAC HIV vCP1521 (env, gag, pol), demonstrated more promising results with a decrease in the rate of acquisition of HIV-1 infection of 31.2% when compared to placebo (Cohen 2009; Rerks-Ngarm, Pitisuttithum et al. 2009). The transient protection from

acquisition of infection that was seen was attributed to non-neutralizing V1/V2 antibodies and not mediated by CD8 cytotoxic T cells.

The main goal of current vaccines against HIV is to induce high avidity CD8+ T cell specific responses of sufficient breadth and magnitude. Moreover CD8+ T cells must be activated and capable of responding at the site of infection in order to limit viral replication. New strategies have evolved to utilize cytokines as adjuvants in HIV vaccine development to enhance antiviral immune responses. Villinger et al. studied influenza and toxoid – specific vaccine-induced responses following IL-2 and/or IL-15 co-administration in rhesus macaques (Villinger, Miller et al. 2004) . In this study they show that the addition of IL-15 alone increased primary effector and memory vaccine responses better than the combination of IL-2 plus IL-15. This suggests that IL-15 may be better than IL-2 for enhancing vaccine responses. This is in contrast with the study of Hryniewicz et al. in which the impact of IL-15 on viral replication and T cell responses after live poxvirus vaccines was assessed in SIV (mac251) infected macaques. They showed that IL-15 had no effect on SIV-specific immune responses or viral control (Chong, Egan et al. 2007; Hryniewicz, Price et al. 2007). This might be due to the timing of delivery of the cytokines. Indeed, Nanjappa et al. have shown, for example, that administration of IL-7 during the contraction phase (7-14 days) and not during the expansion phase has a better effect in the enhancement of antigen-specific CD8+ T cells response after viral infection or vaccination (Nanjappa, Walent et al. 2008).

Further support for IL-15 as good candidate for vaccine strategies stems from its ability to enhance survival of high- avidity CD8+ T cells compared to low-avidity CD8+ T cells (Oh, Perera et al. 2004). At the single cell level, IL-15 also increases surface expression of the TCR coreceptor CD8$\alpha\beta$, leading to better avidity of these cells (Oh, Perera et al. 2004). Because high-avidity CD8+ T cells are more efficient in vaccine strategies, the use of IL-15 as a vaccine adjuvant could improve CD8+ T cell responses both quantitatively and qualitatively.

Moreover the hallmark of vaccine strategies is the ability to generate specific immunological memory that is long-lived and gives rise to central memory and effector

memory CD4+ and CD8+ T cells (Tcm and Tem). Live attenuated viruses such as Yellow fever 17D (YF-17D) and vaccinia viruses (VV) are the most efficient vaccines to date and they have been used as models to study immune protection (Miller, van der Most et al. 2008). Vaccination with YF-17D and VV results in increased expansion of antigen-specific CD8+ T cells with an effector phenotype followed by generation of effector memory cells that are maintained up to 10 years providing long lasting immune protection (Miller, van der Most et al. 2008; Akondy, Monson et al. 2009). Vezys et al. have also shown that vaccination in mice that induce Tem responses induces better immune responses after LCMV infection when compared to Tcm (Vezys, Yates et al. 2009). Thus, vaccine strategies should promote persistence of effector memory CD8+ T cell.

Moreover as HIV-1 transmission occurs in the majority of cases by sexual transmission with the mucosa as the primary site of infection in the majority of HIV-infected subjects, it is important to induce a potent antiviral T cell and antibody responses in these local tissues. Different groups have shown that T cell responses that activate effector cells at the mucosa within the first hours or days following HIV or SIV infection are more protective than responses that activate Tcm populations that have limited migration to peripheral tissues and cannot rapidly respond to infection (Chun, Engel et al. 1998; Haase 2005; Keele, Giorgi et al. 2008; Haase 2010). Indeed, the frequency of Tcm at these peripheral sites is low because of the expression of Lymph nodes homing receptors (CCR7) (Masopust, Vezys et al. 2001; Appay, Douek et al. 2008; Masopust 2009). Although Tcm have self-renewal capabilities, they need around 3 days before undergoing expansion after re-exposure to the pathogen (Whitmire, Eam et al. 2008). Therefore vaccines that are able to induce Tem generation would result in a more efficient CD8+ T cell response at peripheral sites of infection such as the mucosa and could prevent viral dissemination. These hypotheses were confirmed by Hansen et al. where they have shown that the induction of differentiated effector cells (Tem) at such early replication sites by a rhesus CMV-based SIV vaccine improved the efficacy of the vaccine and decreased the acquisition of infection (Hansen, Vieville et al. 2009; Hansen, Ford et al. 2011). Our findings show that the induction of Id2 by IL-15 drives differentiation of effector memory cells, supporting again

the use of IL-15 or molecules that increase Id2 expression as adjuvants in vaccine strategies. The results presented in this thesis clearly demonstrate that Id2 expression levels are significantly increased in both Tem and Temra when compared to naïve and central memory CD8+ T cells (Chapter 4 Figure 1A), suggesting that measurement of Id2 expression levels could be a useful marker to investigate effector function at the mucosal sites in order to assess the levels of effector CD8+ T cells and predict induction of a protective immune response following vaccination.

CONCLUSIONS

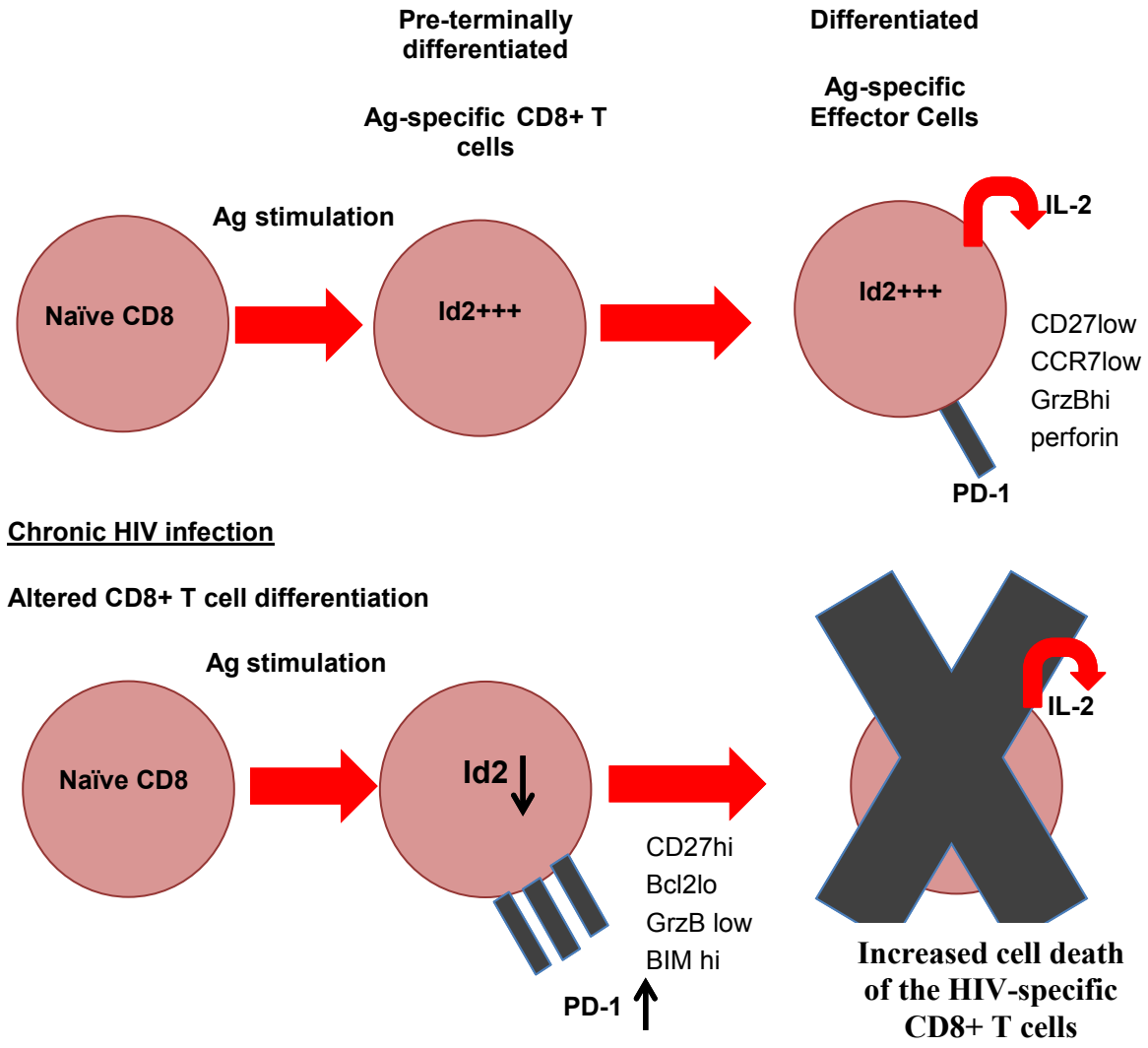

Figure1. Model of CD8+ T cell exhaustion: After antigen priming, activated virus specific CD8+ T cells receive signals that induces Id2 expression. Id2 promotes differentiation of effector cells which are able to exert cytotoxic function, proliferation and cytokine production. However, during chronic HIV infection, the increased levels of PD-1 and its ligand on pre-terminally differentiated cells leads to decreased expression of Id2 which results in decreased capacity of these cells to survive and differentiate into mature effector cells.

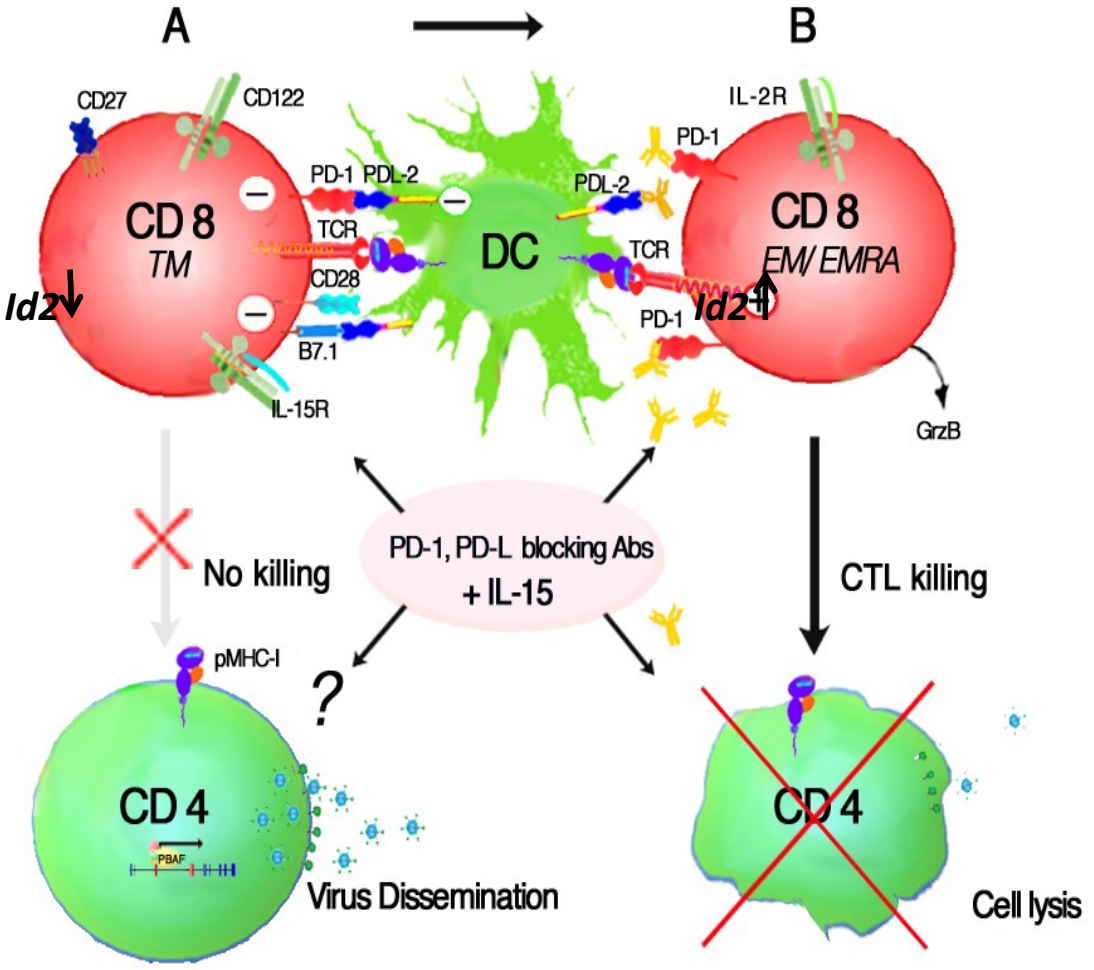

Figure 2. Restoration of exhausted antigen-specific CD8+ T cells by PD-1/PD-L1 blocking antibodies and IL-15 treatment. A) During HIV infection antigen-specific CD8+ T cells express high levels of PD-1. The interaction between PD-1 and PD-L1 on DCs leads to decreased Id2 levels and a block of antigen specific CD8+ T cells in a Ttm phenotype that are not able to exert cytolytic activity. B) Immunotherapy with IL-15 cytokine and blocking PD-1/PD-L1 antibodies can prevent downregulation of Id2 expression and induce proliferation, survival and differentiation of Ttm tetramer+ CD8+ T cells into cytotoxic granzyme producing effector Tem/ Temra. Increased differentiation of CD8+ T cells driven by IL-15 leads to increased killing of CD4+ T cells that are replicating virus. Figure modified from our recent review (Adpapted from (Kulpa, Lawani et al. 2013)).

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APPENDIX

JOURNAL PUBLICATIONS