Effect of antigen load and viral sequence diversification on

HIV-specific CD8$^+$ T cells

McGill University

August 2009

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Ph.D.)

© Loury Janbazian 2009

Abstract

Virus-specific $CD8^+$ T cells have been implicated in the control of acute HIV and SIV infections. Although present in chronic HIV-1 infection, $CD8^+$ T cells exhibit impaired functions due to unidentified molecular signatures. This has urged researchers to revisit parameters that define efficacious $CD8^+$ T cell responses in HIV-1. To this date, polyfunctionality has emerged as the key feature of $CD8^+$ T cell efficacy in chronic infection. Moreover, it is established that T-cell Receptor (TCR) diversity in HIV-specific $CD8^+$ T cells plays a critical role in controlling viremia. Although TCR repertoire studies have been performed in the context of several acute and persistent viral infections including HIV-1, longitudinal studies that aim to study the turnover of the HIV-specific $CD8^+$ TCR repertoire and link HIV-specific $CD8^+$ T cell clonotypes to functional profiles have been limited. We therefore aimed to 1) define a molecular signature of $CD8^+$ T cell exhaustion in HIV-1 infection, 2) study the effect of antigen load and, 3) the effect of viral sequence diversification on the clonality, functionality, and phenotype of HIV-specific $CD8^+$ T cells. Our first set of results identified PD-1 as a molecule of exhaustion on HIV-specific $CD8^+$ T cells and showed a positive correlation with viral load. Interestingly, blocking PD-1 interaction with its ligand alleviated the functional dysfunction of HIV-specific $CD8^+$ T cells. This set of data prompted us to further examine the effect that "antigenic absence" has on the fate of HIV-specific CD8+ T cells. For this, we chose two circumstances; the institution of HAART and emergence of HIV-specific $CD8^+$ T cell epitope escape. Our second set of data provided clear evidence of a HAART-mediated functional reconstitution of the HIV-specific $CD8^+$ T cell compartment on the clonal level. This was attributed by different mechanisms, namely the improvement of function of persisting clonotypes and the recruitment of new clonotypes which were polyfunctional. The functional improvement was in parallel with the significant increase in T_{CM} and T_{EMRA} populations of HIV-specific $CD8^+$ T cells and a drop in PD-1 expression. In our third dataset, we observed functional improvement of HIV wild-type-specific $CD8^+$ T cells concomitant with decay in antigen, due to emergence of escape. This was accompanied by low PD-1 expression of wild-type HIV-specific $CD8^+$ T cells and a selection of high avidity clonotypes that led to the overall bias in the TCR repertoire observed longitudinally. Altogether, our data indicate that

persistence of high loads of antigen could drive the functional impairment of virus-specific $CD8^+$ T cell responses observed during chronic HIV-1 infection and defining the optimal antigenic concentrations that lead to the generation and maintenance of poly functional clonotypes could be promising in therapeutic interventions.

Résumé

Les cellules T $CD8^+$ sont impliquées dans le contrôle des infections virales aiguës tels que les virus VIS et VIH. Bien que ces cellules T $CD8^+$ spécifiques au VIH persistent durant la phase chronique de l'infection, leur fonctionnalité semble être altérée par un mécanisme non identifié. Cela a exhorté les chercheurs à revoir les paramètres qui définissent l'efficacité réelle des lymphocytes T $CD8^+$ en générale et surtout lors d'infection par le VIH. À date, il est admis que la polyfonctionnalité des cellules T $CD8^+$ soit l'élément clé de l'efficacité de la réponse immunitaire durant l'infection chronique. De plus, il est établi que la diversité du récepteur de cellule T (RCT) occupe un rôle important et crucial dans le contrôle de la virémie. Bien que plusieurs études sur le répertoire des RCT aient été menés par plusieurs groupes dans le contexte des infections aigues et chronique par le VIH, il n'existe néanmoins pas des données combinant la fluctuation et la fonction du répertoire spécifique au VIH au cours de la progression de la maladie. De ce fait, il était important de réaliser des études longitudinales qui ont pour but principal l'étude de la régénération du répertoire T $CD8^+$ spécifiques au VIH durant les différentes phases de l'infection. Dans mes travaux de doctorat, nous avons exploré les mécanismes responsables des l'inefficacité de cellules $CD8^+$ cours de l'infection par le VIH. En utilisant plusieurs techniques immunologiques et génétiques, nos objectifs visaient à : 1) Définir une signature moléculaire d'épuisement, 2) Étudier l'effet de la charge virale et, 3) Caractériser l'effet de la diversification des séquences virales, sur la clonalité, la fonctionnalité, et le phénotype des cellules T $CD8^+$ spécifiques au VIH. Nous avons en premier lieu identifié le rôle de la molécule PD-1, une des molécules régulatrices qui joue un rôle critique dans le contrôle de la réponse immunitaire, dans l'inhibition de la fonctionnalité des cellules T $CD8^+$ et que son effet était lié positivement à la charge virale. Néanmoins, le blocage de l'interaction de PD-1 par son ligand restore le fonctionnement des cellules T $CD8^+$ spécifiques au VIH. Ces données nous ont encouragés à poursuivre l'analyse en étudiant l'effet de l'absence de la virémie; le cas du traitement antirétroviral (HAART); sur le sort des cellules T $CD8^+$ spécifiques au VIH. Les données obtenues démontrent le bénéfique rôle de HAART sur l'émergence d'un vaste et nouveau répertoire de clones T $CD8^+$ spécifiques au VIH et qui est polyfonctionnel. A noter que l'amélioration fonctionnelle était accompagnée par une

augmentation significative du pourcentage des cellules effectrices et centrale mémoire dirigées contre le VIH et surtout accompagné par une réduction de l'expression de la molécule PD-1 à la surface de ces cellules T $CD8^+$. Finalement, dans nos dernières données effectuées dans ce projet, nous avons observé que l'amélioration fonctionnelle des cellules CD8 spécifiques au VIH, était accompagnée d'une diminution concomitante de la charge virale. Cela a été aussi relié par de taux faibles d'expression de PD-1 sur les cellules $CD8^+$ spécifiques ayant une forte avidité. Cela a conduit à une polarisation longitudinale dans le répertoire TCR. En conclusion, nos données indiquent que la persistance d'une charge virale pourrait conduire à un état non fonctionnel des cellules T $CD8^+$ spécifiques au VIH. Par contre, la définition d'une charge optimale et adéquate sera requise pour maintenir des cellules T $CD8^+$ polyclonales pourrait avoir des répercussions cliniques importantes.

Table of Contents

List of Figures

Chapter 1: Introduction

Chapter 2: Upregulation of PD-1 expression on HIV-specific $CD8^+$ T cells leads to reversible immune dysfunction

Chapter 3: Highly active antiretroviral therapy (HAART) leads to the reconstitution of HIV-specific $CD8^+$ T cell receptor repertoire

List of Tables

Contributors

Chapter 2: Upregulation of PD-1 expression on HIV-specific $CD8^+$ T cells leads to reversible immune dysfunction

L.T. did the experiments and wrote the paper. L.J., N.C., E.A.S. and S.G. gave previous help with the final experiments and with writing the paper. B.B. generated pMHC monomers. J.-P.R. and R.S.B. provided samples from research subjects. E.D. performed the viral sequencing. H.S. and R.S.B. developed the CBA cytotoxic kit. Co senior authors E.K.H. and R.-P.S. supervised all experiments and wrote the paper.

Chapter 3: Highly active antiretroviral therapy (HAART) leads to the reconstitution of HIV-specific $CD8^+$ T cell receptor repertoire

L.J. did the all the experimental work specifically TCR sequencing, phenotypic, and functional profiling of HIV-specific $CD8^+$ T cell responses. L.J. also analyzed the data and wrote the manuscript. L.T. and J.B. provided help with data analysis and participated in editing the paper. D.A. provided help with initial experiments specifically sorting HIV-specific $CD8^+$ T cell populations. R.H., N.C., and D.A.P. gave previous help to the preparation of figures and participated in editing the manuscript. E.D. performed the HIV-1 bulk sequencing from the research subjects. J.-P.R. and M.R.B. provided samples from research subjects. R.K. provided technical service (sorting facility) at the National Institutes of Health. D.D. and R.P.S. supervised all experiments and wrote the paper.

Chapter 4: The effect of viral sequence diversification on the fate of HIV-specific $CD8^+$ T cells

L.J. did the all the experimental work specifically TCR sequencing, phenotypic, and functional profiling of HIV-specific $CD8^+$ T cell responses and wrote the manuscript. L.T., T.B and B.Y.D. provided help with data analysis. D.A. sorted HIV-specific $CD8^+$ T cell populations. D.A.P. provided help in discussion and in writing the manuscript. E.D. performed the HIV-1 bulk sequencing from study participants. J.-P.R. and M.R.B. provided samples from research subjects. R.K. provided technical service (sorting facility)

at the National Institutes of Health (NIH). D.D. and R.P.S. supervised all experiments and edited the paper.

Editing of the thesis: I would like to thank Ahmad El Sayed and Bader Yassine Diab for editing and formatting the thesis.

Acknowledgments

I would like to thank everyone who was directly and indirectly involved in this work. Special thanks are for my Ph.D. supervisor, Dr. Rafick Sekaly, for allowing me to do research with his group, teaching me scientific rigor and most importantly having faith in me. Special thanks are for Lydie Trautmann, for always being there both scientifically and on the personal level.

My acknowledgements go to Drs. Daniel Douek, David Price, Jason Brenchley, Philip Scheinberg, and Richard Koup from the National Institutes of Health (NIH), for being a major part of this work and for always being there for valuable discussions and feedback.

Special thanks are for my committee members Drs. Hugo Soudeyns, Brian Ward, and Elias Haddad for their support and valuable feedback throughout the years.

None of this would have been possible without the outpouring support of my dear friends Tom Baumgartner, Ahmad El Sayed, Lena Kalfayan, Mehrnoush Douroudchi, Jill Feilhauber, Justin Ries, Oleg Yegorov, Elias Said, Denis Gaucher, Tarek Ayyash, and Luciana Tonelli. I wish you all the best and thank you for being there for me.

Finally yet importantly, special thanks are for my family and loving husband, whose encouragement and love, simply kept me going on.

Thank you all.

I dedicate this dissertation to the loving memory of Raffi Ghougassian, whose laughter and music will always be alive in my heart.

1 Overview of $CD8^+$ T cells in HIV infection

1.1 HIV infection: General considerations

1.1.1 Overview of the HIV pandemic and a historical perspective

Since its isolation in 1983 [1, 2], HIV-1 is a global threat and a leading cause of death. Recent reports by the World Health Organization (WHO), estimated that 33.2 million people live with HIV-1 worldwide. In 2007 alone, 2.7 million new cases were reported, of which 1.9 million occurred in sub-Saharan Africa, the epicenter of the pandemic (http://www.unaids.org/en/KnowledgeCenter/HIVData/GlobalReport/2006). Sadly, the HIV-1 pandemic in sub-Saharan Africa bears a women's face, where 51% of the infected adults are women. This issue poses an additional concern to the pandemic, in regards to increased rates of mother-to-child vertical transmission, where antiretroviral therapy is limited. Moreover, it is estimated that between 2008 and 2010 there will be 45 million new infections in resource-limited regions of the world. Although sub-Saharan Africa remains the epicenter of the pandemic, the prevalence rates of HIV/AIDS in south, south-east Asia and Eastern Europe are becoming major causes of concern. The main route of transmission in these countries is intravenous drug use and men having sex with men (MSM), whereas heterosexual contact is the major mode of transmission in sub-Saharan Africa. Furthermore, the advent of Highly Active Anti Retroviral Therapy (HAART), where available, has increased the life expectancy of HIV-infected patients and decreased mother-to-child transmission. Therefore, despite the general knowledge and awareness, HIV-1/AIDS remains a global threat. HIV-1 challenges the immune system by overcoming innate, adaptive, and intrinsic immunity. Primary HIV-1 infection is characterized by a peak in plasma viral loads, a drop in $CD4^+$ T cell counts, and absence of neutralizing antibodies. Usually primary infection is asymptomatic. This initial viral load drops concomitant with the emergence of $CD8^+$ T cells and a viral set point is reached in chronic infection. Viral set points vary among different individuals and predict disease progression. Risk of transmission is highest at primary infection while viral diversity increases throughout the disease course and poses a major impediment to the development of effective vaccines (Figure 1) [3].

1.1.2 Structure of HIV-1: Genome organization and role of different HIV proteins in infectivity

HIV-1 and HIV-2 belong to the family *Retroviridae*, genus *Lentivirus*. HIV-1 has arisen from cross-species transmission of a chimpanzee's virus to humans [4], whereas HIV-2 is thought to originate from cross-species transmission of a sooty mangabey virus [5]. Unlike HIV-1, HIV-2 is associated with slower disease progression [6]. There are 3 main groups of HIV-1, namely M, N, and O. Group M accounts for the majority of HIV-1 infections worldwide and has 9 described subtypes or clades (A-D, F-H, and J-K), where the clade C has become the most prevalent transmitted virus in Africa [7]. Notably, our knowledge about HIV-1 pathogenesis is often derived from studies with clade B [3].

The HIV-1 genome is diploid, consisting of two positive polarity stranded RNA molecules [8]. The HIV-1 genome is less than 10kb and encodes for structural and accessory proteins. Herein, the functions of each of these proteins are described, as this will help gain insights into the expression kinetics and escape mechanisms from epitopes that originate from these regions.

Gag encodes for a p55 poly-protein that is cleaved into four structural proteins namely, p17 (matrix protein), p24 (capsid), p7 (nucleocapsid) and p6, which has a role in budding. *Env* encodes for SU (surface or gp120) and TM (transmembrane or gp41) needed for cell tropism and fusion, respectively. *Gag* and *Env* together make up the core of the virion and the outer membrane envelope. *Pol* encodes for RT (reverse transcriptase), PR (protease) and IN (integrase). All three are needed for essential enzymatic activity and viral replication. The regulatory proteins of HIV-1 are *Tat* and *Rev* [8, 9]. *Tat* stands for transcriptional activator and is a crucial activator of the long terminal repeat promoter element and initiates transcription of viral genes. *Rev* functions as a nuclear RNA export factor needed for the translocation of unspliced mRNA from the nucleus to the cytoplasm. Therefore, *Tat*, *Rev*, and *Nef* are called early gene products and their expression kinetics are prompt. On the other hand, the accessory proteins of HIV-1 are *Vif*, *Vpr*, *Vpu*, and *Nef* [10]. One of the well-documented functions of *Nef* is its ability to down-regulate MHC-class I on the surface of infected cells and thus provides an evasion mechanism from CD8+ T cell immunity [11]. *Vpu* stands for viral protein U and is needed for the disruption

of CD4-gp160 complexes in the ER [12]. The degradation of CD4-gp160 complexes is advantageous for the virus, as it allows gp160 transport to the cell surface for viral assembly and reduces density of CD4 on the surface of infected cells, thereby limiting its interaction with *Env* proteins, hence facilitating the release of virions [12]. Vif stands for viral infectivity factor and is needed to overcome the antiviral activity of APOBEC3G in the cell cytoplasm [13]. Vif is also needed for the disassembly of virus after infection and in the trafficking of viral pre-integration complex to the nucleus. Moreover, by interacting with Gag, viral protease, and RNA, Vif is needed for the proper particle assembly and stability of the reverse transcription complex. Finally, Vpr, which stands for viral protein R, has several newly identified functions as a critical component in HIV-1 expression from unintegrated HIV-1 DNA, which may play a significant role in HIV-1 pathogenesis [14]. Therefore, since *Gag*, *Env*, and the accessory proteins are all *Rev* dependent, their expression follows a delayed kinetics in contrast to *Tat*, *Rev* and *Nef*.

1.1.3 Cellular tropism of HIV-1

HIV-1 infects activated $CD4^+$ T helper cells through interactions between CD4 and the highly glycosylated gp120. This high tropism of gp120 to the CD4 coreceptor needs also the involvement of the chemokine receptors CXCR4 and CCR5. Viruses using CXCR4 as a coreceptor for entry into target cells occur later in infection and are called R4 viruses, whereas viruses using CCR5 as a coreceptor appear in early infection and are called R5 viruses. Moreover, HIV-1 can also infect Dendritic Cells (DCs), since they express CD4, CXCR4, and CCR5 albeit at low levels [3]. It was also shown that gp120 interacts with dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN). DC-SIGN does not allow fusion nor circumvents the need for coreceptors needed for infectivity. DC-SIGN is needed for the aggregation of HIV-1 particles on the surface of DCs, thus contributing to the cross-infection of $CD4^+$ T cells that are in the vicinity. Similar to DC-SIGN, syndecan is expressed on macrophages and accounts for viral dissemination and tropism by binding gp120 [15]. Natural Killer cells express CD4 and are targets of HIV-1 infection [15, 16]. Recently, it was shown that gp120 interacts and signals though the integrin $\alpha_4\beta_7$ which is a gut homing receptor for peripheral T cells, suggesting a reason for the loss of activated $CD4^+$ T cells from the Gut Associated Lymphoid tissues (GALT) [17].

1.1.4 Overview of innate and adaptive responses to HIV-1: General considerations

The key success of the immune system in fighting a plethora of pathogens is governed by the harmonious orchestration of innate and adaptive immune responses. Innate immune responses are the first line of defense against pathogens and later acquire a role in regulating adaptive responses [18]. Unlike adaptive responses, the cellular components of innate immunity lack specificity to recognize pathogens and develop immunological memory. The cellular components of innate immunity express an array of receptors that sense microbial nucleic acids and generate signaling cascades that ultimately lead to viral clearance. Of these receptors globally called Pattern Recognition Receptors (PRRs), the toll like receptors (TLRs) and intracellular sensors such as retinoic-acid inducible gene-I (RIG-1) and melanoma differentiation-associated antigen 5 (MDA5) sense viral nucleic acids [19, 20]. Bearing in mind the route of transmission of HIV-1 at mucosal sites, it is only expected that HIV-1 would initially interact with cellular components of innate immunity as well as $CD4^+$ T cells [21, 22]. At the same time, it is not surprising that innate responses against HIV-1 would be compromised and greatly hinder the development of robust adaptive responses [23, 24]. Recently, the interactions between HIV-1 and innate responses such as the role of $\gamma^+\delta^+$ T cells in epithelial cells [23], NK cells [16], NK-DC interactions [25], role of APOBEC3G and TRIM5α [26, 27] in different cells of the innate immune system are being unfolded. As DCs play an immense role in shaping adaptive immune responses, attention has been given to these cells to help understand viral transmission and ultimately its impact on generating adaptive responses [18, 28]. Unlike $CD4^+$ T cells, infection of DCs with HIV-1 is limited [22]. In vitro and in vivo studies have addressed the potential reasons for this limited infectivity and point to the presence of host factors. It was shown that R5 viruses replicate better in DCs than R4 viruses, and a potential reason for this could be the fact that immature DCs do not express CXCR4 [29]. Moreover, antiviral actions of APOBEC3G, but not TRIM5α restrict HIV-1 infection in monocyte-derived DCs [30]. However, insights into how DC-SIGN binds gp120 and forms a synapse that leads to transfer of HIV-1 particles to T cells need to be investigated. It is not surprising that the classical DC functions seem to be perturbed in HIV-1 infection. Upon TLR signaling, DCs undergo maturation to elicit adaptive responses [31]. It seems that HIV-1 does not lead to full maturation of DCs probably due

to yet uncharacterized perturbations in PRR signaling pathways. If not matured, DCs could favor tolerance instead of inducing adaptive responses [32, 33]. It is known that HIV-1 interacts with plasmacytoid dendritic cells (pDCs), possibly by triggering TLR7, leads to production of IFN-α in the acute and chronic phases of infection [34, 35] . There has been controversial data surrounding the role of pDCs in HIV-1 infection. It is not yet known whether the low numbers of pDCs in the blood of HIV-1 patients [36] is due to their loss or redistribution. Nonetheless, high levels of functional markers of pDCs, such as IFN-α and the tryptophan-catabolizing enzyme indoleamine 2,3-dyoxigenase (IDO), have been reported in HIV, indicating a potential role for pDCs in HIV-1 infection [37] [38]. It is reasonable that immune activation in immunocompromised hosts could also chronically activate pDCs, and this could result in circumventing adaptive T cell responses by either IDO-mediated mechanisms or IFN-α induced apoptosis of T cells, rather than eliciting effective anti-viral activity. Interestingly, Brenchley et al proposed that microbial translocation could result in systemic immune activation in chronic HIV infection, which is a hallmark of HIV-1 infection [39], and found a positive correlation between plasma lipopolysaccharide (LPS) levels and IFN-α [40]. Although, LPS does not activate pDCs and the source of IFN-α needs to be assessed, this correlation indicates a cross talk between a constantly activated innate response and compromised adaptive T cell responses. Therefore, understanding how HIV-1 is transferred from DCs to T cells, if there are any differences when an infected DC versus an uninfected DC transfers HIV to T cells and the molecular motifs that activate PRRs such as the one identified in HCV [41] need to be addressed. Also, the general milieu that leads to elicitation of the different types of adaptive immune responses, and deciphering innate responses in resistant versus susceptible hosts, can prove efficacious in understanding how innate responses influence adaptive responses and could prove to be potential therapeutic targets.

1.2 Dysfunction of HIV-specific $CD8^+$ T cells

1.2.1 Evidence for the role of $CD8^+$ T cells in HIV infection

HIV-1 infection is characterized by the gradual loss of HIV-specific $CD4^+$ T cells and by the chronic hyper-activation of the immune system [42]. Paradoxically, this hyper-activation of immune cells is often linked to their exhaustion or impaired function.

Evidence for the role of $CD8^+$ T cells was first reported and directly shown to have antiviral effects in the Simian Immunodeficiency Virus (SIV) model. Upon transient depletion of $CD8^+$ T cells in macaques, by the use of monoclonal $CD8^+$ T antibodies, primary viral burdens were high. Viral loads returned to original levels concomitant with the rise of $CD8^+$ T cells. However, this phenomenon was irreversible when depletion of $CD8^+$ T cells exceeded 28 days when the animals died from AIDS-related disease symptoms [43]. Although such direct evidence is lacking in humans, there is strong evidence for the role of $CD8^+$ T cells in exerting selective pressure on the virus. Temporal associations were observed in the acute phase of infection, where the initial drop in viral burdens was concomitant with the rise in $CD8^+$ T cells [44]. Moreover, the association of certain HLA-class I alleles with HIV-1 disease indicated a role for $CD8^+$ T cells in HIV-1 infection. Of these alleles, HLA-B*2705 and HLA-B*5701 have been associated with slow disease progression, whereas HLA-B*3501 and HLA-B*0801 have been linked to more rapid progression to AIDS [3, 45]. Such associations between HLA type and disease course were also observed in SIV infected macaques [46]. Furthermore, the most compelling evidence for the role of $CD8^+$ T cells in HIV-1 infection was supported by the emergence of escape variants in $CD8^+$ T cell epitopes both in acute and chronic HIV-1 infection [47, 48]. Epitope escape has also been reported in Long Term Non Progressors (LTNPs) late in infection and has been associated with loss of virus control and progression to AIDS [49, 50]. Escape however occurred at a fitness cost to the virus. Transmitted escape variants were lost in HLA-mismatched hosts, thus further supporting the existence of $CD8^+$ T cell mediated pressure on the virus [51, 52]. Furthermore, evidence for $CD8^+$ T cell-mediated pressure has been reported to operate even under therapy [53]. Therefore, having provided several lines of evidence of the role for $CD8^+$ T cells in controlling HIV-1 infection, it is intriguing that HIV-1 has evolved mechanisms that lead to the functional impairment of these cells.

1.2.2 Improper processing and presentation of HIV derived antigens

$CD8^+$ T cells target epitopes that are 8-9 amino acids long and that can complex with HLA-class I molecules. Antigen processing and presentation occurs in antigen presenting cells (APCs) by a number of different mechanisms that include; processing and presentation of endogenously-expressed cytosolic proteins (such as gag and pol),

endogenously-expressed membrane proteins (such as env), and cross presentation of exogenously-expressed proteins [54]. Briefly, peptide precursors are cleaved by the proteosome to yield intermediate proteins, which are transported to the Endoplasmic Reticulum (ER) by transporter associated with antigen processing (TAP), trimmed by endoplasmic reticulum aminopeptidase (ERAAP 1,2) and then matured and loaded into MHC-class I molecules [55]. Although it remains a challenge and a tedious task to study the different steps involved, antigen processing and presentation and hence the kinetics of protein expression govern patterns of "immunodominance" in HIV-1 infection and greatly influence the outcome of $CD8^+$ T cell responses [56]. Unfortunately, antigen processing and presentation has not received much attention in the HIV-1 field. However, a few publications have highlighted the impact of escape mutations that flank $CD8^+$ T cell epitopes on antigen processing and presentation [57-59]. In a very ambitious attempt to study the different steps of antigen processing and presentation of an immunodominant HLA-A*0201 restricted gag p17 epitope and its naturally occurring variants, Tenzer et al, reported that all the steps from proteosomic cleavage, TAP binding, ERAAP 1, 2 trimming and affinity of p17 variant peptides for HLA-A*0201 contribute to the hierarchy of $CD8^+$ T cell recognition [60]. Moreover, by resolving the crystal structure of HLA-A*0201 with an elongated p17 core protein, the authors emphasized the "abundance" of peptide structure in influencing $CD8^+$ T cell responses rather than HLA binding affinity. These data provide deeper insights into how mutations that lead to discordant outcomes due to processing and presentation of HIV-1 derived peptides influence $CD8^+$ T cell recognition. Therefore, although it is not very feasible at this time, vaccine strategies should aim to consider which peptide sequences to include in their design; strategies that would ultimately lead to the generation of abundant peptides able to provoke rigorous $CD8^+$ T cell recognition might be the candidates of interest.

1.2.3 Intrinsic defects in signaling cascades upon HIV-derived antigen engagement to $CD8^+$ T cells

Upon encounter with cognate antigen $CD8^+$ T cells become antigen experienced, undergo differentiation, and exert effector functions. These processes begin at the interface

between T cell receptor (TCR) and peptide bound to MHC-class I. Several mechanisms have been put forward to understand how TCR transduces signals through the plasma membrane upon binding peptide-MHC, however these mechanisms remain highly controversial mostly due to the lack of in vitro evidence that support them [61]. Regardless of what mechanism prevails in triggering the TCR, the canonical pathway of TCR triggering results in the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) associated with the TCR / CD3 complex by the Sarc Family Kinases SFK such as Lck and Fyn. Zap-70 binds these phosphorylated ITAMs and becomes phosphorylated itself by SFKs. Phosphorylated Zap-70 activates LAT and SLP-76, which ultimately leads to activation of transcription factors and the induction of effector functions [193]. Despite the widely accepted canonical pathways of TCR triggering, there is evidence for the presence of "non-canonical" TCR triggering pathways, such as the lack of Lck involvement in antigen recognition by memory cells [61].

Notwithstanding the lack of knowledge about TCR triggering mechanisms, it is conceivable that aberrant signaling pathways could contribute to the functional impairment of HIV-specific $CD8^+$ T cells. The most compelling data to this date was that HIV-specific $CD8^+$ T cells had a dual down-modulation of CD28 and CD3ζ, two of the key molecules crucial for signal transduction. Interestingly, this down-modulation seemed to be a characteristic feature of activated $CD8^+$ T cells and was not only limited to HIV-1. Such down-modulation was also reported in T lymphocytes infiltrating tumors in mice and humans [62, 63]. Importantly, incubation of $CD28^-$ $CD3\zeta^-$ $CD8^+$ T cells with exogenous IL-2 resulted in re-expression of CD3ζ. Cytotoxicity was restored after longer incubations with IL-2 indicating that different signaling pathways may be involved. However, this phenomenon was irreversible upon incubation with IL-2 in patients with late stage disease. Therefore, the authors proposed that $CD8^+$ T cell activation leads to CD3ζ down-modulation and production of IFN-γ and that cytotoxicity can be restored only in the presence of $CD4^+$ T helper cells [64]. Although quantification is still lacking, Wherry et al have provided more insights into possible aberrant signaling pathways that are needed for signal transduction via TCR and cytokine receptors [65]. Furthermore, the molecular basis of T cell activation or inactivation is also dictated by the signals delivered by co-stimulatory molecules [66, 67]. Trying to understand signaling pathways delivered by co-

stimulatory molecules in combination with TCR triggering in HIV-specific $CD8^+$ T cells remains a challenging yet worthwhile effort towards understanding regulation of effector functions.

1.2.4 Lack of sufficient $CD4^+$ T cell help in priming efficient HIV-specific $CD8^+$ T cell responses

HIV-1 is characterized by severe decline of activated and memory $CD4^+$ T cells in the periphery and gut associated lymphoid tissues in few weeks of HIV-1 infection [68]. The mechanism behind $CD4^+$ T cell dynamics in HIV-1 infection has been controversial and several hypotheses have been put forward. Such hypotheses include the destruction of mature $CD4^+$ T cells, "appearance of loss" of $CD4^+$ T cells due to redistribution, chronic activation and T cell death, and impaired production of new T cells from precursors [69]. Pitcher et al reported that $CD4^+$ T cells were in fact present in HIV-1 infected individuals at all stages of disease, and that these cells produced IFN-γ in response to Gag p24 [70]. This indicates that the problem is not the lack of $CD4^+$ T cells but a skewing in their quality to produce effector cytokines. In fact, IFN-γ production alone by T cells correlates with antigen persistence, whereas the presence of IFN-γ^+ IL-2^+ dual functional T cells correlates with protracted antigen exposure and drop of viral burdens [71]. The dysfunction of $CD4^+$ T cells has been reported previously [72]. Therefore, how does dysfunction or absence of $CD4^+$ T helper responses affect $CD8^+$ T cell responses? $CD4^+$ T cell help is needed for priming of $CD8^+$ T cells, maintaining $CD8^+$ T cell memory, and maturation of $CD8^+$ T cell function. Aberrant maturation and differentiation of HIV-specific $CD8^+$ T cells may result in impaired in vivo function and is related to lack of $CD4^+$ T cell help [73, 74].

Although the IL-2 dependent restoration of the cytolytic capacity of $CD8^+$ T cells suggests a potential role for $CD4^+$ T cell in mediating $CD8^+$ T cell functions [64], the direct role for HIV-specific $CD4^+$ T cells help in mediating HIV-specific $CD8^+$ T cell proliferation was reported by Lichterfeld et al [75]. In accord with results by Migueles et al, the authors showed that HIV-specific $CD8^+$ T cells from LTNPs exhibited ex vivo proliferative potential, a phenomenon that was absent in HIV-1 progressors [76]. The authors showed that loss of HIV-specific $CD8^+$ T cell proliferation was associated with the parallel loss of

HIV-specific $CD4^+$ help from HIV-1 progressors and help was IL-2 dependent. The proliferative capacity of $CD8^+$ T cells in chronic infection was partially restored upon the addition of autologous HIV-specific $CD4^+$ T cells isolated in acute infection. Interestingly, the presence of IFN-γ^+ IL-2^+ dual functional $CD8^+$ T cells from LTNPs, prompted Zimmerli et al to investigate the potential impact of their loss in HIV-1 progressors. The authors showed that these cells made IL-2 upon antigenic stimulation and proliferated even in the setting of $CD4^+$ T cell depletion, thus indicating that $CD4^+$ T cell help was not crucial for all $CD8^+$ T cells functional compartments. The persistence of these dual functional cells was associated with the resolution of proliferative capacity [77]. It is not surprising that patients chronically infected with HIV-1 had dysfunctional $CD8^+$ T cells as the IL-2 producing helper $CD4^+$ T cells were impaired themselves [78]. Thus delineating the functional heterogeneity in the HIV-specific $CD8^+$ T cell compartment in chronic HIV-1 infection could help understand the role of $CD4^+$ T cell help in maintaining these responses. Such self-proliferating populations could be found under low levels of antigen but maintaining them could be a challenge.

1.2.5 Compromised effector function of HIV-specific $CD8^+$ T cells in chronic infection

$CD8^+$ T cells exert their antiviral activities mainly through two mechanisms; cytokine production and cytolysis of infected cells. $CD8^+$ T cells are believed to contribute to the decline of primary viral load and determination of a viral load set point in HIV and SIV. Ogg et al reported in 1998 that $CD8^+$ T cells persisted in chronic stages of HIV infection. No associations were detected between the viral load and breadth or magnitude of $CD8^+$ T cells in humans [79]. Similar observations were later published by Betts et al and Kiepiela et al [80, 81]. Parallel observations were found in studies of SIV infected sooty mangabeys and African green monkeys [82, 83]. These animals remain healthy despite high viral titers and are being studied to understand correlates of immune protection against disease progression. These studies indicate that neither the magnitude nor breadth of $CD8^+$ T responses correlate with viral loads and $CD4^+$ counts. Taken together, these observations support the conclusion that qualitative rather than quantitative measures of $CD8^+$ T cells are needed to define correlates of immune protection against HIV-1.

$CD8^+$ T cells can inhibit HIV-1 replication in vitro [84]. Similar inhibitory effects were reported in primary infection [85-87] concomitant with the oligoclonal expansion of $CD8^+$ T cells that coincided with containment of the virus [88]. Therefore, after the viral set point is achieved and chronic infection is established, HIV-specific $CD8^+$ T cells lose their ability to lyse infected cells due to aberrant signaling [64], reduced perforin expression [89, 90] and inefficient trafficking to lymphoid sites of infection [91]. The inability of $CD8^+$ T cells to commence infected cell lysis under persisting antigen loads, could be due to high antigenemia, as 10-100 fold less antigen concentrations are needed to promote cytotoxic activity than cytokine production [92]. Moreover, data from HIV controllers indicate that $CD8^+$ T cells from these individuals exhibit potent $CD8^+$ T cell capacity to suppress HIV infection in vivo, although these cells exhibit a unique activation profile that is, HLA-DR^+CD38^- [93].

The proliferative capacity of HIV-specific $CD8^+$ T cells is impaired in chronic infection and is directly linked to the loss of perforin, indicating that loss of proliferative potential occurs in the early stages of exhaustion [76, 89, 94-96]. Loss of proliferation of HIV-$CD8^+$ T cells is often attributed to loss of $CD4^+$ T helper cells [75], although Zimmerli et al found that IFN-γ^+ IL-2^+ dual positive cells from LTNPs have self-proliferating potential despite depleted $CD4^+$ T helper cells [77].

Exhaustion and deletion of antigen-specific $CD8^+$ T cells has also been reported in SIV, in humans infected with Hepatitis B virus (HBV), Hepatitis C Virus (HCV), and melanomas [97-101]. In terms of cytokine production, HIV-specific $CD8^+$ T cells exhibit functional impairment in chronic infection [71, 102, 103]. Interestingly, Wherry et al identified in the lymphocytic choriomeningitis virus (LCMV) model that the exhaustion of antigen-specific $CD8^+$ T cells occurred in a hierarchical manner, such as IL-2 and ability to lyse infected cells was the first function to be lost, followed by TNF-α, and lastly IFN-γ [96]. Therefore, detection of IFN-γ positive cells in chronic infection did not correlate with antiviral activity. Of note, loss of IFN-γ producing $CD8^+$ T cells has been reported during progression to AIDS [104]. Such functional impairments have been attributed to the loss of high avidity $CD8^+$ T cell clonotypes from acute to chronic infection [105], changes in epitope immunodominance [96], and emergence of escape variants [47]. Our group and

others identified the expression of PD-1 on HIV-specific CD8+ T cells as a marker of exhaustion and reported that blocking PD-1 interaction with its ligand PD-L1 relieved the inhibitory signals delivered to CD8+ T cells and restored cytokine production and proliferation [106-109]. Similar observations were later reported in HCV and HBV [110-112]. Furthermore, Wherry et al recently unfolded the molecular signatures of CD8+ T cell exhaustion in micro array analysis and opened potential new targets for investigation in regards to T cell exhaustion [65].

Owing to the fact that $CD8^+$ T cells in HIV-1 infection are functionally exhausted and that mono-functional IFN-γ responses do not correlate with virus containment [71], the key feature for $CD8^+$ T cell antiviral efficacy has now become the assessment of polyfunctional responses. The development of multi-parametric flow cytometry, enabled researchers to measure antigen-specific $CD8^+$ T cell responses for cytokines, chemokines and the degranulation marker CD107a simultaneously on the single cell level. The first two studies that characterized polyfunctionality as a correlate of immune protection came from the mice model of Leishmania major infection and functional assessment of HIV-specific $CD8^+$ T cell responses from LTNPs [113, 114]. Darrah et al reported that the Mean Florescence Intensity (MFI) of IFN-γ was a direct measure of polyfunctionality of both $CD4^+$ and $CD8^+$ T cell responses, indicating that a single polyfunctional cell had higher MFI of IFN-γ compared to single IFN-γ producing cell. Polyfunctional $CD8^+$ T cell responses have also been reported in HIV-2 infection, in HLA-B*2705 slow progressors and were induced upon immunization with vaccinia virus [115-117]. Furthermore, the emergence of polyfunctional $CD8^+$ T cells has been reported to emerge after prolonged suppression of HIV-1 replication by antiretroviral therapy [118]. Recently, it was reported that $CD8^+$ T cell efficacy was a function of antigen sensitivity, in that highly sensitive HIV-specific clonotypes displayed polyfunctional profiles, exhibited efficient proliferation and potent HIV suppressive activity [119]. Taken together, these results pave ways into better understanding the correlates of immune protection in the setting of $CD4^+$ T cell depletion.

1.2.6 Memory $CD8^+$ T cells

1.2.6.1 Memory: historical perspective

Generation of memory is a hallmark of the adaptive immune system. The central feature of immune memory is the ability of memory T cells to mediate faster, stronger, and more effective responses to secondary pathogen challenges than naïve T cells. Thucydides' observation, which mentioned that the deadly plague of Athens never attacked the same man twice, constituted the basis of vaccination. Moreover, Edward Jenner, who demonstrated that individuals immunized with the cowpox virus were protected from the disease caused by smallpox virus, proposed the first scientific proof of vaccination. Protective immune memory can persist for many years after the initial antigenic exposure and in the absence of reexposure to antigen [120].

1.2.6.2 Generation of $CD8^+$ T cell memory in acute infection

The T cell response can be divided into three distinct phases [121]. Upon encounter with antigen, T lymphocytes undergo initial activation, clonal expansion and acquire effector functions. The second phase is the contraction phase where 90-95% of effector cells are lost via apoptosis. The final phase is the generation and maintenance of memory T cells. It has been proposed that all three phases may be programmed shortly following antigenic stimulation [121].

The $CD8^+$ T-cell program can vary depending on the quality of the stimulation received during activation resulting in heterogeneity within the effector and memory populations. Extrinsic factors such as the length of time antigen is available can influence the development and quality of memory $CD8^+$ T cells [120]. Co-stimulatory molecules and cytokines also play a role in the optimal activation and memory differentiation of $CD8^+$ T cells. Recent studies have indicated that $CD4^+$ T cell help during activation plays a role in the formation of competent $CD8^+$ T cell memory [74, 122].

Not all effector cells enter the memory pool. Kaech et al showed that the precursor effector cells that enter the memory pool are $CD127^{hi}$ (IL-7Rα) and that these cells constitute 5-10% of the effector $CD8^+$ T cells, are activated, express high levels of granzyme and low levels of bcl-2 (a gene associated with cell survival). As they enter the

memory pool, they acquire a resting phenotype, express lower levels of granzyme and high levels of bcl-2- and eventually down-regulate CD127 to become $CD127^{lo}$ [121, 123]. Another study demonstrated that the expression of the homotypic form of CD8 molecule (CD8αα) promoted the survival of $CD8^+$ memory T cell precursors [124]. CD8αα expression is also associated with increased expression of Bcl-x_L (a gene associated with cell survival). Mice deficient in the enhancer element that is needed for the expression of CD8αα but have no defect in CD8αβ have severe defects in $CD8^+$ T cell memory development supporting a role for CD8αα in the survival of $CD8^+$ T cell precursors [124].

A number of studies using gene expression analysis and functional profiling have shown that memory development ocuurs gradually, which continues for several weeks until infection is resolved. Accordingly, memory is characterized by the acquisition of resting phenotype, maintenance of ability to produce IFN-γ and TNF-α, and reacquisition of cytotoxic activity. Moreover, the ability to produce more IL-2 increases with memory differentiation (termed homeostatic proliferation of memory), as well as proliferative potential and ability to migrate central lymphoid tissues [122].

1.2.6.3 Maintenance of $CD8^+$ T cell memory in acute infection

One of the characteristics of the memory $CD8^+$ T cell pool is that it can be maintained long-term at constant numbers. This is achieved by a slow homeostatic proliferation of memory $CD8^+$ T cells, which is well balanced with both survival and death, with no net increase in $CD8^+$ T cell numbers. Interestingly, memory $CD8^+$ T cells do not require antigen for survival or homeostasis [125] and do not require the presence of MHC class I molecules [125, 126].

The cytokines IL-7 and IL-15 have been identified in playing a role in the maintenance of memory $CD8^+$ T cells [127]. Evidence that IL-15 was involved in the maintenance of memory $CD8^+$ T cells came from the generation of mice that were deficient in either IL-15 [128] or IL-15 receptor alpha chain (IL-15Rα) [129]. Both mice had decreased numbers of memory phenotype $CD8^+$ T cells. Using antigen-specific systems, it was shown that memory $CD8^+$ T cells were generated, but disappeared over time due to a lack of homeostatic proliferation [130]. LCMV-specific memory $CD8^+$ T cells generated in IL-15

deficient mice regained the ability to undergo homeostatic proliferation when transferred to wild-type mice [130].

On the other hand, IL-7 plays an important role in the survival of memory $CD8^+$ T cells rather than proliferation [131]. Transgenic mice expressing IL-7 driven from MHC-class II promoter had increased numbers of memory $CD8^+$ T cells but underwent proliferation at the same rate as normal cells [131]. As mentioned previously, only effector cells that express high levels of CD127 (IL-7Rα) enter the memory pool. The survival of memory $CD8^+$ T cells by IL-7 could be due to the sustained expression of Bcl-2 induced by IL-7, which could rescue T cell development in IL-7 receptor deficient mice [132]. Altogether, maintenance of memory $CD8^+$ T cells is antigen independent in acute infections and does not require interactions with MHC-class I, but is governed by the combined actions of IL-15 and IL-7 that promote homeostatic proliferation and survival, respectively.

1.2.6.4 $CD8^+$ memory T cell subsets and function

Based on lymphoid homing receptors such as CD62L and CCR7 [133], costimulatory receptors such as CD28 and CD27 [134], and molecules associated with T cell activation such as CD45RA [135], memory $CD8^+$ T cells can be divided into heterogeneous subgroups. Naïve T cells are characterized by the expression of CD45RA, CCR7, CD28, and CD27, and the lack of expression of cytolytic molecules (granzyme, perforin, and CD107a). Central memory T cells (T_{CM}), home preferentially to lymph nodes, share several phenotypic properties of naïve $CD8^+$ T cells, with the exception of CD45RA. T_{CM} can rapidly mediate effector functions after re-stimulation, proliferate, and secrete high levels of IL-2 [136]. Effector memory T cells (T_{EM}) migrate to peripheral tissues and do not express CD45RA or CCR7, and depending on their differentiation state, they may or may not express CD27/CD28. These cells exhibit immediate effector functions such as cytotoxicity and IFN-γ production, but have limited ability to proliferate and produce IL-2. Terminally differentiated T_{EMRA} cells is yet another subset of memory $CD8^+$ T cells that lack the expression of CCR7, CD27, and CD28, but express CD45RA. These cells are endowed with potent effector function but fail to produce IL-2. Therefore, T_{EM} cells can be further subdivided into two groups based on the expression of CD45RA [136]. Importantly, the analyses of new cell surface molecules often result in the identification of

an increasing number of subpopulations; one may find as many T cell subpopulations as there are combination of markers [137]. Therefore, phenotypic markers should be used in correlation with cellular function and survival capacity of each subset.

1.2.6.5 Differentiation pathways of $CD8^+$ T cell lineage

The pathway of T cell differentiation, which is the sequence of development of the different T cell subsets, remains illusive in humans [137]. It remains unclear if the differentiation pathway is linear or branched [137]. Using the LCMV model, Wherry et al were able to track the lineage of T_{CM} and T_{EM} $CD8^+$ T cells using the adaptive transfer approach [138]. They reported that after clearance of antigen during acute infections, there was a gradual conversion from T_{EM} to T_{CM} within the memory $CD8^+$ T cell pool. However, there was always the possibility that a small number of T_{CM} could eventually outgrow and differentiate into other subsets. Therefore, the authors concluded that these two subsets were distinct but were part of a linear naïve ► effector ►T_{EM} ► T_{CM} progressive differentiation pathway [138].

Following antigen priming, naïve T cells eventually give rise to a heterogeneous population of antigen-specific T cells in vitro [139] and in vivo [140]. These studies support a branched model for differentiation and suggest that reversions from one subset to another can occur. In line with the branched differentiation model in humans, murine studies showed that loss of CD27 expression from activated T cells seems to be specifically induced upon engagement with its ligand CD70 [141]. Therefore, loss of CD27 expression in latent infections such as CMV could simply be a reflection of the fact that CMV reactivation induces CD70 upregulation [142]. This indicates that the phenotype of virus-specific T cell populations is determined by repetitive antigenic stimulation and the cytokine milieu. Altogether, integrating the different memory subsets into a single differentiation pathway represents a major challenge.

1.2.6.6 Altered memory T cell differentiation during chronic infections

It is possible that under conditions of chronic infections where antigen persists, a different pattern of memory differentiation occurs when compared to acute infections. One of the

complex and controversial issues regarding the generation and development of memory $CD8^+$ T cell responses in chronic infections is the relationship between virus levels and $CD8^+$ T-cell functions. There are at least three major factors that need to be taken into consideration when evaluating how antigen levels affect $CD8^+$ T-cell function during persistent infection [120].

A) Viral replication patterns: In vivo, these could range from acute infections (where virus is cleared), to latent infections that undergo periodic reactivation (e.g. herpes simplex virus [HSV], varicella-zoster virus [VZV], and Epstein-Barr virus [EBV]), to "smoldering" chronic infections such as CMV, to chronic infections with persistent antigen such as HCV, HBV, and HIV. $CD8^+$ T cell differentiation differs with the type of infection. Acute infections are characterized with the generation of functional memory $CD8^+$ T cells with long-term T-cell persistence [143, 144]. In latent infections, where $CD8^+$ T cells experience cycles of rest and restimulation from antigen, functional $CD8^+$ T cells often develop at high frequencies with long-term T-cell persistence [145]. In "smoldering" chronic infections such as CMV, where ongoing low-level viral replication occurs with infrequent rest from T-cell activation, some impaired effector functions could develop, depending on the frequency of TCR stimulation [146]. Finally, chronic infections with high-level viral replication and continuous TCR stimulation with no rest often result in functional exhaustion and deletion of $CD8^+$ T-cell subsets [135, 147, 148].

B) A second consideration regarding antigen load and T cell function during chronic infection is that, the level of stimulation is not the same for all virus-specific $CD8^+$ T cells. It was reported in the LCMV model, that some epitopes are deleted while others are lost [96]. In a related topic, it is conceivable that escape in viral epitopes such as HIV-1, could lead to differential stimulatory capacities, thus resulting in a range of functional properties for T cells specific for different epitopes [149].

C) $CD4^+$ T cell help in maintaining $CD8^+$ T cell functions: in the presence of adequate $CD4^+$ T cell help during acute infections, efficient effector $CD8^+$ T-cell responses are generated and subsequently form memory $CD8^+$ T cells that persist. Upon reinfection, these "helped" memory $CD8^+$ T cells undergo recall responses and

generate secondary effector T cells. In the absence of $CD4^+$ T cell help in chronic infection, memory $CD8^+$ T cells are generated but respond poorly to restimulation with antigen and generate a suboptimal population of secondary effectors following reinfection compared to that of "helped" $CD8^+$ T cells [102, 120]. Wherry et al proposed a model that possibly mimics the situation in HIV-1 infection. Accordingly, the authors reported that in the context of low antigen load, $CD4^+$ T helper cells increase leading to increased proliferation and cytokine secretion by $CD8^+$ T cells. Conversely, when antigen load increases, $CD4^+$ T cell help becomes limited, resulting in impaired function of $CD8^+$ T cells. Thus, development of memory in the context of chronic infection occurs in the presence of antigen, a situation that is not characteristic of development of memory in acute infection [102]. It will be important to determine the impact of $CD4^+$ deficiency on memory $CD8^+$ T cell differentiation, including the transition from $T_{EM} \blacktriangleright T_{CM}$.

1.2.6.7 Memory $CD8^+$ T cells in HIV-1 infection

It is known that HIV-specific $CD8^+$ T cell responses show a distinct maturation phenotype when compared to other virus-specific CD8+ T cells. Based on the expression of CCR7 and CD45RA, Champagne et al proposed that $CD8^+$ T cells are divided into; Naïve ($CCR7^+CD45RA^+$), T_{CM} ($CCR7^+CD45RA^-$), T_{EM} ($CCR7^-CD45RA^-$), and T_{EMRA} ($CCR7^-CD45RA^+$) [135]. According to the authors HIV-specific $CD8^+$ T cells populate mainly in the pre-terminally differentiated $CCR7^-CD45RA^-$ subset that exhibit IFN-γ production while lacking IL-2 and cytotoxic activity. This was in contrast to the phenotype of CMV-specific $CD8^+$ T cells that mostly populated in the terminally differentiated $CCR7^-CD45RA^+$ subset.

The model proposed by Appay et al, describes $CD8^+$ T cell subsets based on the expression of the costimulatory molecules CD28 and CD27. Naïve and memory $CD8^+$ T cells are identified as $CD28^+CD27^+$, intermediate primed are $CD28^-CD27^+$, and late-primed are $CD28^-CD27^-$ [134]. In regards to this model, HIV-specific $CD8^+$ memory T cells are $CD28^-$ $CD27^+$ show limited proliferative ability and reduced lytic activity in contrast to CMV, which populate in the $CD28^-CD27^-$ compartment and show increased lytic activity. Therefore, defects in maturation and differentiation of HIV-specific $CD8^+$

memory T cells have been attributed to the different memory subtypes and hence provide an explanation to their functional impairment.

In terms of IL-7 expression, it was shown that HIV-specific $CD8^+$ T cells express low levels of CD127 reflecting that this population of effector $CD8^+$ T cells does not transit into memory [123]. Taken together, these observations pinpoint towards a large heterogeneity that exists between different $CD8^+$ T cell subsets in terms of memory and merit further investigation in terms of their lineage and functional efficacy in vivo (Figure 2).

1.2.6.8 Implications for vaccine design and long-term antiviral immunity

In HIV-1 infection, effective $CD8^+$ T cell responses are correlated with the initial drop in viral load. Strategies such as therapeutic vaccination that could boost immune responses and enhance viral control in chronic infection are of great interest. This could slow down disease process and lower viral transmission. Therefore, it is important to determine how to elicit the most successful immune responses after therapeutic vaccination. In fact, therapeutic vaccination has been unsuccessful in providing benefit when antigen load is high [150], and most positive results have been achieved when viral replication is suppressed with drug treatment or latency [151-153].

It is known that effector memory cells present an immediate, but not sustained, defense at pathogen sites of entry, whereas T_{CM} maintain the response by proliferating in the secondary lymphoid organs and producing secondary effector cells. The proliferative potential of responding T cells will be an important factor determining the outcome of therapeutic vaccination [154]. However, in other conditions such as Leishmania major infection, protective immunity was mediated by either short-lived pathogen-dependent effector cells [155] or long-lived pathogen-independent T_{CM} capable of differentiating into tissue homing effector cells upon secondary infection [156]. Determining which type of memory cells provides protective immunity against a specific infection (e.g., T_{EM} at the sites of infection, T_{CM} for systemic infections) would enlighten the way for rationally designing vaccines that induce protective, long-lasting cellular immunity against persistent viral infections. However, before facing the challenges of developing therapeutic vaccines that elicit protective and long-lasting immunity against persistent infections, further

understanding of T cell immunity requires that key issues on differentiation pathways and T cell efficacy are solved and a consensual nomenclature is achieved. Additionally, extrapolation and generalization of data across human and mice models has led to further confusion. Longitudinal studies in humans and changes in T cell attributes upon pathogen-specific settings will be needed for the full understanding of T cell immunity [137]. Finally, the improved ability to implement functional genomic approaches to study memory T cell differentiation should provide rich opportunities to take our understanding of effector and memory T cell differentiation to a new level of practical application [157].

1.3 Viral escape mechanisms from CD8-mediated pressure resulting in $CD8^+$ T cell dysfunction

1.3.1 Non mutational escape from $CD8^+$ T cell responses

HIV-1 has developed several evasion mechanisms that ultimately circumvent $CD8^+$ T cell responses. Globally two evasion mechanisms are exploited by HIV-1, namely mutational and constitutive (non-mutational) escape strategies. Of the constitutive evasion strategies, the establishment of viral reservoirs has been reported. These are anatomical sanctuaries where the virus is able to establish latency in quiescent cells and hence evade cellular immune responses and prove to be obstacles to effective therapeutic strategies [158, 159]. The characteristic feature of HIV-1 infection is the depletion of $CD4^+$ T cells, and this can provide an evasion mechanism from CTL recognition, bearing in mind that $CD4^+$ T cell help is needed for the generation and maintenance of effective $CD8^+$ T cell responses [69, 97, 103]. Moreover, the selective down-regulation of MHC-class I by HIV-1 Nef protein is known to mediate an evasion mechanism from $CD8^+$ T cells and confer resistance to NK recognition [160, 161]. Nef also leads to the up-regulation of FasL on $CD8^+$ T cells, which renders them more susceptible to apoptosis [161]. Furthermore, the Tat protein of HIV-1 has been reportedly involved in interfering with antigen presentation; this could result in aberrant presentation of some HIV epitopes to $CD8^+$ T cells [162].

1.3.2 Mutational escape from $CD8^+$ T cell responses

1.3.2.1 Evidence for the role of $CD8^+$ T cell-mediated escape

HIV and SIV reverse transcriptase is error-prone resulting in viral mutants generated daily during infection [163, 164]. Therefore, although it is challenging to identify mutations that emerge from $CD8^+$ T cell mediated selection pressure or simply are the results of reverse transcriptase infidelity, several lines of evidence support the role of a $CD8^+$ T cell mediated pressure in selecting viral variants. The first observation to support this role of $CD8^+$ T cells was shown in the LCMV model both in vivo and in vitro [165, 166]. Mice infected with a high dose of LCMV could not resolve primary infection and harbored variants, in contrast to mice that received low-dose pathogen that cleared virus and were not associated with escape. Similar observations were reported with SIV. Interestingly, macaques showed different clinical outcomes; rapid and slow progressors differed by a single MHC-class I haplotype [167]. Escape in the SIV model has also been associated with failure of simian-human immunodeficiency virus (SHIV) vaccine. These animals were not protected from infection, but mounted great CTL responses that directly correlated with viral load. Failure to do so was associated with emergence of CTL escape [168].

Evidence for $CD8^+$ T cell-mediated selection of escape in humans was reported in dominant $CD8^+$ T cell epitopes in primary HIV-1 infection [49, 85]. Breadth of $CD8^+$ T cell responses could indicate that overlapping epitopes are presented by different HLA-class I molecules; this implies that it is difficult for the virus to escape. On the other hand, this does not necessarily mean that viral escape has not occurred and been archived [169]; the case of two HLA-identical siblings selecting for different CTL responses due to escape provides robust support to this hypothesis. Furthermore, associations between HLA-class I heterozygosity or homozygosity with disease progression have been established, such that HIV-1 survival rate increases with the increase in HLA-class I heterozygosity in the population, and vice versa. This could possibly be due to the potentially broader $CD8^+$ T cell epitopes being recognized and hence limit the detrimental effects of viral escape [170]. Such differences also point towards the ability of particular epitopes that are associated with slow progression to the ability to tolerate sequence variations. In a cohort

of 400 HIV-1 infected individuals, strong correlations were made between certain HLA molecules and variability in the *Pol* gene. These observations strongly point towards the impact of HLA molecules in molding viral evolution [171]. More compelling evidence for the role of cytotoxic T lymphocyte (CTL)-mediated viral evolution comes from studies of vertical transmission. HLA-B*2705 mothers transmitted a CTL escape mutant to their children that ultimately resulted in lack of responses to this immunodominant epitope. Interestingly, children who had inherited their HLA-B*2705 from their fathers and HIV from their mothers did not harbor this particular CTL mutation, and mounted responses that were associated with slow progression [172]. It is also interesting to note that, although children lacked wild-type virus, they still mounted responses to this index virus similar to the phenomenon of "original sin". Finally, HIV-1 transmission studies in macaques have indicated that virus reverts to wild-type in HLA-mismatched hosts, indicating once again the importance of HLA molecules in driving viral evolution [52, 173].

1.3.2.2 Impact of virus fitness cost on $CD8^+$ T cell-mediated escape

$CD8^+$ mediated escape from immune recognition is not a random process as it occurs at a fitness cost to the virus. Mutations that result in amino acid changes in the virus could create structural restraints to the virus as well as result in crippling its efficient replication. Although attempts to engineer and study variant viruses in terms of their replicative potential have been limited, elegant studies have enhanced our knowledge of escape incurred by HIV-1. The best supporting evidence for cost to viral fitness is indicated by the existence of compensatory mutations and reversion studies in MHC-mismatched host.

In HIV-1 infection, the dominant Gag epitope KK10 (Residues 263-272; KRWIILGLNK) is restricted by HLA-B*2705 individuals and is correlated to slow disease progression, possibly due to the superior functional profile and high avidity of the constituent clonotypes in controlling HIV-1 infection [115]. Escape from this epitope in late infection has been associated with loss of effective immune control. In most HLA-B*2705 individuals a common escape is Arginine (R) to Lysine (K) at position 2 (R264K), an anchor, that results in loss of binding to HLA-class I. It appears that this particular

mutation is always preceded by another mutation from Leucine (L) to Methaionine (M) at position 6 (L268M). R264K mutation poses severe structural restraints to the viral capsid, thus a compensatory mutation L268M is needed to alleviate the fitness cost sustained by the virus [50]. Recently, it was reported that a third mutation from Alanine (A) to Serine (S) designated as A173S, preceded both L268M and R264K mutations. It was shown in vitro that the fitness cost to R264K was also alleviated by A173S [174]. It is possible that the accumulation of such mutations accounts for the late escape that occurs in HLA-B*2705 individuals.

Similar compensatory mutations have been reported in the Mamu-A*01 restricted CM9 epitope (the capsid protein) in SIV infection [173]. Engineered mutants were shown to be viable in vitro only when compensatory mutations were incorporated, thus indicating that such clustered mutations occur to alleviate the detrimental effects of the fitness cost of the mutation itself.

The impact of viral fitness cost has also been shown in a series of reversion studies both in HIV-1 and in SIV infection. Three different escape mutations were engineered and used to infect both HLA-matched and mismatched macaques. These included; a mutant Tat epitope (SL8) that occurs in early infection and is restricted by Mamu-A*01, a Nef mutant (IW9) that occurs intermediate during infection and is restricted by Mamu-B*17, and a Gag mutant (CM9) that occurs late in infection restricted by Mamu-A*01. In HLA-matched macaques all three mutations were retained. Interestingly, all mutations reverted to wild-type virus in HLA-mismatched hosts, with the exception of SL8, indicating that this mutation occurred at a minimum fitness cost to the virus [52, 175-178].

Similar studies have been performed with HIV-1 infection of HLA-B*5701 and B*5801 individuals who have slow disease progression. Seventy five percent (75%) of these individuals harbored a Threonine (T) to Asparagine (N) mutation at position 3 (T242N) in the immunodominant Gag epitope TW10 (Residues 240-249; TSTLQEQIGW). One third of these individuals harbored yet another Glycine (G) to Alanine (A) mutation at position 9 (G248A), either alone or in combination with T242N. It was reported that in HLA-mismatched individuals only T242N reverted to wild-type indicating that it occurred at a high fitness cost [179]. If G248A occurs in combination with T242N, then whether it

conferred a compensatory effect to T242N needs to be established. Altogether, although early escape from the immunodominant Gag epitope TW10 occurs, HLA-B*5701 individuals were still able to control viral loads probably due to the attenuated virus that results post escape in early infection. However, in a recent study by Crawford et al, it was reported that although such mutations occurred in targeted immunodominant Gag epitopes, rapid disease progression was observed in HLA-matched hosts to whom these mutations were transferred. This emphasized on the fact that viral fitness cost is not the only factor shaping viral evolution, but the absence of $CD8^+$ T cell responses in late infection account to the progression of disease [180].

1.3.2.3 Timing of $CD8^+$ T cell-mediated escape

Escape from $CD8^+$ T cell responses can happen in acute and chronic phases of both HIV and SIV infection. Potential mechanism for the timing of escape and its impact on disease progression will be discussed in this section.

CD8+ T cell-mediated escape during acute infection

Several examples of escape from $CD8^+$ T cell responses have been put forward in acute phase of HIV-1 and SIV infection and differences have been noted in terms of disease progression.

One of the most compelling studies that described the role of effective $CD8^+$ T cells in exerting pressure and hence selecting for escape in the acute phase was reported by Mantano et al. In this study, macaques were vaccinated with DNA-prime / Gag expressing Sendai virus vector boost and subsequently challenged with the highly pathogenic $SIVmac_{293}$ strain. Early escape was evident in the macaques, which rapidly reverted to wild-type when transmitted into HLA-mismatched host, indicating that escape occurred at a fitness cost. Interestingly, complete suppression of viral burdens was observed with early escape in Gag [181].

Two $CD8^+$ T cell responses that occur in SIV infection are the Mamu-A*01 restricted Gag CM9 and Tat SL8, that co-dominate the acute phase. Sequence variation in the SL8 epitope was observed as early as 8 weeks post infection coincident with the decline in primary viremia, but not in Gag. These animals were able to achieve effective control of

SIV. Reversion to wild-type did not occur in HLA-disparate hosts indicating that this early mutation does not occur at a fitness cost [173]. Since this escape occurred concomitant with the decline in primary viral burdens, it is not surprising that $CD8^+$ mediated immune selection was highly efficacious, hence resulting in effective control of SIV.

In HIV-1 infection, early escape was observed in the immunodominant response to Gag TW10 in HLA-B*5701 and HLA-B*5801 individuals. The majority of these individuals (75%) harbored the T242N mutation, which was absent in HLA-B*5701 and HLA-B*5801 negative individuals. The mutant virus reverted rapidly to wild-type when transmitted to HLA-mismatched hosts indicating that it occurred at a fitness cost to the virus and in the absence of $CD8^+$ mediated immune pressure it reverted to its fit form. Despite the fact that early mutations occur, these individuals are slow progressors [57].

The above three examples contrast with previous observations that indicated early escape as a cause for rapid disease progression [85, 87]. This is because previous studies were characterizing Env and Nef proteins of HIV-1 that have high sequence variation when compared to Gag. Perhaps in the above two examples, the mutations did not occur at a fitness cost to the virus and subsequent immune control was ineffective.

Why do some mutations occur early in infection and others occur late in the chronic phase? Taking the SL8 escape in SIV as an example, there are three potential explanations. First, Tat is a variable region that harbors several variations. Second, since Tat is synthesized first in an infected cell, the expression kinetics of Tat could explain why it is selected earlier in infection, which makes it more antigenic due to greater protein abundance. Third, it is possible that the $CD8^+$ T cell clones against Tat are functionally different from others in terms of avidity and functional profile [182].

$CD8^+$ T cell-mediated escape during chronic infection

Late escape from $CD8^+$ T cell mediated immune pressure has been described in chronic SIV infection, where the resulting mutations lead to immune control in macaques that received DNA-vaccines [183].

In HIV-1 infection, the best-characterized example is that of the Gag epitope SL9 (Residues 77-85; SLYNTVATL) that is restricted by HLA-A*0201. This particular response is absent in the acute phase and emerges as a dominant response in the chronic phase in nearly 70% of HLA-A*0201 individuals [184]. Interestingly, escape has been reported in this epitope, but does not account for exacerbation of disease [49, 185]. A possible explanation for this is the delayed expression kinetics of Gag. This is not highly likely, as Gag-specific responses have been also selected in acute infection. A more likely explanation is the fact that since this response occurs in chronic phase, i.e. when a viral set point has already been achieved, the $CD8^+$ T cell responses that target this epitope are not as efficacious as they would be in controlling primary viremia.

The case of late escape from the immunodominant epitope KK10 provides yet another complex mechanism to why these mutations occur late in chronic infection in HLA-B*2705 positive individuals [169]. As described above, the KK10 epitope needs to acquire at least three putative compensatory mutations L268M, R264K, and A173S that first compensate for the fitness cost incurred to the virus by the structural constraints elicited by R264K and finally succumb to lack of efficient immune control [50, 174]. Hence, the need for compensatory mutations results in late escape and failure from immune control. Overall, the timing of emergence of escape is a dynamic process that is operated by two forces; the viral fitness cost and the selective pressure of the $CD8^+$ T cells that target it.

1.3.2.4 $CD8^+$ T cell intra epitopic mutations and subversion of $CD8^+$ T cell immune response

Intra-epitopic mutations highly influence the fate of $CD8^+$ T cell responses. Mutations could ultimately lead to abrogation of effective responses via one of the following mechanisms; loss of or poor binding to MHC-class I molecules, abrogation of TCR triggering or antagonism [186], and finally altered processing and presentation of the epitopes to MHC-class I molecules [51, 57].

Intra-epitopic mutations that result in poor binding to MHC-class I occur at anchor positions 2 of the epitope in HLA-A and HLA-B. CTL epitopes that bind HLA-A at anchor position 2 have hydrophobic residues (Leu, Val, Ile, Tyr) and Thr / ser, whereas

epitopes binding HLA-B can have all these in addition to proline, positively and negatively charged amino acids, and Gln. Therefore, in terms of peptide binding, HLA-B recognizes a wider array of peptides than HLA-A. The unique proline residue that makes up the anchor at position 2 for HLA-B is highly conserved. Therefore, polymorphisms at this position could result in viral escape [187]. Moreover, mutations in the anchor residues of epitopes that effect proper binding to HLA-class I abrogate responses.

It is known that mutations at position 2 and 9 of the epitope effect binding to HLA-class I, whereas mutations that occur inside the HLA-class I binding pockets ultimately interfere with TCR. Another mechanism of TCR escape has been proposed but its impact on function has not been determined. The mechanism proposes that similar to the concept of antigenic sin, $CD8^+$ T cell responses are targeted to a wild-type virus, which mutates in TCR contact regions and abrogates response. Original antigenic sin describes a phenomenon whereby a "footprint" of immune responses is established during the first exposure to the virus, and the same specific memory T-cell populations are preferentially re-expanded when re-exposed to the same antigen, thereby limiting the clonal expansion of new specific T cells. While de novo responses are not generated towards the mutant peptide, this variant is still able to stimulate the $CD8^+$ T cells that were originally directed against the wild-type peptide [188].

Reports from Draenert et al [57] and Allen et al [51] have investigated the role of improper processing and presentation in generating effective $CD8^+$ T cell responses. The former group affirmed that a mutation in the carboxy-terminal anchor of Gag HLA-A*0301 restricted RLRPGGKKK, does not only result in loss of binding, but also hinders the efficient processing of the overlapping KIRLRPGGK. In the latter study, the authors acknowledged that a proline to alanine mutation in the N-terminal residue of HLA-B*5701 resulted in escape. Later Serwold et al showed that this particular mutation inhibited ERAAP from cleaving its precursor or influenced TAP binding [189].

Although the proposed mechanisms of intra-epitopic escape have been put forward, direct evidence to support them is still pending. De novo responses to mutants are poorly characterized as well as the extent to which altered TCR triggering results in distorted functions have not been studied. Recently, a mechanism was proposed by Lichterfeld et al,

which highlighted the impact of $CD8^+$ T cell escape in disarming DC function. Leucine (L) to Methiaionin (M) L268M mutation is common to the HLA-B*2705 restricted KK10 and de novo responses with an alternative TCR repertoire are generated against it. The authors found out that this variant has increased binding to immunoglobulin-like transcript 4 (ILT-4), an inhibitory MHC-specific receptor that is expressed on DCs. This resulted in the generation of tolerogenic DCs and indicated that $CD8^+$ T cell escape bias cellular immunity by disarming innate response [190].

1.3.2.5 Implications of $CD8^+$ T cell-mediated escape for vaccine design

The ultimate goal of HIV-1 vaccines would be achieving sterilizing immunity. Since this has proved to be an almost impossible goal, focus has shifted towards a $CD8^+$ T cell induced vaccine. The rationale for the development of a $CD8^+$ T cell-based vaccine is twofold. First, $CD8^+$ T-cell-based vaccines are supposed to elicit $CD8^+$ responses that are more effective qualitatively and quantitatively than the ones generated by natural infection, partly due to the lack of hyper-immune activation in the acute phase. Second, like the rationale for any vaccine, the development of central memory (T_{CM}) $CD8^+$ T cells might occur post vaccination, that are endowed with longevity, protection, and effector functions.

The most important aspect in HIV-1 vaccine development is the reduction of transmission. $CD8^+$ T-cell-based vaccines could either achieve this goal by reducing viral burdens in primary infection or lower viral set points, which are prognostic for disease outcome. This could limit the risk of transmission owing to the fact that risk of transmission is highest when primary viral load peaks.

The question remains which $CD8^+$ T cell epitopes to include in the vaccine design? Clearly, a vaccine that would elicit broad $CD8^+$ T cell responses would be the one most favored. However, this cannot be the sole concern of vaccine design as viral escape from these epitopes could prevail and remain a major impediment in the successful development of vaccines. It is logical to incorporate HIV-1 epitopes that bear structural and functional constraints to the virus, thus when $CD8^+$ T cell selection pressure is applied and mutations arise they would cripple the replicative potential of the virus, thus resulting in reduced viral loads. Unfortunately, all the data on epitopes that occur at a high fitness

cost, come from reversion studies of HLA-B*2705, HLA-B*5701, Mamu-A*01, and Mamu-B*17, alleles associated with immune control. Studies with other HLA-class I alleles, especially ones associated with rapid disease progression, could help identify further mutations that occur at high fitness costs [191].

On the other hand, not all mutations occur at a high fitness cost to the virus. Although it is convenient to keep these epitopes in the vaccine design, one might speculate that these responses take up immunological space and possibly alter the desired responses from functioning efficiently. Therefore, excluding epitopes where mutations occur at the low fitness cost to the virus could be helpful. Another possibility is that, owing to the evolution of the virus, dominant epitopes will escape, leading to new $CD8^+$ T cell responses to new epitopes, which will escape once again, until dominant responses are diminished. It remains uncertain whether subdominant responses aid in immune control against HIV-1. Recently, Friedrich et al reported that after transient depletion of $CD8^+$ T cells and NK cells from Elite Controller (EC) rhesus macaques, previously subdominant $CD8^+$ T cell populations expanded and accounted for viral control. Upon viral sequencing it was shown that Mamu-B*17 positive macaques harbored epitope mutations which were still well tolerated [192]. The data here show that specific subdominant epitopes could be playing a role in controlling viral loads, however similar studies performed in rapid progressors are still pending. Such observations could be lacking in progressors because, as $CD4^+$ responses decline, one might speculate that generating de novo $CD8^+$ T cell responses would be complicated.

1.4 $CD8^+$ TCR repertoire diversity in prominent responses to viral infections

1.4.1 Generation of TCR diversity

There are two types of T lymphocytes α: β T cells and γ: δ T cells that arise from the same progenitor. The molecular mechanisms that lead to the generation of highly diverse α: β T cell receptors will be the focus of this section. The TCR α chain locus is located on Chromosome 14 in humans and consists of 70-80 Variable (V) amino-terminal regions and a cluster of 61 Junctional (J) regions similar to the light chain of immunoglobulins.

The β chain T cell receptor is located on human Chromosome 7 and contains one Diversity region (D) along with a cluster of 52 functional V and 6-7 J and a single Constant region (C), similar to the immunoglobulin heavy chains. T cell receptor rearrangement occurs during the development of T cells in the thymus.

Briefly, the enzymatic steps in RAG-dependent V (D) J rearrangement is as follows: the genes are flanked by 12bp and 23bp spacers that are called Rearrangement Signal Sequences (RSS) that become associated with rearrangement associated genes RAG-1, RAG-2, and a high-mobility group (HMG) proteins between coding sequences to be joined. The 12bp and 23bp spacers are non-conserved regions and are flaked by a heptamer and a nonamer conserved region. Rearrangement follows the 12/23 rule. After binding the two RSSs the two RAGs are put in proximity. The endonuclease activity of RAG cleaves a phosphodiester bond of the DNA backbone to create a 3'-hydroxyl group between the coding segment and its RSS. This new 3'-hydroxyl group reacts with the phosphodiester bond of the facing DNA to yield a blunt 5'-phospholylated DNA end at the heptamer sequence of RSS and a hairpin on the coding end. Therefore, two segments are yielded mainly the coding joints and the signal joints. At the coding ends, Ku70:Ku80 binds the hairpins, followed by DNA-PK:Artemis, which leads to the random opening of the hairpin by its endonuclease activity to yield either a blunt or single-strand extended DNA end. Depending on the site of cleavage, the single-stranded DNA might contain nucleotides that were originally complementary to the double stranded DNA, and therefore form short palindromes. These are called P nucleotides. Terminal deoxynucleotidyl transferase (TdT) modifies these ends and randomly creates diverse and imprecise ends by the addition of non-template nucleotides called N nucleotides. The final step is the ligation of both ends by the DNA ligase IV in association with XRCC4 (DNA Ligase: XRCC4). At the signal joints, the 2 5'-phosphorylated blunt ends at the heptamer sequences will bind Ku70:Ku80 but will not be further modified. DNA ligase IV joins these ends together. Therefore, most of the variability in the TCR chains lies in the junctional regions, by the recombination of V, D, and J segments and is modified by the insertion of P or N nucleaotides. The V gene segments contain hyper-variable loops known as complementary-determining regions (CDR), of which the CDR3 of the α and β

chains, to which the D and J gene segments contribute, form the antigen recognition site of the α: β TCR.

If β chain rearrangements that occur during thymocyte development lead to non-functional β chains, then further rearrangement ensues to provide functional ones, which is possible because of the two clusters of D and J gene segments upstream of the 2 C genes. Once the β chain is rearranged it pairs with the Pre-α chain and forms the pre-TCR that signals to arrest further β chain rearrangements. Then the α chain rearranges and contributes to further diversity since it contains more J regions than the β chain does. Finally, further diversity is reached by pairing the α and β chains together to yield a function TCR that is specific to peptide: MHC complexes. Most of the diversity lies within the CDR3β, due to the availability of additional D elements and the numbers of D, J elements, and N nucleotides [193]. Epitope specific TCR α β repertoires emerge from a pool of naïve precursors estimated to range from 10^7 in mice to 10^8 in humans [194]. However, the epitope-specific repertoire does not appear to be as vast as one would think but expresses a rather biased usage of TCR Vβ usage.

1.4.2 Factors that influence TCR repertoire bias in immunity

1.4.2.1 Definition of TCR repertoire bias

TCR bias refers to the preferential use of Vβ and to a lesser extent Vα gene-segment combinations in response to a particular antigen. TCR bias can be divided into 3 types. Type 1 bias is observed when there is consistent selection of one Vβ with no sequence conservation in the CDR3 domain. Type 2 bias involves the selection of amino acid "motifs" within the CDR3 region. The motif could be comprised of 1 or several amino acids in the antigen-specific repertoire. Finally, type 3 bias is a rare form of bias, which involves the reproducible selection of clonal sequences; it can be defined by a particular TCR Vα or Vβ chain, or by the preferred use of specific TCR Vα and Vβ sequences in combination. Public clonotypes, which are certain TCR sequences that are commonly shared between individuals, are an example of type 3 bias (Figure 3) [195].

1.4.2.2 TCR repertoire bias due to thymic selection: negative and positive selection

Immature thymocytes are positively selected in a way that only those whose receptors engage peptide: self-MHC complexes on the thymic epithelium mature, giving rise to a self-restricted pool of thymocytes. On the other hand, if these interactions between MHC and self-peptide are too strong, thymocytes are deleted by a process called negative selection. This ensures that the mature thymocyte pool is MHC restricted and self-tolerant. Mathematical estimates of the diversity of the TCR naïve pool predicted a range of 10^{12}-10^{15} α and β parings at the population level [196]. A real estimate of TCR αβ parings in individuals is predicted to be $2* 10^7$ in humans and $2 * 10^6$ in mice [197]. Hence, these figures show a bias or limited diversity in the mature naïve TCR pool.

The most compelling evidence that showed the impact of thymic selection on limiting TCR diversity was proposed by Correia-Neves et al where generation of TCR diversity was limited to only a few possible gene rearrangements. In transgenic mice expressing a fixed TCR Vβ and a genomic TCR Vα locus incorporating only a single Vα gene segment and a few Jα gene segments, recombination could occur and diversification is achieved by the insertion of non-template N nucleotides. Analysis of the immature and mature thymic T cells revealed that the mature pool was less diverse in CDR3α than the immature T lymphocytes. Moreover, analysis of the CDR3α amino acids of the mature pool revealed differential selection of amino acids between $CD4^+$ and $CD8^+$ T cells, indicating the positive selection of preferential TCR contacts for the recognition of MHC-class I and II, respectively [198]. Although the Vα can play a role in positive selection, the diversity of the Vβ chain is the main factor that determines TCR αβ bias in the responding repertoire as evidenced by the MHC class-I restricted 2C TCR, which uses Vα 9-4, which is commonly associated with MHC-class II.

Since thymic selection of the T cell repertoire shapes inherent TCR reactivity to self-MHC, it has been also proposed that germline TCR sequences are “hard-wired” for MHC restriction or have a “conserved recognition” for MHC α helices. One such example has been shown for TCR Vβ 13-2 that bound both myelin basic protein complexed with I-Au and conalbumin complexed with I-Ak. Structural analysis revealed that both TCRs made contacts at the same sites with the CDR1 and CDR2 of the Vβ 13-2 [199]. Moreover,

comparison of various I-A allotypes complexed with different peptides revealed conservation of MHC-TCR residues. Thus, thymic selection can shape the TCR repertoire of the mature naïve TCR T cell pool; however, its character is only one factor that affects the TCR bias in antigen-specific T cell responses.

1.4.2.3 TCR repertoire bias in acute and chronic viral infections

TCR repertoire bias in acute viral infections

Influenza A infection in mice: infection of C57B1/6J mice with influenza A leads to an acute respiratory infection that is resolved in 10 days. $CD8^+$ T cell responses are directed towards 2 Influenza A proteins; nucleoprotein Db NP366-374 and the acid polymerase Db PA224-233. Analysis of the 2 responses revealed that the $CD8^+$ TCR repertoire was distinct. The TCR repertoire of Db NP366-374 exhibited a bias towards Vβ 8.3 and had a limited and public profile, with the presence of a 9 amino acid CDR3β loop [200] [201, 202]. This repertoire also contained < 10% of $CD8^+$ T cells that showed private specificities. On the other hand the TCR repertoire against Db PA224-233 was characterized by a more diverse and private repertoire with a preference to Vβ 7, many J segments, and a CDR3 composed of 6 amino acids [200-202]. Importantly there was no tissue-specific distribution of both repertoires in different organs, indicating that all clonotypes were evenly distributed at the site of infection (lung and bronchialveolar lavage), draining lymph nodes, lymphoid (spleen), and non-lymphoid (liver) organs.

Influenza A infection in humans: the dominant $CD8^+$ T cell response is directed against the matrix protein GL9 (Residues 58-66; GILGFVFTL) in HLA-A*0201 individuals [203]. This particular repertoire was a public one, characterized by a bias to Vβ 17 and the presence of IRSSY motif in the CDR3β [204, 205]. Insights into the structural basis of the interaction between HLA-A*0201 complexed with GL9 and Vβ 17 were gained by x-ray crystallography. The "peg-notch" conformation was proposed, suggesting that the side chain of Arginine (R) in the IRSSY motif of the CDR3β loop was the peg, whereas the notch was formed by the cavity between the bound GL9 peptide and MHC-I α2 helix. Thus the central Arginine was inserted into the notch on the antigen surface between the flat peptide and the MHC. This orthogonal orientation is different from the standard TCR / pMHC-I interaction, where an exposed side chain provides a docking structure for TCR

engagement. Therefore such "featureless" peptide-MHC class I interactions generate limited diversity because such structures provide insufficient scope to bind most TCRs with the necessary affinity to promote signaling. Moreover, since "featureless" peptides resemble more "self-like" then it could be possible that many TCRs are deleted in the thymus during negative selection [206].

TCR repertoire bias in chronic viral infections

Human Immunodeficiency Virus-1 infection: The initial T-cell receptor β-chain variable-region (Vβ) repertoire studies in HIV-1 infection combined the use of semi quantitative PCR and flow cytometric analysis using monoclonal antibodies again the different Vβ. To circumvent for the possible impact of HLA-class I alleles, Rebai et al analyzed the TCR Vβ repertoire in 9 pairs of homozygotic twins who were discordant for HIV-1 infection [207]. Perturbations in the $CD4^+$ Vβ repertoire were noted in HIV-1 positive individuals mainly in Vβ13 and Vβ21. Such perturbations were absent in the HIV-1 negative twins. Moreover, correlations between the perturbed Vβs and absolute $CD4^+$ counts were also established, and these perturbations were independent of disease stage. Altogether, these results indicated that the HIV-1 $CD4^+$ Vβ repertoire was biased in HIV-1 infection, possibly due to the expression of a yet unknown superantigenic effect [207]. Expansion of $CD8^+$ TCR Vβ families has been reported in primary infection, was shown to contain HIV-specific $CD8^+$ T cells and was responsible for the resulting control of viral burdens [88]. Interestingly, the same group reported that the ability of HIV-1 to circumvent proper immune control was due to the compromised TCR repertoire in acute infection. The dominantly expanded $CD8^+$ Vβ were shown to be perturbed between primary and chronic phases, finally resulting in rapid disappearance either during primary infection or after transition to chronic phase. The deletions were not due to evolution of the virus but rather due to the high levels of antigen during the primary phase. Interestingly, despite the disappearance of expanded clonotypes, cytotoxic activity was maintained by clonotypes that persisted from primary infection, albeit at subdominant levels. These results however did not directly show the antigen specificity of $CD8^+$ T cell clonotypes [208]. By combining the use of anti-human Vβ chain-specific mAbs and sequence analysis, Wilson et al reported that the majority of expanded $CD8^+$ T cells in chronic asymptomatic HIV-1

infection were antigen-specific in vivo, were oligoclonal in nature, and were able to persist for more than 2 years [209]. Oligoclonal expansions were also observed in the $CD8^+$ T cell compartments of HIV-1 positive children in primary infection, and comprised of persisting clonotypes. Whereas persistence was observed for $CD8^+$ T cells clonotypes, $CD4^+$ repertoires were polyclonal and lacked clonotypic persistence [210]. Therefore, $CD8^+$ T cell repertoires were restricted, as supported by CDR3 size spectra typing to analyze the TCR repertoire in HIV-1 patients [211, 212]. The relative frequency and magnitude of these perturbations have been linked with the rate of HIV-1 disease [213, 214]. In line with these observations, Soudeyns et al hypothesized whether the initiation of HAART during primary HIV infection would influence the patterns of TCR Vβ perturbations that were associated with primary viremia [210]. The authors showed that the TCR Vβ repertoire stabilized more rapidly in treated versus untreated HIV-1 individuals, and this stabilization included reduced oligoclonality in primary infection and an increased polyclonality in line with the results of Gorochov et al [211]. Since the clonality of the initial clinical response influences clinical outcome, possibly by the persistence of $CD8^+$ T cell clonotypes, the representation of $CD8^+$ clonotypes was tested in a longitudinal manner. A biphasic decline in primary $CD8^+$ T cell clonotypes was observed despite which, some $CD8^+$ T cell clonotypes persisted. This stabilization of the repertoire could reflect the transition of effector cells in primary viremia into a memory pool. However, the clonal composition of these diversified repertoires and their contribution to clinical outcome was not assessed. Similar observations were reported by Gorochov et al in 2001, where complete viral suppression by HAART was associated with stabilized CD8+ TCR Vβ repertoires, whereas incomplete suppression of viral load or viral rebound was associated with perturbations in the repertoire. A repertoire switch was reported upon viral rebound and was different from the CD8+ T cell Vβ repertoire at baseline [215]. Importantly, whether this repertoire switch upon viral rebound was secondary to the emergence of viral escape mutants was not assessed. Although many studies have supported a role for clonal deletion to be the cause of CTL-mediated dysfunction in chronic infection, other reports have provided evidence for the persistence of initially expanded CD8+ T cell clonotypes in chronic infection and under viral suppression with HAART [216]. Similar to the LCMV model of infection, these data

indicate that clonal deletion is not necessarily the cause of chronic $CD8^+$ T cell impairment, at least for the time points studied, but rather the result of the persistence of antigenic stimulation, which renders these cells dysfunctional [103].

Epitope-specific $CD8^+$ TCR repertoire studies have been recently characterized. In a cohort of 4 HLA-B*0801 positive delayed progressors, Dong et al reported that the dominant response was directed against the Nef protein FL8 (Residues 90-97; FLKEKGGL). The TCR Vβ repertoire was oligoclonal, encompassed a Vβ 13.2 clonotype with an unusually long CDR3 region that was present only in LTNPs and not progressors. Functional characterization of this clonotype revealed that it was resistant to apoptosis, had a high avidity, and was able to recognize variants, suggesting that this particular TCR could be involved in viral control of these patients [217]. A preference for Vβ 13.2 was not found in B8-FL8 response of HLA-B*0801 individuals who had started therapy in the acute phase of infection [218]. Overall, these data indicated that epitope-specific $CD8^+$ T cell responses were driven by changes in viral load and were dynamic with discordant variations within the epitope-specific TCR repertoire and that this dynamic repertoire was crucial in containing viral variants possibly by containing cross-reactive clonotypes. Turnbull et al reported that poor cross-recognition of variants was associated with a bias to a particular TCR Vβ, while efficient variant cross-recognition was not associated with a bias to particular Vβ and was strongly associated with delayed disease progression [219]. In contrast, it was shown that two closely related HLA-I alleles HLA-B*5701 and HLA-B*5703 that target the immunodominant Gag epitope KF11 (Residues 30-40; KAFSPEVIPMF) epitope, cross-recognize naturally occurring variants, with HLA-B*5703 being less efficient in cross-recognition. Interestingly, HLA-B*5703 restricted KF11 recruited a mutually exclusive and broad TCR repertoire, in contrast to HLA-B*5701 which recruited a highly conserved TCR repertoire and was efficient in cross-recognizing variants. This argues against the general hypothesis that broader TCR repertoires are needed for the proper containment of viral replication, simply by having more TCR specificities to recognize the variants. It is important to mention that the less cross-reactive TCRs recruited to HLA-B*5703-KF11 were variable in terms of TCR β chain recruitment but utilized a single α chain with a conserved CDR3 motif. Thus it is possible that restriction in α chain usage could lead to less cross-reactivity and emergence

of escape [220]. Furthermore, subsequent characterization of TCR repertoire in identical twins infected with the same HIV-1 strain revealed that the twins elicited mutually exclusive repertoires with no biases in CDR3 motifs and Vβ or Vα usage, suggesting that stochastic recruitment of clonotypes contributes to diverse and unpredictable HIV-1 evolution [221].

Simian Immunodeficiency Virus infection: Epitope-specific $CD8^+$ TCR Vβ repertoire studies were initially performed in SIV infection against an immunodominant Mamu-A*01/p11C. This response was oligoclonal, characterized by an average of 4-7 clonotypes in each macaque. There was a dominant Vβ13 usage with a preference for an 11 amino acid CDR3 region and a clonal dominance of particular Vβs. Interestingly, the evolution of the epitope-specific TCR repertoire was either stable or dynamic [222]. In an attempt to identify the molecular signatures behind peptide-MHC interactions with TCR, Price et al studied two codominant responses restricted by Mamu-A*01 in SIV infected macaques; Gag CM9 (Residues 181-189; CTPYDINQM)) and Tat TL8 (Residues 28-35; TTPESANL). Both responses were oligoclonal however, the TCR Vβ repertoire against CM9 had a preferential Vβ 27 usage and exhibited an array of private CDR3 regions in contrast to that of TL8, which expressed Vβ 6-5 and showed discernable motifs within the CDR3 regions. Upon viral sequencing it was shown that such discernable motifs were associated with viral escape within TL8 and suggested that such structural constraints within the hyper-variable regions could facilitate escape [223]. No such identifiable motifs were identified in the CM9 responses, where no variations could be found in this epitope.

Lymphocytic Choriomeningitis Virus infection: Initial $CD8^+$ TCR repertoire studies in LCMV relied on isolation and in vitro expansion of lymphocytes, which introduced biases in TCR usage and yielded discordant results [224, 225]. Sourdive et al demonstrated that the $CD8^+$ TCR repertoire against LCMV infection in mice was oligoclonal in nature and that the primary $CD8^+$ T cell response was structurally and functionally similar to the memory pool and secondary challenge effectors. This supported a role for stochastic selection process where the repertoire of the memory pool reflects the clones present in the effector population [226]. Epitope-specific $CD8^+$ TCR repertoire studies against 3 immunodominant epitopes suggested that there was a preferential bias for Vβ 8.1 and 8.2

usage in acute LCMV infection [227-229]. Interestingly, Cornberg et al showed the presence of strong biases in the TCR repertoires of virus-specific responses challenged with either homologous or heterologous virus and concluded that oligoclonality of persistent viruses could be the result of heterologous immunity encompassing only cross-reactive clones from the memory pool. Such oligoclonal responses could clear virus or not. Oligoclonal responses known to clear virus are reported to be of high avidity [230, 231]. Where oligoclonal responses are unable to clear virus, they will aid at facilitating the emergence of escape. In fact, escape from an epitope-specific response was detected both with LCMV and with HCV infections [229, 232]. Therefore, strategies that aim at proliferating cross-reactive memory CD8+ T cells could have different outcomes in individuals.

Gamma (γ) and Beta (β) Herpes Viruses: TCR repertoire bias in cytomegalovirus (CMV) infection was due to the selection of high avidity clonotypes. These high avidity clonotypes were also endowed with superior functions. The immunodominant response to CMV was to the tegument protein pp65 NV9 (Residues 495-503; NLVPMVATV) in HLA-A*0201 individuals. A dramatic clonal focusing occurred upon CMV reactivation, resulting in the expansion of a single clonotype from the initially expanded T cell pool, which was independent of CD8 for peptide-MHC recognition and function [231]. Similarly, TCR repertoire bias in Epstein barr virus (EBV) infection was due to the selection of high avidity clonotypes from the primary T cell pool and many of the T cell clonotypes detected in individuals with primary EBV infection could be found up to 3 years post infection [230, 233].

1.4.2.4 TCR repertoire bias due to selection of private and public TCR repertoires

Definition of private and public TCR repertoires

Private TCR repertoires describe a scenario in which the repertoires of different individuals against the same peptide-MHC complex have no overlap in the TCR sequences utilized. These repertoires could exhibit broad or limited diversity. In contrast, public TCR repertoires against the same peptide MHC complex are comprised of

clonotypes (either α- or β-chain, or both) that are shared between different individuals. Public TCRs could be the dominant component of an individual's TCR repertoire along with other subdominant clonotypes or they could be the sole TCR used both within and between individuals. Public clonotypes are an example of type 3 TCR repertoire bias [195].

Mechanisms of public TCR repertoire generation

For public TCRs to occur in many individuals the prerequisite is that (i) they are produced by genetic recombinations in the thymus; (ii) they are able to survive thymic selection; (iii) they are able to survive in the periphery; (iv) there is enough precursor frequency and avidity to compete with other TCRs in the repertoire for a given epitope. Several hypotheses have been put forward to explain the mechanisms behind the occurrence of public TCRs. Of these, structural explanations propose that the shape of the peptide-MHC could bias the TCR repertoire and lead to public TCRs. Briefly; "bulged" or "hot and spicy" peptides are proposed to lead to public TCRs, due to inaccessibility of several TCRs to contact the peptide-MHC complex. On the other hand, "featureless" or "vanilla peptides" are known to allow recruitment of many TCRs into the repertoire with no evident bias. This explanation does not seem to be the only determinant, as the TCR repertoire of the "featureless" matrix protein MP58-66 peptide from Influenza virus restricted to HLA-A*0201 exhibits public TCR specificities [206]. Other hypotheses include a sequence-based explanation. An example from mice that lack the TdT enzyme and have no nucleotide additions has suggested that public TCRs are easy to generate since they do not require nucleotide additions. This does not seem to be an exclusive explanation, as multiple nucleotide sequences with differences in nucleotide additions or deletions could encode for the same TCR within and in between individuals. Therefore, biases in V (D) J recombinations or the hypothesis that public TCRs are made without the need for nucleotide insertion does not seem to hold true. Using comprehensive screening on published data from the public TCRs that occur in HLA-A*0201 individuals to the matrix protein of Influenza virus, Venturi et al proposed that public clonotypes emerge due to a mechanism known as "convergent recombination". This suggests that multiple V (D) J recombinations and nucleotide additions occur and converge to produce the same

nucleotide sequence. The second level of convergent recombination encompasses multiple nucleotide sequences that yield the same amino acid sequence. The same protein sequences could be encoded by different nucleotide sequences. The third level of convergent recombination ensures that some of the amino acids conform motif-related amino acid sequences, which have been long associated to public TCRs. However, some TCR sequences responding to the same epitope will be associated with non-motif amino acid sequences [234]. Therefore, public TCRs arise from extremely intermittent TCRs that survive the periphery.

Examples of public TCR repertoire usage in viral infections

Public T-cell responses have been observed in several viral infections including SIV [223], HIV [220, 235], CMV [230, 231], EBV [233, 236], and Influenza virus [204, 206]. What is the relative functional contribution of public TCRs in a virus-specific repertoire? Studies with EBV and CMV have shown that dominant TCR responses elicit public TCRs and are highly avid [230, 231], suggesting that avidity to antigen shapes clonal dominance in an antigen-specific response. Evidence also suggests that such clonotypes could be maintained for long periods of time [233]. Moreover, high avidity clonotypes are often endowed with superior function [119, 237]. Therefore, it is tempting to speculate from these examples that the presence of public clonotypes is associated with better viral control.

More recently, Price et al reported that the number of public clonotypes in both vaccinated and infected macaques is negatively correlated with virological outcome. Further dissecting the mechanisms behind these observations, the authors suggested that although there were no phenotypic and functional profile changes between private versus public TCR responses, public responses were more efficient in cross-recognizing viral variants compared to private specificities [238]. Although naturally occurring variants were absent throughout the study, the authors found such correlations between the frequency of public clonotypes and virologic outcome apparent only at earlier time points thus suggesting that the initial interaction between cognate antigen and adaptive immune responses govern biological outcome. Further studies are necessary to clarify these observations and could be taken into consideration during vaccine design.

1.5 Consequences of TCR diversity on viral immunity and its implications for vaccine design

Having described the different biases that arise in TCR responses due to immunity against viral infections, one can easily acknowledge the consequences of TCR diversity on viral immunity. The association of TCR diversity with escape from immune recognition is a compelling finding and further strengthens how eliciting broad TCR repertoires could contain pathogens in contrast to very limited and restricted repertoires [223, 229, 232]. However, like in CMV and EBV infections [230, 231], TCR diversity is often restricted to oligoclonal responses, yet these responses are able to control infection, possibly due to their high avidity and superior function. Therefore, antigen sensitivity appears to be another major determinant of TCR repertoire diversity such that high avidity clonotypes dominate the response [119]. Moreover, broad responses could also involve cross-reactive clonotypes that could be either beneficial for the containment of viral variants or detrimental in the sense that they might elicit autoimmune responses [239]. Furthermore, the effect of antigen dose seems to be another important determinant of TCR diversity in viral infections. The viral load in CMV and EBV infection is kept steady enough to elicit oligoclonal responses that are of high avidity. It is likely that the high viral load in HIV-1 infection results in the deletion of high avidity clonotypes [105] in acute infection or impairs the function of high avidity clones that survive this deletion process. Hence, longitudinal studies are crucial to understand the evolution and dynamics of TCR clonotypic turnover and the fate of such clonotypes. The effect of antigen load on shaping TCR diversity is also helpful in defining the "optimal concentration" that elicits and importantly maintains the "best fit" responses. Regardless of the advances made in quantifying TCR diversity in viral infections, it needs to be determined what quality of this diversity is most important for optimal control of pathogens; best avidity, best functional effector match, or best prevention of escape? Most importantly, could we modify TCR functional diversity and/or avidity? How could we induce polyclonal responses that do not elicit autoimmune cross-reactivity [240]?

Manipulation of the TCR repertoire in the context of persistent viral infections is a tempting step, yet a field in its infancy. An example of such manipulation has been reported and suggests that vaccination of HAART treated individuals with the

MVA.HIVA that comprises HIV-1 clade A gag p24/p17 sequence fused to multi CTL epitope gene, leads to the expansion of vaccine-specific $CD8^+$ T cell responses to *Gag*, *Nef*, and *Pol*. These expansions were polyclonal in nature in sharp contrast to the oligoclonal TCR repertoires observed in vaccinees who did not elicit any response and unvaccinated controls. Moreover, although vaccination led to the transient upregulation of PD-1, which rapidly returned to base line levels, the polyclonal expansions of vaccine induced $CD8^+$ T cells displayed better proliferative potential and in vivo viral suppression activity. Thus, strategies that aim at inducing polyclonal responses under conditions of HAART appear to be promising [241]. The answers to the above questions could be technically challenging and somehow impossible, however, several studies have unfolded the major determinants or TCR repertoire diversity and if the above questions are successfully resolved, they could provide a powerful rationale for vaccine design to elicit the desired end products of TCR diversity.

1.6 Project rationale and research objectives

HIV-specific $CD8^+$ T cell dysfunction is a major characteristic of chronic HIV infection and a main reason for the failure of cellular immunity to clear virus. The molecular signatures for the dysfunction of HIV-specific $CD8^+$ T cells remained enigmatic. Therefore, the specific research objectives of the work in this dissertation were:

1. Identify PD-1 as an exhaustion marker for HIV-specific $CD8^+$ T cells and manipulate the PD-1 axes to restore HIV-specific $CD8^+$ dysfunction.

2. Study the effect of antigen load and its absence on the functional profile of HIV-specific $CD8^+$ T cells, by longitudinally tracking HIV-specific $CD8^+$ T cell responses in a cohort of four patients who had started HAART and stopped voluntarily.

3. Study the effect of "antigen loss" to wild-type epitopes on the functional profile of HIV-specific $CD8^+$ T cells in a cohort of four chronically infected patients, in the

context of escape, i.e., the effect of viral sequence diversification on the fate of HIV-specific $CD8^+$ T cells against original wild-type epitopes.

Figures

Figure 1

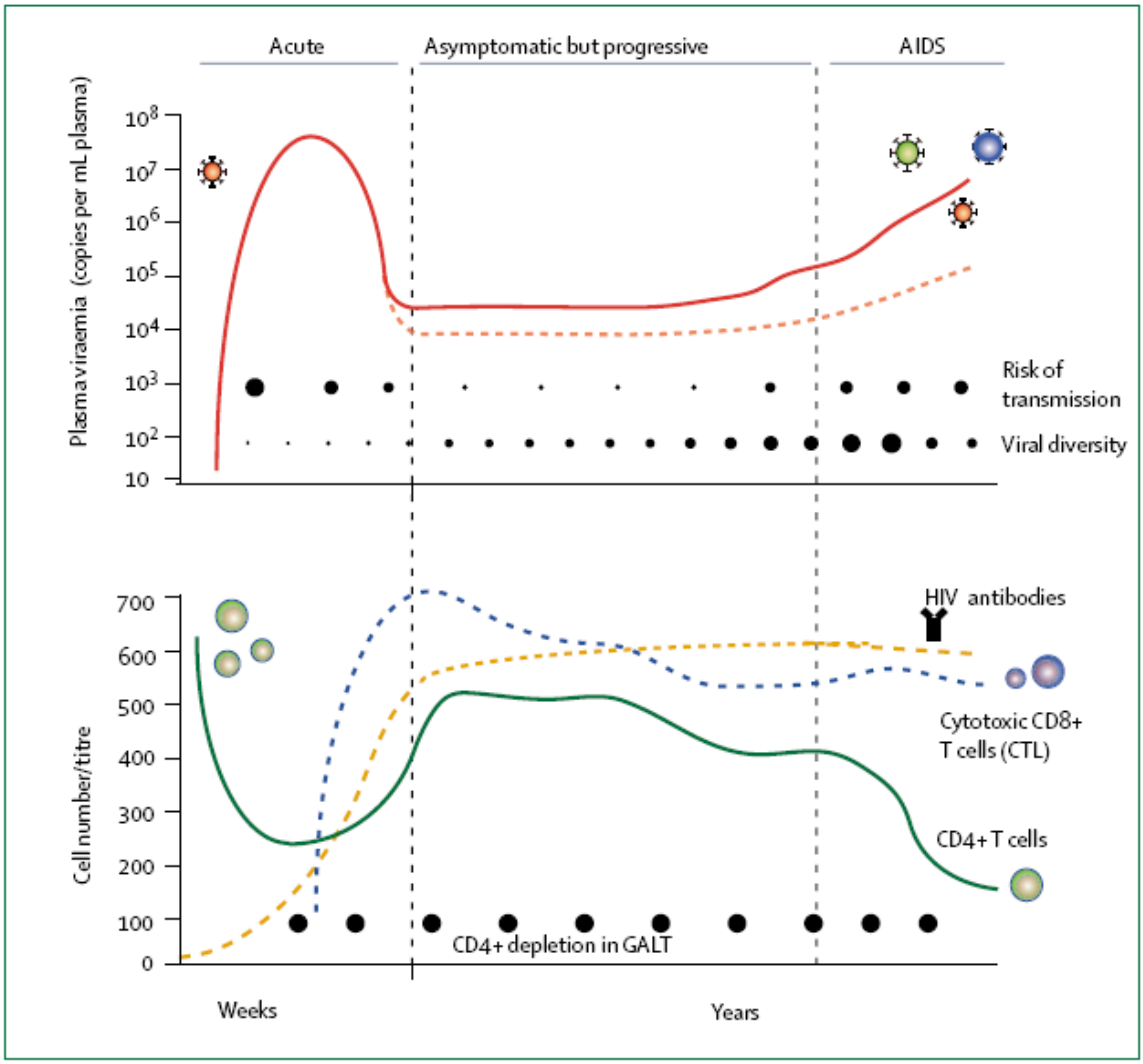


Figure 2

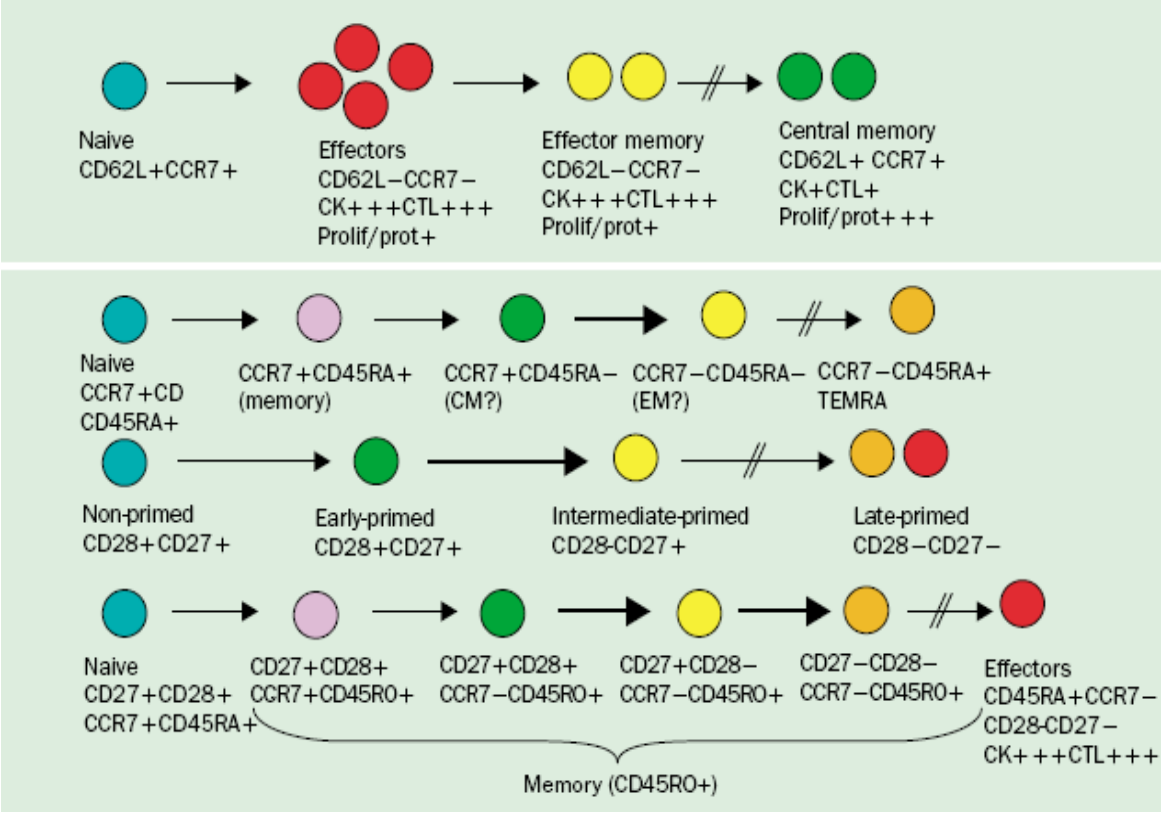
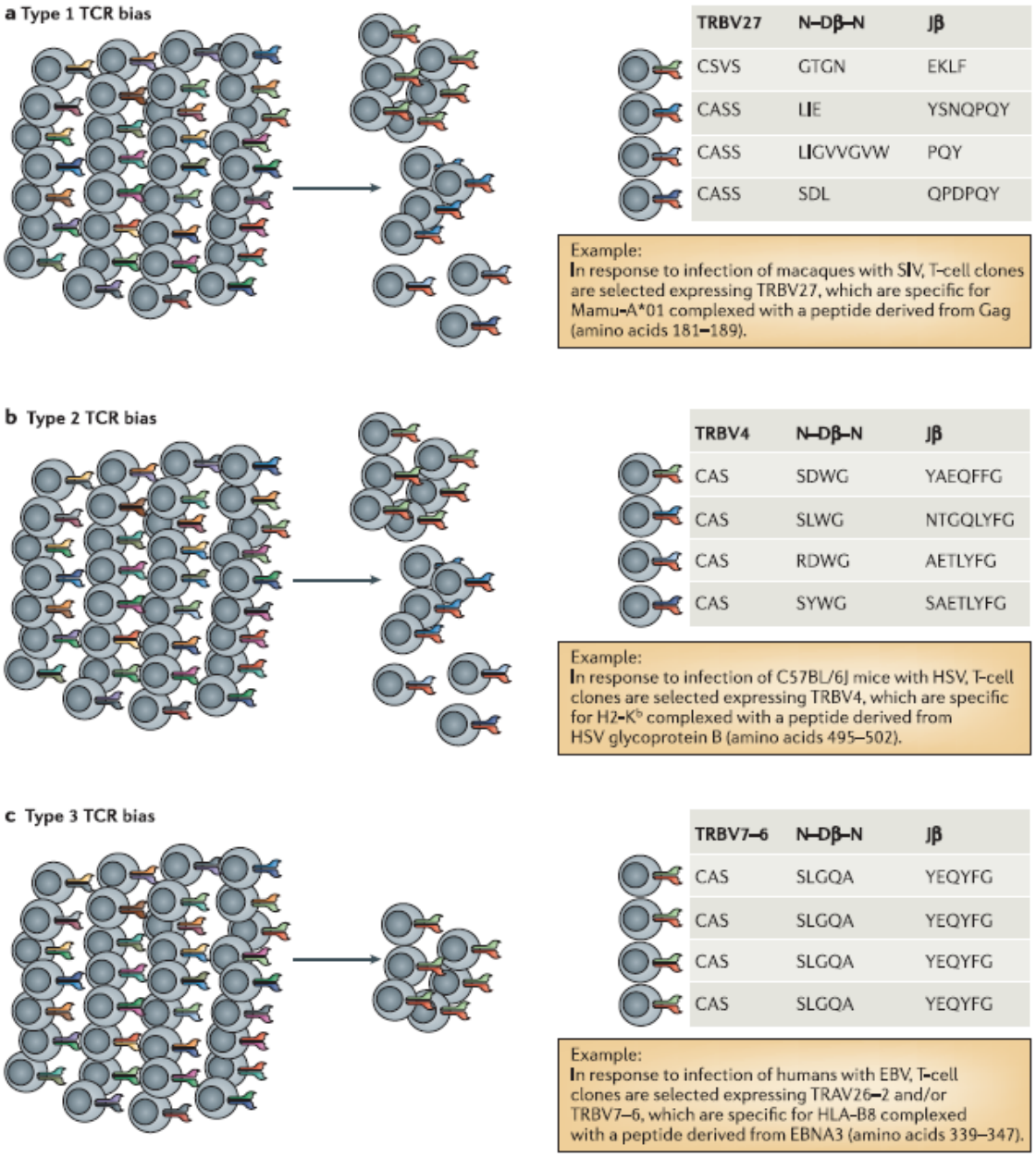

Naive
CD62L+CCR7+
Effectors
CD62L−CCR7−
CK+++CTL+++
Prolif/prot+
Effector memory
CD62L−CCR7−
CK+++CTL+++
Prolif/prot+
Central memory
CD62L+ CCR7+
CK+CTL+
Prolif/prot+++
Naive
CCR7+CD
CD45RA+
CCR7+CD45RA+
(memory)
CCR7+CD45RA−
(CM?)
CCR7−CD45RA−
(EM?)
CCR7−CD45RA+
TEMRA
Non-primed
CD28+CD27+
Early-primed
CD28+CD27+
Intermediate-primed
CD28-CD27+
Late-primed
CD28−CD27−
Naive
CD27+CD28+
CCR7+CD45RA+
CD27+CD28+
CCR7+CD45RO+
CD27+CD28+
CCR7−CD45RO+
CD27+CD28−
CCR7−CD45RO+
CD27−CD28−
CCR7−CD45RO+
Effectors
CD45RA+CCR7−
CD28-CD27−
CK+++CTL+++
Memory (CD45RO+)

Figure 3


a Type 1 TCR bias
TRBV27 N–Dβ–N Jβ
CSVS GTGN EKLF
CASS LIE YSNQPQY
CASS LIGVVGVW PQY
CASS SDL QPDPQY
Example:
In response to infection of macaques with SIV, T-cell clones are selected expressing TRBV27, which are specific for Mamu-A*01 complexed with a peptide derived from Gag (amino acids 181–189).
b Type 2 TCR bias
TRBV4 N–Dβ–N Jβ
CAS SDWG YAEQFFG
CAS SLWG NTGQLYFG
CAS RDWG AETLYFG
CAS SYWG SAETLYFG
Example:
In response to infection of C57BL/6J mice with HSV, T-cell clones are selected expressing TRBV4, which are specific for H2-Kb complexed with a peptide derived from HSV glycoprotein B (amino acids 495–502).
c Type 3 TCR bias
TRBV7–6 N–Dβ–N Jβ
CAS SLGQA YEQYFG
CAS SLGQA YEQYFG
CAS SLGQA YEQYFG
CAS SLGQA YEQYFG
Example:
In response to infection of humans with EBV, T-cell clones are selected expressing TRAV26–2 and/or TRBV7–6, which are specific for HLA-B8 complexed with a peptide derived from EBNA3 (amino acids 339–347).

Legends of figures

Figure 1: The Course of HIV-1 infection defined by the level of viral replication

The figure summarizes the major immune players in HIV-1 disease. Plasma viremia and dynamic changes of the CD4+ T-lymphocyte compartments are depicted in the top and bottom panels, respectively. Primary HIV infection is characterized by high plasma viral load (red line, top), low CD4+ T cells (green line, bottom), and absence of HIV-1 specific antibodies (orange line, bottom). Viremia drops concomitant with the emergence of cytotoxic CD8+ T-lymphocytes (CTL) (blue line, bottom) and an individual viral-load set point is reached during chronic infection. Viral set points vary greatly among individuals (red dotted line, top) and predict disease progression. Viral diversity increases throughout the disease (closed circles, top); whereas the risk of transmission is highest in the first weeks when viremia peaks (closed circles, top). GALT= gut-associated lymphoid tissues. This figure is adapted from Simon, V. et al, *HIV/AIDS epidemiology, pathogenesis, prevention, and treatment.* Lancet, 2006. **368**(9534): p. 489-504.

Figure 2: Models of memory $CD8^+$ T-cell differentiation in mice and humans

The figure summarizes advances in understanding $CD8^+$ T cell functional differentiation and maturation, and how the process can be compromised during chronic viral infections. Top panel: in the murine lymphocytic choriomeningitis virus (LCMV) model, memory $CD8^+$ T-cell maturation follows antigenic priming of naive $CD8^+$ T cells, which differentiate first into $CD8^+$ effector/effector-memory cells and subsequently into central memory $CD8^+$ T-cells. Effector $CD8^+$ T cells downregulate the lymphoid homing receptors CD62L and CCR7 and mediate effector functions such as cytokine production (CK) and CTL activity. These cells do not proliferate in response to ex vivo antigenic restimulation and are less effective than central memory cells in mediating protection from in vivo virus challenge. Memory (central-memory) $CD8^+$ T-cells re-express CD62L and CCR7, mediate only limited effector function, but can proliferate vigorously in response to ex vivo antigenic restimulation and are highly effective in mediating proliferation and protection (prolif/prot) from in vivo virus. Bottom panel: in the human system, a number of models have been proposed. In the first and based on the expression of CCR7 and

CD45RA, naive $CD8^+$ T-cells differentiate into central-memory cells (CM), then into effector memory cells (EM), and finally into terminally differentiated effectors or $CD45RA^+$ effector-memory cells (TEMRA). In the second and based on the expression of CD27 and CD28, naive (i.e., nonprimed) $CD8^+$ T cells differentiate into early primed, intermediate-primed, and late-primed cells. In the third model which integrates all the above markers, naive $CD8^+$ T cells differentiate into several stages of memory cells, and finally in effector T cells. Defects in maturation and differentiation of antigen-specific memory $CD8^+$ T-cells have been reported during chronic viral infections. Such defects are represented by both bold arrows that indicate increased/excessive production and broken arrows that indicate defective differentiation/maturation. In the human system, the defects in differentiation/maturation are particularly described for HIV-specific $CD8^+$ T cells in HIV-infected individuals. This figure is adapted from Garber, D.A. et al, *Prospects for an AIDS vaccine: three big questions, no easy answers.* The Lancet Infectious Diseases, 2004. **4**: p. 397-413.

Figure 3: Types of T-cell receptor bias in antigen-specific T-cell repertoires

a) Type 1 TCR bias occurs when responding T cells in an individual use the same TCR α-chain variable (Vα) region or β-chain variable (Vβ) region, but have little or no similarity in the complementarity-determining region 3 (CDR3)- or junctional (J)-region sequences. N–Dβ–N indicates the CDR3 for the TCR β-chain, which is formed by non-templated nucleotides (N) and imprecise joining at the V (D) J junctions. **b)** Type 2 TCR bias occurs when responding T cells in an individual use the same TCR Vα or Vβ region, and share amino acids at the same position in the CDR3 region (a CDR3 motif).The motif can vary depending on the number of amino acids utilized. The example shown here encompasses a two amino-acid motif, WG. **c)** Type 3 TCR bias can be defined as when responding T cells in an individual use the same TCR Vα or Vβ region, CDR3 and J-region sequence. It can refer to a single TCR α- or β-chain, or both. EBV, Epstein–Barr virus; EBNA3, Epstein–Barr virus nuclear antigen 3; HSV, herpes simplex virus; SIV, simian immunodeficiency virus. TCR Vα and Vβ gene segments are here referred to as TRAV and TRBV according to the ImMunoGeneTics (IMGT) database2.

This figure is adapted from Turner, S.J. et al, *Structural determinants of T-cell receptor bias in immunity.* Nature Reviews Immunology, 2006. **6**: p. 883-894.

2 Upregulation of PD-1 expression on HIV-specific $CD8^+$ T cells leads to reversible immune dysfunction.

In 2006, Barber et al [106] showed that PD-1 was upregulated on LCMV-specific $CD8^+$ T cells in chronic infection, and that blocking the interaction of PD-1 with its ligand; PD-L1 restores antigen-specific $CD8^+$ T cell function. Therefore, we wanted to see whether PD-1 was upregulated on HIV-specific $CD8^+$ T cells and whether similar manipulations would lead to restored function of HIV-specific $CD8^+$ T cells.

Upregulation of PD-1 expression on HIV-specific CD8$^+$ T cells leads to reversible immune dysfunction.

Lydie Trautmann[1,2,3], Loury Janbazian*[1,4], Nicolas Chomont*[1,2,3], Elias A. Said[1,2,3], Sylvain Gimmig[1], Benoit Bessette[1], Mohamed-Rachid Boulassel[5], Eric Delwart[6], Homero Sepulveda[7], Robert A. Balderas[7], Jean-Pierre Routy[3,5], Elias K. Haddad[1,2,3,4,8] and Rafick-P. Sekaly[1,2,3,4,8].

[1]Laboratoire d'Immunologie, Centre de Recherche CHUM Saint-Luc, Montréal, Québec, Canada.

[2]Laboratoire d'Immunologie, Département de Microbiologie et d'Immunologie, Université de Montréal, Québec, Canada.

[3]INSERM U743, CR-CHUM, Université de Montréal, Montréal, Québec, Canada.

[4]Department of Microbiology and Immunology, McGill University, Montréal, Québec, Canada.

[5]Immunodeficiency Service and Division of Haematology, Royal Victoria Hospital, McGill University Health Centre, McGill University, Montréal, Québec, Canada.

[6] Blood Systems Research Institute, San Francisco, California, USA.

[7] BD Biosciences, San Diego, CA, USA.

[8] Co-senior authors

* These authors contributed equally to this work

Abstract

The engagement of Programmed Death 1 (PD-1) to its ligands, PD-L1 and PD-L2 [1-4], inhibits proliferation and cytokine production mediated by antibodies to CD3 [5-7]. Blocking the PD-1/PD-L1 pathway in mice chronically infected with lymphocytic choriomeningitis virus restores the capacity of exhausted $CD8^+$ T cells to undergo proliferation, cytokine production and cytotoxic activity and, consequently, results in reduced viral load [8]. During chronic HIV infection, HIV-specific $CD8^+$ T cells are functionally impaired [9-11], showing a reduced capacity to produce cytokines and effector molecules as well as an impaired capacity to proliferate [12-15]. Here, we found that PD-1 was upregulated in HIV-specific $CD8^+$ T cells; PD-1 expression levels were significantly correlated with both viral load and with the reduced capacity for cytokine production and proliferation of HIV-specific $CD8^+$ T cells. Notably, cytomegalovirus (CMV)-specific $CD8^+$ T cells from the same donors did not upregulate PD-1 and maintain the production of high levels of cytokines. Blocking PD-1 engagement to its ligand (PD-L1) enhanced the capacity of HIV-specific $CD8^+$ T cells to survive and proliferate and led to an increased production of cytokines and cytotoxic molecules in response to cognate antigen. The accumulation of HIV-specific dysfunctional $CD8^+$ T cells in the infected host could prevent the renewal of a functionally competent HIV-specific $CD8^+$ repertoire.

We monitored the expression of PD-1 in HIV-, CMV- and Epstein-Barr virus (EBV)-specific $CD8^+$ T cells for six different epitopes using staining for tetramers of peptide-major histocompatibility complex class I (pMHC) complexes from 12 viremic, 7 aviremic highly active antiretroviral therapy (HAART)-treated HIV-infected individuals and 9 healthy donors. Percentages of tetramer-positive $CD8^+$ T cells for each individual are listed in Supplementary Table 1. Representative dot plots for tetramer and PD-1 staining show that HLA-A2 and HLA-B7 HIV tetramer-positive cells obtained from HIV viremic individuals express much higher levels (3.5-fold) of PD-1 than do HLA-A2 and HLA-B7 CMV tetramer-positive cells (Figure 1a). Fig.1b illustrates such differences for 7 viremic individuals and further shows variations in the expression levels of PD-1 on HIV-specific $CD8^+$ T cells for different specificities in the same individual. Of note, we did not observe differences in cytotoxic T lymphocyte-associated protein 4 (CTLA-4) expression levels between HIV-specific T cells and CMV- and EBV-specific $CD8^+$ T cells (data not shown). Analysis of 12 viremic individuals (38 tetramers) showed that HIV-specific $CD8^+$ T cells expressed higher levels of PD-1 when compared to 7 aviremic HAART-treated individuals (10 tetramers, $P<0.0001$; Figure 1c). PD-1 expression was consistently higher on HIV-specific $CD8^+$ T cells in viremic individuals than on T cells specific for CMV ($n=19$, $P<0.0001$) or EBV ($n=15$, $P<0.0001$; Figure 1c). Moreover, PD-1 expression was higher on HIV-specific $CD8^+$ T cells in aviremic individuals than CMV-specific $CD8^+$ T cells in the same subjects ($P<0.0001$; Figure 1c). We then examined the levels of PD-1 on total $CD8^+$ T cells in a larger group of individuals. The levels of PD-1 on total $CD8^+$ T cells were significantly higher in 20 viremic and 18 aviremic individuals than those in 14 healthy individuals ($P<0.0001$ and $P=0.0003$ respectively; Figure 1d). Notably, PD-1 expression on total $CD8^+$ T cells in viremic individuals correlated significantly with HIV viral load ($P=0.0009$;Figure 1e). Taken together, our results show that PD-1 expression is higher in HIV-specific $CD8^+$ T cells (viremic or aviremic) than in CMV- and EBV-specific $CD8^+$ T cells. Moreover, PD-1 upregulation on HIV-specific $CD8^+$ T cells correlates with high viral load and consequently high antigenemia.

Longitudinal analyses of PD-1 expression on HIV-, EBV-, and CMV-specific $CD8^+$ T cells were performed on 5 individuals followed for up to six years. Over this period,

individuals showed marked fluctuations in viral load, largely as a consequence of voluntary treatment interruptions. This allowed us to correlate the magnitude of PD-1 expression to viral load in the same person. The analysis of PD-1 expression in three of these participants is shown in Figure 2. For subjects #026 and #319, PD-1 expression by HIV-specific $CD8^+$ T cells was already upregulated as early as five months after infection; it decreased substantially following treatment, whereas high expression was induced by the resurgence of viremia shortly after cessation of HAART (Figure 2a,b). Subject #316, who maintained high viral loads, consistently displayed elevated PD-1 expression levels on HIV-specific $CD8^+$ T cells (Figure 2c). In contrast, PD-1 expression levels on CMV- and EBV-specific tetramer positive cells were initially low in those individuals and never changed over the study period (Figure 2a, b, c). Similar observations were made in two other individuals (Supplementary Figure 1). Notably, those who are under HAART treatment can have residual HIV replication, despite HIV being undetectable by routine viral load assays; hence, the magnitude of the residual viremia could differ from person to person, thereby explaining the differential decrease of PD-1 expression levels on HIV specific T cells in aviremic stages [16, 17]. Taken together, these results show that the correlation between viral load and PD-1 expression was present both cross-sectionally (Figure 1) and longitudinally (Figure 2).

The longitudinal analysis of subject #026 allowed us to follow B7NEF-specific (see methods for peptide definition) $CD8^+$ T cells and their repertoire over time. To exclude the possibility that HIV-specific $CD8^+$ T cell impairment was caused by altered peptide effects of cytotoxic T lymphocyte escape mutations, we sequenced this HLA-B7-restricted epitope (TPGPVRYPL). Because this epitope was not mutated over the course of infection, we can conclude that $CD8^+$ dysfunction was not attributed to an altered peptide ligand effect [18,19]. Moreover, sequencing the T cell antigen receptor (TCR) complementarity-determining region 3 CDR3β at each time point (35 to 50 clones) showed a strong conservation of CDR3β motifs for the most frequent clonotypes (Figure 2a). Similar findings were also observed for CMV-specific $CD8^+$ T cells (data not shown). The conservation of TCR sequences in PD-1^{hi} HIV-specific T cells indicates that PD-1 upregulation may induce, at least over this study time course, the functional impairment of antigen-specific cells rather than their clonal depletion.

The phenotypes of PD-1^{hi} and PD-1^{low} cells were assessed by determining the expression levels of CCR7, CD45RA, CD27, CD28, CD57, CD127 and CD279 (PD-1) on CD3^{+}, CD8^{+} HIV, and CMV-specific tetramer positive cells using ten color-flow cytometric analysis (Supplementary Table 2). All PD-1^{hi} HIV-specific CD8^{+} T cells from viremic individuals had a significantly different phenotype compared to PD-1^{low} CMV-specific CD8^{+} T cells in that they were CD45RA^{-} (P=0.001; Figure 3a), and expressed low levels of CD127 (P=0.003; Figure 3b), higher levels of CD27 (P<0.0001; Figure 3c), low levels of senescence marker CD57 (P=0.001; Figure 3d), and low levels of CD28 (P=0.019; Figure 3e). Therefore, PD-1^{hi} HIV-specific CD8^{+} T cells in viremic individuals have a poorly differentiated phenotype (CD27^{hi}, CD28^{low}, CD57^{low}, CD127^{low}, CCR7^{-} and CD45RA^{-}) previously reported to accumulate in dysfunctional HIV-specific CD8^{+} T cells[20,21]. As expected, this phenotype is in sharp contrast to that of CMV-specific tetramer-positive cells, which are more differentiated, as characterized by the expression of high levels of CD45RA and CD57 and low levels of CD27 [20,21].

We confirmed the dysfunction of PD-1^{hi} HIV-specific CD8^{+} T cell first by investigating the capacity of these cells to produce interferon (IFN)- γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α upon stimulation with cognate peptides (See representative experiment in Figure 3f). The frequency of HIV-specific CD8^{+} T cells producing IL-2 and TNF- α was significantly lower than the corresponding frequencies of CMV- and EBV-specific CD8^{+} T cells (P=0.006 and P=0.015, respectively; Figure 3g,h). On the other hand, HIV-, EBV- and CMV-specific CD8^{+} T cells produced similar levels of IFN-γ (Figure 3i). Moreover, there was a significant inverse correlation between PD-1 expression levels and IL-2 and TNF- α production (P=0.01 and P=0.04, respectively; Supplementary Figure 2) unlike for IFN- γ whose expression showed no correlation with PD-1 levels (Supplementary Figure 2). Altogether, the relative levels of PD-1 were negatively correlated with the CD8^{+} T cells' capacity to produce cytokines.

We next determined whether interfering with PD-1 ligation by PD-L1 would allow HIV-specific CD8^{+} T cells to secrete increased amounts of effector molecules upon TCR triggering. We focus on cytokines and molecules associated with cytotoxic T-lymphocyte function, a hallmark of CD8^{+} T cells [20,22,23]. Peripheral blood mononuclear cells (PBMCs) were stimulated with their cognate peptides and secretion of TNF- α, Granzyme

B (GzB), and Lymphotoxin-α (LT-α) was measured in the culture supernatant at day 6 using cytometric bead array (CBA). In the absence of peptide or in addition of anti-PD-L1 blocking antibody alone, we observed a background secretion of effector molecules in HIV viremic individual (Figure 4a, b). Addition of HIV cognate peptide induced moderate amounts of TNF- α, Granzyme B and Lymphotoxin-α However, in the presence of blocking antibody to PD-L1, we observed a significant increase in the production of these molecules in 19 HIV peptide-specific stimulations (using cells from 10 participants) for TNF- α ($P=0.0001$; Figure 4c), for Granzyme B ($P<0.0001$; Figure 4d), and for Lymphotoxin-α ($P=0.0003$; Figure 4e). These results were confirmed by intracellular cytokine staining, in which 3 out of 3 patients showed increased frequencies of tetramer positive cells producing TNF- α after 6 d stimulation in the presence of antibody to PD-L1 (Supplementary Figure 3). These results provide further evidence that disruption of the PD-1-PD-L1 interaction can increase cytokine production.

We then determined whether interrupting the ligation of PD-1 by its ligand PD-L1 could increase the capacity of HIV-specific $CD8^+$ T cells to proliferate, as monitored by the frequency of carboxyfluorescein succinimidyl ester $(CFSE)^{low}$ tetramer labeled $CD8^+$ T cells, after stimulation with their cognate peptide in a 6 d assay. The results of these experiments (see Figure 4 f for representative experiment) show that the addition of the PD-L1 antibody was able to augment the proliferation of HIV-specific $CD8^+$ T cells as indicated by the increase in the number of tetramer-positive HIV-specific $CD8^+$ T cells ($P=0.0015$; Figure 4 g) and in the number of proliferating $CFSE^{low}$ HIV-specific $CD8^+$ T cells ($P=0.0035$; Figure 4 h). Notably, addition of IL-2 alone or in combination with antibody to PD-L1 did not induce proliferation of these cells. Proliferation and effector molecule secretion were assayed in the same culture medium. Moreover, we have shown that all proliferating cells expressed even higher levels of PD-1 as compared to baseline; this is expected because PD-1 is a T cell activation marker ($n=4$, Supplementary Figure 3). Despite these high levels, proliferation and cytokine production were increased in the presence of a blocking antibody to PD-L1. Similar data were published in a previous study, where it was clearly shown that $PD\text{-}1^{hi}$ cells remained $PD\text{-}1^{hi}$ after 6 d of culture even in the presence of antibodies to PD-L1 [9]. Taken together, these data show that

blocking PD-1-PD-L1 interaction increases the capacity of HIV-specific $CD8^+$ T cells to proliferate and survive.

HIV-specific $CD8^+$ T cells have been known to lack effector T cell functions [20,21]. Several strategies have attempted to rescue the dysfunction of HIV-specific T cells including HAART and the addition of functionally competent HIV-specific $CD4^+$ T cells [22]. Although the latter was successful in restoring the capacity of $CD8^+$ T cells to proliferate, restoration of effector function was not observed. In this paper, we report for the first time, that disruption of the molecular interaction of PD-1 to its ligand can increase the proliferation and the secretion of effector molecules by HIV-specific $CD8^+$ T cells. This suggests that the presence of blocking antibodies to PD-L1 relieves these cells from the previously reported skewed maturation phenotype. Although all HIV-specific $CD8^+$ T cells expressed high levels of PD-1 at the onset of cultures, and all $CFSE^{low}$ cells at the end of the 6 d culture also expressed high levels of PD-1, it is nonetheless impossible however to rule out that hyporesponsive cells could be responsible for the increased cytokine production. Identification at the single-cell and at molecular levels of the mechanisms which lead to the PD-1 induced $CD8^+$ dysfunction will help in resolving this issue.

PD-1 upregulation on HIV-specific $CD8^+$ T cells leads to the failure of the cells to enter into cell cycle and to produce cytokines such as IL-2 and TNF- α possibly by down-regulating the TCR signal. PD-1 is associated with SHP-1 and SHP-2, two phosphatases that negatively regulate T cell signaling [7]. By analogy, SHP-1 and SHP-2, which are also associated intracellularly with CTLA-4 are required for CTLA-4 mediated T cell regulation of proliferation and cytokine production [24]. This could provide a mechanism by which PD-1 exerts its negative effect on T cell proliferation and cytokine production. Of note, the fact that $PD\text{-}1^{hi}$ cells also do not express the co-stimulatory molecule CD28 could further contribute to their reduced growth potential and their lack of IL-2 production [25]. The capacity of HIV-specific T cell to produce IFN- γ, even under conditions of high PD-1 expression, is not surprising since as in other models of chronic infection, the ability to secrete IFN-γ is the last T cell function to be affected [26].

Our results show a differential increase in proliferation and effector molecule production in different $CD8^+$ T cells' HIV specificities, sometimes within the same person. The existence of an "exhaustion gradient" within the dysfunctional population [26] could provide a likely explanation for this heterogeneity. Several factors including TCR avidity and HIV epitope abundance could be responsible for the different levels of dysfunction. Understanding the mechanisms underlying different stages of dysfunction would be of great interest. Finally, our results clearly demonstrate that PD-1^{hi} HIV-specific $CD8^+$ T cells can persist throughout the course of infection. This could be advantageous for the virus because it hampers the renewal of functional virus-specific $CD8^+$ T cells by accumulating a functionally impaired population and preventing the reestablishment of a niche of functionally competent T cells.

Methods

Subjects

38 HIV-1-infected patients (20 viremic and 18 aviremic) and 14 healthy donors included in this study, signed informed consent approved by the Royal Victoria Hospital and the CR-CHUM hospital review boards. We measured plasma viral load using the Amplicor HIV-1 Monitor UltraSensitive Method (Roche). Supplementary Table 1 summarizes the 12 viremic and 7 aviremic subjects who were assessed for tetramer staining. Subjects' HLA haplotypes, plasma viral loads, $CD4^+$ T-cell and $CD8^+$ T-cell counts were determined at various time points.

Tetramers

We obtained pMHC monomers from the CANVAC core facility (Montreal, Canada). The different peptides used to analyze the CMV-, EBV- and HIV-specific CD8 T cell responses are as follows: for CMV, NLVPMVATV (A2CMV) and TPRVTGGGAM (B7CMV); for EBV, GLCTLVAML (A2EBV); for HIV, TPGPGVRYPL (B7NEF), SLYNTVATL (A2p17), and FLGKIWPSYK (A2p15). Soluble pMHC monomers were generated as described [27].

Phenotypic analysis

We resuspended PBMCs in RPMI-10% FCS at $5x10^7$ cells in 100µl. We added phycoerythrin (PE) or allophycocyanin (APC) tetramers at 0.3µg per 10^6 cells and incubated at 37°C for 15 min. We incubated Antibodies to cell surface markers at 4° C for 20 min. Two cocktails of antibodies and tetramers were used: Tetramer-PE or Tetramer-APC, CD28-APC or CD127-PE, CD57-PE-Cy5 or CD28-PE-Cy5, CD3-Alexa700, CD8-PE-Texas Red (ECD), CD27-Pacific blue, CCR7-PE-Cy7, CD45RA-APC-Cy7, and PD-1-FITC. All antibodies were purchased from BD Biosciences, except for CD8-ECD (Cedarlane). Live cells were gated by forward and side scatter. We acquired $2.5x10^6$ events for each sample using the BD LSRII flow cytometer (BD Biosciences) and analyzed data using the DIVA software (BD Biosciences).

Intracellular cytokine staining assay

We stained three million cells with the appropriate tetramers for 15 min at 37°C and stimulated with 10µg/ml of the cognate peptides for 6 h in RPMI containing 8% human serum in the presence of 5µg/ml of Brefeldin A (Sigma-Aldrich). Unstimulated cells were used as controls. We then stained the cells with surface specific antibodies for 15 min at 4°C and fixed for 20 min in 100µl 2% paraformaldehyde (PFA) at room temperature (25°C). To stain cells with antibodies specific for intracellular cytokines (IFN-γ APC, IL-2-FITC, TNF-α Alexa700) (BD Biosciences), we incubated the cells with antibodies in 0.25% saponin (Sigma-Aldrich) for 30 min at 25°C and analyzed them on the BD LSRII flow cytometer as described above.

CFSE Proliferation assay

CFSE-based proliferation assays were performed as previously described [28]. We incubated $2x10^6$/ml CFSE labeled PBMCs in the presence of 5µg/ml of corresponding peptides in RPMI containing 8% human serum, 10U/ml of IL-2 (eBiosciences) for 6 d to substitute for the lack of helper cells. We added blocking antibody specific to PD-L1 (eBiosciences) to cell cultures at a concentration of 10µg/ml. After six d of incubation, we collected culture medium and stained cells with tetramers for 15 min at 37°C, then with CD3-Alexa700 and CD8-ECD, and analyzed them on the BD LSRII flow cytometer as described above. We assayed supernatant from day 6 for cytokines and cytolytic molecules using a custom-made cytometric bead array (see below).

Multiplex cytometric bead assay

Was used A BD™ Cytometric Bead Array (CBA) assay (BD Biosciences, San Diego, CA) to measure Fas ligand, Lymphotoxin-α, Granzyme A, Granzyme B, TNF-α and IFN- γ. Briefly, six bead populations with distinct fluorescence intensities were coated with capture antibodies specific for each molecule. The six bead populations were mixed together and were resolved in the FL3 channel of a BD LSR-II flow cytometer. The capture beads were mixed with the PE-conjugated detection antibodies and then incubated with recombinant standards or test samples. Following acquisition of sample data using

the flow cytometer, the sample results were tabulated and graphed using the BD CBA Analysis Software.

TCR sequencing

We sorted Tetramer$^+$, CD8$^+$ and CD3$^+$ cells on the BDAria cell sorter (BD Biosciences). We lysed Sorted peptide-specific CD8$^+$ T cells and extracted mRNA from them using the oligotex mRNA mini kit (Qiagen). Anchored RT-PCR was performed using a modified version of the switching mechanism at 5' end of RNA template (SMART) procedure and a TCR-β constant region 3' primer for the PCR using the SMART Race cDNA Amplification Kit (Clontech), as described [29].

Statistics

We performed Statistical analyses using Instat 2.00 software. To assess differences, associations, and trends, and to first-order approximate *P* values for rejecting null effect hypotheses, we used straight-forward, established conservative standard statistical tests appropriate for relatively small numbers of samples. For Figure 1c, d, and 3, we used unpaired t-test assuming independent samples and unknown unequal variances for the underlying populations; Paired *t* tests were applied to log-transformed concentration values when measured concentrations spanned many orders-of-magnitude in dynamic range as in Figure 4 c, d, e. A paired t test was also used to show the effect of treatment with antibodies to PD-L1 on the number of HIV-specific tetramer T cells (Figure 4 g, h). The data met the assumption of the *t* tests. Notably, equivalent non parametric tests yielded similar results. The Pearson correlation test was used to demonstrate significant correlation between PD-1 expression and viral load (Figure 1e) as well as the inverse correlation between PD-1 expression and cytokine production (Supplementary Figure 3).

Acknowledgments

We thank P. Wilkinson, L. Greller and R. Somogyi for the statistical analyses and M. Lainesse for her technical assistance. This work was supported by grants awarded to R.P.S. from the National Institutes of Health, the Canadian Institutes of Health Research, the Canadian Network for Vaccine and Immunotherapeutics, Genome Canada and the Réseau SIDA FRSQ R.P.S. is the Canada Research Chair in Human Immunology. J.-P.

Routy is a scientific scholar receiving support from the FRSQ. We also thank Bruce Walker, Rick Koup and Rafi Ahmed for discussions.

Figures

Figure 1

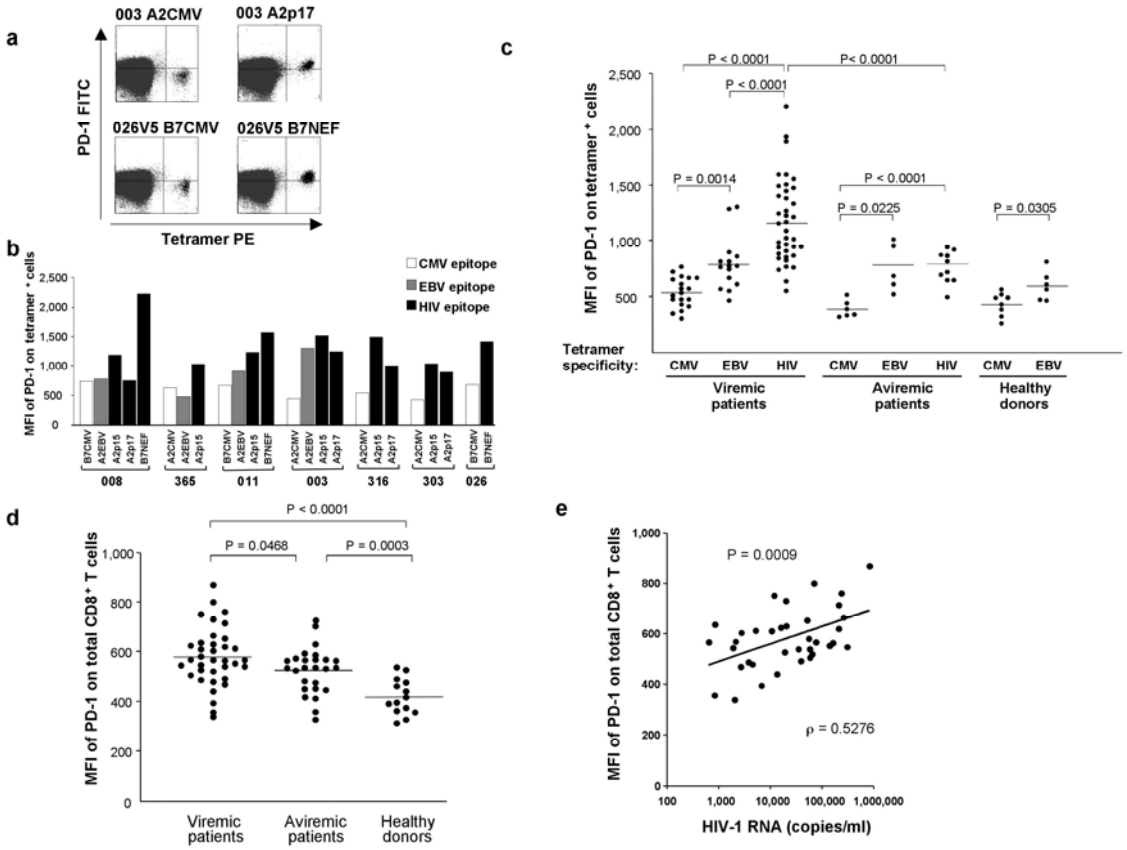


Figure 2

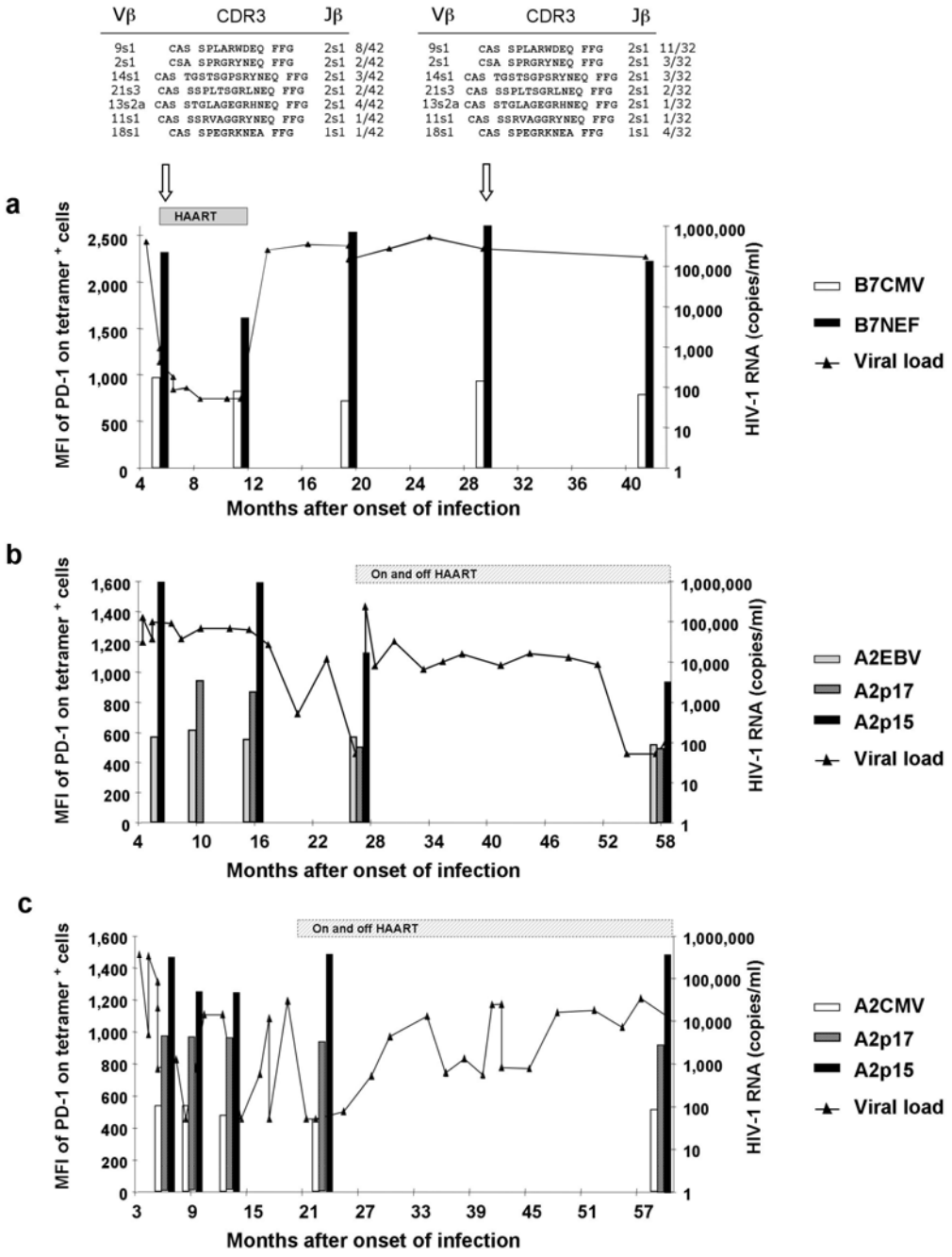

Vβ CDR3 Jβ
9s1 CAS SPLARWDEQ FFG 2s1 8/42
2s1 CSA SPRGRYNEQ FFG 2s1 2/42
14s1 CAS TGSTSGPSRYNEQ FFG 2s1 3/42
21s3 CAS SSPLTSGRLNEQ FFG 2s1 2/42
13s2a CAS STGLAGEGRHNEQ FFG 2s1 4/42
11s1 CAS SSRVAGGRYNEQ FFG 2s1 1/42
18s1 CAS SPEGRKNEA FFG 1s1 1/42
Vβ CDR3 Jβ
9s1 CAS SPLARWDEQ FFG 2s1 11/32
2s1 CSA SPRGRYNEQ FFG 2s1 3/32
14s1 CAS TGSTSGPSRYNEQ FFG 2s1 3/32
21s3 CAS SSPLTSGRLNEQ FFG 2s1 2/32
13s2a CAS STGLAGEGRHNEQ FFG 2s1 1/32
11s1 CAS SSRVAGGRYNEQ FFG 2s1 1/32
18s1 CAS SPEGRKNEA FFG 1s1 4/32
a
HAART
MFI of PD-1 on tetramer + cells
HIV-1 RNA (copies/ml)
Months after onset of infection
B7CMV
B7NEF
Viral load
b
On and off HAART
MFI of PD-1 on tetramer + cells
HIV-1 RNA (copies/ml)
Months after onset of infection
A2EBV
A2p17
A2p15
Viral load
c
On and off HAART
MFI of PD-1 on tetramer + cells
HIV-1 RNA (copies/ml)
Months after onset of infection
A2CMV
A2p17
A2p15
Viral load

Figure 3

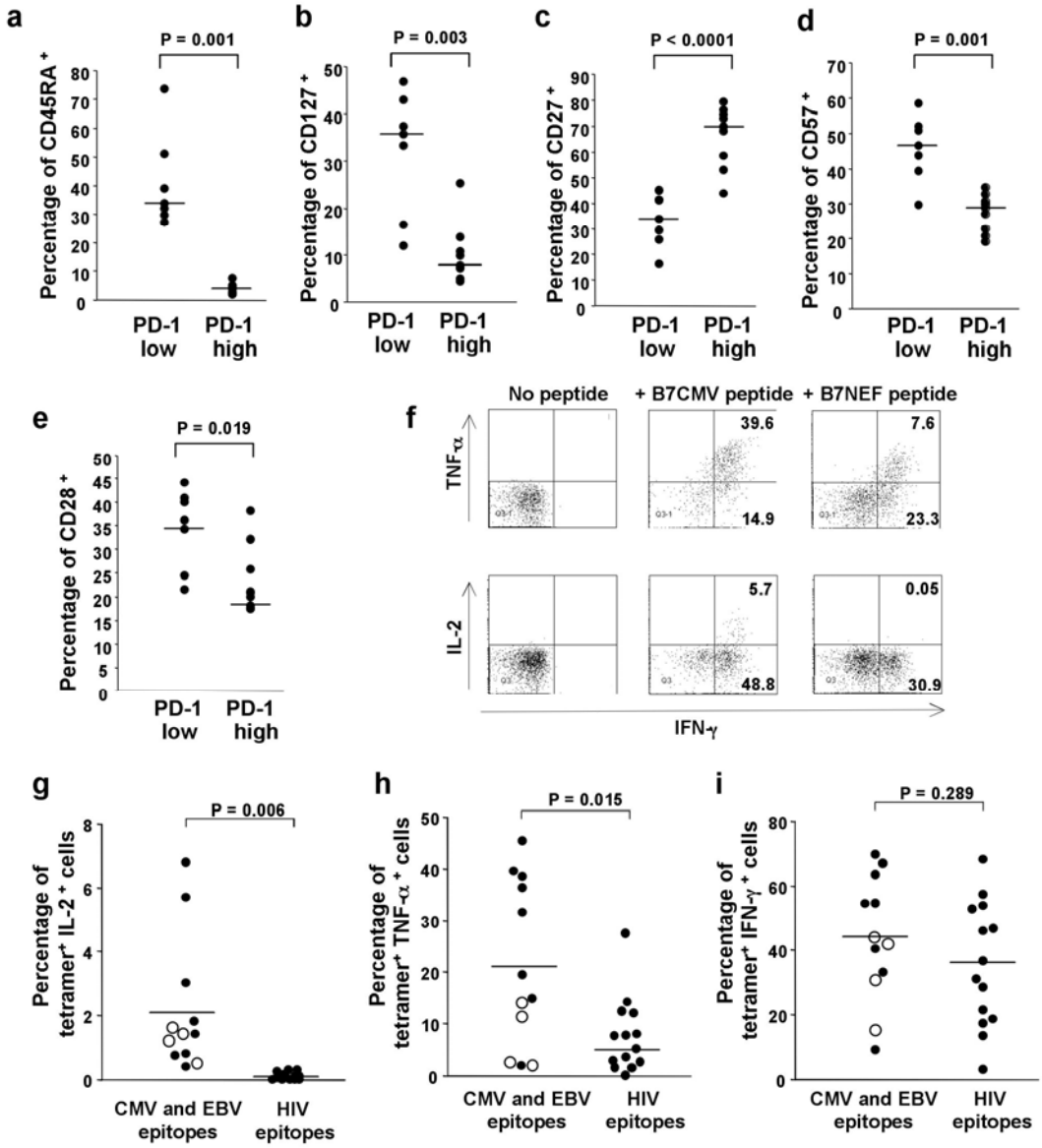
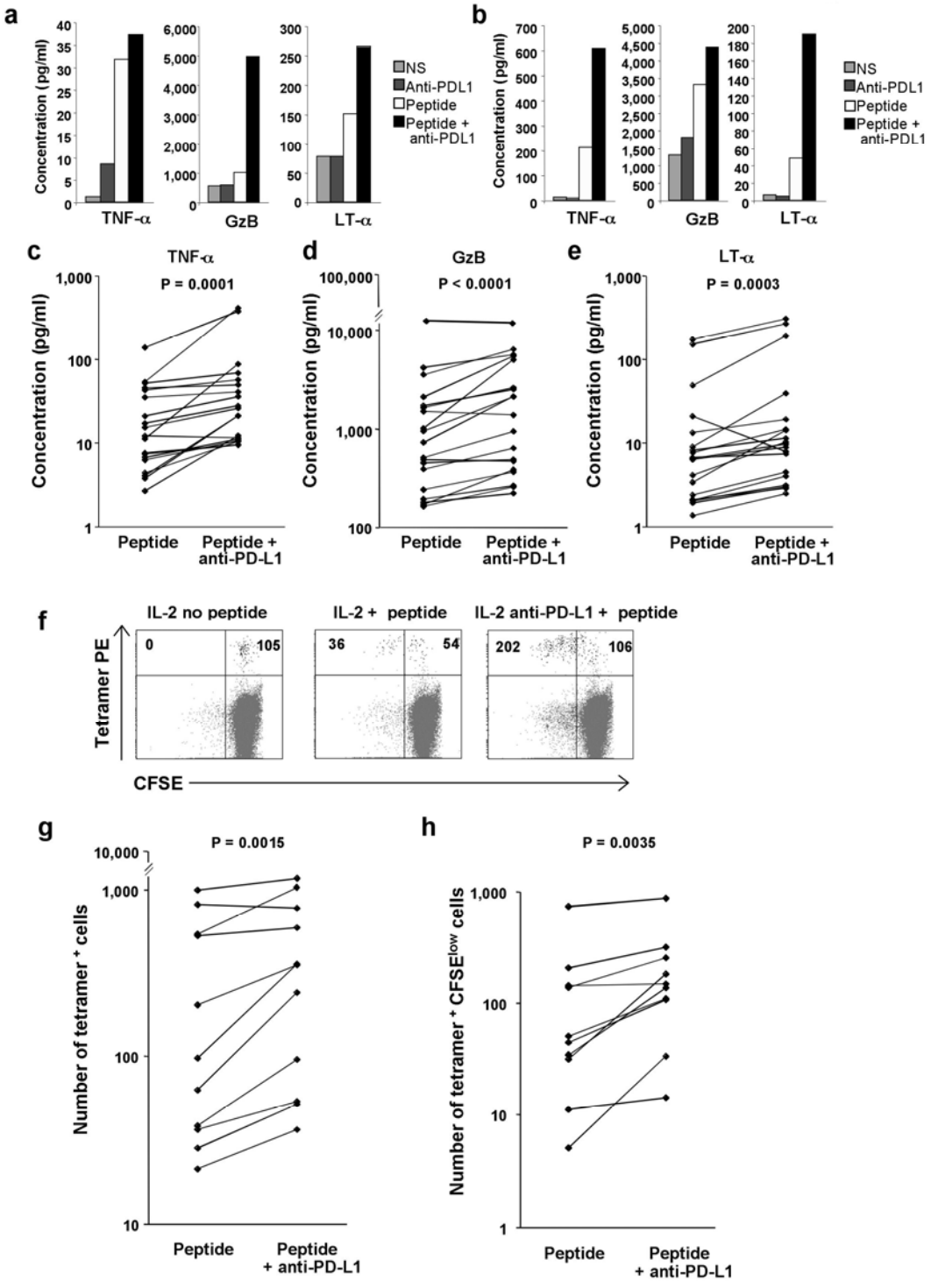
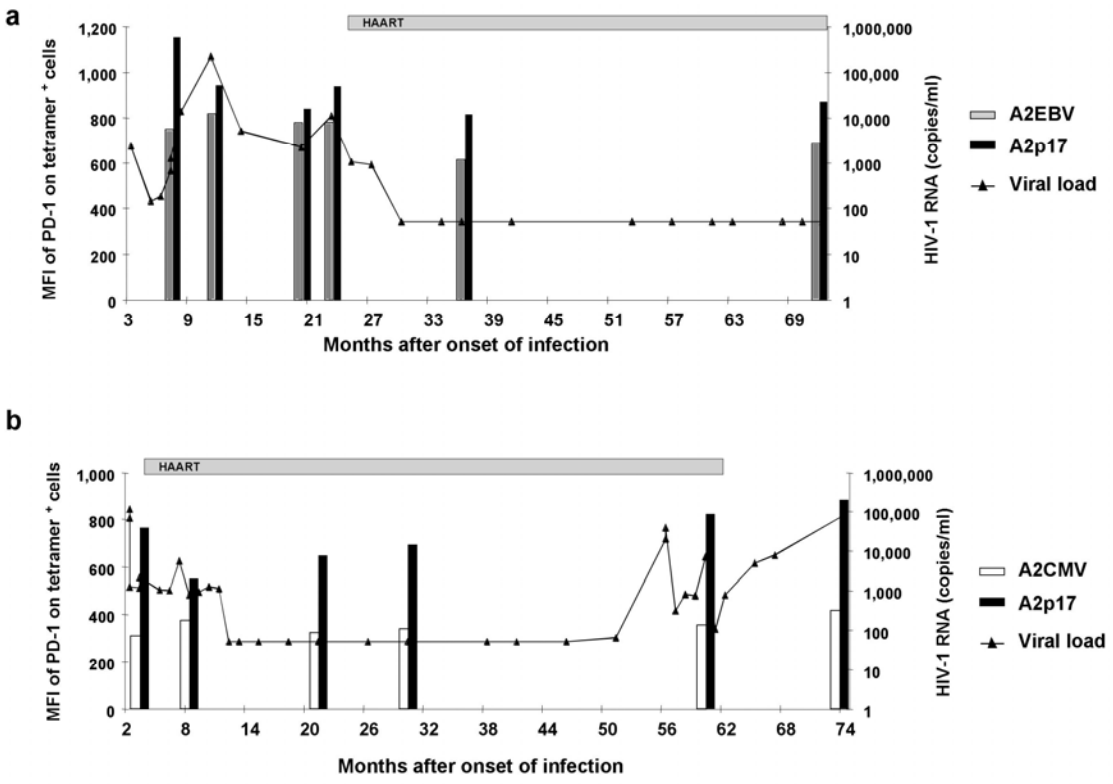

a
P = 0.001
Percentage of CD45RA+
PD-1 low
PD-1 high
b
P = 0.003
Percentage of CD127+
PD-1 low
PD-1 high
c
P < 0.0001
Percentage of CD27+
PD-1 low
PD-1 high
d
P = 0.001
Percentage of CD57+
PD-1 low
PD-1 high
e
P = 0.019
Percentage of CD28+
PD-1 low
PD-1 high
f
No peptide
+ B7CMV peptide
+ B7NEF peptide
TNF-α
39.6
14.9
7.6
23.3
IL-2
5.7
48.8
0.05
30.9
IFN-γ
g
P = 0.006
Percentage of tetramer+ IL-2+ cells
CMV and EBV epitopes
HIV epitopes
h
P = 0.015
Percentage of tetramer+ TNF-α+ cells
CMV and EBV epitopes
HIV epitopes
i
P = 0.289
Percentage of tetramer+ IFN-γ+ cells
CMV and EBV epitopes
HIV epitopes

Figure 4

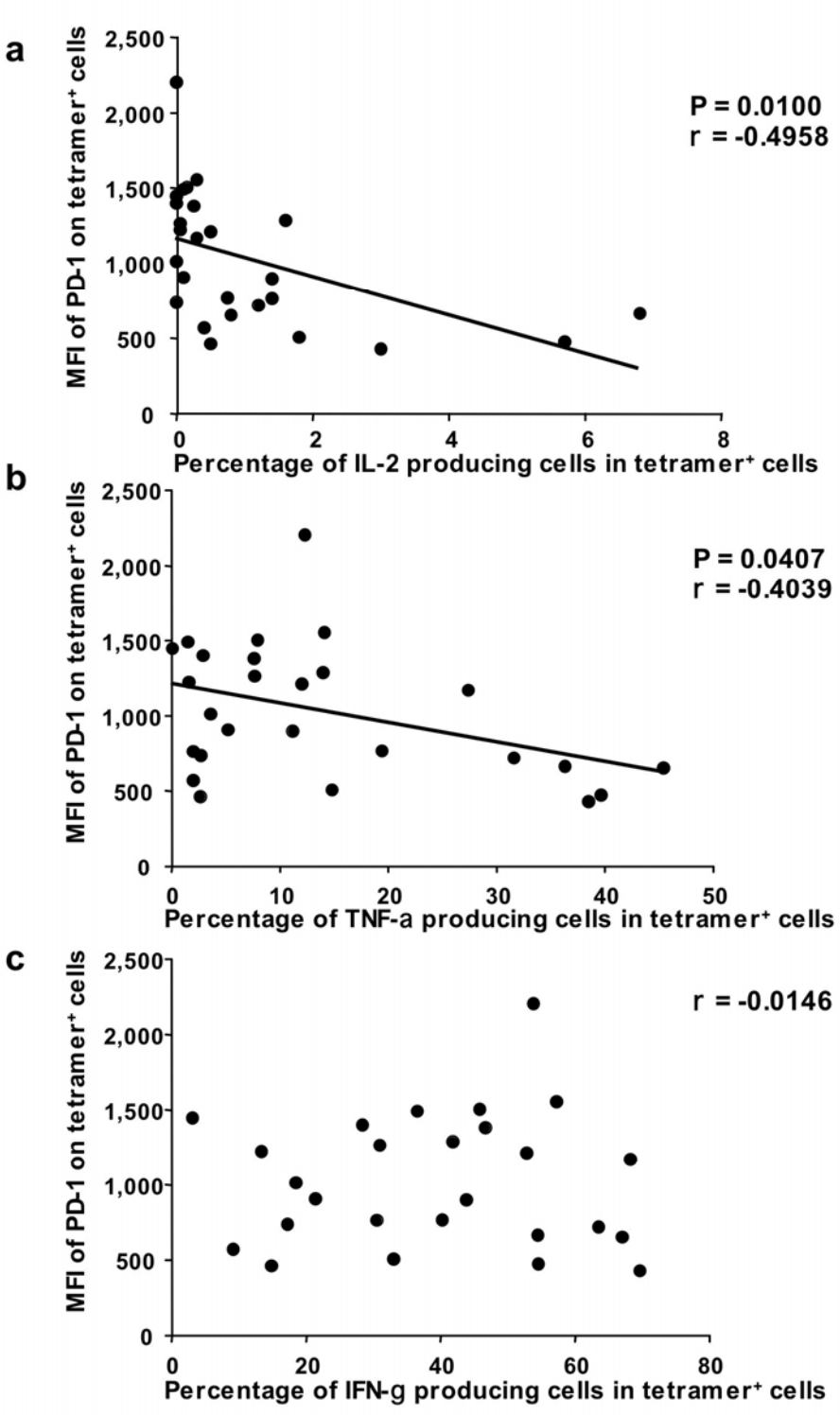

a
Concentration (pg/ml)
TNF-α
GzB
LT-α
NS
Anti-PDL1
Peptide
Peptide + anti-PDL1
b
c
TNF-α
P = 0.0001
Peptide
Peptide + anti-PD-L1
d
GzB
P < 0.0001
e
LT-α
P = 0.0003
f
IL-2 no peptide
IL-2 + peptide
IL-2 anti-PD-L1 + peptide
Tetramer PE
CFSE
0
105
36
54
202
106
g
P = 0.0015
Number of tetramer+ cells
h
P = 0.0035
Number of tetramer+ CFSElow cells

Supplementary Figures

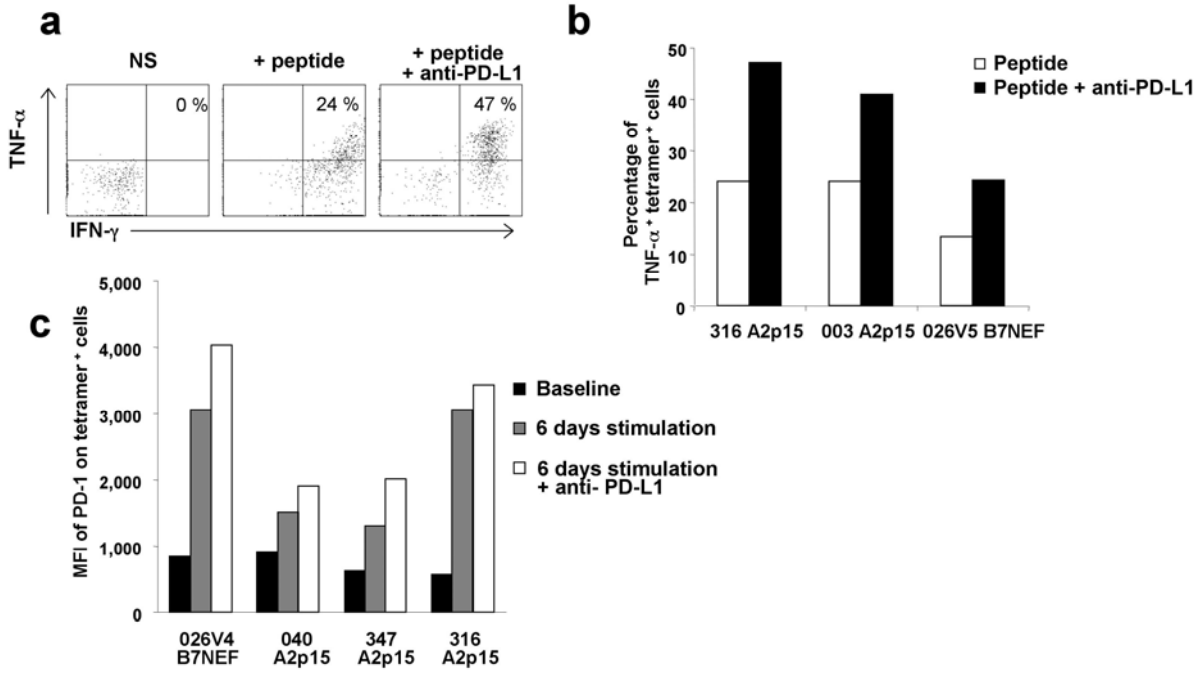
Supplementary figure1



Supplementary figure2



Supplementary figure3



Supplementary Tables

Supplementary Table 1

							Percentage of tetramer + cells in CD8+ cells					
Patient	HLA-A type	HLA-B type	Viral load*	Time of infection§	CD4 (n/µl)	CD8 (n/µl)	B7CMV	B7NEF	A2CMV	A2EBV	A2p15	A2p17
026V1	A3/A29	B7/B44	871	6	870	1080	0.095	0.334				
026V2	A3/A29	B7/B44	<50	12	985	787	0.149	0.063				
026V3	A3/A29	B7/B44	144544	19	599	858	0.095	0.366				
026V4	A3/A29	B7/B44	269153	30	na	na	0.109	0.686				
026V5	A3/A29	B7/B44	165958	42	437	814	0.186	0.581				
003	A2/A3	B7/B15	16625	16	850	1100	1.542	0.004	0.213	0.157	0.281	0.171
316V1	A1/A2	B7/B37	19596	5	569	797			0.162		0.513	0.335
316V2	A1/A2	B7/B37	13803	12	527	655			0.277		0.432	0.680
316V3	A1/A2	B7/B37	12100	60	771	888			0.185		0.744	0.434
365	A2/A29	B18/B44	3890	15	313	354			0.017	0.058	0.108	
008	A2/A3	B7/B57	2754	166	514	555	1.088	0.064		0.110	0.309	0.636
011	A2/A3	B7/B44	5370	180	325	941	2.959	0.260		0.077	0.790	
013	A2/A32	B18/B35	316227	5	483	930			0.011		0.040	0.114
040	A2/A29	B40/B52	71910	9	345	511				0.314	0.076	
303V1	A2/A3	B38/B44	2093	4	371	402			0.013			0.361
303V2	A2/A3	B38/B44	858	9	na	na			0.019			1.953
303V3	A2/A3	B38/B44	<50	22	538	516			0.018			0.035
303V4	A2/A3	B38/B44	<50	31	254	772			0.017			0.105
303V5	A2/A3	B38/B44	6901	61	500	540			0.163		0.124	1.823
303V6	A2/A3	B38/B44	78196	74	na	na			0.122		0.221	1.361
347	A2	B41/B50	4621	10	855	915				0.054	0.011	0.019
319V1	A2/A3	B27/B40	35692	4	1029	1631				0.174	0.008	
319V2	A2/A3	B27/B40	65683	9	563	923				0.236		0.015
319V3	A2/A3	B27/B40	60011	14	na	na				0.185	0.023	0.382
319V4	A2/A3	B27/B40	100	57	364	499				0.117	0.021	0.019
322	A2/A68	B24/B44	<50	94	235	399				0.166	0.016	
332V1	A2/A33	B53/B58	659	6	555	765				0.137		0.132
332V2	A2/A33	B53/B58	217207	9	368	734				0.124		1.032
332V3	A2/A33	B53/B58	2187	18	281	822				0.158		0.547
332V4	A2/A33	B53/B58	10845	21	239	769				0.121		0.495
332V5	A2/A33	B53/B58	<50	34	356	626				0.102		0.047
332V6	A2/A33	B53/B58	<50	70	396	645				0.107		0.049
102	A3/A23	B7/B38	<50	19	662	1051	0.547	0.015				
Gol8	A3/A11	B7/B15	<50	20	463	376	0.047	0.035				

* RNA copies/ml

§ Months

Supplementary Table 2

Viremic patients	CCR7	CD45RA	CD27	CD28	CD57	CD127	CD279
026V1 B7CMV	-	+	+	+	++	+	-
026V3 B7CMV	-	+	+	+	++	+	-
026V4 B7CMV	-	+	+	+	+	+	-
026V5 B7CMV	-	+	+	+	+	+	-
003 B7CMV	-	++	-	-	++	-	-
003 A2CMV	-	++	+	+	+	+	-
316 A2CMV	-	+	+	-	+	-	-
003 A2EBV	-	-	++	+	++	-	++
026V1 B7NEF	-	-	++	+	+	-	+++
026V3 B7NEF	-	-	++	+	-	-	+++
026V4 B7NEF	-	-	+	-	-	-	+++
026V5 B7NEF	-	-	++	-	+	-	+++
003 B7NEF	-	-	+++	+	-	+	++
003 A2p15	-	-	++	-	+	-	+++
316 A2p15	-	-	++	-	+	-	+++
003 A2p17	-	-	+++	-	+	-	++
316 A2p17	-	-	++	-	+	-	+

Aviremic patients	CCR7	CD45RA	CD27	CD28	CD57	CD127	CD279
026V2 B7CMV	-	+	+	+	++	+	-
Gol8 B7CMV	-	+	++	+	++	nd	-
319 A2EBV	-	-	+++	-	++	nd	++
322 A2EBV	-	-	+++	+	-	nd	+
332 A2EBV	-	-	++	+	+	nd	+
026V2 B7NEF	-	-	++	-	+	+	++
Gol8 B7NEF	-	-	+++	+	++	nd	+
319 A2p15	-	-	+++	-	+	nd	+++
322V2 A2p15	-	-	++	+	+	nd	++
319 A2p17	-	++	+	+	+	nd	+
332 A2p17	-	+	+++	-	-	nd	++

Legends of figures

Figure 1: PD-1 is upregulated on HIV-specific CD8$^+$ T cells. (a) PD-1 staining representative dot plot for patient #003 (A2CMV and A2p17 tetramers) and for patient #026V5 (B7CMV and B7NEF tetramers). Dot plots are on a 5log scale axis. (**b)**, Mean Fluorescence Intensity (MFI) of PD-1 expression on HIV-, CMV- and EBV-specific CD8$^+$ T cells in seven representative viremic individuals, we measured PD-1 expression levels on more than one HIV epitope. **(c)** MFI of PD-1 expression on tetramer-positive (tetramer+) T cells specific for HIV, EBV and CMV in 12 viremic, 7 infected aviremic and 9 healthy individuals. Each dot represents one tetramer specificity. **(d)** MFI of PD-1 expression on total CD8 T cells in 20 viremic (36 measurements), 18 infected aviremic (26 measurements) and 14 healthy individuals. Each dot represents one individual at one time point. **(e)** MFI of PD-1 expression on total CD8$^+$ T cells in function of viral load (36 measurements).

Figure 2: Longitudinal analysis of PD-1 expression on HIV-specific CD8$^+$ T cells correlates with viremia. (a) MFI of PD-1 expression for B7CMV and B7NEF tetramer-positive (tetramer+) cells in subject no. 026 at five time points. White arrows indicate the points where sequencing of the CDR-3 region was performed. The line represents measurements of viral load at the different time points. Conserved CDR-3 TCR-□sequences from time points 1 and 4 are represented for B7NEF specific CD8$^+$ T cells in this individual. The frequency of each clonotype on the total number of analyzed sequences is indicated. **(b)** MFI of PD-1 expression for A2EBV, A2p17 and A2p15 tetramer-positive cells in subject no. 319 are represented at five time points. **(c)** MFI of PD-1 expression for A2CMV, A2p17 and A2p15 tetramer-positive cells in subject no. 316 at five time points.

Figure 3: Pre-terminally differentiated phenotype and functional impairment of PD-1^{hi} HIV-specific CD8$^+$ T cells. (a-e) Phenotypic characterization of PD-1^{hi} and PD-1^{low} antigen-specific cells using HIV and CMV tetramers. PD-1^{hi} were only observed in HIV-

specific $CD8^+$ T cells from viremic individuals and PD-1^{low} cells were only observed in CMV-specific $CD8^+$ T cells from the same patients. Expression levels of PD-1^{hi} (n=9) and PD-1^{low} (n=7) cells of CD45RA **(a),** CD127 (IL-7R□) **(b),** CD27 **(c),** CD57 **(d)** and CD28 **(e)**. **(f-i)** Cytokine production in HIV-, CMV- and EBV-specific $CD8^+$ T cells. **(f)** Representative dot plots of IL-2, IFN- γ and TNF-α intracellular staining gated on B7CMV and B7NEF tetramer-positive (tetramer+) cells in individual no 026V5. Dot plots are on a 5-log-scale axis. **(g-i)** Cytokine production in six viremic patients for 8 B7CMV and A2CMV tetramers, 4 A2EBV tetramers (empty dots) and 14 B7NEF, A2p15 and A2p17 tetramers: IL-2 (**g**), TNF-(**h)** and IFN- γ **(i)**. Each dot represents one single tetramer staining.

Figure 4: Blocking the PD-1-PD-L1 pathway increases effector molecules production and proliferation of HIV-specific $CD8^+$ T cells. A 6-d stimulation assay was performed on CFSE labeled cells in the presence of the peptide alone or with blocking antibody to PD-L1. A CBA assay was performed on day 6 supernatants to quantify effector molecules production. **(a-b)** Production of TNF-α, Granzyme B and Lymphotoxin-α in the supernatants of 6-d cultures of two representative viremic subjects, nos 303 **(a)** and 026 **(b). (c-e)** Effector molecules production by cells incubated with peptide alone or peptide and blocking antibody to PD-L1: TNF-α **(c),** Granzyme B **(d)**, and Lymphotoxin- α **(e)**. (**f-h)** Increased proliferation in the presence of blocking antibody to PD-L1 on HIV-specific $CD8^+$ T cells. **(f)** Representative dot plots of a CFSE experiment using anti-PD-L1 blocking antibody on subject no. 003, who carries the A2p17 epitope. Number of proliferating $CFSE^{low}$ and $CFSE^{hi}$ cells are indicated in each quadrant. **(g)** Number of HIV specific tetramer-positive (tetramer+) T cells after 6 d of stimulation with peptide alone or in presence of anti-PD-L1 blocking antibody. **(h)** Number of proliferating $CFSE^{low}$ tetramer-positive cells in the presence or absence of anti-PD-L1 antibody.

Legends of Supplementary Figures

Supplementary Figure1: Longitudinal analysis of PD-1 expression on HIV-specific CD8$^+$ T cells correlates with viremia. **(a),** MFI of PD-1 expression for A2EBV (grey bars) and A2p17 (black bars) tetramer positive cells in patient #332 are presented for six time points. **(b),** MFI of PD-1 expression for A2CMV (white bars) and A2p17 (black bars) tetramer positive cells in patient #303 are represented for six time points. The line represents measurements of viral load at the different time points. The viral load of the patients decreased to undetectable levels exclusively during HAART treatment. Expression levels of PD-1 on HIV-specific CD8$^+$ T cells follow the fluctuations of viremia, and are always higher at the viremic time points. EBV and CMV-specific CD8$^+$ T cells display constant PD-1 expression during the study period.

Supplementary Figure 2: Percentage of cytokine production of tetramer positive cells is a function of levels of PD-1 expression. ICS was performed on 6 patients for 14 HIV-specific tetramers and 12 CMV and EBV-specific tetramers. **(a),** Percentages of IL-2 producing tetramer$^+$ cells are correlated with the MFI of PD-1 on the tetramer$^+$ cells. **(b),** Percentages of TNF-α producing tetramer$^+$ cells tend to correlate with the MFI of PD-1 on the tetramer$^+$ cells. **(c),** Percentages of IFN-γ producing tetramer$^+$ cells do not correlate with the MFI of PD-1 on the tetramer$^+$ cells.

Supplementary Figure 3: Increase in TNF-α production in HIV-specific CD8$^+$ T cells in a 6 d culture stimulation in presence of anti-PD-L1 antibody. **(a),** Representative dot plots of TNF-α intracellular staining gated on A2p15 tetramer positive cells in patient number 316. PBMCs were stimulated at day zero with cognate peptide and incubated for 6 d in absence or presence of blocking anti-PD-L1 antibody. At day six, cells were re-stimulated with the peptide alone or in presence of anti-PD-L1 antibody for 6 h and then assayed by ICS. Dot plots are on a 5log scale axis. The frequency of HIV-specific CD8$^+$ T cells producing TNF-α is indicated. **(b),** Percentage of TNF-α producing cells in tetramer$^+$ cells. In the presence of blocking anti-PD-L1 antibody, the number of tetramer$^+$ cells secreting TNF-α increased by a 2 fold average for the three tested patients. **(c),** PD-1 expression levels are high on HIV-specific tetramers in a 6 d stimulation culture in the presence of PD-L1 blocking antibody. A six day CFSE stimulation culture assay was

performed in the presence of anti-PD-L1 blocking antibody. Cells were then analyzed for the expression of PD-1 on HIV specific tetramer^{+} T cells.

Legends of Supplementary Tables

Supplementary Table 1: Characteristics of patients with percentages of tetramer positive CD8$^+$ T cells. The frequency of HIV specific CD8$^+$ T cells was significantly higher in HIV viremic patients (between 0.004% and 1.953%) as compared to aviremic patients (between 0.015% and 0.105%). The frequency of EBV-specific CD8$^+$ T cells was varying in the different donors from 0.054% to 0.236% and the frequency of CMV-specific CD8$^+$ T cells was on a range of 0.011% and 2.959. V corresponds to the visits of patients that were followed longitudinally. The time of infection is indicated for each patient and corresponds to the number of months between the onset of infection and the visit.

Supplementary Table 2: Phenotypic analysis of CMV, EBV and HIV tetramer positive cells in viremic and aviremic patients. Patient numbers and tetramers used are indicated in the first column. Symbols represent: - = 0-25%; + = 25-50%; ++ = 50-75%; +++ = 75-100% of positive cells as defined by flow cytometry. All histograms showed a bimodal distribution. CD279 = PD-1.

References

1. Chen, L. et al. *Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity*. Nat Rev Immunol, (2004).**4**: p336-47.

2. Ishida, Y. et al. *Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death*. Embo, (1992) **11**: p3887-95.

3. Agata, Y. et al. *Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes*. Int Immunol, (1996). **8**: p765-72.

4. Latchman, Y. et al. *PD-L2 is a second ligand for PD-1 and inhibits T cell activation*. Nat Immunol**, (2001). 2**: p261-8.

5. Freeman, G.J. et al. *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation*. J Exp Med, (2000). **192**: p1027-34.

6. Parry, R.V. et al. *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Mol Cell Biol, (2005). **25**: p9543-53.

7. Chemnitz, J.M., Parry, R.V., Nichols, K.E., June, C.H. & Riley, J.L. *SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation.* J Immunol, (2004). **173**: p945-54.

8. Barber, D.L. et al. *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature, (2006).**436: p**682-7.

9. Zajac, A.J. et al. *Viral immune evasion due to persistence of activated T cells without effector function*. J Exp Med, (1998). **188**: p2205-13.

10. Wherry, E.J. et al. *Antigen-independent memory CD8 T cells do not develop during chronic viral infection*. Proc Natl Acad Sci U S A, (2004). **101**: p16004-9.

11. Pantaleo, G. et al. *Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know*. Nat Med, (2004). **10:** p806-10.

12. Wherry, E.J. et al. *Low CD8 T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination*. J Virol, (2005). **79**: p8960-8.

13. Shankar, P. et al. *Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection*. Blood, (2000). **96**: p3094-101.

14. Migueles, S.A. et al. *HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors*. Nat Immunol, (2002). **3**: p1061-8.

15. Zhang, D. et al. *Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic*. Blood, (2003). **101**: p226-35.

16. Zhang, L. et al. *Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy*. N Engl J Med, (1999). **340**: p1605-13.

17. Dornadula, G. et al. *Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy*. Jama (1999). **282**: p1627-32.

18. Geels, M.J. et al. *Identification of sequential viral escape mutants associated with altered T-cell responses in a human immunodeficiency virus type 1-infected individual*. J Virol (2003). **77**: p12430-40.

19. Price, D.A. et al. *T cell receptor recognition motifs govern immune escape patterns in acute SIV infection*. Immunity, (2004). **21**: p793-803.

20. Champagne, P. et al. *Skewed maturation of memory HIV-specific CD8 T lymphocytes*. Nature, (2001). **410**: p106-11.

21. Appay, V. et al. *Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections*. Nat Med (2002). **8**: p379-85.

22. Lichterfeld, M. et al. *Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells.* J Exp Med, (2004). **200**: p701-12.

23. Yang, O.O. et al. *Decreased perforin and granzyme B expression in senescent HIV-1-specific cytotoxic T lymphocytes*. Virology, (2005). **331**: p16-9.

24. Lee, K.M. et al. *Molecular basis of T cell inactivation by CTLA-4*. Science, (1998). **282**: p2263-6.

25. Almanzar, G. et al. *Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons*. J Virol, (2005). **79**: p3675-83.

26. Wherry, E.J. et al. *Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment*. J Virol, (2003). **77**: p4911-27.

27. Altman, J.D. et al. *Phenotypic analysis of antigen-specific T lymphocytes*. Science, (1996). **274**: p94-6.

28. Younes, S.A. et al. *HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity*. J Exp Med, (2003). **198**: p1909-22.

29. Douek, D.C. et al. *A novel approach to the analysis of specificity, clonality, and frequency of HIV-specific T cell responses reveals a potential mechanism for control of viral escape*. J Immunol, (2002). **168**: p3099-104.

3 Highly active antiretroviral therapy (HAART) leads to the reconstitution of the HIV-specific CD8+ T cell receptor repertoire

The study presented in Chapter 2 identified PD-1 as a marker of exhaustion on HIV-specific $CD8^+$ T cells and showed that blockade of the PD-1-PD-L1 pathway restores proliferation and cytokine secretion of HIV-specific $CD8^+$ T cells. Moreover, a positive correlation was found between PD-1 expression and viral load. This led us to study the effect of antigen load on the clonality, functionality, and maturation status of HIV-specific $CD8^+$ T cells.

We chose four chronically HIV-infected patients who received highly active retroviral therapy (HAART) temporarily and chose to terminate it voluntarily. We studied the above characteristics of HIV-specific $CD8^+$ T cells in comparison to CMV-specific $CD8^+$ T cells pre, during, and, post HAART time points. Our model is unique in that we were able to study the profile of HIV-specific $CD8^+$ T cells and compare it to available viral resurgence time points, thereby measuring the effect of HAART and its absence on HIV-specific $CD8^+$ T cells. Our results indicate a HAART-mediated restoration of the HIV-specific $CD8^+$ T cell compartment.

Highly active antiretroviral therapy (HAART) leads to the reconstitution of HIV-specific CD8$^+$ T cell receptor repertoire

Loury Janbazian[1,2], Lydie Trautmann[1,3], David Ambrosac[4], Jason Brenchley[4], David Price[4,5], Rabih Halwani[1], Nicolas Chomont[1,2], Eric Delwart[6], Richard Koup[4], Mohamad Rachid Boulassel[7], Jean-Pierre Routy[7], Daniel Douek[4], and Rafick-Pierre Sekaly[1,2,3].

[1]Laboratory of Immunology, Department of Microbiology and Immunology, Université de Montréal.

[2]Faculty of Medicine, Department of Microbiology and Immunology, McGill University, Montreal, Quebec, H3A 2B4, Canada.

[3]CR-CHUM,INSERM U743 and CUSM, Montréal, Canada.

[4]Immunology Laboratory, Vaccine Research Centre, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

[5]Department of Medical Biochemistry and Immunology, Cardiff University School of Medicine, Cardiff CF14 4XN, UK

[6]Blood Systems Research Institute, 270 Masonic Ave., San Francisco, California 94118, USA.

[7]Division of Haematology, Royal Victoria Hospital, McGill University Health Centre, 687 Pine Avenue West, Montreal, Quebec, H3A 1A1, Canada.

Abstract

Virus-specific $CD8^+$ T cells have been implicated in the control of acute HIV-1 and SIV infection both in vitro and in vivo and current vaccine strategies are being pursued that focus upon eliciting antiviral $CD8^+$ T cell responses to control the level of HIV replication in vivo. Although present in chronic HIV infection, $CD8^+$ T cells are dysfunctional. Several factors may account for decreased functionality of HIV-specific $CD8^+$ T cells, such as their skewed phenotypic maturation status, emergence of CTL escape mutants and PD-1 up regulation. Recently, it was suggested that polyfunctional HIV-specific $CD8^+$ T cells, that are endowed with the ability to produce many cytokines, chemokines and have the ability to degranulate, are competent in controlling viral replication in chronic HIV infection. We hypothesized that the initiation of highly active antiretroviral therapy (HAART), improves the functional quality of HIV-specific $CD8^+$ T cells by influencing the antigen-specific $CD8^+$ T cell TCR repertoire. We specifically followed responses to conserved HIV-1 epitopes longitudinally in 4 chronically infected patients at pre, during, and post HAART.

Our foremost conclusions were: (a) the dynamic turnover of HIV-specific $CD8^+$ T cells was in contrast to that of CMV-specific $CD8^+$ T cells. Under HAART and post-HAART, we observed the emergence of new HIV-specific $CD8^+$ T cell clonotypes. (b) HAART led to a significant increase in T_{CM} and T_{EMRA} populations of HIV-specific $CD8^+$ T cells. (c) HAART led to the functional improvement of HIV-specific $CD8^+$ T cells, as observed by the increase of IFN-γ^+ TNF-α^+ $CD107a^+$ tri-functional cells, whereas CMV-specific $CD8^+$ T cells were always polyfunctional and their TCR repertoire remained static. Moreover, the increased polyfunctionality observed under HAART persisted when patients were taken off HAART. (d) HIV-specific $CD8^+$ T cell clonotypes that persisted over time underwent changes in functionality and phenotype with variations in viral load; they were functionally superior under HAART. Our data provide clear evidence of a HAART-mediated functional reconstitution of the HIV-specific $CD8^+$ T cell compartment, which can be attributed by different mechanisms, namely the improvement of function of existing clonotypes and the recruitment of new clonotypes under HAART, which are polyfunctional.

Introduction

Several observations suggest that virus-specific $CD8^+$ T cells are implicated in the control of HIV-1 infection [1-4]. In vivo evidence comes from SIV infection of rhesus macaques, where depletion of $CD8^+$ T cells by monoclonal antibodies leads to increased viremia [2, 4]. Evidence for a role of $CD8^+$ T cells in controlling HIV-1 infection in humans remains indirect. Temporal associations have been reported between emergence of HIV-specific $CD8^+$ T cells and the decline in viral load in acute infection [3]. Moreover, selection of escape variants by $CD8^+$ T cells both in acute and chronic infection suggests the presence of $CD8^+$ T cell-mediated immune pressure on the virus [5, 6]. Further evidence that supports the role of $CD8^+$ T cells in HIV-1 infection is the association of different HLA molecules with differential disease progression [7].

It has been demonstrated that under chronic antigen persistence, HIV-specific $CD8^+$ T cells are exhausted or dysfunctional, as characterized by their inability to produce cytokines, their compromised proliferative capacity and reduced cytotoxic activity [8-13]. Moreover, it was previously reported that quantitative measures, such as the frequency of HIV-specific $CD8^+$ T cells, do not correlate with plasma viral loads [14, 15]. Recently, two studies demonstrated the role of polyfunctional $CD8^+$ T cells as correlates of immune protection in mice and humans [16, 17]. HIV-specific $CD8^+$ T cell responses in long-term non-progressors (LTNP) have been shown to be polyfunctional [18]. Furthermore, $CD8^+$ T cells that target HIV-1 epitopes restricted by protective alleles such as HLA-B*2705, have shown superior polyfunctional responses when compared to other HLA-class I alleles [19].

Although T cell receptor (TCR) repertoire studies have been performed in the context of several acute and persistent viral infections including HIV-1 [20, 21], longitudinal studies that aim to study the evolution of the HIV-specific $CD8^+$ TCR repertoire and further couple HIV-specific $CD8^+$ T cell clonotypes to functional profiles have been limited [22]. Therefore, to better define the qualitative features of HIV-specific $CD8^+$ T cells, we hypothesized that the initiation of highly active antiretroviral therapy (HAART) / absence of antigen would lead to the improvement of the functional quality of HIV-specific $CD8^+$

T cell responses by influencing the antigen-specific $CD8^+$ T cell TCR repertoire. More importantly, we wanted to determine how antigen load influences $CD8^+$ T cell function.

In this study, we undertook a comprehensive analysis of $CD8^+$ T cell clonal diversity (TCRB gene usage), functional profile (degranulation and cytokine production), and maturation status (phenotyping) of both HIV and CMV-specific $CD8^+$ T cells. This analysis was performed longitudinally in four patients at time points prior to, during, and after HAART. Our data provide clear evidence of a HAART-mediated reconstitution of the HIV-specific $CD8^+$ T cell compartment, which can be attributed by different mechanisms, namely the improvement of function of existing clonotypes and the recruitment of new clonotypes under HAART, which are polyfunctional.

Results

Virological and immunological characteristics of the study cohort

We studied antigen-specific $CD8^+$ T cell responses longitudinally in four patients. This provided us with the opportunity to track the clonality, functionality, and phenotype of HIV-specific $CD8^+$ T cells under conditions of high and low antigen load. Figure 1 A shows the treatment scheme and time points studied for each patient. All patients had stopped HAART voluntarily, except for patient 1, who underwent a structured treatment interruption for 1 month. Month zero indicates the estimated date of HIV-1 infection. The viral loads, peripheral CD4 and CD8 counts for each patient are listed in Supplementary Figure 1. HIV plasma viral loads were reduced to less than 50 RNA copies / ml with therapy, for almost all patients. However, viral loads rebounded, to pre HAART levels, in all four individuals soon after termination of HAART. Most patients had maintained almost normal peripheral blood CD4 T cell counts (Supplementary Figure 1 A) under therapy. Both HIV and CMV responses were studied in each patient, except for patient three, where a CMV response was not detected.

We focused our analysis on conserved epitopes, to exclude the possibility that viral mutations led to the observed changes in phenotype, functionality, and clonality of HIV-specific $CD8^+$ T cells. Therefore, we sequenced viral epitopes from plasma obtained at the Pre-HAART and the last Post-HAART time points for each patient. Autologous viral

sequences did not show any escape from any epitope-specific $CD8^+$ T cells at the different time points (Supplementary Figure 1 B) and hence, autologous peptides were folded into peptide-MHC I (pMHCI) tetrameric complexes. $CD8^+$ T cell responses were specific for HLA-A*0201-FLGKIWPSHK (Gag FK10, residues 435-444) and HLA-A*0201-NLVPMVATV (pp65) in Patient 1, HLA A*0301-RLRPGGKKR (Gag RR9, residues 20-28) and HLA-B*0702-TPRVTGGGAM (pp65) in patient 2, HLA-B*0801-FLKEKGGL (Nef FL8, residues 90-97) in patient 3, and HLA-B*0702-TPGPGVRYPL (Nef TL10, residues 134-143) and HLA-B*0702-TPRVTGGGAM (pp65) in patient 4. Figure 1 B is an example of a longitudinal HIV and CMV-specific tetramer co-staining from patient 2. Frequencies of HIV-specific $CD8^+$ T cells decreased after initiation of HAART (Figure 1 C) (by an average of 10.40 fold; range 3.62 to 26.88), consistent with decay in antigen load, whereas frequencies of CMV-specific $CD8^+$ T cells remained constant. Taken together, the selected cohort with its characteristic trend of variable antigen load helped us decipher the correlates of immune protection in the setting of low antigen load.

HAART leads to the emergence of new HIV-specific $CD8^+$ T cell clonotypes

HIV-specific $CD8^+$ T cell receptor (TCR) repertoires tend to be oligoclonal in nature in chronic HIV-1 infection, most likely as a consequence of the disappearance of several HIV-specific T cells through deletion [23] and the lack of replacement of T cell diversity due to decreased thymic output [24]. To investigate the potential impact of HAART on the HIV-specific $CD8^+$ T cell compartment, and to understand better the impact of antigen load on the clonal diversity of antigen-specific responses to conserved epitopes, we undertook a comprehensive clonotypic analysis of $CD8^+$ T cell antigen-specific responses (CDR3 amino acid region, TCRBV and TCRBJ usage and the relative frequency of the clonotypes). We observed the dynamic evolution of HIV-specific $CD8^+$ T cell clonotypes in contrast to CMV-specific $CD8^+$ T cell clonotypes after initiation of HAART (Table 1). Although both antigen-specific repertoires were oligoclonal, the CMV-specific repertoire was significantly less diverse than that of the HIV-specific repertoire.

The HIV-specific $CD8^+$ TCR repertoire was dynamic in all 4 patients with important changes in the turnover of HIV-specific T cell receptor clonotypes (Table 1 A). On average, 2-13 clonotypes were observed pre-HAART. With the institution of HAART, we observed emergence of new clonotypes and loss of some that were present pre-HAART. On average, the HIV-specific repertoire during HAART was comprised of 1 to 13 clonotypes. Post-HAART, 1 to 11 clonotypes constituted the HIV-specific repertoire. Between the pre and post-HAART time points, we observed a contraction in the number of HIV-specific clonotypes longitudinally in each patient. The contraction of the repertoire post-HAART involved the recruitment of new clonotypes, together with the maintenance of some clonotypes that persisted post-HAART. Globally, the HIV-specific $CD8^+$ TCR repertoire was oligoclonal, comprising several clonotypes. On average 1 to 5 junctional (J) regions and 2 to 9 variable (V) regions were used by HIV-specific $CD8^+$ T cells Pre-HAART, 1 to 5 J's and 1 to 8 V's during HAART, and 1 to 5 J's and 1 to 9 V's Post-HAART. Of note, we did not observe a selection for common motifs in the CDR3β regions, or a preference towards selected variable (V) and junction (J) region usage.

The HIV-specific TCR repertoire for conserved epitopes fluctuated with changes in viral load; with several clonotypes persisting and others being lost. Clonotypes using Vβ 6.2 in patient 1, Vβ 13 and Vβ 6.6 in-patient 2, and Vβ 20.1 CSA SPRGRYNEQF Jβ 2.1 and Vβ 20.1 CSA SSRGRVDEQF Jβ 2.1 in patient 4, persisted until the termination of HAART. Persisting clonotypes exhibited fluctuations in their immunodominance pattern with variations in viral load, as in patient 1, where clonotype Vβ 6.2, which was the second most dominant clonotype in the repertoire pre-HAART (28%), increased in frequency during therapy, and became the immunodominant clonotype after termination of HAART (88%). Importantly, we noted the emergence of new clonotypes with the initiation of HAART; some of these persisted after therapy cessation (Vβ 14 in patient 2 and Vβ 27 in patient 4) and others were lost (Vβ 27 in patient 1 and all the other clonotypes except for Vβ 25.1 in patient 3). Furthermore, after termination of HAART, new clonotypes were observed, although preexisting clonotypes contributed to the dominant HIV-specific clonotypes. These clonotypes were the following; Vβ 6.4 (at 20 months) in patient 1 (12.35%), Vβ 5.6 CAS SAGWGSYEQY Jβ 2.7 (at 11 months) in patient 2 (14.7%), Vβ 5.6 CAS SWGWGIAEAF Jβ 1.1 (at 24 months) (16.66%), and Vβ

9 CAS SPRGRIDEQY Jβ 2.7 (at 20 months) in patient 4 (9.25%). Patient three differed from the other patients. One month after the estimated date of infection, the HIV-specific $CD8^+$ TCR repertoire consisted of the major clonotype using Vβ 25.1. Emerging clonotypes were detected with the initiation of HAART at 5 months, but were lost at 8 months. The dominant clonotype was still Vβ 25.1. Interestingly, after termination of HAART, a new clonotype Vβ 7.2 emerged and replaced Vβ 25.1. Taken together, our results indicate that the HIV-specific $CD8^+$ TCR repertoire against conserved epitopes is highly dynamic and that the initiation and termination of HAART leads to the emergence of new HIV-specific $CD8^+$ T cell clonotypes.

Viral load fluctuations result in phenotypic changes of HIV-specific $CD8^+$ T Cells

Having shown that the HIV-specific $CD8^+$ T cell repertoire was dynamic, we evaluated the impact of antigen load on the maturation status of HIV-specific $CD8^+$ T cells. We stained the tetramer positive ($tetramer^+$) $CD8^+$ T cells longitudinally in all 4 patients, for the following phenotypic markers: CCR7, CD45RA, CD27 and CD127.

According to previous reports and based on the surface markers CCR7 and CD45RA; we identified four T cell subsets within the $CD8^+$ compartment: Naïve ($CCR7^+$ $CD45RA^+$), Central Memory (T_{CM}) ($CCR7^+CD45RA^-$), Effector Memory (T_{EM}) ($CCR7^-CD45RA^-$) and Effector Memory RA+ (T_{EMRA}) ($CCR7^-CD45RA^+$) (Figure 2 A) [25]. In agreement with previous results, HIV-specific $CD8^+$ T cells under high viral load, express low levels of CD127 (IL-7Rα) [26]. In our longitudinal analysis and in contrast to CMV-specific $CD8^+$ T cells, HIV-specific CD8+ T cells exhibited a distinct maturation phenotype $CCR7^-$ $CD27^+$ $CD45RA^-$ $CD127^-$. CMV-specific $CD8^+$ T cells were $CCR7^-CD45RA^+$ [27]. At the pre-therapy time points, HIV specific $CD8^+$ T cells were mostly $CD45RA^-$ $CCR7^-$, with few $CCR7^+CD45RA^-$ cells in the repertoire. Initiation of HAART, led to a significant increase in T_{CM} $CD8^+$ T cells and T_{EMRA} cells (Figure 2 B). CMV-specific $CD8^+$ T cells exhibited a terminally differentiated phenotype $CCR7^-$ $CD27^-$ $CD45RA^+$ $CD127^-$ (Supplementary Figure 3).

Interestingly, we observed a sharp increase in CD127 expression on HIV-specific $CD8^+$ T cells with the initiation of HAART, indicating a transition from effector to a resting memory phenotype [28]. CD127 levels immediately decreased at the first time point

following termination of HAART in all four patients. In contrast, CD127 expression was stable for CMV-specific $CD8^+$ T cells (data not shown).

The different phenotypic subsets were pooled for the 4 patients, and statistical significance was derived when all time points were compared to pre-HAART. The following combinations yielded statistical significance ($p < 0.05$; Figure 2 B) when HAART time points were compared to pre-HAART: the increase in T_{CM} (CCR7+CD27+CD45RA-CD127+), increase in T_{EMRA} (CCR7-CD27+CD45RA+CD127-, CCR7+CD27-CD45RA+CD127-, CCR7-CD27-CD45RA+CD127-), increase in T_{EM} (CCR7-CD27+CD45RA-CD127+), the decline of T_{EM} (CCR7-CD27-CD45RA-CD127-). Moreover, the decline of T_{EM} (CCR7-CD27+CD45RA-CD127+ and CCR7-CD27-CD45RA-CD127+) was also statistically significant when the last Post-HAART time points were compared to Pre-HAART time points ($P< 0.05$). Taken together, our results indicate that HAART leads to a significant increase in T_{CM} and T_{EMRA} populations of HIV-specific $CD8^+$ T cells.

HAART improves the functionality of HIV-specific $CD8^+$ T cell responses

Cells endowed with multiple functions and producing high levels of cytokines as measured by intracellular cytokine staining (ICS), have been described as correlates of immune protection [12]. In this study, we investigated the impact of antigen load on the functionality of both HIV and CMV- specific $CD8^+$ T cells. Specifically, we performed a comprehensive multiparametric flow cytometric analysis to investigate the functional evolution of the antigen- specific $CD8^+$ T cells pre, during, and post- HAART. Using ICS, we measured the effect of HAART on the ability of HIV and CMV-specific $CD8^+$ T cells to produce cytokines (IFN-γ, IL-2 and TNF-α) as well as their ability to degranulate (CD107a expression). Figure 3 A shows a representative flow data for the production of IFN-γ, TNF-α, IL-2, and CD107a by HIV-specific $CD8^+$ T cells with and without peptide stimulation.

Our results showed the dynamic changes in the functionality of HIV-specific $CD8^+$ T cells (Figure 3 B) in contrast to CMV-specific $CD8^+$ T cells (Supplementary Figure 4). CMV- specific $CD8^+$ T cells were polyfunctional, exhibiting 4 ($CD107a^+$ IFN-γ^+ TNF-α^+ IL-2^+), 3 ($CD107a^+$ IFN-γ^+ TNF-α^+) and 2 (IFN-γ^+ TNF-α^+, and $CD107a^+$ IFN-γ^+)

functions. Although there were mono-functional cells in the total CMV-specific repertoire as exhibited by IFN-γ production, these cells were minor contributors to the total functional pool (Supplementary Figure 4). In contrast, HIV-specific $CD8^+$ T cells comprised mostly mono-functional cells pre-HAART (except for Patient 4, since we were unable to analyze the stimulation data, due to very small number of events); IFN-γ producing cells were the main contributors to the mono-functional pool along with $CD107a^+$ cells, which contributed to the functional pool with lower frequencies. More importantly, we observed functional improvement of HIV- specific $CD8^+$ T cells upon institution of HAART. This was consistent in 3 out of 4 patients where functional improvement was restored and further augmented by the second time point on HAART. The polyfunctional cells during HAART were mainly $CD107a^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ (3+) producing $CD8^+$ T cells. Cells producing all 3 cytokines and CD107a (4+) were observed on HAART, but did not reveal any statistical significance when compared to the pre-HAART time points ($P > 0.05$) (Figure 4 A). Furthermore, the functional improvement observed under HAART persisted partially after its termination. The increase in $IFN\text{-}\gamma^+CD107a^+TNF\text{-}\alpha^+$ (3+) cells was statistically significant ($p<0.05$) for all time points when compared to pre-HAART and the decline of this population was significant when post-HAART was compared to on-HAART time points. $IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$ (2+) cells statistically increased under HAART when compared to Pre-HAART ($p<0.05$). Importantly, the increase in $IFN\text{-}\gamma^+CD107a^+TNF\text{-}\alpha^+$ (3+) observed during HAART and post-HAART, was accompanied by a statistically significant ($p<0.05$) decrease of mono-functional $CD107a^+$ expressing cells (Figure 4 A).

Recent publications have shown a positive correlation between polyfunctionality and increased production of IFN-γ [16]. Our results are in line with these reports, where we observed a clear correlation between mean florescence intensity (MFI) of IFN-γ and increased polyfunctionality (Figure 4 B). This correlation tended towards statistical significance when the MFI of IFN-γ was compared between pre-HAART (n=3) and second time points on HAART (n=4, P=0.06) (Figure 4 B). Although the increase in the MFI of IFN-γ tended towards statistical significance during HAART when compared to pre-HAART (P=0.06), this increase in MFI was not as high as the MFI of IFN-γ observed with CMV-specific $CD8^+$ T cells (data not shown). Collectively, our results indicate that

HAART augments the functional profile of HIV- specific $CD8^+$ T cells and this augmentation is partially sustained even after the termination of anti retroviral therapy, however this phenomenon did not result in improved control of viral replication.

HAART improves the functional quality of HIV-specific $CD8^+$ T cell responses at the clonal level

Our next objective was to determine if the restoration in T cell function of the oligoclonal populations of HIV-specific $CD8^+$ T cells was due in part to the emergence of new clonotypes or whether clonotypes that persisted before HAART showed improved T cell function.

Our studies initially focused on clonotype Vβ 6.2 from patient 1 that persisted throughout the study. This clonotype changed in terms of frequency and became the dominant clonotype in the repertoire at 17 (58.66%) and 20 months (87.64%). Since no commercial Vβ antibodies were available for any of the emerging clonotypes to allow a robust comparison of functional profiles between persisting and emerging clonotypes, we measured the degranulation marker CD107a and the cytokines IL-2, TNF-α, and IFN-γ in both HLA-A*0201-FK10 $tetramer^+$ specific $CD8^+$ T cells encompassing several clonotypes and in Vβ 6.2^+ $CD8^+$ T cells, in the same multiparametric intracellular cytokine staining experiment after 6 hours of stimulation with autologous cognate peptide. At 1 month, HLA-A*0201-FK10 tetramer-specific $CD8^+$ T cells were predominantly IFN-γ^+. However, at 17 months (which corresponded to the second time point under HAART), HLA-A*0201-FK10-specific $CD8^+$ T cells exhibited multiple functions, as seen by the increase in the frequency of $CD107a^+$ IFN-γ^+ TNF-α^+ (3+) cells (Figure 5 B). At 20 months (after cessation of HAART), HLA-A*0201-FK10 tetramer-specific $CD8^+$ T cells were mainly IFN-γ^+ with a lower frequency of $CD107a^+$ IFN-γ^+ cells (2+). $CD107a^+$ IFN-γ^+ TNF-α^+ (3+) cells were absent at 20 months. Interestingly, the functional profile of Vβ 6.2-specific $CD8^+$ T cells was similar to that of the HLA-A*0201-FK10-specific $CD8^+$ T cells, specifically under HAART, where polyfunctionality was restored as manifested by the increase in $CD107a^+$ IFN-γ^+ TNF-α^+ (3+) (Figure 5 D). $CD107a^+$ IFN-γ^+ TNF-α^+ (3+) producing Vβ 6.2-specific $CD8^+$ T cells

were absent at 20m. Hence, we conclude that persisting clonotypes, such as Vβ 6.2, contribute to the major changes in functionality of the antigen specific $CD8^+$ T cells.

In patient 2, we were able to compare the functional profile between a persisting clonotype Vβ 6.6 and clonotype Vβ 14 that emerged under HAART. The experiment was performed on available samples from 1, 4, and 24 months. An irrelevant Vβ 6.2 was used in the assay as a negative control. We assessed the production of CD107a, IFN-γ and TNF-α by $CD8^+$ T cells expressing Vβ 6.6, Vβ 14, and Vβ 6.2 (irrelevant Vβ). The persistent clonotype Vβ 6.6 inversed in frequency and became dominant at 24 months (31.25%). At 1 month, Vβ 6.6^+ $CD8^+$ T cells were TNF-α^+, IFN-γ^+, and $CD107a^+$ (1+) cells. With the initiation of HAART, Vβ 6.6^+ $CD8^+$ T cells were mostly IFN-γ^+ TNF-α^+ and IFN-γ^+ $CD107a^+$ (2+) cells. The mono-functional cells under HAART were mainly TNF-α^+ (Figure 5 F). Interestingly, the increased polyfunctionality observed under HAART was preserved after termination of therapy specifically for the IFN-γ^+ $CD107a^+$ (2+) population. IFN-γ^+ TNF-α^+ $CD107a^+$ (3+) were also observed after termination of HAART. The functional profile of Vβ 6.6-specific $CD8^+$ T cells was similar to that of the total HIV-specific $CD8^+$ T cells at 1,4 and 24 months (Figure 3 B). No cytokine production was observed from the irrelevant Vβ 6.2 (data not shown).

The emerging Vβ 14 clonotype was detected in the HIV-specific $CD8^+$ T cell repertoire at 4 months, when patient 2 was on HAART and was present at 24 months, when patient 2 was off HAART. The clonal frequency of this clonotype did not alter between the two time points (Table 1). At 4 months, Vβ 14^+ $CD8^+$ T cells emerged as polyfunctional, mainly $CD107a^+$ IFN-γ^+ TNF-α^+ (3+) cells. This could contribute to the increase of $CD107a^+$ IFN-γ^+ TNF-α^+ (3+) cells observed under HAART, for the HIV-specific $tetramer^+$ population (Figure 3 B). At 24 months and with the resurgence of viremia, this clonotype had undergone changes in functional profile, and became mono-functional (50%) and bi functional (IFN-γ^+ $CD107a^+$ and IFN-γ^+ TNF-α^+) (50%). The irrelevant Vβ 6.2 clonotype was negative for cytokine secretion in this co-staining experiment (data not shown).

The clonotypic and functional analysis from patient three further supported our observations. Vβ 25.1 was the major clonotype at 1 and 8 months in this patient. The

functional profile of the HIV-specific $CD8^+$ T cells at the 2 time points was different, as we observed an improvement of function at 8 months (under HAART), as seen by increase $CD107a^+$ IFN-γ^+ TNF-α^+ (3+) cells (Figure 3 B). Therefore, this clonotype underwent changes in functional profile with changes in viral load.

Taken together, our data indicate that persistent clonotypes contribute to the functional changes of the HIV-specific repertoire and are highly influenced by changes in viral load. These clonotypes become functionally superior upon institution of HAART and this improved functionality is partially maintained with viral rebound, at least for the time points studied. Emerging clonotypes could also be contributing to the superior functional repertoire at HAART, but their functional superiority is lost with the resurgence of viral load.

Discussion

Although several observations suggest that virus specific $CD8^+$ T cells are implicated in the control of HIV-1 infection [1-4], it has been demonstrated that under chronic antigen persistence, HIV-specific $CD8^+$ T cells are exhausted or dysfunctional, as characterized by their inability to produce cytokines, their compromised proliferative capacity and reduced cytotoxic activity [8-13]. Polyfunctional HIV-specific $CD8^+$ T cell responses have been reported in long term non progressors (LTNPs) [18] and in HLA-B*2702 individuals and shown to exhibit superior functions [19]. Moreover, it was recently documented that antigen load and viral sequence diversification affect the functional profile of antigen specific $CD8^+$ T cells [39] and that HAART leads to the emergence of polyfunctional $CD8^+$ T cells [28].

Wilson et al reported that HIV-specific CD8 TCR repertoire was oligoclonal [29]. These oligoclonal TCR repertoires are thought to mediate a better control of viral infections, perhaps by containing high affinity clones, which are presumably the “best-fit” [30], [22]. The lack of viral sequence diversification as obtained in our analysis, together with the lack of selective variable and junctional gene segment usage, and the absence of motifs within the CDR3β region, ruled out the possibility that the dynamic TCR repertoire observed with HIV-specific $CD8^+$ T cells was driven by viral escape variants. This was in agreement with data obtained from the SIV model by Price et al, where the authors

affirmed that a highly focused TCR repertoire with discernable motifs within the CDR3β regions facilitated "escape" from the recognition of viral mutants, whereas broader TCR repertoires were associated with conserved epitopes [20].

The most crucial question raised by our data was what was driving this clonal turnover? One of the reasons for observing such a dynamic HIV-specific $CD8^+$ T cell repertoire was the fact that we analyzed time points from acute and chronic phases of infection. It has been shown in the persistent lymphocytic choriomeningitis virus (LCMV) model that there is a switch in $CD8^+$ T cell clones from acute to chronic infection [31]. Moreover, results from Pantaleo et al, showed that a significant number of clones are lost during the transition from acute to chronic HIV infection [21]. Furthermore, Rees et al showed that antigen load shaped the T cell repertoire, and that high antigen load leads to the emergence of low avidity clonotypes [32].

The most striking result in our longitudinal analysis was the emergence of new clonotypes under HAART, which clearly contributed to the overall clonal turnover of HIV-specific $CD8^+$ T cells. It is possible that the clonotypes detected under HAART were already present, and that institution of therapy led to a redistribution of these clonotypes from secondary lymphoid organs back to the periphery, rather than their emergence [33], [34]. A thorough phenotypic characterization of the clonotypes within the HIV-specific $CD8^+$ T cell compartment, would better address their origins. It is also possible that reconstitution of the $CD4^+$ compartment during antiretroviral therapy contributed to the priming of additional HIV-specific $CD8^+$ T cells from the naïve pool and that new $CD8^+$ T cells were primed to enter the HIV-specific memory $CD8^+$ T cell pool [35-37]. Moreover, our data indicate that the functional changes of the HIV-specific $CD8^+$ T cells could mostly be attributed to increased functionality of pre-existing clonotypes, but emerging clonotypes could also contribute to the increased polyfunctionality.

Increased function of HIV-specific $CD8^+$ T cells in persistent HIV infection has been attributed to the ability of HIV-specific $CD8^+$ T cells to simultaneously produce cytokines, chemokines, and have the ability to degranulate [38]. Such studies were performed on samples from LTNP's [18, 19]. We therefore, reasoned that HAART would reverse the functional profile of HIV-specific $CD8^+$ T cells, towards a superior function.

Our results indicated that, while the overall magnitude of HIV-specific responses declined under HAART, there was a clear functional improvement of HIV-specific $CD8^+$ T cells, which was partially sustained after its cessation.

The gain of function of HIV-specific $CD8^+$ T cells observed under HAART was primarily a gain in TNF-α production. The results from Rehr et al were in agreement with ours, pointing at the fact that the emergence of polyfunctional $CD8^+$ T cells after prolonged HAART, was attributed by a statistically significant increase in TNF-α and IL-2 production of total and HIV-specific $CD8^+$ T cells, in contrast to the untreated control group [28]. Furthermore, Streeck et al also reported that the exhausted phenotype of HIV-specific $CD8^+$ T cells significantly decreased upon removal of antigen, either by anti-retroviral therapy or by the emergence of viral escape variants [39]. Interestingly, in our results, we noted that although viral loads were similar at pre-HAART and after cessation of HAART, HIV-specific $CD8^+$ T cells exhibited different functional and phenotypic profiles. It seems likely that the gain in TNF-α production was HAART-mediated, since no gain of this cytokine was observed from acute to chronic infection in untreated patients (to genetically conserved epitopes), and loss of TNF-α production has been described as an early loss of effector function [8, 40]. Furthermore, HAART-mediated $CD8^+$ T cell compartment restoration was specific for HIV, as CMV-specific $CD8^+$ T cells exhibited similar functionality and effector phenotype over the time points studied [41-43]. Therefore, what we observed under HAART was a reversion from an exhausted phenotype and this was further supported by the sharp decrease of PD-1 levels on HIV-specific $CD8^+$ T cells during HAART (data not shown).

Restoration of the HIV-specific $CD8^+$ T cell compartment under HAART was evident as early as 3 months after institution of HAART for patient 2, 5 months for patients 1 and 3, and a month for patient 4. However, patient 4 started HAART in the chronic phase of infection, which could account for the distinct functional, maturation status, and clonotypic profile that this patient exhibited when compared to the other studied subjects. Altogether and notwithstanding patient 4, restoration of HIV-specific $CD8^+$ T cells under HAART encompassed functional improvement as observed by the significant increase of IFN-γ^+ TNF^+ $CD107a^+$ 3^+ cells, emergence of new clonotypes and a switch towards T_{CM}

and T_{EMRA} phenotypes. Hence, treatment interventions that aim at either expanding or maintaining these polyfunctional HIV-specific $CD8^+$ T cell responses could be promising. An example of such manipulation of the TCR repertoire in the context of persistent viral infections has been shown with vaccination. These reports suggested that vaccination of HAART treated individuals with the MVA.HIVA, which comprises HIV-1 clade A gag p24/p17 sequence fused to multiCTL epitope gene, led to the expansion of vaccine-specific $CD8^+$ T cell responses to *Gag*, *Nef*, and *Pol* [44]. These expansions are polyclonal in nature in sharp contrast to the oligoclonal TCR repertoires observed in vaccinees who did not elicit any response and unvaccinated controls. Moreover, although vaccination led to the transient upregulation of PD-1, which rapidly returned to base line levels, the polyclonal expansions of vaccine-induced $CD8^+$ T cells displayed better proliferative potential and in vivo viral suppression activity. Although the clonality of these oligoclonal/polyclonal populations was not assessed, strategies that aim at inducing polyclonal responses under conditions of HAART appear to be promising [44].

Materials and Methods

Study participants

Four patients were enrolled in the study. Peripheral blood mononuclear cells were available from each patient longitudinally. Three of the participants had started HAART during acute phase HIV-1 infection. Patient 4 started HAART 6 months after estimated date of infection. All patients had stopped HAART voluntarily. The participants were recruited from Hospital Notre Dame, Montreal, Quebec, Canada, and they all provided written consent for participation in the study.

Tetrameric pMHCI complexes

Soluble biotinylated pMHCI monomers were obtained from the CANVAC tetramer core facility (Montreal, Canada) and tetramerized with fluorochrome-conjugated streptavidine at a 4:1 molar ratio. The following HIV and CMV specific pMHCI tetramers were produced; HLA-A*0201-FLGKIWPSHK (Gag FL10, residues 435-444) and HLA-A*0201-NLVPMVATV (pp65) in patient 1, HLA-A*0301-RLRPGGKKR (Gag RR9, residues 20-28) and HLA-B*0702-TPRVTGGGAM (pp65) in patient 2, HLA-B*0801-FLKEKGGL (Nef FL8, residues 90-97) in patient 3, HLA-B*0702-TPGPGVRYPL (Nef TL10, residues 134-143) and HLA-B*0702-TPRVTGGGAM (pp65) in patient 4. Soluble pMHC monomers were generated as previously described [45].

Autologous Viral Sequencing

Amplification and sequencing of the near complete HIV genomes has been described in detail [46]. In brief, viral RNA was extracted from plasma samples using the QIAamp viral RNA minikit (Qiagen, Valencia, CA) in a final volume of 50 ul of H_2O containing 40 U of RNase inhibitor (Protector RNase Inhibitor, Roche Diagnostic Corporation, Indianapolis, IN). First strand cDNA synthesis was initiated with 5-10 ug of RNA and the primers Tat2 or FB12 for the 5' and 3' half genome, respectively. For amplification of each half genome, a nested PCR was performed using Takara ExTaq DNA polymerase (Takara Bio Inc., Shiga, Japan), following the manufacturer's instructions. The sets of nested primers and the PCR cycle conditions that were used have been previously reported [46]. The final PCR products were purified using the QIAquick PCR purification

(Qiagen) and directly sequenced with sense or antisense primers [46]. Sequencing reactions were performed with ABI big Dye terminators and run on an ABI 3700 automated capillary sequencer. Sequences were edited using EditView and were assembled into a single contig and then aligned using the Seqman and Megalign programs, respectively (DNAstar Inc., Madison, WI). When sequence indeterminations due to insertion/deletion mutations could not be resolved, purified PCR products were cloned in pGEM-T easy vector (Promega, Madison, WI). Six to twelve white colonies were randomly picked and directly used for a second round PCR. The final products were then purified and sequenced as described above.

T cell Receptor (TCR) Clonotype Analysis

For RNA-based clonotype analysis, HIV and CMV- specific $CD8^+$ T cells were conjugated to pMHC-PE and pMHC-APC tetrameric complexes respectively and sorted. mRNA was extracted from the tetramer positive cells using the Oligotex mRNA mini kit (Qiagen). The extracted mRNA was subjected to template switched anchor RT-PCR by using a 3' TCRB constant region primer (5'- TGCTTCTGATGGCTCAAACACAGCGA CCT-3') as described previously [24]. The amplicons were ligated into pGEM-T Easy vector (Promega) and cloned by transformation of competent DH5-α *E.coli*. A minimum of 50 white colonies were picked and screened for the insert by the standard M13 primers and then sequenced. All sequences were analyzed by VectorNTI (Invitrogen). Non-functional sequences were discarded from the analysis.

Functional profiling of antigen-specific $CD8^+$ T cells by polychromatic flow cytometry.

Cryopreserved Peripheral Blood Mononuclear Cells (PBMCs) from all four patients at all time points were thawed and rested for an hour in R-10 prior to stimulation (RPMI 1640 medium supplemented with 10% Fetal Calf Serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml), and L-glutamine (2mM)). Corresponding HIV and CMV tetramers were conjugated with PE and tetramer staining at 37°C for 15 minutes for samples that received stimulation. After 15 minutes of incubation, tetramer stained samples were washed with wash buffer. For each sample at each time point, non-

stimulated and SEB stimulated samples were used as negative and positive controls respectively. Co-stimulatory monoclonal antibodies αCD28 and αCD49d (10 μg/ml; BD Biosciences), monensin (0.7 μl/ml; BD Biosciences), brefeldin A (10 mg/ml; Sigma Aldrich), and αCD107a-Alexa 680 were added. Cells were stimulated with corresponding autologous peptides at a concentration of 5μg/ml. After 6 hours of stimulation, the non-stimulated tubes from each sample were stained for tetramer at 37°C for 15 minutes. All samples were washed and stained for the following extra cellular markers; αCD3-Cy7-APC, αCD4-Cy5.5-PE, αCD8-Qdot 705. The cells were washed and fixed. Afterwards, the cells were washed twice in permibalization buffer and stained with the following antibodies to intracellular cytokines: αIL-2-APC, αTNF-α-Cy7-PE, and αIFN-γ-FITC (BD Biosciences). After another wash, the cells were fixed and data was acquired. Polychromatic flow cytometric analysis was performed using an LSRII flow cytometer (Becton Dickinson, San Jose, CA). All live lymphocytes were collected and files were analyzed using FlowJo, version 8.7.3 (Tree Star Inc., Ashland, OR). The gating strategy was set to eliminate doublets according to forward-scatter area-versus-height properties. Dead cells (Cascade blue bright) and CD14+/CD19+ cells were gated out against side scatter and lymphocytes were selected according to standard side scatter properties after confirming that any functional response was originated from this population. Values used for analyzing a proportionate representation of responses were background subtracted. Functional capacity was determined after Boolean gating, and subsequent analysis was performed using Simplified Presentation of Incredibly Complex Evaluations SPICE (version 2.9; Mario Roederer, VRC, NIAID, NIH). For functional data on the clonotypes, PBMCs from patient 1 (at 1, 17, and 20 months) and patient 2 (at 1, 4, and 24 months) were studied. The following Vβ surface antibodies were used; Vβ 6.2- PE in patient 1, Vβ 6.6- FITC and Vβ 14 -FITC in patient 2.

Antigen specific $CD8^+$ T cell phenotyping

Thawed PBMCs from all patients were surface stained with corresponding tetramers-PE, viability dye, αCD3-PB, αCD8-TR-PE, αCCR7-PECY7, αCD45RA-APCCY7, αCD127-PECy5, and αCD27-Alexa 700. Data were collected using an LSRII flow cytometer and analyzed using FLowJo software version 8.7.3 (Tree Star Inc., Ashland, OR). The

Boolean platform was used to create an array of 16 different combinations for the four phenotypic markers used.

Statistics

We used the Wilcoxon test from SPICE to derive the statistical data for all the functional and phenotypic analysis, for both CMV and HIV. $P < 0.05$ was considered significant. A paired t test by Prism was used to for the correlations between poly functionality and MFI of IFN-γ in Figure 4 B.

Figures

Figure 1

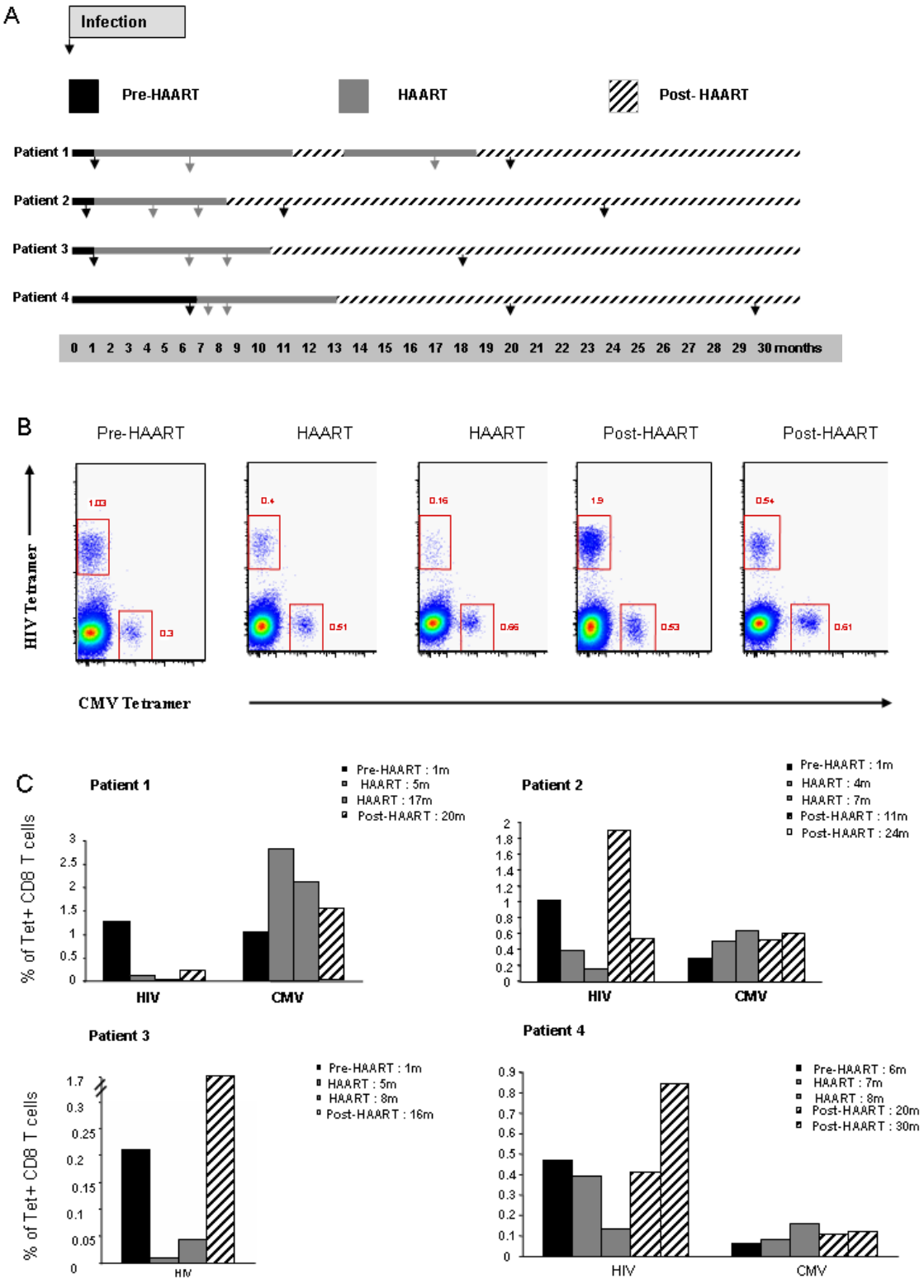


Figure 2

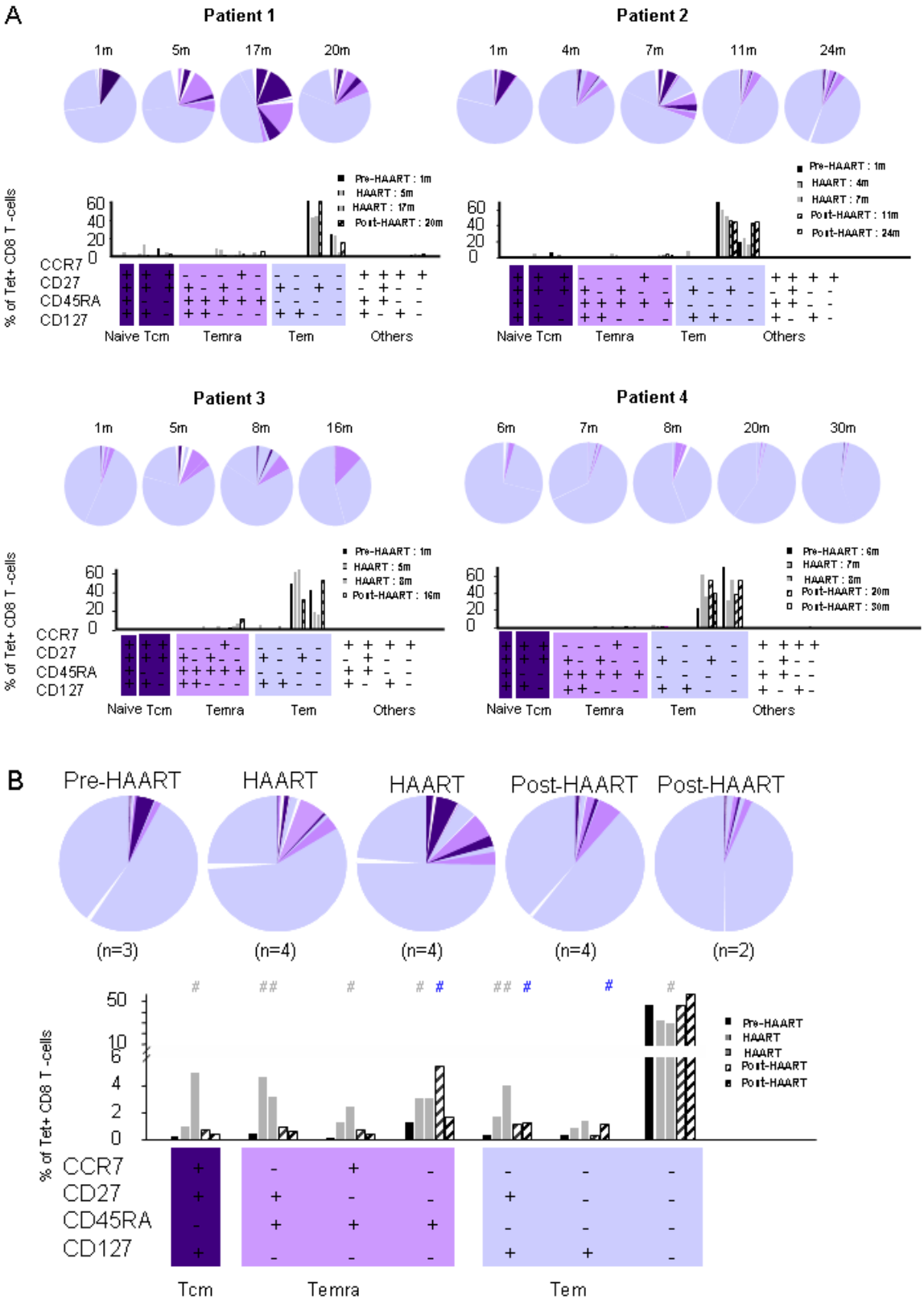

A
Patient 1
1m
5m
17m
20m
Patient 2
1m
4m
7m
11m
24m
% of Tet+ CD8 T -cells
Pre-HAART : 1m
HAART : 5m
HAART : 17m
Post-HAART : 20m
Pre-HAART : 1m
HAART : 4m
HAART : 7m
Post-HAART : 11m
Post-HAART : 24m
CCR7
CD27
CD45RA
CD127
Naive Tcm
Temra
Tem
Others
Patient 3
1m
5m
8m
16m
Patient 4
6m
7m
8m
20m
30m
Pre-HAART : 1m
HAART : 5m
HAART : 8m
Post-HAART : 16m
Pre-HAART : 6m
HAART : 7m
HAART : 8m
Post-HAART : 20m
Post-HAART : 30m
B
Pre-HAART
HAART
HAART
Post-HAART
Post-HAART
(n=3)
(n=4)
(n=4)
(n=4)
(n=2)
% of Tet+ CD8 T -cells
Pre-HAART
HAART
HAART
Post-HAART
Post-HAART
CCR7
CD27
CD45RA
CD127
Tcm
Temra
Tem

Figure 3

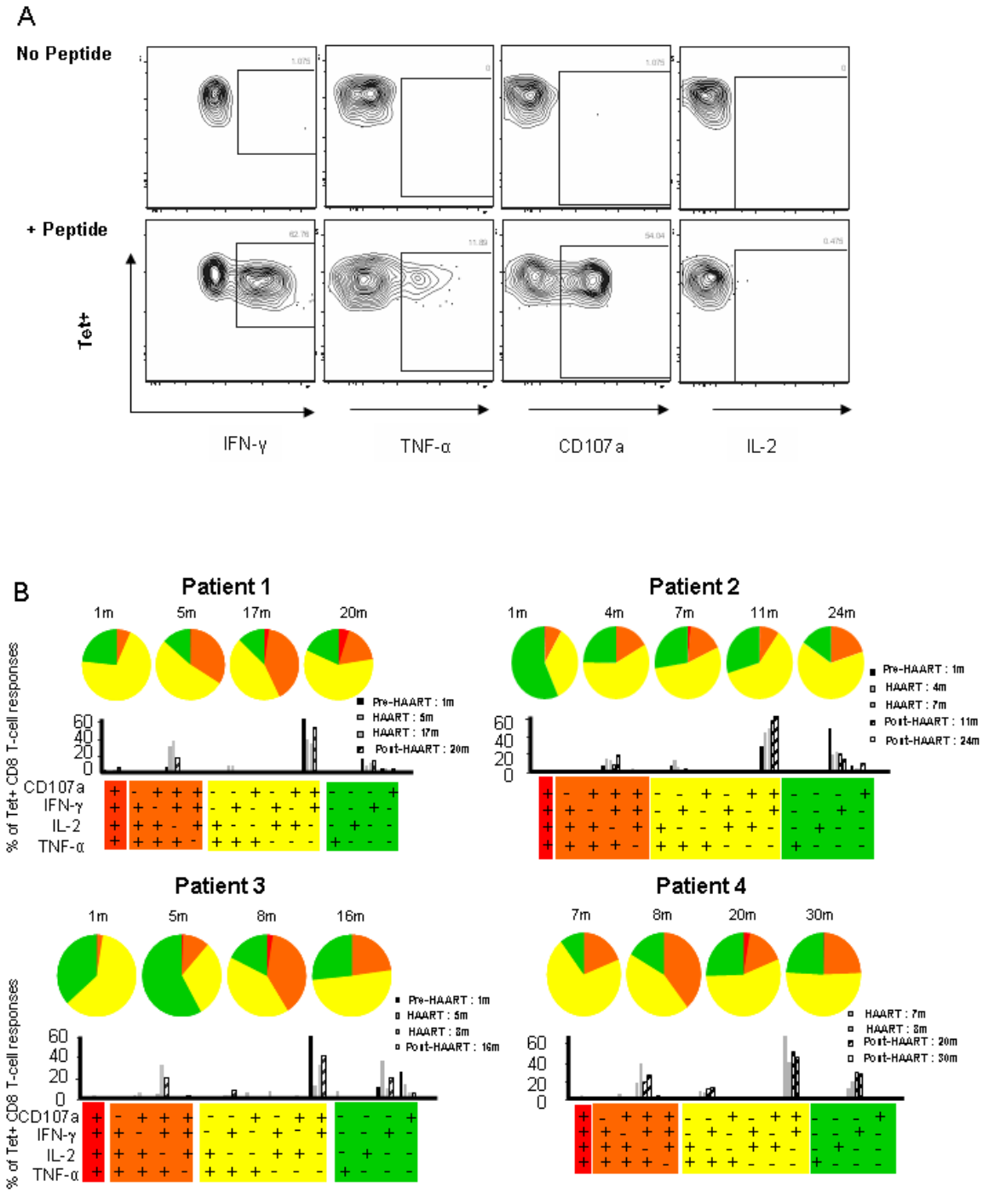
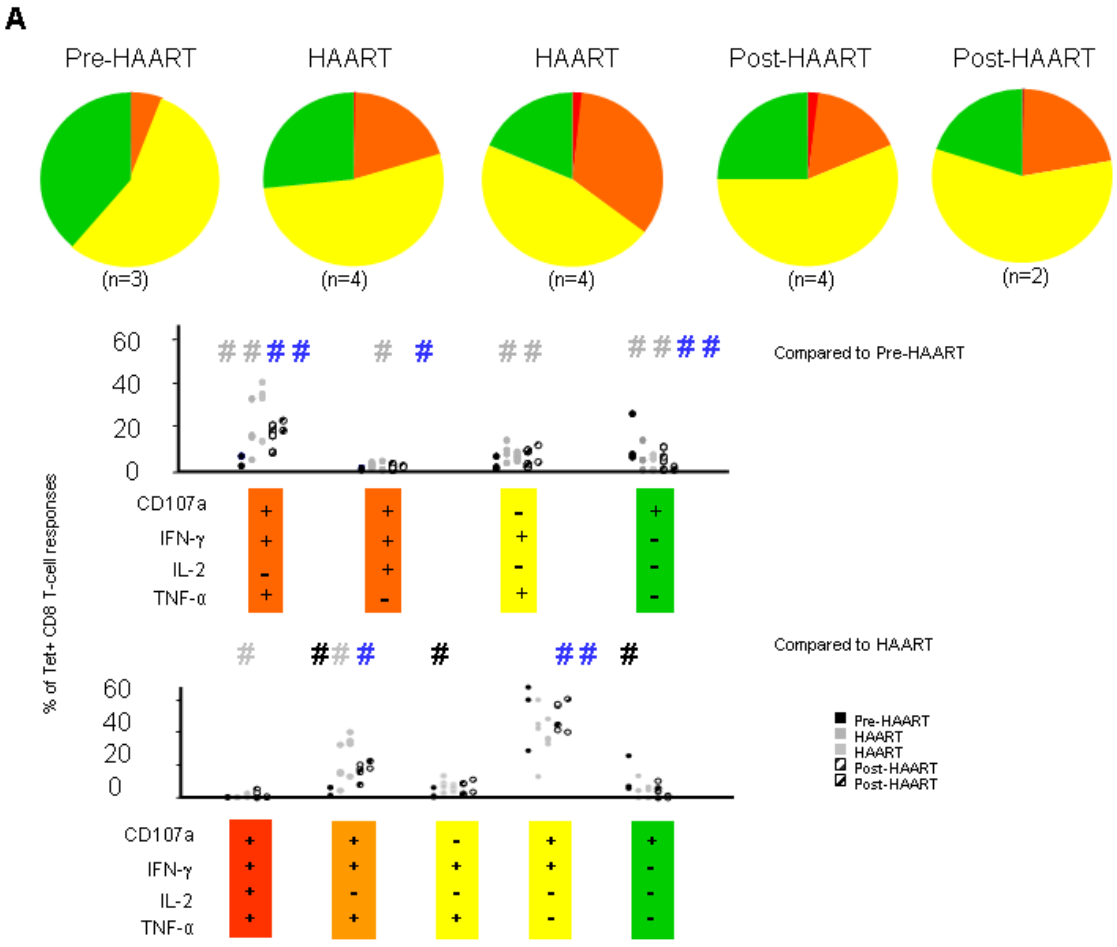

A
No Peptide
+ Peptide
Tet+
IFN-γ
TNF-α
CD107a
IL-2
B
Patient 1
1m
5m
17m
20m
Pre-HAART : 1m
HAART : 5m
HAART : 17m
Post-HAART : 20m
% of Tet+ CD8 T-cell responses
60
40
20
0
CD107a
IFN-γ
IL-2
TNF-α
Patient 2
1m
4m
7m
11m
24m
Pre-HAART : 1m
HAART : 4m
HAART : 7m
Post-HAART : 11m
Post-HAART : 24m
Patient 3
1m
5m
8m
16m
Pre-HAART : 1m
HAART : 5m
HAART : 8m
Post-HAART : 16m
Patient 4
7m
8m
20m
30m
HAART : 7m
HAART : 8m
Post-HAART : 20m
Post-HAART : 30m

Figure 4


A
Pre-HAART
HAART
HAART
Post-HAART
Post-HAART
(n=3)
(n=4)
(n=4)
(n=4)
(n=2)
% of Tet+ CD8 T-cell responses
60
40
20
0
CD107a
IFN-γ
IL-2
TNF-α
Compared to Pre-HAART
Compared to HAART
Pre-HAART
HAART
HAART
Post-HAART
Post-HAART

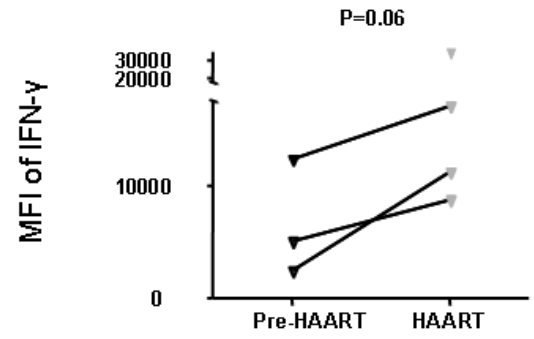

B
P=0.06
MFI of IFN-γ
30000
20000
10000
0
Pre-HAART
HAART

Figure 5

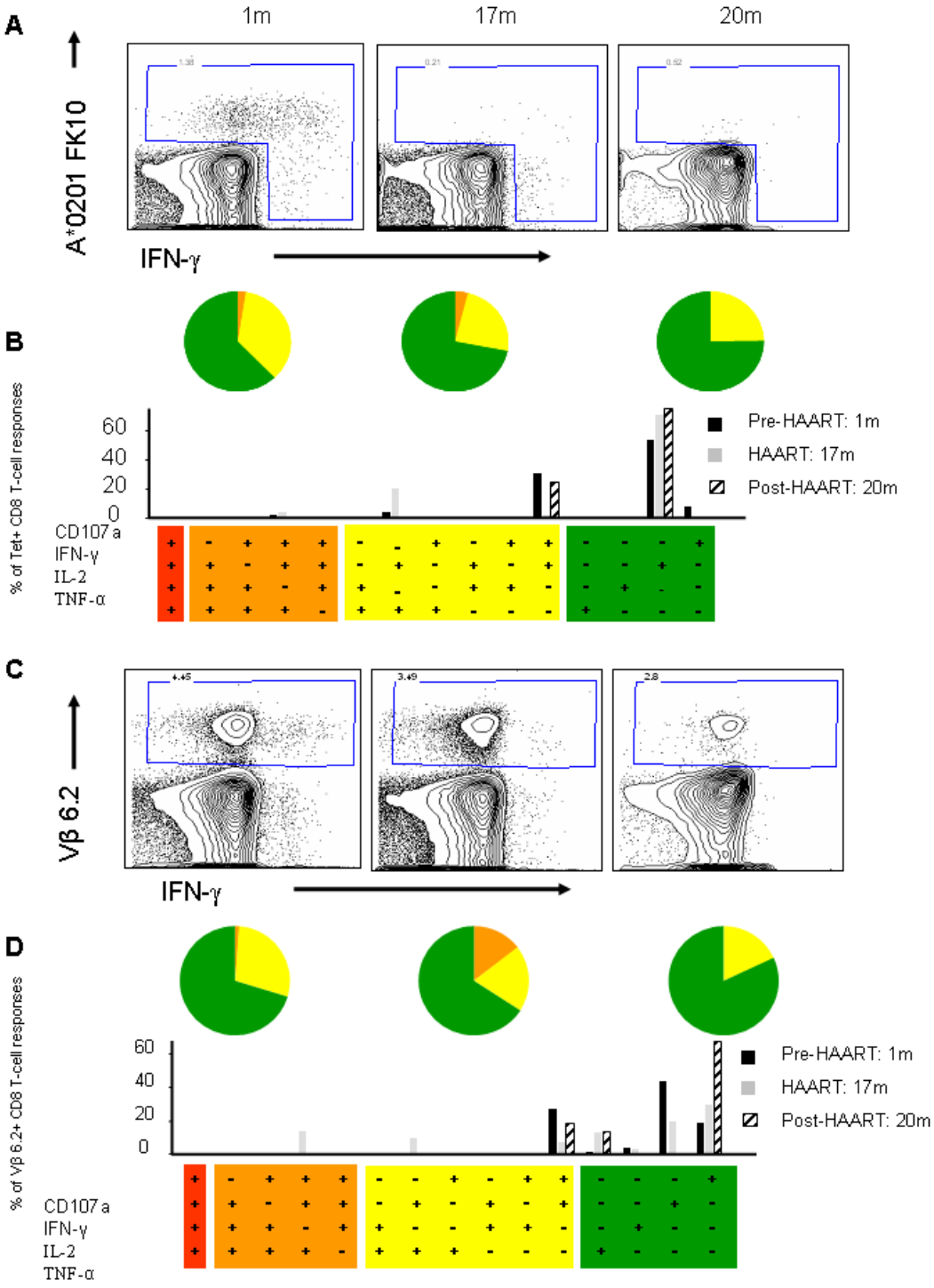
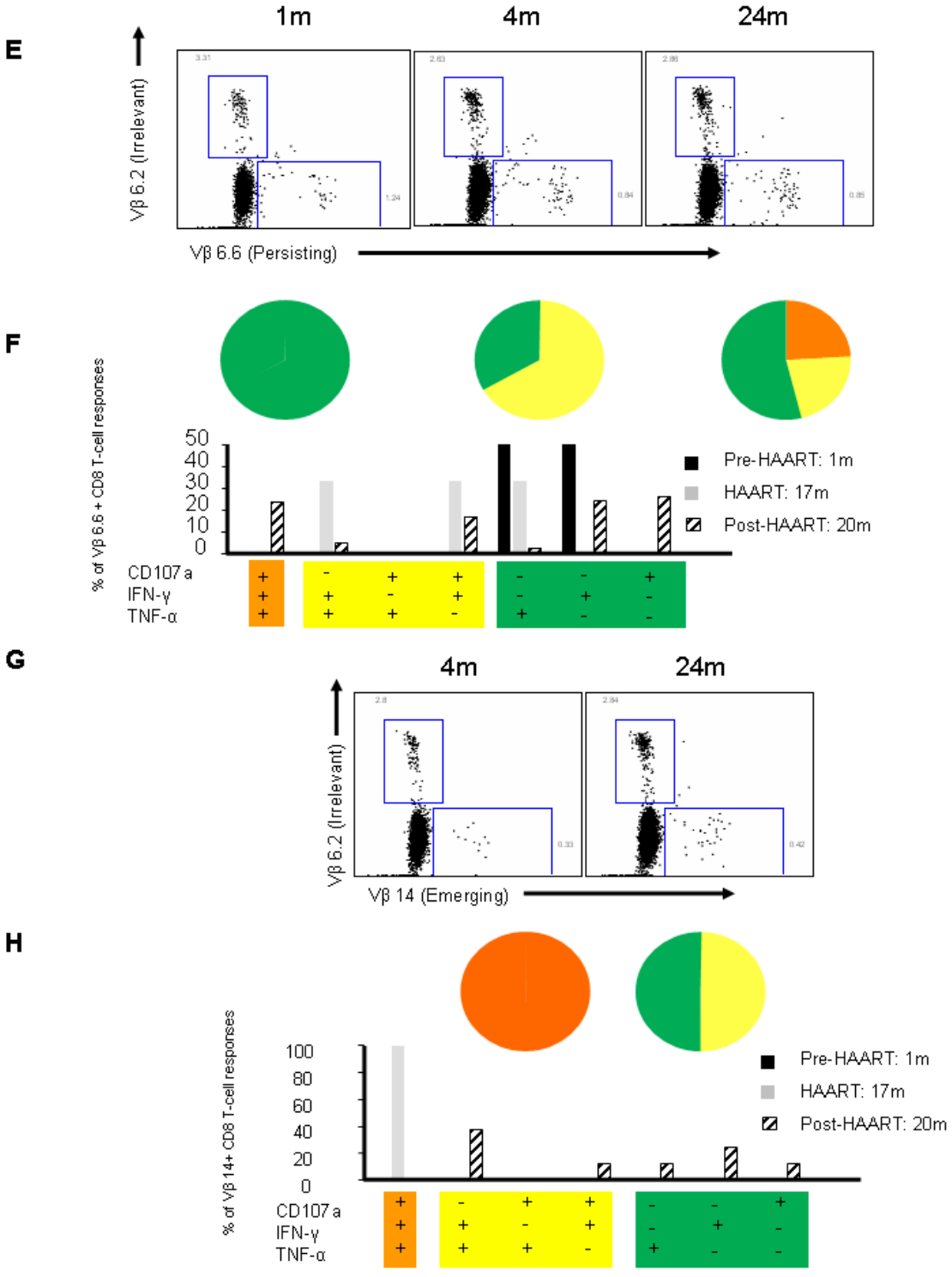

A
1m
17m
20m
A*0201 FK10
IFN-γ
B
% of Tet+ CD8 T-cell responses
60
40
20
0
Pre-HAART: 1m
HAART: 17m
Post-HAART: 20m
CD107a
IFN-γ
IL-2
TNF-α
C
Vβ 6.2
IFN-γ
D
% of Vβ 6.2+ CD8 T-cell responses
60
40
20
0
Pre-HAART: 1m
HAART: 17m
Post-HAART: 20m
CD107a
IFN-γ
IL-2
TNF-α

Figure 5 (continued)



Tables

Table 1

A

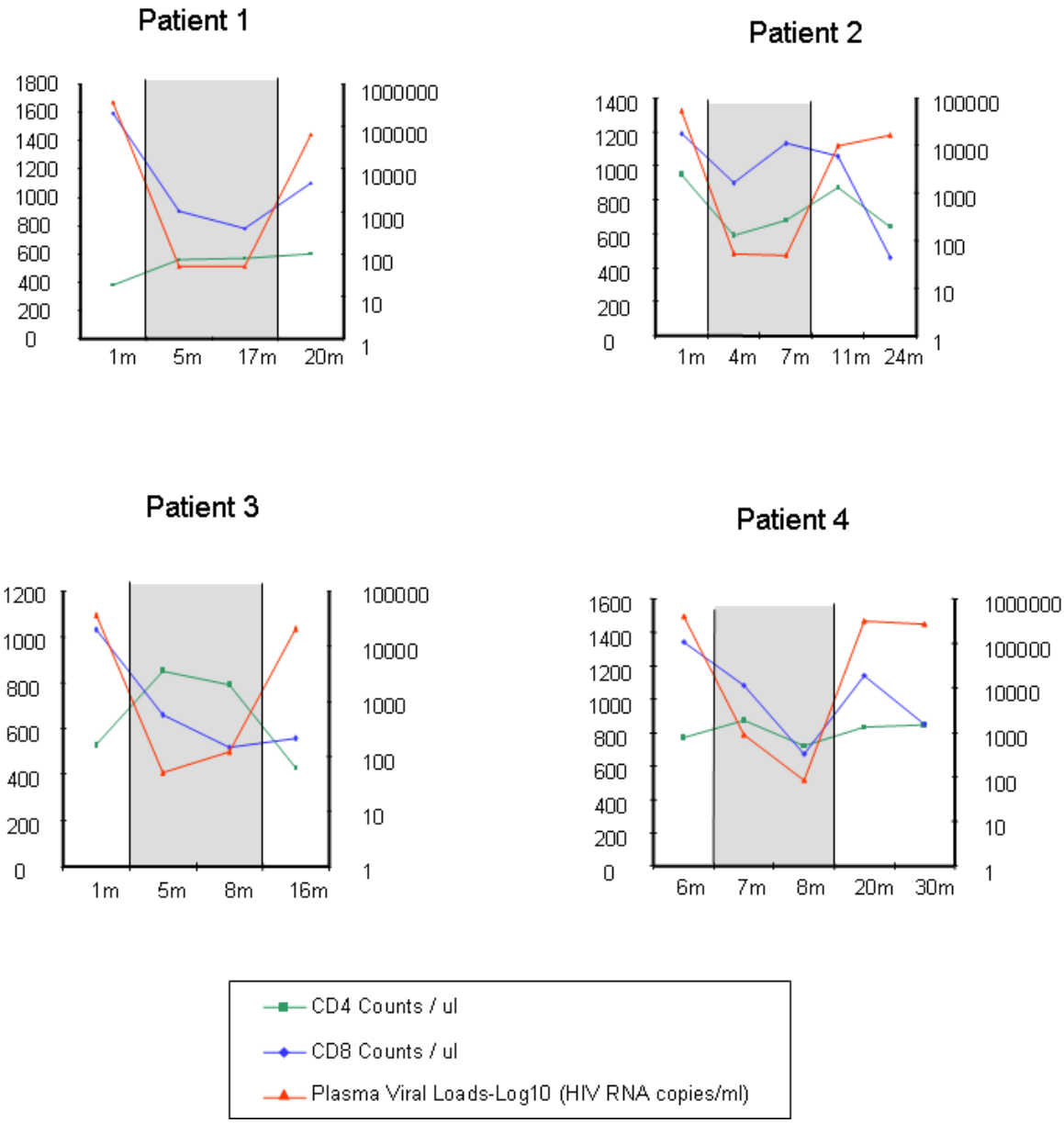
	TCRVB	CDR3	TCRJB	% freq	TCRVB	CDR3	TCRJB	% freq	TCRVB	CDR3	TCRJB	% freq	TCRVB	CDR3	TCRJE	% freq	TCRVB	CDR3	TCRJB	% freq
Patient 1	**1 month**				**5 months**				**17 months**				**20 months**							
	4-2	CAS SFTSGTDTQY	2-3	46	6-2	CAS SYVGGDGYT	1-2	31	6-2	CAS SYVGGDGYT	1-2	44	6-2	CAS SYVGGDGYT	1-2	78				
	6-2	CAS SYVGGDGYT	1-2	23	27	CAS SLSGGGDTQY	2-3	30	7-9	CAS SEGSQEYF	2-1	16	6-4	CAS SLTGSADTQY	2-3	11				
	5-1	CAS SLAAGGDEQY	2-7	6	19	CAS SIAIADEKLF	1-4	16	4-1	CAS SFTSGTDTQY	2-3	6								
	19	CAS SIAIADEKLF	1-4	5					6-2	CAS SEGLQEYF	2-1	5								
	4-1	CAS HGSGTGELF	2-2	3					19	CAS SIAIADEKLF	1-4	4								
				83				77				75				89				
Patient 2	**1 month**				**4 months**				**7 months**				**11 months**				**24 months**			
	13	CAS SLERETEAF	1-1	20	5-6	CAS SLDWGTFGEQY	2-7	18	13	CAS SLERETEAF	1-1	10	13	CAS SLERETEAF	1-1	20	6-6	CAS SEDESYEQY	2-7	15
	5-6	CAS SLDWGTPGEQY	2-7	7	14	CAS SQDPAGWGTDTQY	2-3	15	5-6	CAS SLDWGTPGEQY	2-7	7	5-6	CAS SLDRAGDTQY	2-3	5	5-6	CAS SLDRAGDTQY	2-3	11
	6-6	CAS SEDESYEQY	2-7	6	24-1	CAT SDDGASDGNEQF	2-1	13	6-6	CAS SEDESYEQY	2-7	7	5-6	CAS SAGWGSYEQY	2-7	5	14	CAS SQDPAGWGTDTQY	2-3	10
	10-2	CAS SEWNTEAF	1-1	5	5-6	CAS SLGWDSLTDTQY	2-3	6	5-6	CAS SLDRAGDTQY	2-3	6	6-6	CAS SEDESYEQY	2-7	3	5-6	CAS SWGWGIAEAF	1-1	8
	5-6	CAS SLDRAGDTQY	2-3	5	13	CAS SLERETEAF	1-1	5	10-1	CAS SEGQLYSYEQY	2-7	2	20-1	CSA REPSDRDVEQY	2-7	1	13	CAS SLERETEAF	1-1	2
	5-6	CAS SLGWDSLTDTQY	2-3	4	7-6	CAS SQGWGADTQY	2-3	3	24-1	CAT SDDGASDGNEQF	2-1	1					20-1	CSA EFRELRGAEAF	1-1	2
	24-1	CAT SDDGASDGNEQF	2-1	3	10-1	CAS SEGQLYSYEQY	2-7	1	10-2	CAS SEWNTEAF	1-1	1								
	7-6	CAS SQGWGADTQY	2-3	1	6-6	CAS SEDESYEQY	2-7	1	5-6	CAS SPGWGGETQY	2-5	1								
	5-6	CAS SPGWGEDTQY	2-3	1	12-4	CAS SDPGGSGEQY	2-7	1												
	27	CAS SPQWVEDGVS	1-2	1	5-6	CAS SWGWGGDTQY	2-3	1												
				55				64				35				34				48
Patient 3	**1 month**				**5 months**				**8 months**				**16 months**							
	25-1	CAS SVLRAAF	1-1	86	25-1	CAS SVLRAAF	1-1	26	25-1	CAS SVLRAAF	1-1	56	7-2	CAS SLSQLWETQY	2-5	75				
	20-1	CSA RDTMTTGGVFYEAF	1-1	2	2	CAS RTGGAYNEQF	2-1	7												
					2	CAS SESPGTSGSPLNEQF	2-1	5												
					5.1	CAS SLKGTYNEGF	2-1	4												
					27	CAS SRGQTLSYEQY	2-1	3												
					18	CAS SPQSVGDTQY	2-3	2												
					12-3/ 12-4	CAS RSTPTDIQY	2-4	1												
					29-1	CSV AVNYGYT	1-2	1												
				88				49				56				75				
Patient 4	**6 month**				**7 months**				**8 months**				**20 months**				**30 months**			
	3-1	CAS SPLARVDEQF	2-1	23	3-1	CAS SPLARVDEQF	2-1	8	20-1	CSA SPRGRYNEQF	2-1	17	20-1	CSA SSRGRVDEQF	2-1	18	11-3	CAS STPGLAGRHNEQF	2-1	16
	20-1	CSA SPRGRYNEQF	2-1	20	20-1	CSA SSRGRVDEQF	2-1	7	11-3	CAS STPGLAGRHNEQF	2-1	9	20-1	CSA SPRGRYNEQF	2-1	11	20-1	CSA SSRGRVDEQF	2-1	8
	4-3	CAS SQVGRRDTQY	2-3	6	6-2/6-3	CAS STGLAGEGRHNEQF	2-1	4	3-1	CAS SPLARVDEQF	2-1	8	3-1	CAS SPLARVDEQF	2-1	6	20-1	CSA SPRGRYNEQF	2-1	2
	15	CAT SSPGTSGRHNEQF	2-1	5	27	CAS TGSTSGPSRYNEQF	2-1	3	24-1	CAT SDEVAGGRHNEQF	2-1	8	9	CAS SPRGRIDEQY	2-7	5				
	5-1	CAS SPRGGYNEQF	2-1	5	11-2	CAS SSPLTSGRLNEQF	2-1	2	20-1	CSA SSRGRVDEQF	2-1	6	9	CAS SPLGRVDEQF	2-1	4				
	18	CAS SPEGRKNEAF	1-1	3	20-1	CSA SPRGRYNEQF	2-1	2	4-3	CAS SQEGRRDEQF	2-1	2	18	CAS SPEGRKNEAF	1-1	3				
	20-1	CSA SSRGRVDEQF	2-1	3	24-1	CAT SDEVAGGRHNEQF	2-1	2	25-1	CAS SSRVAGGRYNEQF	2-1	2	27	CAS TGSTSGPSPSRYNEQF	2-1	2				
	20-1	CSV SPRGRYNEQF	2-1	1	24-1	CAT RDRQGRYNEQF	2-1	2	4-3	CAS SQSGRQNEQF	2-1	1	11-3	CAS STPGLAGRHNEQF	2-1	2				
	6-2/6-3	CAS SGTSGRARHNEQF	2-1	2	7-9	CAS SPPPGGLGGYT	1-2	2	2	CAS SERGRYNEQF	2-1	1	6-1	CAS SVTGQSNSPLH	1-6	1				
	20-1	CSA SPRGRHNEQF	2-1	1	9	CAS SPLGRVDEQF	2-1	1					24-1	CAT SGLAGGPRHNEQF	2-1	1				
	20-1	CSA SPRGRYNEQF	2-1	1	25-1	CAS SSRVAGGRYNEQF	2-1	1					9	CAS GPRGRIDEQY	2-7	1				
	25-1	CAS SSRVAGGRYNEQF	2-1	1	9	CAS SPRGRTGELF	2-2	1												
	11-2	CAS SSRGRIVEQY	2-7	1	20-1	CSA SSRGRVDEQF	2-1	1												
				72				37				54				54				26

B

	TCRVB	CDR3	TCRJB	% frec	TCRVB	CDR3	TCRJB	% freq	TCRVB	CDR3	TCRJB	% freq	TCRVB	CDR3	TCRJE	% freq	TCRVB	CDR3	TCRJB	% freq
Patien 1	**1 month**				**5 months**				**17 months**				**20 months**							
	6-5	CAS SPRTGTTDTQ YFG	2-3	91	6-5	CAS SPRTGTTDTQ YFG	2-3	74	6-5	CAS SPRTGTTDTQ YFG	2-3	56	6-5	CAS SPRTGTTDTQ YFG	2-3	50				
Patient 2	**1 month**				**4 months**				**7 months**				**11 months**				**24 months**			
	6-2/ 6-3	CAS SYSPLNTEA FFG	1-1	79	6-2/ 6-3	CAS SYSPLNTEA FFG	1-1	38	6-2/ 6-3	CAS SYSPLNTEA FFG	1-1	43	6-2/ 6-3	CAS SYSPLNTEA FFG	1-1	57	6-2/ 6-3	CAS SYSPLNTEA FFG	1-1	65
	7-9	CAS SLIGISSYNEQ FFG	2-1	4	7-9	CAS SLHDYTGFGTEA FFG	1-1	3		CAS SLIGEGWDDEQ YFG		2	6-2	CAS SLVGESAFPNEQ YFG	2-7	1				
				83				41				45				58				65
Patient 3	**6 month**				**7 months**				**8 months**				**20 months**				**30 months**			
	27	CAS SLGSSPREQ FFG	2-1	5	27	CAS SLGSSPREQ FFG	2-1	18	27	CAS SLGSSPREQ FFG	2-1	93	7-9	CAS SLHGQGVRSPL HFG	1-6	16	27	CAS SLGSSPREQ FFG	2-1	58
	12-3/12-4	CAS SLGTPNEQ FFG	2-1	5	6-2/6-3	CAS SYSSGEL FFG	2-2	8					6-2/6-3	CAS SYSSGEL FFG	2-2	13	6-2/6-3	CAS SYSSGEL FFG	2-2	5
	7-9	CAS SLVGVSKDNEQ FFG	2-1	3	12-3/12-4	CAS SLGTPNEQ FFG	2-1	4					27	CAS SLGSSPREQ FFG	2-1	5	12-3/12-4	CAS SLGTPNEQ FFG	2-1	9
					7-9	CAS SLRDPSYEQ YFG	2-7	10					4-3	CAS SSAEPYNSPL HFG	1-6	3	7-9	CAS SLHTQGAR FFG	1-1	6
					7-9	CAS SLHGQGVRSPL HFG	1-6	12					7-9	CAS SSHDRAGINSPL HFG	1-6	3	7-2	AS SLGGAGTPQETQ YF	2-5	3
					7-9	CAS SLIGVSVRNEQ FFG	2-1	1					7-9	CAS SNRTGVPTGEL FFG	2-2	3	2	CAS SDNTGFYGY TFG	1-2	1
					7-9	CAS SLKGQGPINEQ FFG	2-1	1					5-1	CAS SLIEANTGEL FFG	2-2	1	29-1	CSV EKRSNIAKNIQ YFC	2-4	1
					28	CAS SFALVSGEL FFG	2-2	1					28	CAS SPLGLAGPNEQ FFG	2-1	1				
					7-9	CAS SLHGDPSYEQ YFG	2-7	1					29-1	CSV DVIANEQ FFG	2-1	1				
													2	CAS TSTGGYGY TFG	1-2	1				
													28	CAS KVPAGLEQ YFG	2-7	1				
				13				56				93				48				83

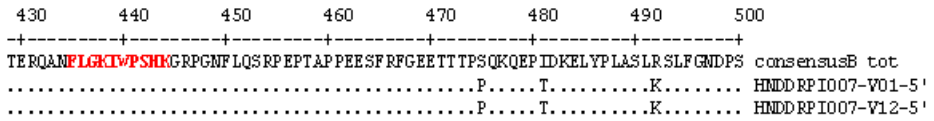
Supplementary Figures

Supplementary figure1

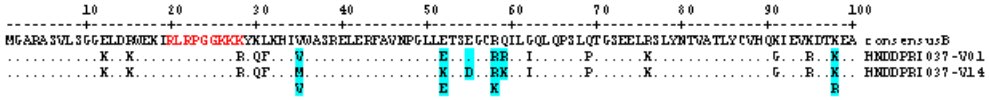


Supplementary figure2

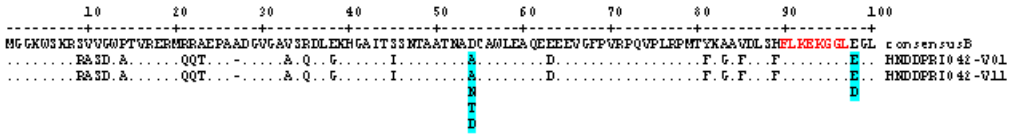
Patient 1



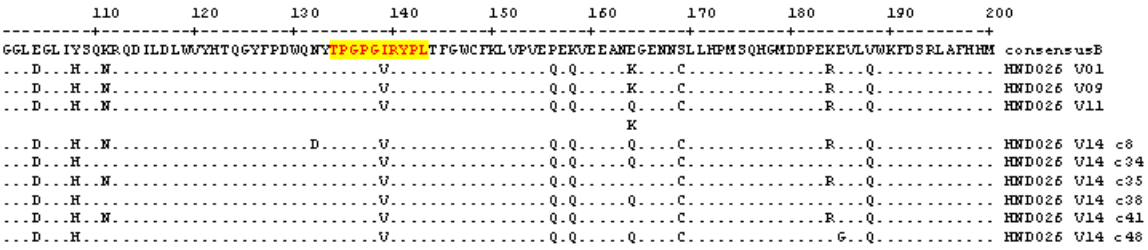
Patient 2



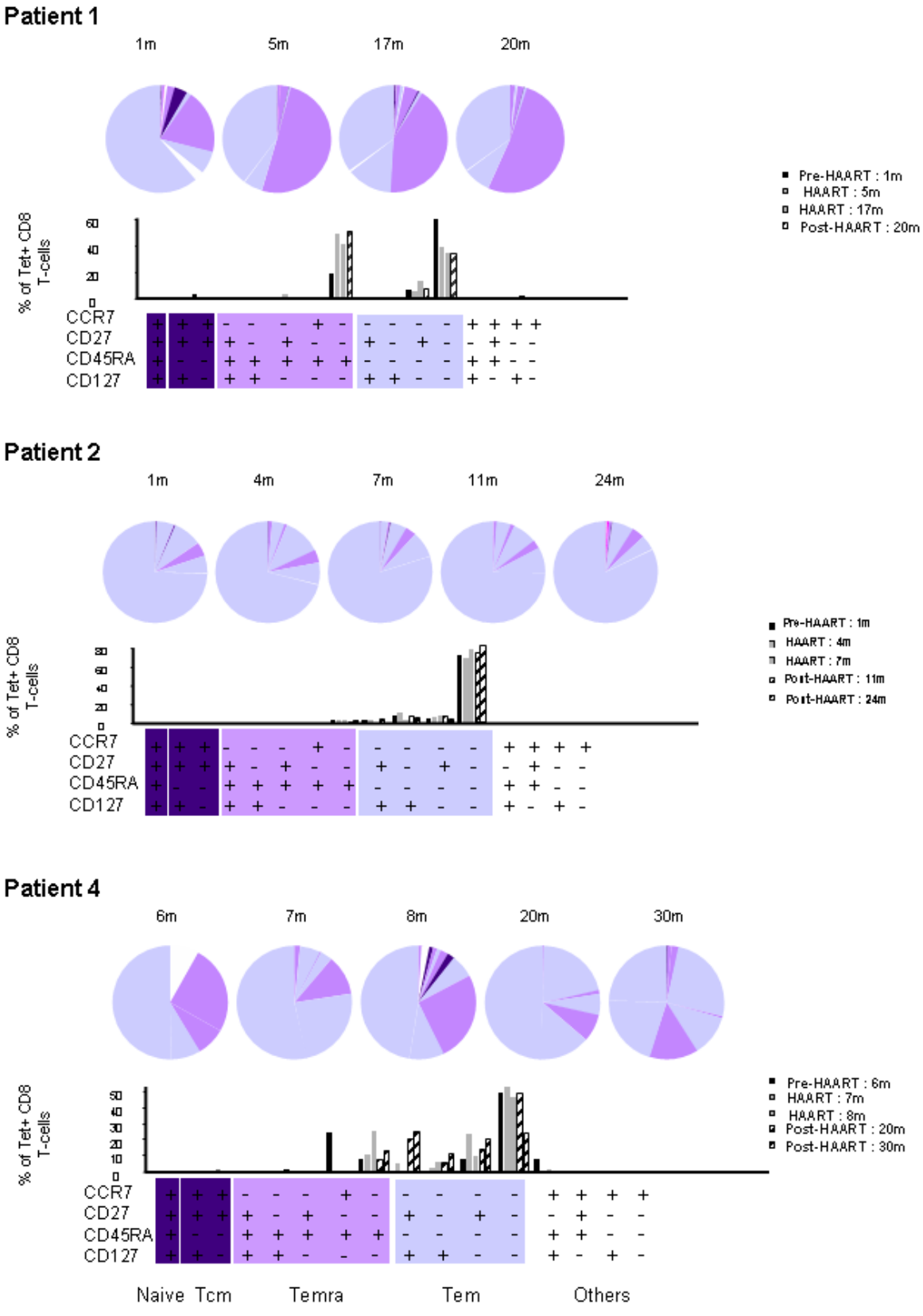
Patient 3



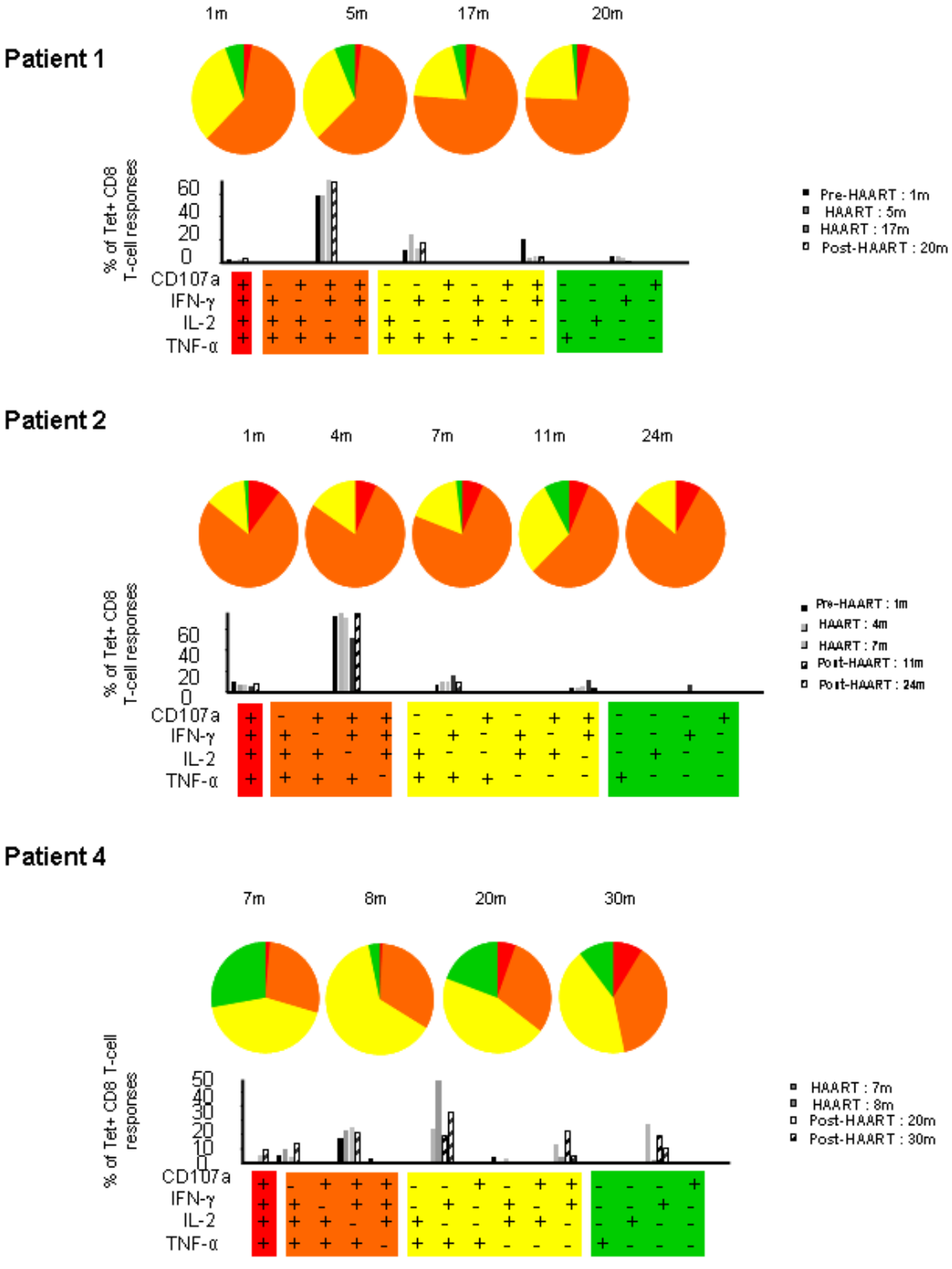
Patient 4



Supplementary figure3



Supplementary figure 4



Legends of Figures

Figure 1: Representation of the study cohort. **(A)** Four patients were studied longitudinally. A color code is assigned for the 3 main treatment criteria studied for each patient; Pre-HAART (black), on HAART (grey) and Post-HAART (diagonal lines). Month zero indicates the estimated date of infection. The arrows are indicative of the studied time points for each patient. Antigen-specific $CD8^+$ T cell responses were studied as measured by tetramer staining and were as follows; HLA-A*0201-FLGKIWPSHK (Gag FK10, residues 435-444) and HLA-A*0201-NLVPMVATV (pp65) in patient1, HLA-A*0301-RLRPGGKKR (Gag RR9, residues 20-28) and HLA-B*0702-TPRVTGGGAM (pp65) in patient 2, HLA-B*0801-FLKEKGGL (Nef FL8, residues 90-97) in patient 3, and HLA-B*0702-TPGPGVRYPL (Nef TL10, residues 134-143) and HLA-B*0702-TPRVTGGGAM (pp65) in patient 4. **(B)** Representative tetramer co staining showing the frequency of HIV and CMV-specific $CD8^+$ T cells longitudinally in patient 2**. (C)** Frequency of HIV and CMV-specific $CD8^+$ T cells. The black, grey and diagonally lined bars indicate Pre-HAART, HAART, and Post-HAART time points, respectively. The time points studied and their corresponding treatment schedules are indicated in the right panels for each patient.

Figure 2: HAART leads to an increase in HIV-specific $CD8^+$ T_{CM} and T_{EMRA} Cells. **(A)** Representative phenotypic composition of HIV-specific $CD8^+$ T cells. After the gates for four phenotypes were created (CCR7, CD27, CD45RA, and CD127), the boolean gate platform was used to create 16 different combinations. The colors on the pie indicate the different memory subsets; Naïve and T_{CM} in purple, T_{EM} in light blue, T_{EMRA} in lilac and others / not defined in white. The bars demonstrate the mean frequencies of cells belonging to a particular subset. HAART time points are depicted in grey bars. The time points studied and their corresponding treatment schedule are represented in the insert on the right of each patient. **(B)** Using the Wilcoxon test, statistical significance was reached when all time points were pooled and compared to the first Pre-HAART time point for the different memory subsets. Only memory subsets that yielded statistical significance are depicted in the figure. The number sign **(#)** indicates statistical significance. Black,

grey and blue number signs indicate Pre-HAART, on HAART, and Post-HAART time points, respectively.

Figure 3: HAART improves the functionality of HIV-specific CD8$^+$ T cells. (A) Representative example of simultaneous multifunctional assessment of HIV-specific CD8$^+$ T cells by multi parametric flow cytometry at a given time point. Cells were stimulated for 6 hours with corresponding cognate peptide before intracellular staining. Percentages of cytokine producing HIV-specific tetramer $^+$ cells are shown. Plots are gated on CD3$^+$ and CD8$^+$ cells. Dead cells were excluded using a viability dye. **(B)** Functional assessment of HIV-specific CD8$^+$ T cells performed longitudinally for all four patients. After the gates for four cytokines were created (CD107a, IFN-γ, IL-2, and TNF-α), the boolean gate platform was used to create an array of 16 different combinations. The pies represent the functional profiles of the antigen-specific CD8$^+$ T cells. The slices in each pie with their different colors represent different functional combinations; 4+ (red), 3+ (orange), 2+ (yellow) and 1+ (green). The bars on the axis represent the response frequency for each combination. The insert on the right indicates the time points studied with their corresponding treatment schedule. Black, grey and diagonally lined bars indicate Pre-HAART, HAART, and Post-HAART time points, respectively.

Figure 4: **(A) Restoration of polyfunctionality of HIV- specific CD8$^+$ T cells under HAART is statistically significant compared to Pre-HAART and Post-HAART**. All Pre-HAART, on HAART, and post-HAART time points were pooled for all four patients. Using the Wilcoxon test, statistical significance was reached when all time points were compared to the first Pre-HAART time point for different combinations. Functional subsets that yielded statistical significance are depicted in the figure. The number sign (#) indicates statistical significance. Black, grey and blue number signs indicate statistical significance for Pre-HAART, HAART, and Post-HAART time points, respectively. **(B)** Correlation between polyfunctionality and MFI of IFN-γ was performed for Pre-HAART (n=3) and second time point on HAART (n=4). P values were calculated using the Wilcoxon test.

Figure 5: HAART improves the functionality of HIV-specific CD8$^+$ T cells on the clonal level (A) and (C) Gating strategy showing HLA-A*0201 FK10 tetramer and Vβ

6.2 staining respectively at 1m: Pre-HAART, 17m: HAART, and 20m: Post-HAART from patient 1. All gates were created on $CD3^+$ and $CD8^+$ T cells. **(B) and (D)** Simultaneous multi functional assessment of HLA-A*0201 FK10 tetramer and Vβ 6.2 positive $CD8^+$ T cells respectively, from patient 1 at 1, 17 and 20 months. **(E)** Representative data showing gating strategy of Vβ 6.6 (persisting clonotype) and Vβ 6.2 (irrelevant Vβ) at 1m: Pre-HAART, 4m: HAART, and 24m: Post-HAART from patient 2. All gates were created on $CD3^+$ and $CD8^+$ T cells. **(F)** Simultaneous multi functional assessment of Vβ 6.6 positive cells at 1, 4, and 24 months. **(G)** Representative data showing gating strategy of Vβ 14 (emerging clonotype) and Vβ 6.2 (irrelevant Vβ) at 4 and 24 months. **(H)** Simultaneous multi functional assessment of Vβ 14 positive $CD8^+$ T cells at 4 and 24 months. An irrelevant Vβ 13.2 was used in the assay as a negative control.

Legends of Tables

Table 1: Clonotypic composition of antigen-specific CD8$^+$ T cells. The table shows the CDR3 amino acid sequence, TCRBV and TCRBJ usage, and the relative frequency of HIV-specific CD8$^+$ T cell clonotypes at Pre-HAART, on HAART (grey) and Post-HAART for the time points studied for each patient. Colored backgrounds indicate persisting clonotypes over time, whereas non-colored backgrounds indicate non-persisting clonotypes. The Imunogenetics (IMGT) nomenclature was used for the TCR clonotypic analysis. The above table summarizes TCR clonotypic data obtained from **(A)** HIV-specific CD8$^+$ T cells for all four patients, while the lower table **(B)** summarizes TCR clonotypic data from CMV-specific CD8$^+$ T cells from three patients.

Legends of Supplementary Figures

Supplementary figure1: Disease course in four longitudinally studied HIV-infected patients. A) HIV plasma viral load, peripheral blood CD4 T cell counts, and peripheral blood CD8 counts are depicted longitudinally for each patient. Plasma viral loads are represented in red. Green squares indicate CD4 counts / ul, while blue diamonds indicate CD8 counts / ul. **B)** Autologous virus sequences obtained by population / bulk sequencing at Pre-HAART and post-HAART for each patient. The epitopes of interest are depicted in red. Where indicated, clonal sequencing is performed (patient 4).

Supplementary figure2: Autologous viral sequencing of the studied epitopes. Population / bulk sequencing performed at pre-HAART and post-HAART time points for all patients. Highlighted regions indicate the studied epitopes.

Supplementary figure3: Phenotypic assessment of CMV-specific $CD8^+$ T cells. Representative example of simultaneous phenotypic assessment of CMV-specific $CD8^+$ T cells by multi parametric flow cytometry for 3 patients. The colors on the pie indicate the different memory subsets while the bars demonstrate the mean frequencies of cells showing a particular memory subset. The insert on the right indicates the time points studied with their corresponding treatment schedule

Supplementary figure4: Functional assessment of CMV-specific $CD8^+$ T cells. Representative example of simultaneous multifunctional assessment of CMV-specific $CD8^+$ T cells by multi parametric flow cytometry for three patients. Cells were stimulated for 6 hours with corresponding cognate peptide before intracellular staining. The colors on the pie indicate the different functional subsets while the bars demonstrate the mean frequencies of cells showing a particular functional combination. The insert on the right indicates the time points studied with their corresponding treatment schedule.

References

1. Borrow, P., et al., *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.* J Virol, 1994. **68**(9): p. 6103-10.
2. Jin, X., et al., *Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques.* J Exp Med, 1999. **189**(6): p. 991-8.
3. Koup, R.A., et al., *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.* J Virol, 1994. **68**(7): p. 4650-5.
4. Schmitz, J.E., et al., *Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes.* Science, 1999. **283**(5403): p. 857-60.
5. Goulder, P.J., et al., *Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS.* Nat Med, 1997. **3**(2): p. 212-7.
6. Price, D.A., et al., *Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection.* Proc Natl Acad Sci U S A, 1997. **94**(5): p. 1890-5.
7. Kiepiela, P., et al., *CD8+ T-cell responses to different HIV proteins have discordant associations with viral load.* Nat Med, 2007. **13**(1): p. 46-53.
8. Wherry, E.J., et al., *Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment.* J Virol, 2003. **77**(8): p. 4911-27.
9. Wherry, E.J. and R. Ahmed, *Memory CD8 T-cell differentiation during viral infection.* J Virol, 2004. **78**(11): p. 5535-45.
10. Appay, V., et al., *HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function.* J Exp Med, 2000. **192**(1): p. 63-75.
11. Migueles, S.A., et al., *HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors.* Nat Immunol, 2002. **3**(11): p. 1061-8.

12. Shankar, P., et al., *Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection.* Blood, 2000. **96**(9): p. 3094-101.
13. Trautmann, L., et al., *Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction.* Nat Med, 2006. **12**(10): p. 1198-202.
14. Betts, M.R., et al., *Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection.* J Virol, 2001. **75**(24): p. 11983-91.
15. Gea-Banacloche, J.C., et al., *Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors.* J Immunol, 2000. **165**(2): p. 1082-92.
16. Darrah, P.A., et al., *Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major.* Nat Med, 2007. **13**(7): p. 843-50.
17. Precopio, M.L., et al., *Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses.* J Exp Med, 2007. **204**(6): p. 1405-16.
18. Betts, M.R., et al., *HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells.* Blood, 2006. **107**(12): p. 4781-9.
19. Almeida, J.R., et al., *Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover.* J Exp Med, 2007. **204**(10): p. 2473-85.
20. Price, D.A., et al., *T cell receptor recognition motifs govern immune escape patterns in acute SIV infection.* Immunity, 2004. **21**(6): p. 793-803.
21. Pantaleo, G., et al., *Evidence for rapid disappearance of initially expanded HIV-specific CD8+ T cell clones during primary HIV infection.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9848-53.
22. Turner, S.J., et al., *Structural determinants of T-cell receptor bias in immunity.* Nat Rev Immunol, 2006. **6**(12): p. 883-94.
23. Lichterfeld, M., et al., *Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection.* J Virol, 2007. **81**(8): p. 4199-214.

24. Douek, D.C., et al., *Evidence for increased T cell turnover and decreased thymic output in HIV infection.* J Immunol, 2001. **167**(11): p. 6663-8.
25. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.
26. Bradley, L.M., L. Haynes, and S.L. Swain, *IL-7: maintaining T-cell memory and achieving homeostasis.* Trends Immunol, 2005. **26**(3): p. 172-6.
27. Champagne, P., et al., *Skewed maturation of memory HIV-specific CD8 T lymphocytes.* Nature, 2001. **410**(6824): p. 106-11.
28. Rehr, M., et al., *Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy.* J Virol, 2008. **82**(7): p. 3391-404.
29. Wilson, J.D., et al., *Oligoclonal expansions of CD8(+) T cells in chronic HIV infection are antigen specific.* J Exp Med, 1998. **188**(4): p. 785-90.
30. Nikolich-Zugich, J., M.K. Slifka, and I. Messaoudi, *The many important facets of T-cell repertoire diversity.* Nat Rev Immunol, 2004. **4**(2): p. 123-32.
31. Welsh, R.M., *Private specificities of heterologous immunity.* Curr Opin Immunol, 2006. **18**(3): p. 331-7.
32. Rees, W., et al., *An inverse relationship between T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9781-6.
33. Bucy, R.P., et al., *Initial increase in blood CD4(+) lymphocytes after HIV antiretroviral therapy reflects redistribution from lymphoid tissues.* J Clin Invest, 1999. **103**(10): p. 1391-8.
34. Fleury, S., et al., *Long-term kinetics of T cell production in HIV-infected subjects treated with highly active antiretroviral therapy.* Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5393-8.
35. Carcelain, G., P. Debre, and B. Autran, *Reconstitution of CD4+ T lymphocytes in HIV-infected individuals following antiretroviral therapy.* Curr Opin Immunol, 2001. **13**(4): p. 483-8.
36. Smith, B.A., et al., *Persistence of infectious HIV on follicular dendritic cells.* J Immunol, 2001. **166**(1): p. 690-6.

37. Autran, B., et al., *Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease.* Science, 1997. **277**(5322): p. 112-6.

38. Makedonas, G. and M.R. Betts, *Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection.* Springer Semin Immunopathol, 2006. **28**(3): p. 209-19.

39. Streeck, H., et al., *Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells.* PLoS Med, 2008. **5**(5): p. e100.

40. Lichterfeld, M., et al., *Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells.* J Exp Med, 2004. **200**(6): p. 701-12.

41. Komanduri, K.V., et al., *Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1.* Nat Med, 1998. **4**(8): p. 953-6.

42. O'Sullivan, C.E., et al., *Decrease of cytomegalovirus replication in human immunodeficiency virus infected-patients after treatment with highly active antiretroviral therapy.* J Infect Dis, 1999. **180**(3): p. 847-9.

43. Deayton, J., et al., *Loss of cytomegalovirus (CMV) viremia following highly active antiretroviral therapy in the absence of specific anti-CMV therapy.* AIDS, 1999. **13**(10): p. 1203-6.

44. Yang, H., et al., *Broad TCR usage in functional HIV-1-specific CD8+ T cell expansions driven by vaccination during highly active antiretroviral therapy.* J Immunol, 2007. **179**(1): p. 597-606.

45. Altman, J.D. et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**, 94-6. (1996).

46. Bernardin, F., et al., *Primary infection of a male plasma donor with divergent HIV variants from the same source followed by rapid fluctuations in their relative frequency and viral recombination. AIDS Res Hum Retroviruses*. **19**, p. 1009-15. (2003).

4 The effect of viral sequence diversification on the fate of HIV-specific $CD8^+$ T cell clonotypes

In chapter 3, we studied the effect of HAART on the clonality, functional profile, and maturation status of HIV-specific $CD8^+$ T cells, and provided mechanistic evidence for the HAART-mediated functional restoration of HIV-specific $CD8^+$ T cells. In the current chapter, we chose another model for "antigen loss" in the context of intra-epitopic escape. We chose to study the effect of epitope escape on the fate of residual wild-type-specific $CD8^+$ T cells.

Work performed by *Price et al* [223] had previously shown that the T cell receptor (TCR) repertoires that are narrow and associated with motifs within their complementarity-determining region 3 (CDR3) are more likely to facilitate escape in SIV infection. Therefore, we chose not only to characterize the TCR repertoires against HIV-1 epitopes that escape, but also studied the effect of escape on the functional profile, maturation status of the residual $CD8^+$ T cells specific to wild-type antigen. Our data show that coincident with epitopic escape, the functional profile of wild-type-specific $CD8^+$ T cells is enhanced, PD-1 expression drops and CD127 expression increases. Highly avid clonotypes endowed with polyfunctionality persist in the context of limited sources of antigen and contribute to the overall functional enhancement of the residual wild-type-specific $CD8^+$ T cell population.

The effect of viral sequence diversification on the fate of HIV-specific CD8+ T cells

Loury Janbazian[1,2], Lydie Trautmann[1,3], David Price[4,5], David Ambrosac[4], Tomas Baumgartner[1], Eric Delwart[6], Richard Koup[4], Mohamad Rachid Boulassel[7], Jean-Pierre Routy[7], Bader Yassine Diab[1], Daniel Douek[4], and Rafick-Pierre Sekaly[1,2,3].

[1]Laboratory of Immunology, Department of Microbiology and Immunology, Université de Montréal.

[2]Faculty of Medicine, Department of Microbiology and Immunology, McGill University, Montreal, Quebec, H3A 2B4, Canada.

[3]CR-CHUM,INSERM U743 and CUSM, Montréal, Canada.

[4]Immunology Laboratory, Vaccine Research Centre, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

[5]Department of Medical Biochemistry and Immunology, Cardiff University School of Medicine, Cardiff CF14 4XN, UK

[6]Blood Systems Research Institute, 270 Masonic Ave., San Francisco, California 94118, USA.

[7]Division of Haematology, Royal Victoria Hospital, McGill University Health Centre, 687 Pine Avenue West, Montreal, Quebec, H3A 1A1, Canada.

Abstract

Escape from CTL-mediated immunity has long been reported in HIV-1 infection. It is known that when viral epitopes mutate, they abrogate $CD8^+$ specific responses, and hence provide a robust way to evade immune recognition. It was also suggested that broader T Cell Receptor (TCR) repertoires mediate better control of viral load. This led us to hypothesize that HIV-specific $CD8^+$ TCR repertoires against epitopes that eventually escape are narrow and restricted. Moreover, we reasoned that as the frequency of wild-type virus decays due to emergence of viral escape mutants, the functional profile of antigen specific $CD8^+$ T cells to the wild-type epitopes should be enhanced. Therefore, these "selected" clonotypes should be endowed with increased function.

In this study, we longitudinally followed HIV-specific $CD8^+$ T cell responses to wild-type epitopes that escaped in four chronically HIV-infected patients. We chose $CD8^+$ epitopes that mutate at MHC-I anchors and TCR contact sites. The frequency of HIV-specific $CD8^+$ T cells decayed over time for all wild-type epitopes as viral escape mutants emerged. The TCR repertoire evolved from being polyclonal to oligoclonal or sometimes even monoclonal responses. The focusing of the repertoire was accompanied by enhanced functional profile of HIV-specific $CD8^+$ T cells to the wild-type peptide. Therefore, our data showed that as the TCR repertoire to the wild-type epitope focuses due to escape; some clones are selected that are endowed with increased function. The persistence of high avidity clonotypes that are polyfunctional contributed to the enhanced functionality of the wild-type-specific $CD8^+$ T cells observed coincident with the emergence of escape.

Introduction

Immune escape through mutation of targeted antigens is a cardinal feature of infection with HIV-1 and remains a major impediment to the development of an effective AIDS vaccine [1-5]. In the case of $CD8^+$ T cell responses, viral mutations within the epitopic region can diminish or abrogate immune recognition through three non-mutually exclusive mechanisms: (i) loss of peptide presentation due to altered antigen processing or epitope deletion; (ii) loss of peptide binding to the restricting major histocompatibility complex class I (MHC-I) molecule; and, (iii) loss of recognition due to altered T cell receptor (TCR) engagement of the peptide-MHCI (pMHCI) complex [1, 6]. In the latter scenario, effective escape has been shown in other systems to be facilitated by restricted TCR repertoires and/or at prevalence of oligomorphic clonotypes that likely reflect limited antigen binding modes [7-10]. Although complete escape is rare for any given variant, even partial diminution of immune recognition and subtle differences in T cell activation are often sufficient to confer substantial survival advantages in the dynamic setting of viral quasispecies that continuously adapt to prevailing immune pressures at both the individual and population level within the confines of replicative fitness [1, 11].

Despite the fundamental significance of perpetual viral evolution in relation to disease pathogenesis, however, relatively little is known of reciprocal effects; i.e. the impact of immune escape on HIV-specific $CD8^+$ T cell populations. It is known that de novo variant-specific responses can emerge, presuming a degree of epitope presentation persists, and that new clonotypes can be incorporated within such $CD8^+$ T cell populations [12, 13]. Furthermore, it is known that wild-type specific responses wane over time due to diminished antigenic drive, yet this process does not continue to

extinction. Thus, residual populations of memory $CD8^+$ T cells with wild-type antigen specificity persist in some form and appear to play an important role in the maintenance of escape mutations. Strong evidence for this assertion comes from HIV and SIV transmission studies, in which selected escape mutations revert rapidly to optimize viral fitness in the absence of the presenting MHC-I molecule and remain relatively stable in the presence of the appropriate restriction element due to the induction of wild-type-specific $CD8^+$ T cell populations by viral revertants [14-17]. Indeed, with the application of deep sequencing approaches to the evaluation of mutational immune escape, it is becoming clear that variants bearing wild-type antigen frequently persist to some extent within complex mixtures of immune escape variants and it is probable that these viral forms establish an equilibrium with the residual cognate $CD8^+$ T cell population in accordance to standard predator-prey models [18].

Given that immune escape variants frequently carry associated fitness cost, which in turn can be beneficial for the host with respect to virologic outcome, it is reasonable to postulate that residual wild-type antigen-specific $CD8^+$ T cell populations play an important role in the overall immune control of HIV replication in vivo by preventing mass reversion to more prolific forms. On this basis, we undertook a comprehensive analysis of wild-type antigen-specific $CD8^+$ T cell responses in the face of ongoing viral replication and immune escape in a cohort of four HIV infected individuals. In each case we conducted a longitudinal examination of the clonal composition, phenotypic status, functional profile and antigen sensitivity of $CD8^+$ T cell populations specific for autologous wild-type antigen and, where present, the corresponding variant specific populations.

Results

Virological and immunological characteristics of the study cohort

Antigen-specific $CD8^+$ T cell responses were studied longitudinally in four HIV infected patients. Figure 1 A depicts the time points studied in each patient and the autologous viral sequences detected at each time point for which plasma was available; Figure 1 B depicts the viral load trajectories with concomitant $CD4^+$ and $CD8^+$ T cell counts for each patient. Viral loads were high in all patients throughout the study period with the exception of the first time point (7 mo) in patient 1 and the last time point (27 mo) in patient 2; antiretroviral therapy (ART) was administered at these time points (grey backgrounds).

The following $CD8^+$ T cell specificities were studied: (i) HLA-B*0702-FPQGEAREL (Pol FL9, residues 7-15) and HLA-B*0702-TPGPGVRYPL (Nef TL10, residues 128-137) in patient 1; (ii) HLA-A*0301-RLRPGGKKK (p17 Gag RK9, residues 20-28) in patient 2 and 3; and, (iii) HLA- B*0801-GEIYKRWII (p24 Gag GI9, residues 127-135) in patient 4. Antigenic sequences from each patient displayed directional changes over time (Figure 1 A and Supplementary figure1 A), consistent with epitope escape through mutation at MHC-I anchor positions (patients 1, 2 and 3) and/or putative TCR contact residues (pateints1, 3 and 4); the only exception to this pattern was the Nef TL10 epitope in patient 1, which remained unchanged throughout. In patient 1, we observed a mutation from E to K at position 8 (E8K) and a L9F mutation in the Pol FL9 epitope; these variants were present at frequencies of 34% and 66% by the end of the study period. In patient 2, 100% of the analyzed sequences acquired a K9Q mutation in the p17 Gag RK9 epitope

over the course of the study. In patient 3, a dual K7R and K9R mutation was observed. However, at the final time point of study (26 mo), the majority of the viral sequences contained the K9R mutation either alone or in combination with additional variants, thereby indicating reversion to the wild-type residue at position 7. In patient 4 a universal R6K and I3V mutation emerged in the p24 Gag GI9 epitope of the latest time points studied (29 mo) (Supplementary figure1 A). Consistent with the loss of these wild-type epitope sequences in all 4 patients, we observed a progressive decay in the corresponding HIV epitope-specific CD8+ T cell frequencies quantified by the binding of pMHCI tetramers refolded around the original cognate peptides (Figure 1 C and Supplementary figure1 C), except for the Nef TL10 epitope in patient 1, where the original cognate peptide remained unchanged throughout the study period.

The CD8 T cell receptor (TCR) repertoire against epitopes that escape are restricted

In this study we undertook a comprehensive clonotypic analysis (CDR3 amino acid region, TCRBV and TCRBJ usage and the relative frequency of the clonotypes) of HIV-specific $CD8^+$ T cells against peptides that escaped by mutating amino acids either in TCR contact regions or MHC-I anchor sites or both. Table 1 summarizes the TCR clonotypic analysis that was performed longitudinally in three patients.

The oligoclonal nature of HIV-specific $CD8^+$ T cells has been reported previously [19]. Our results show the oligoclonal nature of the CD8 clonotypes involved. On average, 4-6 clonotypes were observed at the first time points studied, which corresponded to acute phases of HIV infection. Interestingly, the TCR repertoire contracted over the time points studied for patients 1 and 2, coincident with the emergence of viral escape mutants and

decay in the frequency of wild-type tetramer positive $CD8^+$ T cells. The TCR repertoire in patient 3 exhibited similar patterns of non-biased Vβ and Jβ usage and clonal hierarchy at 2 and 16 months against wild-type (RLRPGGKKK) and mutant epitopes (RLRPGGRKR).

In patient 1, we observed a major bias in the $CD8^+$ TCR repertoire. We noticed a preferential usage of Vβ 18 and Jβ 2.5 and the presence of a discernable RGR motif in the CDR3β region. Moreover, clonotype Vβ 18 CAS SPRGREETQY Jβ 2.5, that was presented as a subdominant clonotype at 7 months (4.87%) increased in frequency (9.19% at 21 m) and became the dominant clonotype at 31 and 43 months (100% and 98.03%, respectively). The dominant clonotype at 7 and 21 months, Vβ 18 CAS SPRGRDETQY Jβ 2.5, was lost from the tetramer positive pool at 31 and 43 month. Hence, for this particular escape mutation at P8 and P9, we observed a very biased TCR repertoire with preferential Vβ and Jβ usage, identifiable motifs in the CDR3 regions and finally a selection of 1 particular clonotype that persisted over time.

In patient 2, at 2 months, 4 clonotypes dominated the HIV-specific $CD8^+$ T response. At 21 months, none of these clonotypes was preserved, and the TCR repertoire was comprised of one clonotype Vβ 13 CAS PGLDGEQFG Jβ 2.7. Therefore, we observed a selection towards one clonotype concomitant with escape at P9. Interestingly at 27 months, when the patient started therapy, we observed a broadening of the TCR repertoire, with new specificities recruited. Clonotype Vβ 13 CAS PGLDGEQFG Jβ 2.7 was present at a frequency of 19.75% along with other three clonotypes. We have previously observed such broadening of epitope-specific $CD8^+$ TCR repertoires under antiviral therapy (Janbazian *et al.*, manuscript in preparation).

In patient 3, the TCR repertoire was analyzed against both wild-type (RLRPGGKKK) and variant epitopes (RLRPGGRKR). Responses to both epitopes were detected and decayed over time at 2 and 16months. We did not observe similar biases in the TCR repertoires of wild-type and variant epitopes between 2 and 16 months, as we did for patients 1 and 2. The TCR repertoire of the wild-type epitope at 2 and 16 months was unaltered, as it exhibited similar patterns of clonal dominance; the 2 dominant clonotypes namely Vβ 5.6 CAS SLDRNTGELF Jβ 2.2 and Vβ 28 CAS RDSSYEQY Jβ 2.7 were presented at both time points. Moreover, no bias was observed in terms of Vβ and Jβ usage, and no identifiable motifs were present in the CDR3 regions. The TCR repertoire against the variant epitope (RLRPGGRKR) displayed a similar unaltered profile at 2 and 16 months. The dominant clonotype Vβ 28 CAS RDSSYEQY Jβ 2.7 at 2months was 60% and dropped to 13.33% at 16months. The dominant clonotype at 2months was Vβ 10 CAS SDTLNTEAF Jβ 1.1 (29.33%). This particular clonotype was detected at 16months and was shared between the 2 repertoires. Therefore, a degree of cross-reactivity was observed between wild-type and variant-specific TCR repertoires. The cross-reactive clonotypes at 2 and 16 months were Vβ 28 CAS RDSSYEQY Jβ 2.7 and Vβ 24.1 CAT SDDGTPNNEQF Jβ 2.1. Interestingly, the dominant clonotype in the HLA-A*0301 RLRPGGKKK repertoire was not a cross-reactive clonotype. It is worth mentioning that both repertoires also contained mutually exclusive clonotypes.

To further decipher the cross-reactive nature of $CD8^+$ T cell clonotypes recognizing wild-type and variant forms of the peptide, we performed a co-staining experiment with 2 tetramers that were specific for HLA-A*0301 RLRPGGKKK (wild-type) and HLA-A*0301 RLRPGGRKR (variant), by coupling them to PE and APC, respectively (Figure

2). This co-staining experiment helped us define a very well clustered population that was double positive for both tetramers. Indeed, the clonotypic analysis of this double positive population showed that Vβ 28 CAS RDSSYEQY Jβ 2.7 was the major clonotype found in this population. Hence, our observations were further strengthened by the fact that Vβ 28 CAS RDSSYEQY Jβ 2.7 was a cross-reactive clonotype recognizing both wild-type and variant forms of the peptide. The dominant clonotype Vβ 5.6 CAS SLDRNTGELF Jβ 2.2 that recognized the wild-type peptide was not a cross-reactive clonotype.

Interestingly, we observed sharp differences between the TCR repertoires of conserved epitopes and escaping epitopes that were restricted by the same HLA-B*0702 in patient 1 (Table 2). This analysis was performed at 7, 21, and 31 months. The conserved epitope studied was HLA B*0702- TL10 (nef) and the escaping epitope was HLA B*0702-FL9 (pol). The TCR repertoire directed against HLA B*0702-TPGPGVRYPL was broader than that of HLA B*0702-FPQGEAREL in the number of clonotypes utilized. Moreover, we did not observe any preferential usage of and Jβ, and no observable motifs within the CDR3β regions in the HLA-B*0702- TL10 specific TCR repertoire. It is also noteworthy that initiation of HAART at 7m did not alter the bias observed in the HLA B7-FL9 repertoire. Altogether, our data suggest that the TCR repertoires against $CD8^{+}$ epitopes that mutate to escape immune recognition are highly biased and restricted, and this bias might be relieved when there are cross-reactive clonotypes that recognize circulating variants.

Decay of wild-type antigen due to emergence of virus variants improves the functionality of residual wild-type HIV-specific $CD8^+$ T cells

To study the functional profile of HIV-specific $CD8^+$ T cells to epitopes that escape, we undertook a comprehensive mutiparametric flow cytometric analysis, encompassing three different cytokines (IL-2, IFN-γ, and TNF-α) and the degranulation marker CD107a in all four patients. After the gates were created for each function, we used the Boolean platform to create an array of different combinations of function. We report here the improvement in the functional profile of tetramer positive ($tetramer^+$) $CD8^+$ T cells to wild-type epitopes coincident with the loss of antigen, due to the emergence of epitope variants (Figure 3 A and Supplementary figure1 D).

For all four patients, the acute time points were predominated by mono-functional responses. These mono-functional responses were mainly IFN-γ producing cells except for patient 2, where CD107a producing $CD8^+$ T cells dominated the mono-functional response. CD107a producing CD8+ T cells were the second highest in the frequency of mono-functional cells. IL-2 producing $CD8^+$ T cells also contributed to the mono-functional pool, albeit in low frequencies. Polyfunctional cells were present at the acute points studied; they were mainly $CD107a^+$ IFN-γ^+ (2+), $CD107a^+$ IFN-γ^+ TNF-α^+ and $CD107a^+$ $IL2^+$ TNF-α^+ (3+) cells in patients 4, 3 and patient 2, respectively. Interestingly, the functional profile of the tetramer+ $CD8^+$ T cells to the wild-type epitope improved following decay in epitope-specific antigen load. In patient 1 and 4, we observed the presence of $CD107a^+$ IFN- γ^+ $IL\text{-}2^+$ TNF-α^+ (4+) $CD8^+$ T cells at 43 and 7m, respectively. Therefore, the functionality of $tetramer^+$ $CD8^+$ T cells to the wild-type epitopes enhanced with the decay in antigen load.

Furthermore, we characterized the functional profile of RLRPGGRKR-specific $CD8^+$ T cells at 2 and 16m in patient 3. The functionality of these cells remained unaltered between 2 and 16 months. Although polyfunctional cells were present in small frequencies, responses to this variant epitope were mainly mono-functional IFN-γ^+ $CD8^+$ T cells (Figure 3 B).

Decay in wild-type antigen load leads to increased frequencies of $CCR7^-CD27^-$ $CD45RA^+$ (T_{EMRA}) and $CD28^-$ $CD127^+$ $PD\text{-}1^-$ of wild-type-specific $CD8^+$ T cells

We assessed the maturation status (Figure 4 A and Supplementary figure1 F) and activation profile (Figure 4 B and Supplementary figure1 G) of epitope-specific responses to wild-type epitopes longitudinally in all four patients. Maturation status was measured by looking at different combinations of CCR7, CD27, and CD45RA expression, whereas activation was measured by looking at the expression of CD28, CD127, and PD-1 of the wild-type-specific $CD8^+$ T cells.

In accord with previous results and based on the expression of CCR7, CD27, and CD45RA, HIV-specific $CD8^+$ T cells are mostly $CCR7^-$ $CD27^+$ $CD45RA^-$ [20]. In our analyses we observed that the frequency of $CCR7^-$ $CD27^+$ $CD45RA^-$ cells declined with the decay of wild-type antigen in all patients, while $CCR7^-$ $CD27^-$ $CD45RA^+$ frequencies were enhanced. Similarly, coincident with emergence of escape, the frequency of $CD28^-$ $CD127^-PD\text{-}1^+$ $CD8^+$ T cells dropped, while frequencies of $CD28^-CD127^+PD\text{-}1^-$ $CD8^+$ T cells increased.

Since we observed a degree of cross-reactivity between KKK and RKR-specific $CD8^+$ T cells on the clonal level (Table 1 and Figure 2), we assessed the maturation and activation

status of epitope-specific $CD8^+$ T cells in the context of a tetramer co-staining experiment, in order to discriminate between the 3 different $tetramer^+$ populations (Figure 5. A). We did not observe major differences in terms of memory markers between the 3 different tetramer populations. In fact, all three populations exhibited a $CCR7^-$ $CD27^+CD45RA^-$ phenotype, which dropped in all three populations, but mostly for the KKK-specific population. Similar to the data obtained in Figure 4 A, the frequencies of $CCR7^-CD27^-CD45RA^+$ increased at 16 months. Interestingly, we observed differences in the mean fluorescence intensity (MFI) of PD-1 and CD127 between the different $tetramer^+$ populations. MFI of PD-1 KKK-specific tetramer+ $CD8^+$ T cells dropped from 1835 (at 2 months) to 1757 (at 16 months), whereas the MFI of PD-1 increased from 1089 to 1669 and from 1405 to 1725 in the RKR and double tetramer populations respectively, between 2 and 16 months. On the other hand, the MFI of CD127 increased from 2 to 16 months, for all the tetramer populations (Figure 5 B). The differences in MFI of PD-1 and CD127 observed between the three $tetramer^+$ populations, was particular to $tetramer^+$ cells.

Improved functionality could be attributed to the persistence of high avidity clonotypes that display superior function

We next wanted to determine whether improved functionality in the context of epitope escape is due to the persistence of functionally superior clonotypes or a gain of function. The clonotypic and functional data from patient 1 allowed us to address this issue. We reported earlier the functional improvement of HLA-B*0702-FL9-specific $CD8^+$ T cells

at 43 months over the earlier months studied. Interestingly, the TCR repertoire of HLA B*0702-FL9-specific $CD8^+$ T cells was comprised of 1 clonotype, namely, Vβ 18 CAS SPRGREETQY Jβ 2.5 at 43 months. Therefore, we wanted to see in which functional compartment this clonotype belonged to at earlier time points. Hence, we sorted 4 different functional populations originating from the HLA-B*0702-FL9-specific $CD8^+$ T cells at 21 months and performed DNA-based clonotypic analysis as previously described [21]. We chose this time point simply because the frequency of Vβ 18 CAS SPRGREETQY Jβ 2.5 clonotype was higher than at 7 months. The four functional populations sorted were the following; 3+ (INF-γ^+TNF-α^+IL-2^+), 2+ (INF-γ^+TNF-α^+ and INF-γ^+IL-2^+), and 1+ (IFN-γ^+) $CD8^+$ T cells. Analysis of at least 96 white bacterial colonies indicated that clonotype Vβ 18 CAS SPRGREETQY Jβ 2.5 was present in all four functional populations sorted (Supplementary figure2). Therefore, we conclude that the persistence of this particular clonotype could attribute to the superior functionality of the HLA-B*0702-FL9-specific $CD8^+$ T cells at 43 months.

We next wanted to determine whether there were differences in antigen sensitivity of wild-type-specific $tetramer^+$ responses between the different time points. These experiments were performed on available samples of peripheral blood from patients 1, 3 and 4. Our results indicate that the antigen sensitivity, as measured by the EC50 of IFN-γ, of wild-type-specific tetramer $CD8^+$ T cell responses stimulated by wild-type peptides increased over time, concomitant with escape in these epitopes. In patient 1, EC50 of IFN-γ ranged from 0.24 to 0.12 at 7 and 31 months respectively. In patient 3 and 4, there was a sharp drop in antigen sensitivity from 2 to 16 (0.59 to 0.08) and 1 to 7 (0.6 to 0.09) months respectively (Figure 3 C and Supplementary figure1 E).

Interestingly, the antigen sensitivity of HLA-A*0301-RLRPGGKKK-specific $CD8^+$ T cells to the variant peptide RLRPGGRKR was lower at 16 months (2.362) than 2 months (0.4098). Moreover, in patient 1, HLA-B*0702-FL9-specific $CD8^+$ T cells did not respond to the variant peptide FPQGEAREL. Taken together, our results indicate that the enhanced functionality observed under limited sources of wild-type antigen could be due to the persistence of high avidity clonotypes that have superior function.

Discussion

The aim of the current study was to investigate the effect of HIV viral sequence diversification on the fate of residual epitope-specific $CD8^+$ T cell responses. Particularly, we wanted to determine whether the TCR repertoire of HIV epitope-specific responses that eventually escape, bear molecular signatures that facilitate evasion mechanisms from immune responses, similar to previously published observations from SIV, HCV, and LCMV infection [7, 9, 10]. We further aimed to characterize the functionality and maturation status of epitope-specific $CD8^+$ T cells that declined over time, coincident with the emergence of escape. Our results indicated that in the context of escape, the TCR repertoires against the original wild-type epitopes were narrow and restricted. Interestingly, this phenomenon was alleviated in the presence of cross-reactive clonotypes that recognized variant epitopes. Moreover, concurrent with the emergence of escape, wild-type-specific $CD8^+$ T cells were functionally enhanced, displayed effector (T_{EMRA}) phenotype, expressed low PD-1, and were highly avid.

The oligoclonal nature of HIV-specific $CD8^+$ TCR repertoire has been reported previously [19]. It was hypothesized that efficient antiviral activity is correlated with

broader TCR repertoires and that narrower repertoires failed to prove efficacious in viral control [22, 23]. In other words, the more $CD8^+$ T cells recognize a particular HIV-1 antigen, the better the chances of containing it. The TCR repertoire in patient 2 was restricted and eventually dominated by one clonotype at 21 months, coincident with the emergence of escape (Table 1). Unfortunately, we did not have earlier time points that allowed us to track when this clonotype was detected. At 27 months, when the patient was on HAART, we observed a broadening of the epitope-specific repertoire. This was in agreement with our previous observations that HAART leads to the recruitment of new $CD8^+$ T cell specificities (Janbazian et al; manuscript in preparation). This indicates that antigen load could influence the nature of the responding cells.

It was shown by Price et al that the SIV-specific $CD8^+$ TCR repertoire against CM9 (that does not escape) and TL8 (that escapes) differ drastically in terms of their $CD8^+$ TCR repertoires. Particularly, TCR repertoires that have a preferential Vβ and Jβ usage along with identifiable motifs within their complemantarity determining region 3 CDR3β regions were associated with escape (TL8), whereas no such biases were observed in the TCR repertoires of CM9 [10]. Similar observations were reported in HCV and LCMV infection [7, 9]. The TCR repertoire of patient 1 displayed a preference to Vβ 18 and Jβ 2.5 and a discernable RGR motif within the CDR3 region (Table 1). Such preference in Vβ and Jβ and motifs have been reported within the CDR3β region in the influenza matrix protein (HLA-A2+-MP58-66) and SIV (Mamu-A*01 TL8) epitope-specific $CD8^+$ TCR repertoires [10, 24, 25]. Therefore, at least partially, structural determinants such as peptide-MHC complex topology could influence the nature of the responding repertoires [26].

In patient 3, we observed a degree of cross reactivity between wild-type and variant epitopes (Table 1 and Figure 2). Both KKK and RKR-specific $CD8^+$ T cell repertoires were not biased and remained oligoclonal over time. We did not detect any motifs within the CDR3β regions. Thus, although there was a discrepancy between the sequencing techniques used to evaluate viral diversification between the time points, we report here that even in the face of escape, such biases in the TCR repertoires could be alleviated due to the presence of cross-reactive clonotypes. Similar results were published by Turnbull et al., where the authors reported that poor cross-recognition of variants was associated with a bias to a particular TCR Vβ, while efficient variant cross-recognition was not associated with a bias to particular Vβ [27].

However, to this point, correlations between epitope-specific $CD8^+$ T cell repertoires and efficient antiviral activity remain unclear. We clearly cannot make such correlations given our small cohort and number of epitopes studied per patient. Larger cohorts and multiple epitopes followed longitudinally could help investigate such correlations. We could however reason that, given the relationship between antigen availability and TCR avidity, lack of antigen shapes the TCR repertoire. Our data indicated that removal of wild-type antigen, due to emergence of escape, leads to a focusing of the repertoire due to the selection of high avidity clonotypes (Supplementary Table 2). The selection of high avidity clonotypes was in parallel with the improved functionality observed over time, coincident with emergence of escape. It was previously reported that high avidity CTLs exhibit superior functions resulting in more efficient antiviral activity compared to low avidity CTLs [28-31]. Recently, Almeida et al showed that highly sensitive epitope-specific clonotypes display superior function, efficient proliferation, and HIV-1

suppressive activity [32, 33]. We also observed a drop in PD-1 expression of epitope-specific responses over time similar to observations in the rhesus macaque SIV model [34], as well as increased frequencies of CCR7-CD27-CD45RA+ (T_{EMRA}) cells. One could argue that high avidity clonotypes persisted under limiting sources of antigen similar to observations in EBV and CMV infection [35, 36]. Altogether, our results point at the detrimental effect of persistent antigenic-specific stimulation on $CD8^+$ T cells and entail the need for defining optimal concentration of antigen that could generate and maintain high avidity clonotypes that are endowed with superior function. Finally, due to the small cohort studied, we acknowledge that our observations are descriptive and hypothesis generating and require the need for further investigation for more robust conclusions.

Materials and Methods

Study participants

Four HIV-infected patients were enrolled from Hospital Notre Dame, Montreal, Quebec, Canada. All patients were male; patient 1 was infected through intravenous drug use, and patients 2, 3 and 4 were infected through homosexual contact. The estimated date of infection in each case was based on clinical history and Western Blot HIV Test analysis. Longitudinal cryopreserved peripheral blood mononuclear cell (PBMC) samples were available from each patient and written informed consent for sample use was obtained in all cases.

Autologous viral sequencing

For bulk analysis of autologous viral populations, amplification and sequencing of the near complete HIV genomes was performed as described previously [37]. In brief, viral RNA was extracted from plasma samples using the QIAamp viral RNA minikit (Qiagen) in a final volume of 50 ul of H_2O containing 40 U of RNase inhibitor (Protector RNase Inhibitor, Roche Diagnostic Corporation). First-strand cDNA synthesis was initiated with 5-10 ug of RNA and the primers Tat2 or FB12 for the 5' and 3' half-genome, respectively. For amplification of each half-genome, a nested PCR was performed using Takara ExTaq DNA polymerase (Takara Bio Inc), following the manufacturer's instructions. The sets of nested primers and the PCR cycle conditions that were used have been reported previously [37]. The final PCR products were purified using the QIAquick PCR purification kit (Qiagen) and directly sequenced with sense or antisense primers [37]. Sequencing reactions were performed with ABI Big Dye terminators and run on an

ABI 3700 automated capillary sequencer. Sequences were edited using EditView, then assembled into a single contig and aligned using the Seqman and Megalign programs, respectively (DNAstar Inc.). When sequence indeterminations due to insertion/deletion mutations could not be resolved, purified PCR products were cloned in pGEM-T Easy vector (Promega). Six to 12 white colonies were then randomly picked and directly used for a second round PCR; the final products were then purified and sequenced as described above.

For clonal sequencing, viral RNA was extracted using the QIAamp viral RNA minikit (Qiagen) and reverse transcribed using the SuperScript One-Step RT-PCR kit (Invitrogen). Amplification was performed using the following sets of outer primers (5' to 3'); ATGAGGAAGCTGCAGAATGGG and AGGGGTCGTTGCCAAAGA in patient 1, GACTAGCGGAGGCTAGAAG and GGTATTACTTCTGGGCTRAAAGC in patients 2

and 3, and TCCACATACCTAGAAGAATAAGACA and ACTGGTACTAGCTTGTAG

CACCATCCA in patient 4. Products were then further amplified in a nested PCR using the following sets of inner primers (5' to 3'); CAGCATTATCAGAAGGAGCC and ATCATCTGCTCCTGTATCTA in patient 1, GACTAGCGGAGGCTAGAAG and GGTATTACTTCTGGGCTRAAAGC in patients 2 and 3, and TGTGGAACTTCTGGG

AC and CTAACCAGAGAGACCCAGTA in patient 4. Amplicons were ligated into pGEM T vector (Promega) and cloning was performed by transformation of competent DH5-α E.coli. For each amplicon 24 white colonies were picked, screened using standard M13 primers and then sequenced. All sequences were analyzed using Codon Code Aligner Version 3.0 (Codon Code Corporation).

Tetrameric pMHCI complexes

Soluble biotinylated pMHCI monomers were obtained from the CANVAC tetramer core facility (Montreal, Canada) and tetramerized with fluorochrome-conjugated streptavidin at a 4:1 molar ratio. The following pMHCI tetramers were produced: (i) HLA-A*0301-RLRPGGKKK (p17 Gag RK9, residues 20-28); (ii) HLA-B*0702-FPQGEAREL (Pol FL9, residues 7-15), a novel epitope predicted on the basis of binding algorithms and autologous viral sequence data; (iii) HLA-B*0702-TPGPGVRYPL (Nef TL10, residues 128-137); and, (iv) HLA-B*0801-GEIYKRWII (p24 Gag GI9, residues 127-135). The corresponding $CD8^+$ T cell populations, with the exception of those specific for the invariant Nef TL10 epitope, were selected for study based on progressive epitope sequence variation within the autologous viral population. We also folded tetrameric complexes with the variant epitopes HLA-B*0702-FPQGEARKL and HLA-A*0301-RLRPGGRKR for experiments conducted with samples from patients 1 and 3 respectively.

Functional analysis of antigen-specific $CD8^+$ T cells

Cryopreserved PBMC were thawed and rested for 1 hr in R10 (RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2mM L-glutamine) prior to stimulation with cognate peptide. Stimulated samples were pre-stained with the corresponding pMHCI tetrameric complexes conjugated to phycoerythrin (PE) for 15 min at 37°C. After addition of the costimulatory monoclonal antibodies (mAbs) αCD28 and αCD49d (1 μg/ml each; BD Biosciences), monensin (0.7 μg/ml; BD Biosciences), brefeldin A (10 μg/ml; Sigma-

Aldrich) and αCD107a-Alexa 680, cells were stimulated with the relevant autologous peptides at a concentration of 5 μg/ml for 6 hr at 37°C. Costimulation alone and staphylococcal enterotoxin B (SEB; 1 μg/ml; Sigma-Aldrich) were used as negative and positive controls, respectively, in all cases; cells in the negative control tubes were stained with pMHCI tetramers at the end of the stimulation period. After a single wash, the cells were stained with αCD3-Pacific Blue and αCD8-PE-Texas Red (ECD), and then washed again prior to fixation/permeabilization (2% PFA-FCS and 0.05% Saponine-FCS) and intracellular staining with the following mAbs (BD Biosciences): αIL-2-fluorescein isothiocyanate (FITC), αTNF-α-Alexa700 and αIFN-γ-allophycocyanin (APC). Antigen sensitivity was assessed using a similar protocol for the measurement of intracellular IFN-γ after stimulation with a ten-fold dilutional series of cognate peptide from 10 μg/ml to 0.0001 μg/ml; experiments were performed in triplicate and the EC50 was defined as the peptide concentration that yielded 50% of the maximum IFN-γ response. For all experiments, data were acquired immediately using BD LSRII flow cytometer (BD Biosciences) after a final wash step and fixation with 2% paraformaldehyde. All live lymphocyte events were collected and files were analyzed using FlowJo software (version 8.7.3; Tree Star Inc.) after electronic compensation. Functional capacity was determined after Boolean gating and subsequent analysis was performed using SPICE software (version 2.9; Mario Roederer, VRC, NIAID, NIH). Values used for the analysis of proportionate response representation were background subtracted.

Phenotypic analysis of antigen-specific $CD8^+$ T cells

Thawed PBMC were stained with PE-conjugated pMHCI tetrameric complexes for 15 min at 37°C, washed and then surface stained with the following mAbs (BD Biosciences)

except for CD8-PE Texas-Red (ECD) (Cedarlane): αCD3-Pacific Blue, αCD8-PE Texas-Red (ECD), αCCR7-PECy7, αCD27-Alexa700, αCD28-FITC, αCD45RA-APCCy7, αCD127-PECy5, and αPD-1-APC. For the tetramer co-staining experiment shown Figure 5, the KKK- and RKR-specific $CD8^+$ T cells were stained with the corresponding pMHCI tetramers conjugated to PE and APC, respectively, then surface stained with the following mAbs (BD Biosciences): αCD3-AmCyan, αCD8-PE Texas-Red (ECD), αCCR7-PECy7, αCD27-Alexa700, αCD28-PECy5, αCD45RA-APCCy7, αCD127-Pacific Blue, and PD-1 FITC. Data were collected using BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 8.7.3; Tree Star Inc.); the Boolean platform was used to create an array of different marker combinations. Subsequent analysis was performed using SPICE software (version 2.9; Mario Roederer, VRC, NIAID, NIH).

T cell receptor (TCR) clonotype analysis

For RNA-based clonotype analysis, HIV-specific $CD8^+$ T cells were identified by pMHCI tetramer staining and sorted viably to >98% purity by flow cytometry directly into 1.5 ml Sarstedt tubes containing 100 µl RNAlater (Applied BioSystems). mRNA was extracted using the Oligotex mRNA mini kit (Qiagen) and subjected to a template-switch anchored RT-PCR using a 3' TCRB constant region primer as described previously [38]. The amplicons were ligated into pGEM-T Easy vector (Promega) and cloned by transformation of competent DH5-α E.coli. At least 50 white colonies were amplified by PCR for each sorted population using standard M13 primers and then sequenced.

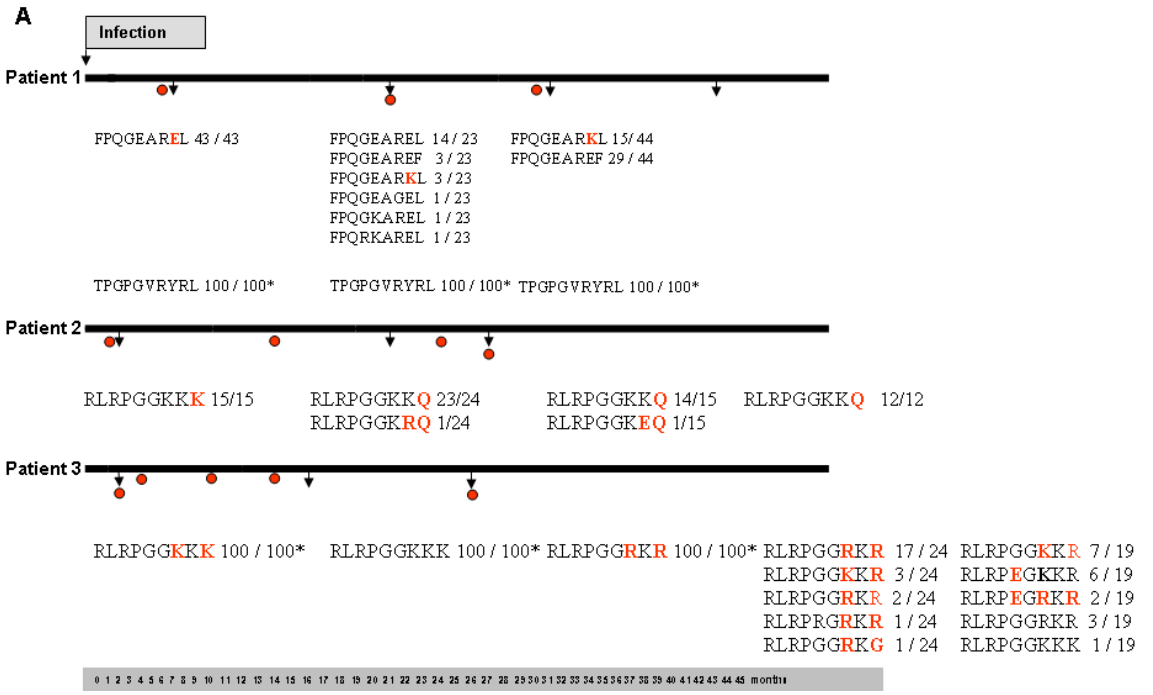
For DNA-based clonotype analysis, distinct functional $CD8^+$ T cell subsets were sorted to >98% purity by flow cytometry after intracellular cytokine staining. DNA was extracted

by lysis of sorted T cells in 100 μg/ml proteinase K (Boehringer) for 1 h at 56 °C and then 10 min at 95 °C. A heminested multiplex touchdown PCR was then performed using previously described TCRBV/TCRBJ primer combinations and PCR conditions [21]. Amplicons were then subcloned using the pGEM-T vector system and sequenced.

All sequences were analyzed using Vector NTI (Invitrogen); non-functional sequences were discarded from the analysis. The ImMunoGeneTics (IMGT) nomenclature was used throughout.

Figures

Figure 1



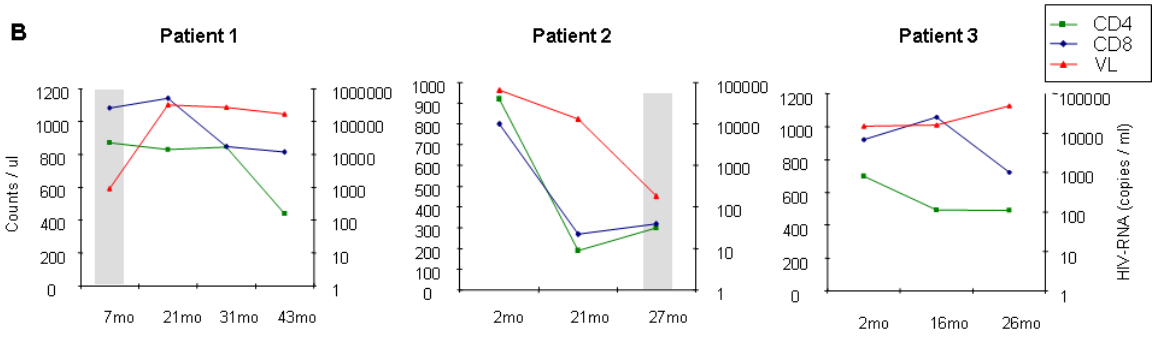


Figure 1 (continued)

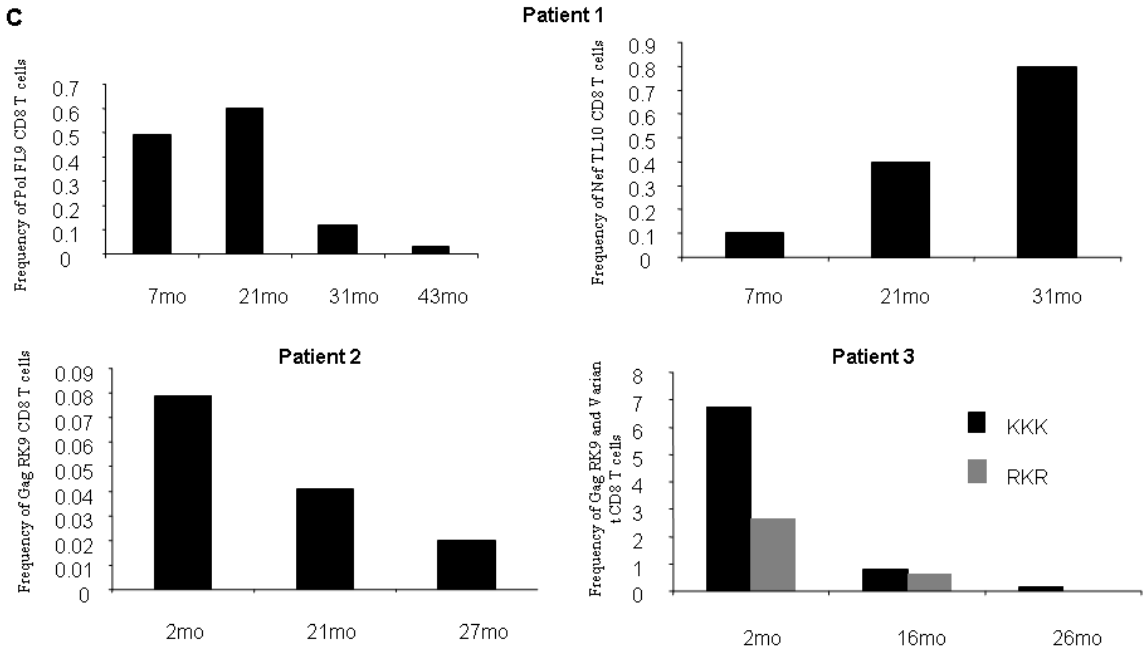


Figure 2

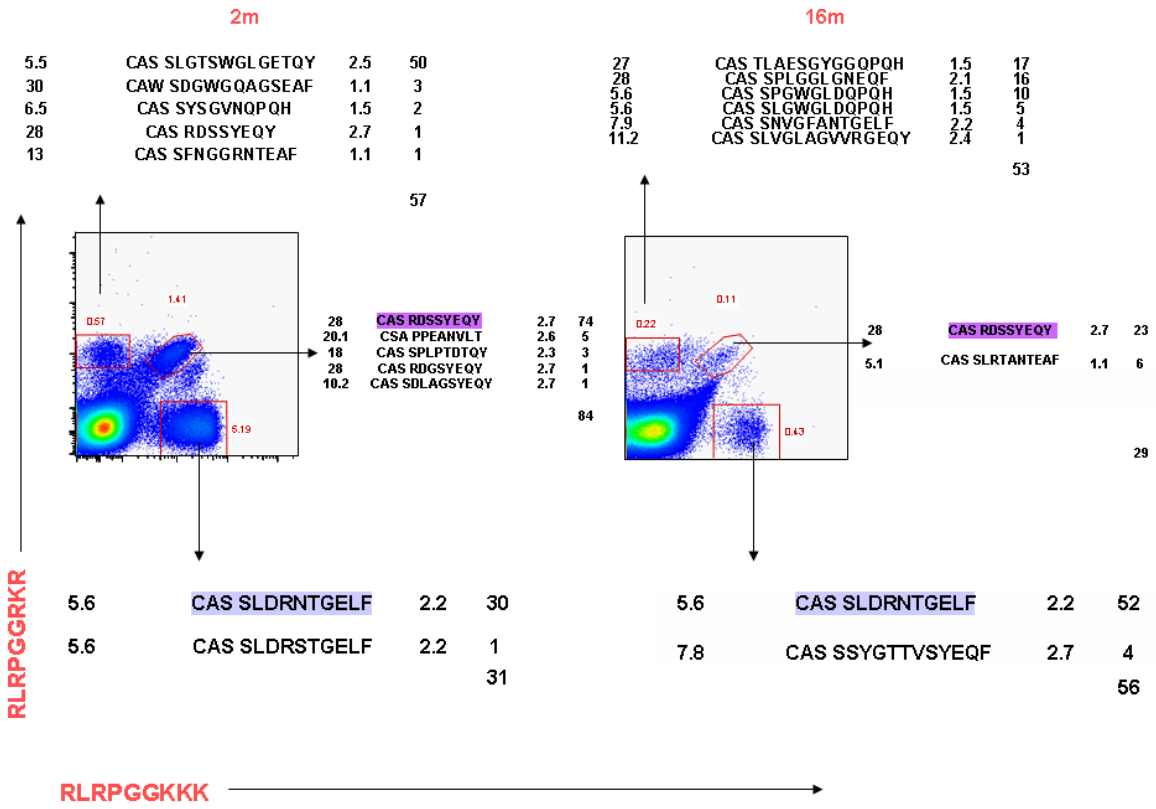
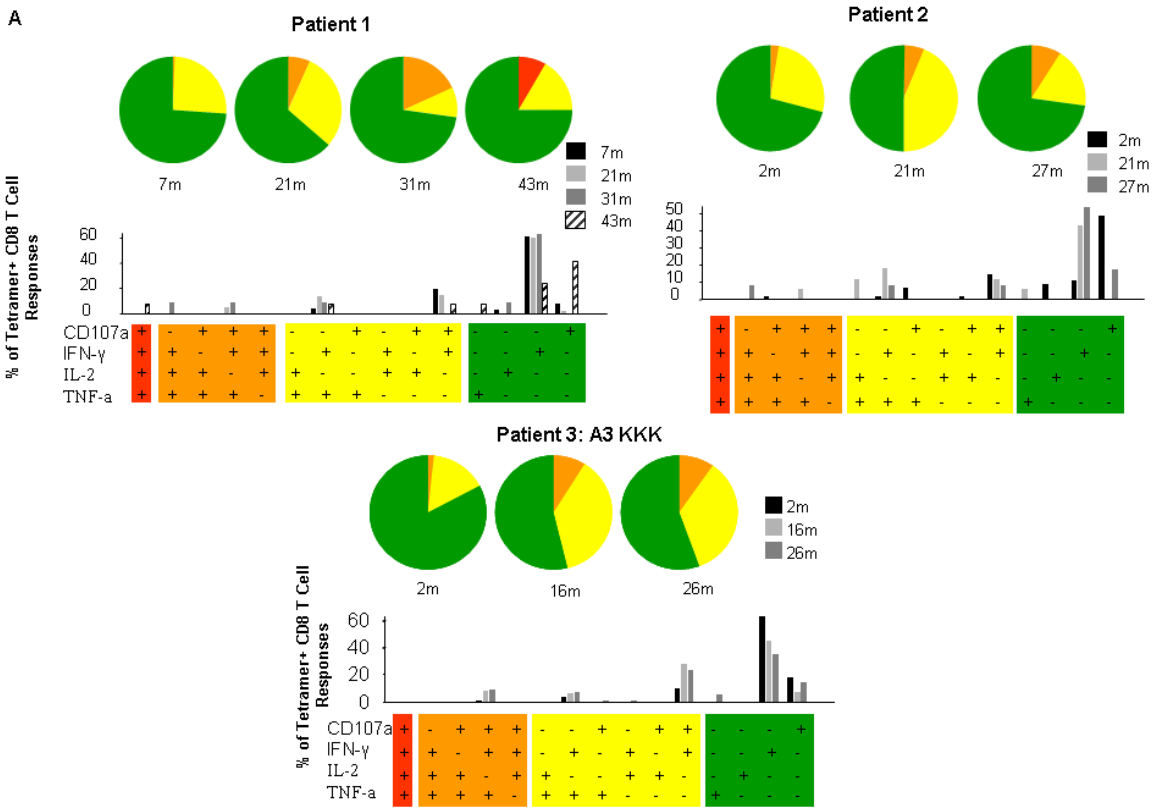
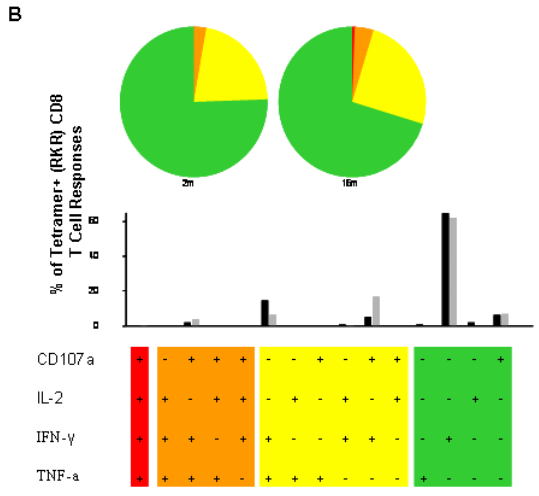

2m
16m
5.5 CAS SLGTSWGLGETQY 2.5 50
30 CAW SDGWGQAGSEAF 1.1 3
6.5 CAS SYSGVNQPQH 1.5 2
28 CAS RDSSYEQY 2.7 1
13 CAS SFNGGRNTEAF 1.1 1
57
27 CAS TLAESGYGGQPQH 1.5 17
28 CAS SPLGGLGNEQF 2.1 16
5.6 CAS SPGWGLDQPQH 1.5 10
5.6 CAS SLGWGLDQPQH 1.5 5
7.9 CAS SNVGFANTGELF 2.2 4
11.2 CAS SLVGLAGVVRGEQY 2.4 1
53
1.41
0.57
5.19
28 CAS RDSSYEQY 2.7 74
20.1 CSA PPEANVLT 2.6 5
18 CAS SPLPTDTQY 2.3 3
28 CAS RDGSYEQY 2.7 1
10.2 CAS SDLAGSYEQY 2.7 1
84
0.11
0.22
0.43
28 CAS RDSSYEQY 2.7 23
5.1 CAS SLRTANTEAF 1.1 6
29
RLRPGGRKR
5.6 CAS SLDRNTGELF 2.2 30
5.6 CAS SLDRSTGELF 2.2 1
31
5.6 CAS SLDRNTGELF 2.2 52
7.8 CAS SSYGTTVSYEQF 2.7 4
56
RLRPGGKKK

Figure 3





C

1

patient 1: B7-FPQGEAREL	7m	21m	31m
	0.24	0.13	0.12
patient 3: A3-RLRPGGKKK	2m	16m	
	0.5964	0.0839	

2

patient 1: B7-FPQGEARKL	7m	21m	31m
	0	0	0
patient 3: A3-RLRPGGRKR	1m	7m	
	0.4098	2.362	

Figure 4

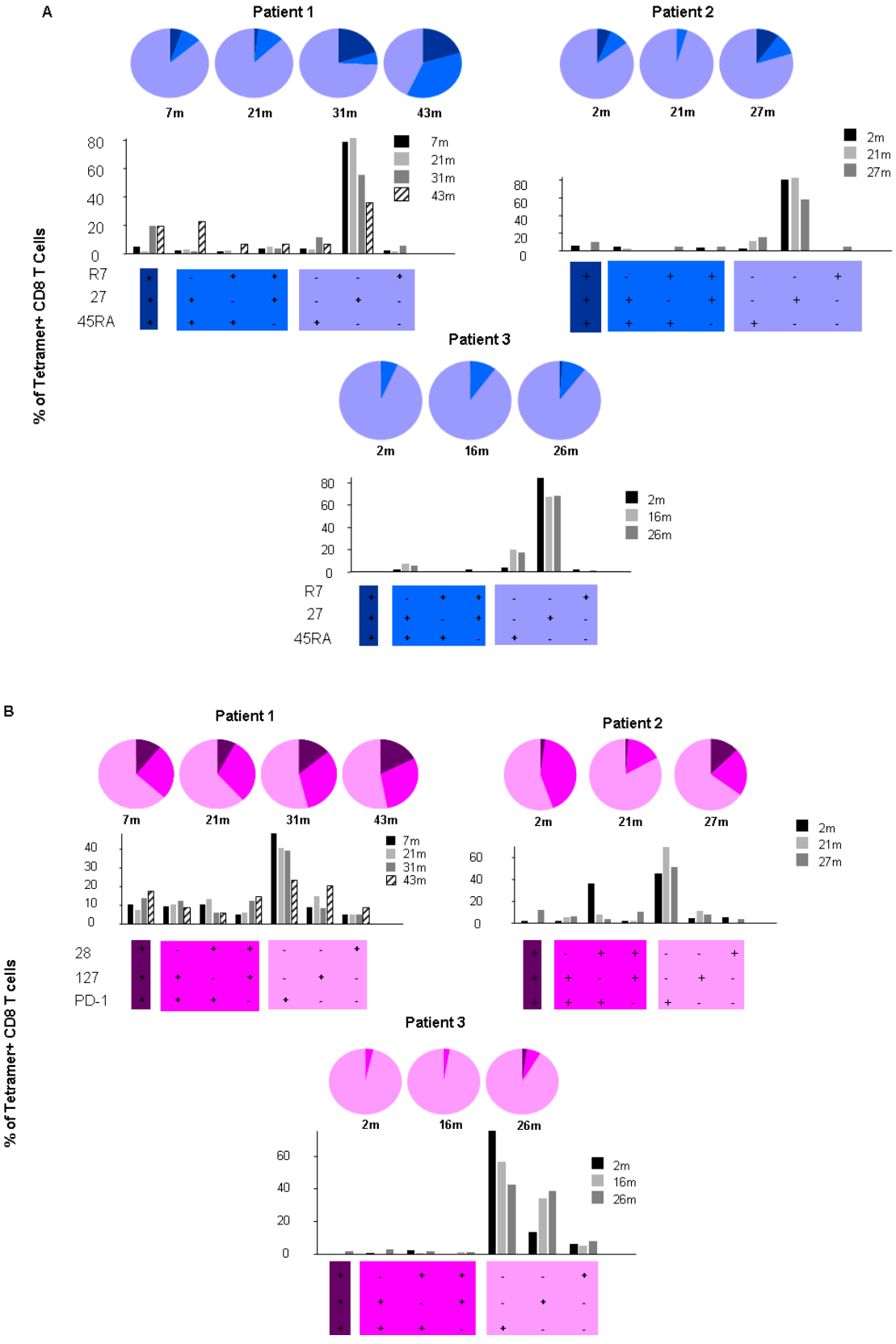
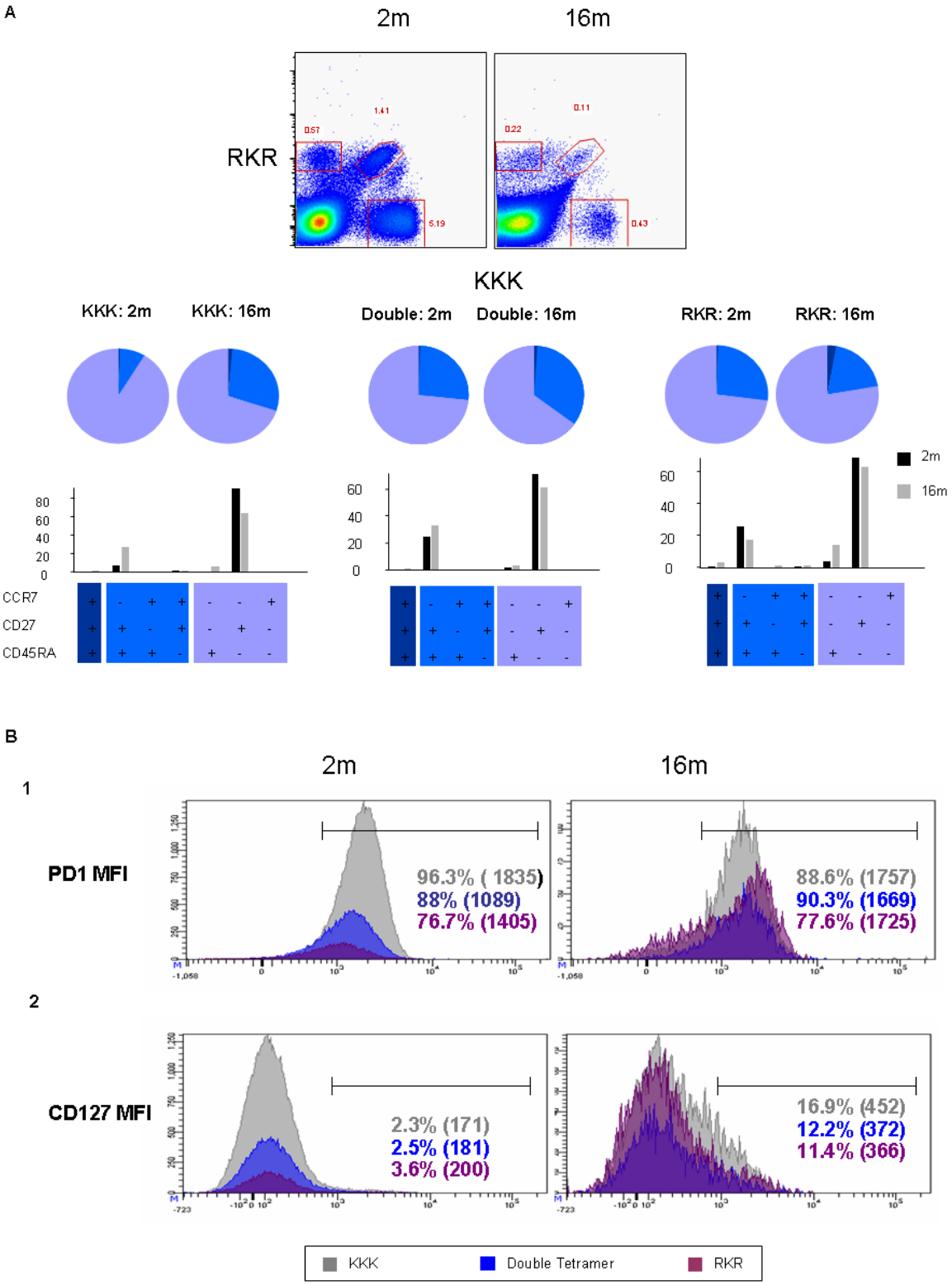
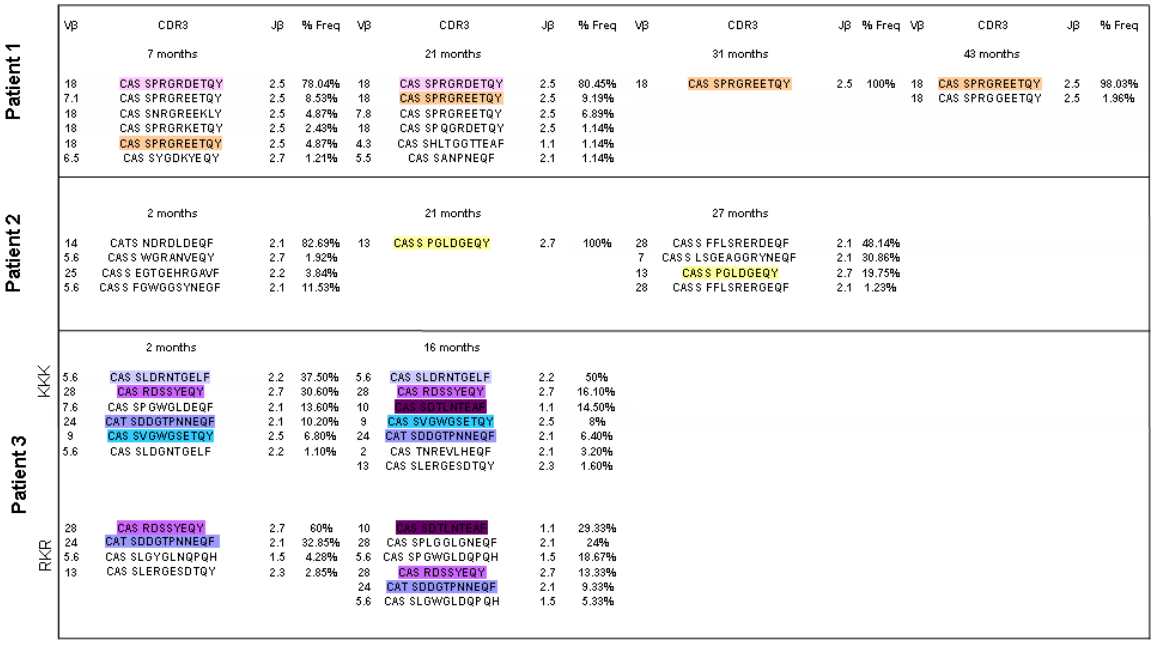

A
Patient 1
7m
21m
31m
43m
Patient 2
2m
21m
27m
% of Tetramer+ CD8 T Cells
R7
27
45RA
Patient 3
2m
16m
26m
B
Patient 1
Patient 2
Patient 3
28
127
PD-1
% of Tetramer+ CD8 T cells

Figure 5


A
2m
16m
RKR
1.41
0.57
5.19
0.11
0.22
0.43
KKK
KKK: 2m
KKK: 16m
Double: 2m
Double: 16m
RKR: 2m
RKR: 16m
2m
16m
CCR7
CD27
CD45RA
B
2m
16m
1
PD1 MFI
96.3% (1835)
88% (1089)
76.7% (1405)
88.6% (1757)
90.3% (1669)
77.6% (1725)
2
CD127 MFI
2.3% (171)
2.5% (181)
3.6% (200)
16.9% (452)
12.2% (372)
11.4% (366)
KKK
Double Tetramer
RKR

Tables

Table 1



Patient 1

Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq
	7 months				21 months				31 months				43 months		
18	CAS SPRGRDETQY	2.5	78.04%	18	CAS SPRGRDETQY	2.5	80.45%	18	CAS SPRGREETQY	2.5	100%	18	CAS SPRGREETQY	2.5	98.03%
7.1	CAS SPRGREETQY	2.5	8.53%	18	CAS SPRGREETQY	2.5	9.19%					18	CAS SPRGGEETQY	2.5	1.96%
18	CAS SNRGREEKLY	2.5	4.87%	7.8	CAS SPRGREETQY	2.5	6.89%								
18	CAS SPRGRKETQY	2.5	2.43%	18	CAS SPQGRDETQY	2.5	1.14%								
18	CAS SPRGREETQY	2.5	4.87%	4.3	CAS SHLTGGTTEAF	1.1	1.14%								
6.5	CAS SYGDKYEQY	2.7	1.21%	5.5	CAS SANPNEQF	2.1	1.14%								

Patient 2

Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq
	2 months				21 months				27 months		
14	CATS NDRDLDEQF	2.1	82.69%	13	CASS PGLDGEQY	2.7	100%	28	CASS FFLSRERDEQF	2.1	48.14%
5.6	CASS WGRANVEQY	2.7	1.92%					7	CASS LSGEAGGRYNEQF	2.1	30.86%
25	CASS EGTGEHRGAVF	2.2	3.84%					13	CASS PGLDGEQY	2.7	19.75%
5.6	CASS FGWGGSYNEQF	2.1	11.53%					28	CASS FFLSRERGEQF	2.1	1.23%

Patient 3

KKK

Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq
	2 months				16 months		
5.6	CAS SLDRNTGELF	2.2	37.50%	5.6	CAS SLDRNTGELF	2.2	50%
28	CAS RDSSYEQY	2.7	30.60%	28	CAS RDSSYEQY	2.7	16.10%
7.6	CAS SPGWGLDEQF	2.1	13.60%	10	CAS SDTLNTEAF	1.1	14.50%
24	CAT SDDGTPNNEQF	2.1	10.20%	9	CAS SVGWGSETQY	2.5	8%
9	CAS SVGWGSETQY	2.5	6.80%	24	CAT SDDGTPNNEQF	2.1	6.40%
5.6	CAS SLDGNTGELF	2.2	1.10%	2	CAS TNREVLHEQF	2.1	3.20%
				13	CAS SLERGESDTQY	2.3	1.60%

RKR

Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq
28	CAS RDSSYEQY	2.7	60%	10	CAS SDTLNTEAF	1.1	29.33%
24	CAT SDDGTPNNEQF	2.1	32.85%	28	CAS SPLGGLGNEQF	2.1	24%
5.6	CAS SLGYGLNQPQH	1.5	4.28%	5.6	CAS SPGWGLDQPQH	1.5	18.67%
13	CAS SLERGESDTQY	2.3	2.85%	28	CAS RDSSYEQY	2.7	13.33%
				24	CAT SDDGTPNNEQF	2.1	9.33%
				5.6	CAS SLGWGLDQPQH	1.5	5.33%

Table 2

Conserved epitope

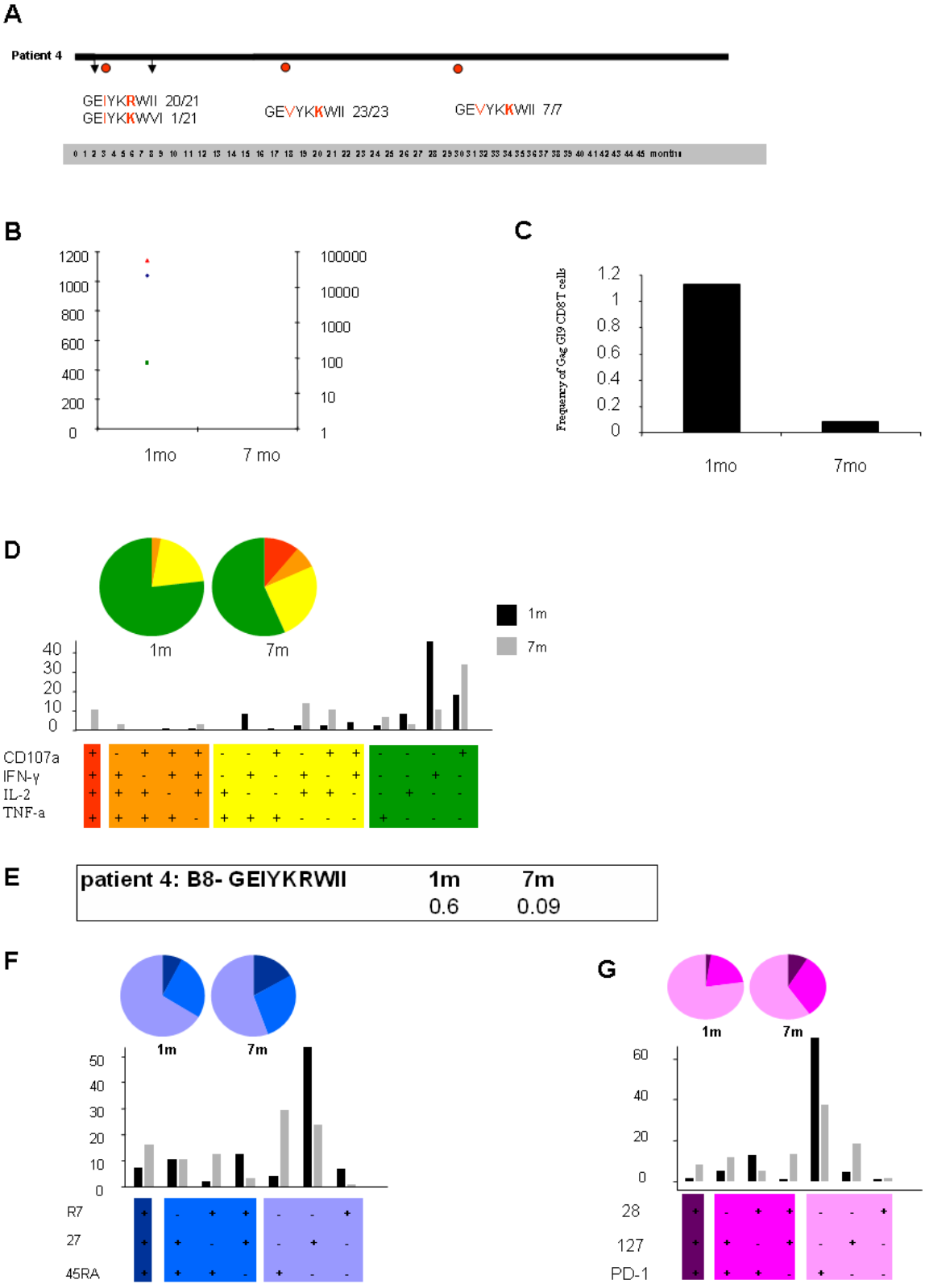
TCRVB	CDR3	TCRJB	% freq	TCRVB	CDR3	TCRJB	% freq	TCRVB	CDR3	TCRJB	% freq
	7 months				21 months				31months		
3.1	CAS SPLARWDEQF	2.1	8	20.1	CSA SSRGRVDEQF	2.1	18	11.3	CAS STPGLAGRHNEQF	2.1	16
6.2/6.3	CAS STGLAGEGRHNEQF	2.1	4	20.1	CSA SPRGRYNEQF	2.1	11	20.1	CSA SSRGRVDEQF	2.1	8
20.1	CSA SSRGRVDEQF	2.1	5	3.1	CAS SPLARWDEQF	2.1	6	20.1	CSA SPRGRYNEQF	2.1	2
27	CAS TGSTSGPSRYNEQF	2.1	3	9	CAS SPRGRIDEQY	2.7	5				
11.2	CAS SSPLTSGRLNEQF	2.1	2	9	CAS SPLGRVDEQF	2.1	4				
20.1	CSA SSRGRVDEQF	2.1	2	18	CAS SPEGRKNEAF	1.1	3				
20.1	CSA SPRGRYNEQF	2.1	2	27	CAS TGSTSGPSPSRYNEQF	2.1	2				
24.1	CAT SDEVAGGRHNEQF	2.1	2	11.3	CAS STPGLAGRHNEQF	2.1	2				
24.1	CAT RDRQGRYNEQF	21	2	6.1	CAS SVTGQSNSPLH	1.6	1				
7.9	CAS SPPPGGLGGYT	1.2	2	24.1	CAT SGLAGGPRNNEQF	2.1	1				
9	CAS SPLGRVDEQF	2.1	1	9	CAS GPRGRIDEQY	2.7	1				
25.1	CAS SSRVAGGRYNEQF	2.1	1								
9	CAS SPRGRTGELF	2.2	1								
20.1	CSA SSRGRVDEQF	2.1	1								
			37				54				26

Escaping epitope

7.8	CAS SPRGREETQY	2.5	7	7.8	CAS SPRGREETQY	2.5	6	18	CAS SPRGREETQY	2.5	60
18	CAS SPRGRDETQY	2.5	64	18	CAS SPRGRDETQY	2.5	70				
18	CAS SNRGREEKLY	2.5	4	18	CAS SPRGREETQY	2.5	8				
18	CAS SPRGRKETQY	2.5	2	18	CAS SPQGRDETQY	2.5	1				
18	CAS SPRGREETQY	2.5	4	4.3	CAS SHLTGGTTEAF	1.1	1				
6.5	CAS SYGDKYEQY	2.7	1	5.5	CAS SANPNEQF	2.1	1				
			82				87				60

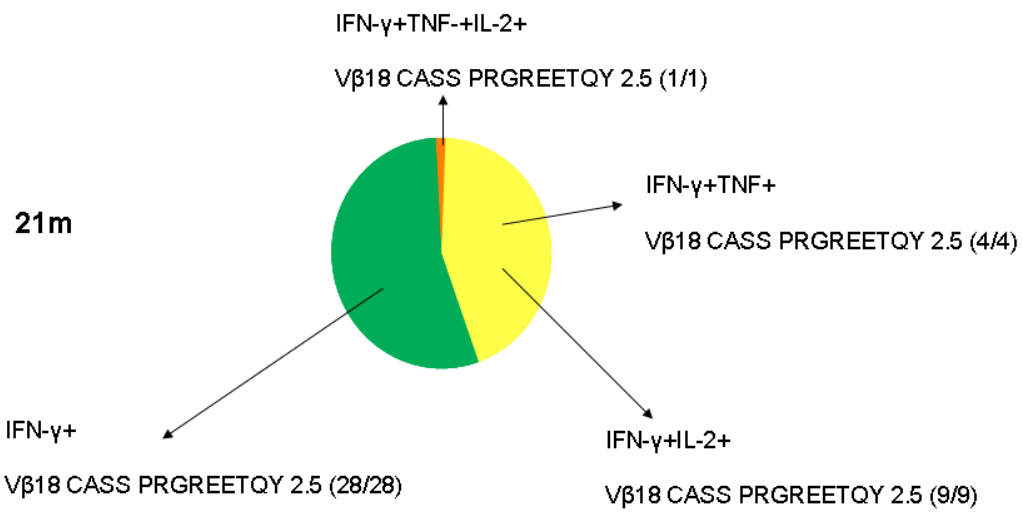
Supplementary Figures

Supplementary figure 1



Supplementary figure 2

A



B

IFN-γ+ TNF+				IFN-γ+ IL-2+			
Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq
18	CAS SPRGREETQY	2.5	100%	18	CAS SPRGREETQY	2.5	100%
IFN-γ+ TNF+ IL-2+				IFN-γ+			
Vβ	CDR3	Jβ	% Freq	Vβ	CDR3	Jβ	% Freq
18	CAS SPRGREETQY	2.5	100%	18	CAS SPRGREETQY	2.5	100%

Legends of Figures

Figure 1: Representation of the study cohort and its immunological and virological characteristics. (A) A schematic showing the disease course in 3 patients who were followed longitudinally. Month zero indicates the estimated date of infection. Black arrows indicate the time points studied in each individual, while red bullets indicate the time points where viral sequences were obtained. Epitope-specific responses are depicted in each patient and viral sequence diversification was observed longitudinally. Viral sequences were obtained by clonal sequencing except for the ones highlighted by an asterisk, where bulk / population sequencing was performed. **(B)** HIV plasma viral load, peripheral blood CD4 T cell counts, and peripheral blood CD8 counts are depicted longitudinally for patients 1, 2, and 3. Plasma viral loads are represented in red. Green squares indicate CD4 counts / ul, while blue diamonds indicate CD8 counts / ul. **(C)** Epitope-specific responses were the following; B7-FPQGEAREL (pol) and B7-TPGPGVRYRL in patient 1, A3-RLRPGGKKK (gag) in patient 2 and patient 3. The black bars indicate the frequencies of the wild-type epitope-specific $CD8^+$ T cell responses longitudinally in each patient, whereas the grey bars indicate frequencies of variant epitope-specific $CD8^+$ T cell responses where applicable (patient 3).

Figure 2: TCR clonotypic composition of cross-reactive responses. The figure represents a co-staining plot with KKK- and RKR-specific tetramers from patient 3. The x and y-axes indicate KKK- and RKR-specific responses, respectively. The double tetramer population indicates cross-reactive clonotypes. Arrows emerging from each $tetramer^+$ population describe their clonotypic composition showing CDR3 amino acid sequence, TCRBV and TCRVJ usage, and the relative frequency of HIV-specific $CD8^+$ T

cell clonotypes at 2 and 16 months. Colored backgrounds indicate persisting clonontypes over time, while non-colored backgrounds indicate non-persisting clonotypes. The ImmunoGeneTics (IMGT) nomenclature was used for the TCR clonotypic analysis.

Figure 3: Improvement of functionality of wild-type HIV-specific CD8$^+$ T cells concomitant with decay in wild-type antigen load, due to emergence of escape variants. (A) Multifunctional assessment of wild-type HIV-specific CD8$^+$ T cell responses by multi parametric flow cytometry performed longitudinally for each patient. Cells were stimulated for 6 hours with corresponding cognate peptide before intracellular staining. Percentages of cytokine producing HIV-specific tetramer$^+$ cells are shown. Plots are gated on CD3$^+$ and CD8$^+$ T cells. After the gates for four cytokines were created (CD107a, IFN-γ, IL-2, and TNF-α), the boolean gate platform was used to create an array of 16 different combinations. The pies represent the functional profiles of the antigen-specific CD8$^+$ T cells. The slices in each pie with their different colors represent different functional combinations; 4+ (red), 3+ (orange), 2+ (yellow) and 1+ (green). The bars on the x-axis represent the response frequency for each combination. The insert on the right indicates the time points studied. Black, light grey, dark grey and diagonally lined bars indicate the first, second, third, and fourth time points studied, respectively. **(B)** Multifunctional assessment of variant HIV-specific (RKR) CD8$^+$ T cell responses by multi parametric flow cytometry performed longitudinally for patient 4. **(C)** The table represents antigen sensitivity measurements by IFN-γ EC50 for the following peptide stimulations **(1)** Wild-type epitopes B7-FPQGEAREL in patient 1 and A3-RLRPGGKKK in patient 3 **(2)** Variant epitopes B7-FPQGEARKL in patient 1 and A3-RLRPGGRKR in patient 3 longitudinally. The values are representatives of three independent experiments.

Figure 4: Wild-type HIV-specific $CD8^+$ T cells express $CCR7^-CD27^-CD45RA^+$ and are $CD28^-PD\text{-}1^-CD127^+$ concomitant with loss of wild-type antigen. (A) Maturation status of HIV-specific $CD8^+$ T cells to wild-type epitopes, based on the expression of CCR7, CD27, and CD45RA. After the gates for the three markers were created, the boolean gate platform was used to create seven different combinations. The colored slices in each pie represent different phenotypic combinations mainly, 3+ (dark blue), 2+ (blue) and 1+ (light blue). **(B)** Activation status of HIV-specific $CD8^+$ T cells to wild-type epitopes, based on the expression of CD28, CD127, and PD-1. The colored slices in each pie represent different phenotypic combinations mainly, 3+ (purple), 2+ (pink) and 1+ (light pink). The bars demonstrate the mean frequencies of cells belonging to a particular combination. The insert on the right indicates the time points studied. Black, light grey, dark grey and diagonally lined bars indicate the first, second, third, and fourth time points studied, respectively.

Figure 5: Maturation and activation status of cross-reactive clonotypes in patient 3. (A) Representative co-staining with KKK- and RKR-specific tetramers at 2 and 16 months. The x and y-axes indicate KKK- and RKR-specific responses, respectively. The double tetramer population indicates cross-reactive clonotypes. Maturation status of KKK, RKR and double tetramer-specific CD8+ T cells were measured based on the expression of CCR7, CD27, and CD45RA. After the gates for the three markers were created, the boolean gate platform was used to create seven different combinations. The colored slices in each pie represent different phenotypic combinations mainly, 3+ (dark blue), 2+ (blue) and 1+ (light blue). The insert on the right indicates the time points studied. Black and light grey bars indicate 2 and 16 months respectively. **(B)** The

histograms represent the Mean Fluorescence Intensity (MFI) of **(1)** PD-1 and **(2)** CD127 for KKK (grey), RKR (magenta) and double tetramer $CD8^+$ T cells (blue) performed at 2 and 16 months. The insert in each graph represents the frequency and MFI values for the three tetramer populations at 2 and 16 months.

Legends of Tables

Table 1: Evolution of T cell receptor (TCR) repertoire in the context of viral sequence diversification: The table shows the CDR3 amino acid sequence, TCRBV and TCRVJ usage, and the relative frequency of HIV-specific $CD8^+$ T cell clonotypes at the time points studied, for each patient. Colored backgrounds indicate persisting clonontypes over time, while non-colored backgrounds indicate non-persisting clonotypes. The IMGT nomenclature was used for the TCR clonotypic analysis. Rows represent the TCR clonotypic evolution for each patient, whereas columns indicate the different time points studied. Patient names are indicated on the left side of the table. KKK and RKR represent wild-type and variant epitopes in patient 4, respectively.

Table 2: Clonotypic composition of an escape versus a non-escape HIV-specific $CD8^+$ T cell epitope restricted by the same HLA. The table shows the CDR3 amino acid sequence, TCRBV and TCRVJ usage, and the relative frequency of HIV-specific $CD8^+$ T cell clonotypes at the time points studied, for patient 1. Colored backgrounds indicate persisting clonontypes over time, while non-colored backgrounds indicate non-persisting clonotypes. The IMGT nomenclature was used for the TCR clonotypic analysis. Rows represent the TCR clonotypic evolution for a conserved and an escaping HIV-specific $CD8^+$ T epitope, whereas columns indicate the different time points studied.

Legends of Supplementary Figures

Supplementary figure1: Composite data for patient 4. The figure represents **(A)** time points studied and autologous viral sequences performed. **(B)** Viral load and concomitant CD4/CD8 counts at 1 month. **(C)** The frequencies of BI*0801-GI9 decay over time. **(D)** Multiparametric flow cytometry showing different combinations of function at 1 and 7 mo. **(E)** EC50 for IFN-γ at 1 and 7 mo. **(F)** Maturation status of B*0801-GI9 specific $CD8^+$ T cells at 1 and 7 mo. **(G)** Activation status of B*0801-GI9 specific $CD8^+$ T cells at 1 and 7 months.

Supplementary figure2: Improved functionality of wild-type HIV-specific $CD8^+$ T cells is due to the persistence of functionally superior clonotypes rather than a gain of function. (A) The colors represent different functional combinations that were sorted; 3+ (orange), 2+ (yellow) and 1+ (green). The table shows CDR3 amino acid sequence, TCRBV and TCRVJ usage, and the relative frequency of HIV-specific $CD8^+$ T cell clonotypes at 2 and 16 months. The IMGT nomenclature was used for the TCR clonotypic analysis. **(B)** The table represents the clonotypic composition of the different functional profiles of total HLA B*0701-FPQGEAREL tetramer response at 21 months, as determined by DNA-based clonotypic analysis.

References

1. Goulder, P.J. and D.I. Watkins, *HIV and SIV CTL escape: implications for vaccine design.* Nat Rev Immunol, 2004. **4**(8): p. 630-40.

2. Garber, D.A., G. Silvestri, and M.B. Feinberg, *Prospects for an AIDS vaccine: three big questions, no easy answers.* Lancet Infect Dis, 2004. **4**(7): p. 397-413.

3. Borrow, P., et al., *Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus.* Nat Med, 1997. **3**(2): p. 205-11.

4. Goulder, P.J., et al., *Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS.* Nat Med, 1997. **3**(2): p. 212-7.

5. Price, D.A., et al., *Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection.* Proc Natl Acad Sci U S A, 1997. **94**(5): p. 1890-5.

6. Price, D.A., et al., *The influence of antigenic variation on cytotoxic T lymphocyte responses in HIV-1 infection.* J Mol Med, 1998. **76**(10): p. 699-708.

7. Cornberg, M., et al., *Narrowed TCR repertoire and viral escape as a consequence of heterologous immunity.* J Clin Invest, 2006. **116**(5): p. 1443-56.

8. Davenport, M.P., D.A. Price, and A.J. McMichael, *The T cell repertoire in infection and vaccination: implications for control of persistent viruses.* Curr Opin Immunol, 2007. **19**(3): p. 294-300.

9. Meyer-Olson, D., et al., *Limited T cell receptor diversity of HCV-specific T cell responses is associated with CTL escape.* J Exp Med, 2004. **200**(3): p. 307-19.

10. Price, D.A., et al., *T cell receptor recognition motifs govern immune escape patterns in acute SIV infection.* Immunity, 2004. **21**(6): p. 793-803.

11. Kawashima, Y., et al., *Adaptation of HIV-1 to human leukocyte antigen class I.* Nature, 2009. **458**(7238): p. 641-5.

12. Allen, T.M., et al., *De novo generation of escape variant-specific CD8+ T-cell responses following cytotoxic T-lymphocyte escape in chronic human immunodeficiency virus type 1 infection.* J Virol, 2005. **79**(20): p. 12952-60.

13. Oxenius, A., et al., *HIV-specific cellular immune response is inversely correlated with disease progression as defined by decline of CD4+ T cells in relation to HIV RNA load.* J Infect Dis, 2004. **189**(7): p. 1199-208.

14. Barouch, D.H., et al., *Dynamic immune responses maintain cytotoxic T lymphocyte epitope mutations in transmitted simian immunodeficiency virus variants.* Nat Immunol, 2005. **6**(3): p. 247-52.

15. Friedrich, T.C., et al., *Reversion of CTL escape-variant immunodeficiency viruses in vivo.* Nat Med, 2004. **10**(3): p. 275-81.

16. Goulder, P.J., et al., *Evolution and transmission of stable CTL escape mutations in HIV infection.* Nature, 2001. **412**(6844): p. 334-8.

17. Leslie, A.J., et al., *HIV evolution: CTL escape mutation and reversion after transmission.* Nat Med, 2004. **10**(3): p. 282-9.

18. Bimber, B.N., et al., *Ultra-deep pyrosequencing detects complex patterns of CD8+ T-lymphocyte escape in SIV-infected macaques.* J Virol, 2009.

19. Wilson, J.D., et al., *Oligoclonal expansions of CD8(+) T cells in chronic HIV infection are antigen specific.* J Exp Med, 1998. **188**(4): p. 785-90.

20. Champagne, P., et al., *Skewed maturation of memory HIV-specific CD8 T lymphocytes.* Nature, 2001. **410**(6824): p. 106-11.

21. Scheinberg, P., et al., *The clonal composition of human CD4+CD25+Foxp3+ cells determined by a comprehensive DNA-based multiplex PCR for TCRB gene rearrangements.* J Immunol Methods, 2007. **321**(1-2): p. 107-20.

22. Nikolich-Zugich, J., M.K. Slifka, and I. Messaoudi, *The many important facets of T-cell repertoire diversity.* Nat Rev Immunol, 2004. **4**(2): p. 123-32.

23. Kedzierska, K., et al., *Tracking phenotypically and functionally distinct T cell subsets via T cell repertoire diversity.* Mol Immunol, 2008. **45**(3): p. 607-18.

24. Lehner, P.J., et al., *Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V beta 17 gene segment.* J Exp Med, 1995. **181**(1): p. 79-91.

25. Moss, P.A., et al., *Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 8987-90.

26. Turner, S.J., et al., *Structural determinants of T-cell receptor bias in immunity.* Nat Rev Immunol, 2006. **6**(12): p. 883-94.

27. Turnbull, E.L., et al., *HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently.* J Immunol, 2006. **176**(10): p. 6130-46.

28. Alexander-Miller, M.A., G.R. Leggatt, and J.A. Berzofsky, *Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy.* Proc Natl Acad Sci U S A, 1996. **93**(9): p. 4102-7.

29. Derby, M., et al., *High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL.* J Immunol, 2001. **166**(3): p. 1690-7.

30. Sedlik, C., et al., *In vivo induction of a high-avidity, high-frequency cytotoxic T-lymphocyte response is associated with antiviral protective immunity.* J Virol, 2000. **74**(13): p. 5769-75.

31. Bihl, F., et al., *Impact of HLA-B alleles, epitope binding affinity, functional avidity, and viral coinfection on the immunodominance of virus-specific CTL responses.* J Immunol, 2006. **176**(7): p. 4094-101.

32. Almeida, J.R., et al., *Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV suppressive activity.* Blood, 2009.

33. Almeida, J.R., et al., *Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover.* J Exp Med, 2007. **204**(10): p. 2473-85.

34. Petrovas, C., et al., *SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection.* Blood, 2007. **110**(3): p. 928-36.

35. Price, D.A., et al., *Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses.* J Exp Med, 2005. **202**(10): p. 1349-61.

36. Trautmann, L., et al., *Selection of T cell clones expressing high-affinity public TCRs within Human cytomegalovirus-specific CD8 T cell responses.* J Immunol, 2005. **175**(9): p. 6123-32.

37. Bernardin, F., et al., *Primary infection of a male plasma donor with divergent HIV variants from the same source followed by rapid fluctuations in their relative frequency and viral recombination.* AIDS Res Hum Retroviruses, 2003. **19**(11): p. 1009-15.

38. Douek, D.C., et al., *A novel approach to the analysis of specificity, clonality, and frequency of HIV-specific T cell responses reveals a potential mechanism for control of viral escape.* J Immunol, 2002. **168**(6): p. 3099-104.

5 Original contribution to scientific knowledge

The contribution of the work described in this thesis is original to scientific knowledge in several aspects. The exhaustion of HIV-specific $CD8^+$ T cells had been described by many investigators, however, a mechanistic explanation for this dysfunction was lacking. In chapter 2, we described that the upregulation of PD-1 was associated with the dysfunction of HIV-specific $CD8^+$ T cells, and that manipulating PD-1 interaction with its ligand PD-L1 led to reversible functional restoration of HIV-specific $CD8^+$ T cells. Thus, in chapter 2, we provided evidence for molecular signatures of HIV-specific $CD8^+$ T cells exhaustion. This provides insights into potential therapeutic strategies that aim at restoring $CD8^+$ T cell function in HIV-1 infection.

Moreover, having established a positive correlation between the level of PD-1 expression and viral load, we chose to study the impact of antigen load on the functional profile and maturation status of HIV-specific $CD8^+$ T cells in 2 distinct models for "antigenic loss", mainly application of HAART and its voluntary cessation in chapter 3, and the characterization of HIV-specific $CD8^+$ T cell epitopes that escape immune recognition in chapter 4. The originality of both models lies within the comprehensive TCR clonotypic analysis that was coupled to the phenotypic and functional changes observed with varying viral loads. Furthermore, our results in chapter 3 highlight the mechanisms behind the HIV-specific $CD8^+$ T compartment reconstitution under HAART, whereas our results in chapter 4 emphasize the effect of viral escape mutations on the fate of residual wild-type-specific $CD8^+$ T cells.

6 Discussion

6.1 Restoration of HIV-specific CD8$^+$ T cell dysfunction: "The PD-1 axis"

6.1.1 What is exhaustion of CD8$^+$ T cells and how does it differ from anergy?

A characteristic feature of HIV-specific CD8$^+$ T cells is their impaired ability to produce cytokines, exhibit cytotoxicity and defects in proliferative potential [76, 89, 102]. Therefore, the terms "dysfunction" or "exhaustion" have been used to define these functional defects. What prevents a chronically stimulated T cell from functioning properly? There are several reasons for these functional defects which include lack of CD4$^+$ T cell mediated help [75], aberrant proximal TCR signals [64], upregulation of inhibitory molecules [107-109, 242], downregulation of costimulatory molecules [64], defects in cytokine signals [96] and the impact of antigen load [118, 243]. We and others identified PD-1 as a marker of "exhaustion" on antigen-specific CD8$^+$ T cells in chronic infections [107-109]. Interestingly, the expression of PD-1 was found to be highest on HIV-specific CD8$^+$ T cells when compared to EBV- and CMV-specific CD8$^+$ T cells. Importantly, a positive correlation was established between PD-1 expression on HIV-specific CD8$^+$ T cells and viral load, indicating that high levels of antigen load could be driving the dysfunction of HIV-specific CD8$^+$ T cells. This however, cannot be the absolute reason for CD8$^+$ T cell dysfunction, as EBV-specific CD8$^+$ T cells express high to intermediate levels of PD-1. The reasons for these differences could potentially lie at the interface of TCR and peptide (signal 1) and signals delivered by costimulatory molecules (signal 2). Anergy occurs when initial TCR signals are received in the absence of optimal costimulatory molecules leading to a state of immunological hypo-responsiveness. Anergy could also result if T cells use the inhibitory receptor for B7 molecules such as CTLA-4 [244]. Therefore, are dysfunction and anergy of CD8$^+$ T cells in persistent chronic infection similar or distinct processes? It is possible that anergy is defined as the level of highest dysfunction of CD8$^+$ T cells. In contrast to its expression on CD4$^+$ T cells, blocking CTLA-4 interaction with its ligands by the administration of blocking antibodies in LCMV infection, did not result in partial restoration of function of antigen-specific CD8$^+$ T cells [106]. Therefore, it is temping to speculate that CTLA-4 expressing HIV-specific CD8$^+$ T cells are anergic rather than exhausted. It was also reported that anergic cells are refractory to anti-CD3 stimulation and express low levels

of transcription factors for *tnf*, *il2*, and *Infg* [245]. Interestingly, exhausted HIV-specific $CD8^+$ T cells produce IFN-γ, a characteristic lost in anergic T lymphocytes [96]. On the other hand, PD-1 expression varies between different antigen-specific $CD8^+$ T cells and leads to functional restoration of HIV-specific $CD8^+$ T cells, indicating that exhaustion could be a reversible process. Macian et al had previously identified 14 anergy-associated genes both in vivo and in vitro [245]. In an effort to decipher whether anergy and exhaustion of $CD8^+$ T cells are distinct or similar processes, Wherry et al studied the expression of these 14 anergy-associated genes in exhausted cells and found out that the two are distinct processes [65]. Although quantification for this micro array data is imminent and awaits much more intense investigation to reach robust conclusions, this could indicate that the differential expression of inhibitory molecules such as CTLA-4 and PD-1 on T lymphocytes could reflect distinct signaling pathways, leading to different degrees of exhaustion, anergy being the "most exhausted".

6.1.2 CTLA-4 and PD-1 expression on HIV-specific $CD8^+$ T cells: synergy or different functions?

CTLA-4 and PD-1 are inhibitory molecules expressed upon T cell activation and are needed to circumvent the detrimental effects that could result from autoimmunity and inflammation. Previous reports stated that CTLA-4, unlike PD-1, was not involved in the exhaustion of antigen-specific $CD8^+$ T cells [106] and blocking CTLA-4 did not lead to improved $CD8^+$ T cell function. Kaufmann et al reported however, that CTLA-4 was not expressed on $CD8^+$ T cells and its expression was mainly restricted to HIV-specific $CD4^+$ T cells. These contrasting views need to be further elucidated and could be due to the different antigen-specific $CD8^+$ T cells studied. Therefore, these results indicated that PD-1 operates on both $CD4^+$ and $CD8^+$ T cells while CTLA-4 operates on $CD4^+$ T cells; blocking the interaction of PD-1 with its ligand PD-L1 was associated with increased proliferation and cytokine production after restimulation with cognate peptide in a 6 day $CD8^+$ T cell culture assay, whereas blocking this interaction on CD4+ T cells correlated positively with viral loads and disease progression [107]. CTLA-4 blockade on HIV-specific $CD4^+$ T cells was associated with increased proliferation and decline in viral

burdens. Interestingly, the simultaneous blockade of PD-1 and CTLA-4 on HIV-specific $CD8^+$ T cells did not show any additive effect in vitro [106, 242]. Such simultaneous blockade experiments should also be addressed on $CD8^+$ T cells in order to decipher the mutually exclusive roles exhibited by each inhibitory molecule. This is consistent with the current report stating that T cell dysfunction is regulated by multiple inhibitory signals in chronic viral infection [246] thus indicating that our knowledge in the field is still in its infancy.

In vivo blockade of PD-1 in SIV-infected macaques was effective in controlling viral loads both shortly after early infection (week 10) and later in the chronic phase (week 90). Virus-specific $CD8^+$ T cell function was improved both in the periphery and gut associated lymphoid tissue (GALT), along with expansions in virus-specific $CD4^+$ T cells, memory B cells, and increases in envelope-specific antibodies [247]. Additionally, blocking PD-1 together with therapeutic vaccination enhanced functional $CD8^+$ T cell responses and improved viral control in chronically LCMV infected mice [248]. These results are striking owing to the fact only one inhibitory pathway was blocked and resulted in clearance of viral loads and survival of the macaques. In contrast, in vivo blockade of CTLA-4 in SIV-infected macaques did not show any benefit in terms of viral load clearance [249], possibly through exacerbating immune activation, which ultimately led to increased viral replication. Synergistic in vivo blockade of PD-1 and CTLA-4 has not been yet reported. Altogether, whether these 2 inhibitory molecules exhibit mutually exclusive functions in regulating T cell activity in chronic viral infections, their differential tissue distribution, the ligands they utilize, and the distinct signaling pathways they elicit need to be further evaluated to implicate the use of these molecules as promising therapeutic targets.

6.1.3 The "exhaustion gradient" of HIV-specific $CD8^+$ T cells

The functional exhaustion of antigen-specific $CD8^+$ T cells is not an "all-or-nothing" process [250]; instead, there appears to be a gradient in functional exhaustion and antigen load is responsible in shaping it.

6.1.3.1 Do TCR-peptide avidity and magnitude / kinetics of antigen burden drive the "exhaustion gradient" of HIV-specific CD8$^+$ T cells?

It is conceivable that after a positive correlation was established between the expression levels of PD-1 on HIV-specific CD8$^+$ T cells and viral load [107, 109], that this latter factor influences the functional profile of HIV-specific CD8+ T cells. This is applicable when HIV-specific CD8$^+$ T cell responses are compared to other persistent viral infections such as EBV- and CMV-specific CD8$^+$ T cells [230, 231]. PD-1 expression levels vary largely between different HIV tetramer positive (tetramer$^+$) populations, indicating that sensitivity to peptide at the interface of TCR defines the exhaustion of these HIV-specific CD8$^+$ T cell populations. It is also well established that avidity for antigen shapes the TCR repertoire of persistent DNA viruses such as EBV and CMV, in such a way that these repertoires are dominated by highly avid clonotypes that are endowed with superior function [230, 231]. Therefore, even though EBV viral loads are not comparable to that of HIV, EBV-specific CD8$^+$ T cells still express high levels of PD-1 and are highly functional. Thus, if affinity of peptide to MHC-class I and its interaction with TCR is strong enough to override the negative signals delivered by PD-1, then one would expect that even though PD-1 is expressed on these cells, these cells would exhibit some degree of functional activity. If TCR-pMHC-class I interactions are weak, then the negative signals delivered by the expression of PD-1 on these cells would attenuate activating signals. Thus, understanding the expression levels of PD-1 on different tetramer$^+$ CD8$^+$ T cells and correlating it with antigen sensitivity would contribute to understanding how PD-1 can impact negatively the signals delivered by TCR-pMHC-class I interactions on the single-cell level.

6.1.3.2 Correlation between the "exhaustion gradient" and effector function of HIV-specific CD8$^+$ T cells: Are we restoring the "least-exhausted" cells after PD-1 blockade? Are the "least-exhausted" cells more polyfunctional and terminally differentiated?

As previously described by Wherry et al, exhaustion of antigen-specific CD8$^+$ T cells occurs as a gradient; ranging from highly exhausted to least exhausted cells [96]. In terms

of cytokine production, exhaustion occurs in a hierarchical fashion, where the first function to be lost is proliferative potential and IL-2 production, followed by loss of TNF-α production, and finally IFN-γ production. Our observations indicate that the loss of CD28 on PD-1 high HIV-specific $CD8^+$ T cells, may contribute to their limited growth and lack of IL-2 production [109, 251]. Moreover, IFN-γ producing cells define exhausted cells and have been shown to dictate HIV-specific $CD8^+$ T cell responses in chronic infection, where their eventual loss correlates with progression to AIDS [104].

Recently, polyfunctionality has emerged as a key feature for measuring efficacy of virus-specific $CD8^+$ T cell responses in controlling viral loads [252]. Polyfunctional cells are those that simultaneously produce multiple cytokines, chemokines, and elicit cytotoxic activity, which is often measured by CD107a expression levels. Therefore, by blocking PD-1 and PD-L1 interactions, which degree of exhaustion are we able to restore? A mathematical estimate from the lymphocytic choriomeningitis virus (LCMV) model by Barber et al, suggests that upon blockade of the PD-1 axis after 7-14 days of treatment, the rescue should yield a net increase of at least 2^7 or 128 fold. However, the actual blockade yielded a net increase of 2 to three fold, indicating that a fraction of these cells is restored [106, 250]. It is possible that the most-exhausted cells are accompanied by apoptosis thus contributing to the smaller number of cells rescued. Furthermore, in terms of what is being restored, several questions call for further investigation. Is the restoration of function an increase in the number of precursors that are induced to proliferate or an actual improvement in functional profile? What is the phenotype of the "rescued" cells? Are they terminally differentiated? Are the rescued cells polyfunctional? Such questions can be answered in experiments that measure the degree of polyfunctionality and maturation status, along with a comprehensive TCR repertoire analysis pre and post PD-1 blockade. These issues need to be addressed carefully to fully understand whether restoration leads to the rescue of polyfunctional cells.

6.1.4 Potential reasons and mechanisms for the survival of dysfunctional HIV-specific $CD8^+$ T cells in chronic infection.

One of the major reasons for the survival advantage of PD-1^{high} $CD8^+$ T cells in chronic infection could be a method exploited by the virus to perturb homeostasis. The generation

of potentially functional cells could be limited or even of too low frequency to contribute to antiviral effects. The mechanisms for this however remain unknown. One study investigated the link between PD-1 expression and apoptosis [108]. The authors indicated that high levels of PD-1 expression rendered these HIV-specific $CD8^+$ T cells more susceptible to both spontaneous and Fas-mediated apoptosis. Interestingly, cross-linking PD-1 with anti-PD-1 resulted in the apoptosis of HIV-specific CD8+ T cells with the highest levels of PD-1 expression. Thus, the level of expression and hence the degree of exhaustion of HIV-specific $CD8^+$ T cells determines their fate, where PD-1 high cells die by apoptosis, and cells with lower expression of PD-1 survive and proliferate. Although proliferation is more than just resistance to apoptosis, it is reasonable to speculate how the varying degrees of PD-1 expression effect cell survival and proliferation [108]. Therefore, this could explain why there are increased numbers of cells after PD-1 blockade [109]. This could be due to the preferential expansion of a subset of PD-1 expressing cells that is not highly susceptible to pro-apoptotic factors.

Other factors that could also contribute to the susceptibility of HIV-specific $CD8^+$ T cells to apoptosis include the cytokine milieu, the level of T cell activation, maturation status, and possibly the preferential expression of cell survival genes. In line with the latter factor, it was shown that ligation of the inhibitory molecule CTLA-4 expressed on $CD4^+$ T cells, delivers signals to resting $CD4^+$ T cells that prevents IL-2 production but does not alter the CD28-mediated induction of bcl-X_L, a gene associated with cell survival [253]. In parallel to CTLA-4, perhaps PD-1 expressing $CD8^+$ T cells have developed a potential to attenuate partial signals triggered by TCR, while preserving other signals that induce cell survival. Hence, by surviving and accumulating in the antigen-specific $CD8^+$ T cell pool, they are able to perturb homeostasis by preventing the re-establishment of a niche of functionally competent T cells.

6.1.5 Insights into the positive correlation between PD-1 expression on HIV-specific $CD8^+$ T cells and viral load

Having established a positive correlation between HIV viral burdens and PD-1 expression on HIV-specific $CD8^+$ T cells, we sought to determine how the "absence of antigen" impacts the polyfunctional profile of HIV-specific $CD8^+$ T cells on the clonal level. We

chose two scenarios for the "absence of antigen" model; Chapter 3 encompasses the longitudinal analysis of epitope-specific $CD8^+$ T cell responses followed in 4 patients treated temporarily with HAART, while Chapter 4 comprises the longitudinal analysis of epitope-specific $CD8^+$ T cell responses that mutate over time in 4 chronically infected patients thus resulting in wild-type antigenic loss.

Our results in Chapter 3 showed that the frequency of HIV-specific $CD8^+$ T cells dropped with viral load and increased with viral resurgence (Figure 1. C). Interestingly, the expression of PD-1 on HIV-specific $CD8^+$ T cells correlated positively with viral load (data not shown), even though differential decreases were observed between the different tetramer positive cells under HAART. This could simply be explained by the possibility that residual virus replication was still ongoing under HAART but was undetectable by routine screening methods. The levels of residual viral replication differ among individuals and could explain why differential decreases in PD-1 expression were observed between the different tetramers under HAART [254, 255].

Moreover, bearing in mind the positive correlation between PD-1 and viral load, we performed a robust polyfunctional profiling of the tetramer positive cells along with the clonal composition of each HIV-specific response pre, during, and post HAART (Chapter 3; Table 1 and Figure 3 B). We observed that HIV-specific $CD8^+$ T cells were functionally superior under therapy where PD-1 expression was lowest. After cessation of HAART, some degree of polyfunctionality was maintained even though PD-1 levels peaked concomitant with resurgence of viral loads. This indicated that, within the HIV-specific $CD8^+$ T cell population, individual clonotypes contribute to the total functional profile differently. This emphasizes the need to evaluate the expression of PD-1 on individual clonotypes. It is very tempting to speculate that within the HIV-specific $CD8^+$ T cell repertoire; there exists a hierarchy of different functions, attributed by different clonotypes. It could be that the clonotypes expressing the highest levels of PD-1 are mono-functional, whereas those with lower PD-1 expression are polyfunctional. It is also possible that the same clonotype exhibits different functional profiles, thus indicating that the level of antigen / sensitivity to it dictates differential functional outcomes. The second hypothesis is more likely to occur; as seen Chapter 3; Table 1, the functional profile of

HIV-specific $CD8^+$ T cells for patient 3, exhibited different "functional flavors" contributed by only a single clonotype (Chapter 3; Table 1 and Figure 3 B). Moreover, the DNA-based multiplex analysis performed in Chapter 4 indicated that the same clonotype is found in the different functional groups (Chapter 4; Supplementary Figure S2 A and B). Therefore, this indicates that the same clonotype could have different sensitivity to peptide and/or PD-1 expression and hence result in different functional profiles.

In Chapter 4, we examined how decay of wild-type antigen influences the functional profile of residual wild-type HIV-specific $CD8^+$ T cells. Our results in Chapter 4 (Figure 4. B) were similar to the reports suggesting that the expression of PD-1 declined on antigen-specific $CD8^+$ T cell epitopes that had undergone mutational escape in humans and SIV infected macaques [243, 256]. Therefore, although viral loads were still high except for patient 1 and patient 2, at 43 and 27 months, respectively (Chapter 4; Figure 1. B), PD-1 expression declined on the HIV-specific $CD8^+$ T cells concomitant with decay in wild-type antigen. This decline in PD-1 was accompanied by enhanced function, decline in CCR7-CD27+CD45RA- HIV-specific $CD8^+$ T cells and an increase in the frequency of terminally differentiated T_{EMRA} cells (CCR7-CD27-CD45RA+) (Chapter 4; Figure 3. A, Figure 4. A and B). Altogether, our results indicate that the expression of PD-1 could vary between different epitope-specific $CD8^+$ T cell responses and suggests how antigen load could be driving the different functional and phenotypic outcomes observed.

Importantly, the mechanisms of PD-1 regulation in activated and exhausted cells are still poorly defined. Sauce et al performed a detailed analysis of PD-1 expression pattern on various $CD8^+$ T cell subsets from healthy or HIV infected donors. They reported that PD-1 expression had two facets in vivo. On one hand, it was linked to T-cell differentiation: PD-1 was up-regulated on early/intermediate differentiated subsets, which include HIV and Epstein-Barr virus-specific $CD8^+$ T-cell populations, but was down-regulated during late stages of differentiation. On the other hand, it was linked to T-cell activation: PD-1 over-expression occurred along with the up-regulation of activation markers such as

CD38 or HLA-DR. Therefore, PD-1 expression on $CD8^+$ T cells, including those specific for HIV, can be related to both their differentiation stage and their activation status [257].

Although, further studies are needed to better understand the regulation of PD-1 expression and function in activated and exhausted cells, it may be possible that HIV-specific CD8+ T cells express high PD-1 levels as a result of T cell activation due to high viral load. In this case, PD-1 upregualtion could reflect a mechanism of retro-regulation of cell activity as T cells are stimulated. Examination of nonpathogenic SIV infection in sooty mangabeys indicated that their typically low immune activation was associated with an early increased level of PD-1 expression on T cells of lymphoid tissue, suggesting that PD-1 upregulation on bulk T cell subsets may exert a protective effect [258]. It is possible that downregulation of PD-1 expression on HIV-specific CD8+ T cells as a result of antiretroviral therapy (Chapter 3) or epitope escape (Chapter 4), simply reflects a less activated phenotype due to the loss of antigenic stimulus. In this regard, it is possible that the observed functional restoration of HIV-specific CD8+ T cells in both models, could be due to the fact that these cells are no longer activated and are more responsive to antigenic stimulation in ex vivo functional assays.

6.2 Restoration of HIV-specific $CD8^+$ T cell dysfunction: "Absence of antigen"

As mentioned previously, after having established a correlation between antigen load and expression of PD-1 on HIV-specific $CD8^+$ T cells, we sought to decipher the effect of antigen load on the clonotypic composition, functional profile, and maturation status of HIV epitope-specific $CD8^+$ T cells. The two models we chose to study the "absence of antigen" were the administration of HAART and emergence of escape from epitope-specific response, in Chapters 3 and 4, respectively.

6.2.1 Absence of antigen in the context of HAART: potential caveats

Our longitudinal analyses of HIV epitope-specific $CD8^+$ T cell responses in Chapter 3 focused on the interpretation of data from conserved epitopes. We specifically chose conserved epitopes to rule out the possibility that changes accompanying HIV-specific $CD8^+$ T cell responses pre, during, and post HAART, were elicited by viral escape

mutants. Therefore, we sequenced plasma virus pre and post HAART time points for the epitopes of interest. Supplementary figure 1 B of Chapter 3 showed the sequences of the epitope of interest for each patient and indicated that there were no signs of intra-epitopic mutational escape. However, a potential caveat in this sequencing technique is the fact that this analysis was generated using bulk or population sequencing, a method that is not overly sensitive, in contrast to clonal sequencing. Moreover, epitope sequencing was not performed under HAART due to limitations in accessing sensitive sequencing techniques that were able to detect viral variants under conditions of less than 50 viral RNA copies / ml. Therefore, our model for "absence of antigen" is not absolute and since viral escape mutants have been reported to emerge under therapy [53, 255, 259]; we cannot rule out the possibility that viral variants are present, and therefore this represents a potential caveat in our study model.

6.2.2 Absence of antigen in the context of emergence of $CD8^+$ T cell-mediated escape from wild-type epitopes: potential caveats

Potential caveats in the "absence of antigen" model described in Chapter 4, could arise from differences in sequencing techniques. The conserved epitope HLA-B*0702 TL10 at all time points studied in patient 1 and the escaping epitope HLA-A*0301 RK9 at 2, 16, and 26 months in patient 3, have been obtained by bulk or population sequencing, a technique that is not as sensitive as clonal sequencing (Chapter 4; Figure 1. A). The reason for not generating these sequences by clonal sequencing on these samples was the lack of plasma available from these time points.

Moreover, data on patient 4 poses a potential caveat in terms of interpreting results (Chapter 4; Supplementary figure 1). For all the other 3 patients the frequency of wild-type epitope declines because it relates to the effective reduced antigenic drive following the emergence of escape (Chapter 4; Figure 1 C). We cannot give the same explanation for patient 4 as we do not know whether the decay in wild-type $CD8^+$ T cell frequencies (Chapter 4; Supplementary figure 1 C) is due to the loss of recognized antigen or to a falling plasma viral load. Unfortunately, we do not have the plasma viral load determined for the last time point in patient 4 (7 months) and since we do not have functional data

with the variant epitopes for this patient, we cannot assume that the loss of wild-type antigen is due to escape.

The major limitation of the work presented in Chapters 3 and 4 is the small number of subjects studied. In Chapter 3, we studied HIV-specific CD8+ T cell responses longitudinally pre, during, and post HAART. The four patients studied in this chapter had stopped HAART voluntarily, thereby allowing us to study time points under viral recrudescence. Since current treatment strategies do not favor the voluntary cessation of HAART in HIV-1 infected patients, a small number of subjects were included in the analysis. The reasons for having a small cohort of patients in Chapter 4 are twofold. Since HIV-1 epitope escape operates under high viral load, we sought to obtain samples from highly viremic time points. This was not easily accessible since the majority of HIV-1 infected patients had received antiretroviral therapy. Moreover, epitope mutation was the main criterion for the selection of HIV-specific CD8+ T cell epitopes in Chapter 4. This caused a major limitation to the number of epitopes studied. Selecting regions from the HIV-1 genome that undergo mutations longitudinally could be highly limited by the HLA-I profile of the patient (whether the epitope is represented or not) and the availability of MHC-class I tetramers. Finally, the small number of patients of our cohorts in both models has made us acknowledge the fact that our results are hypothesis generating and need to be confirmed on bigger cohorts and with multiple epitopes.

6.3 Reconstitution of the HIV-specific $CD8^+$ T cell repertoire in the context of HAART: clonal emergence or redistribution?

The absence of durable virological benefit of short-term HAART initiated during acute HIV-1 infection has been reported and suggests that durable maintenance of low-level viremia is difficult to achieve [260, 261]. However, studying HIV-specific $CD8^+$ T cell efficacy could help understand how these cells would otherwise function when antigen is absent.

What is most striking in our clonotypic analysis of antigen-specific $CD8^+$ T cell responses is the dynamic turnover of HIV-specific $CD8^+$ T cell clonotypes between pre, during, and post HAART time points (Chapter 3; Table 1). It was previously reported that initial $CD8^+$ T cell responses to HIV-1 in acute infection, although low in magnitude [262],

comprised of oligoclonal expansions that were indeed antigen-specific [209], and were lost due to the transition from acute to chronic infection [208]. Loss of antigen-specific $CD8^+$ T cell clones from the acute to chronic phase of infection has also been described in LCMV infection of mice [239]. The deletion of clonotypes from acute to chronic phase could be due to the disappearance of high avidity clonotypes [105] or the loss of cross-reactive clonotypes that are lost due to heterologous immunity [239].

Soudeyns et al reported that the initiation of HAART during primary HIV-1 infection stabilized the TCR Vβ repertoire more rapidly in treated versus treatment naïve individuals and that this stabilization included reduced oligoclonality and increased polyclonality [210]. Similar observations were reported by Gorochov et al, where complete viral suppression by HAART was associated with stabilized $CD8^+$ TCR Vβ repertoires, whereas incomplete viral suppression or viral resurgence was associated with perturbed repertoires [211]. These studies although limited in terms of determining the clonality of the HIV-specific $CD8^+$ T cell responses, indicate nonetheless the reconstitution that follows HAART treatment.

Our results are in line with these previous reports indicating that perturbations in HIV-specific $CD8^+$ TCR Vβ repertoire occurred at pre and post HAART. However, we observed changes even under HAART, given by the fact that clonotypes were lost and others emerged (Chapter 3; Table 1). This discrepancy could simply be addressed by the different methods used for studying TCR Vβ repertoire diversity used by our group and others. Spectratyping does not determine the individual identity of the clonotypes that comprise the $CD8^+$ TCR repertoire.

The origins of these clonotypes under HAART remain to be elucidated. It could be possible that these clonotypes are being reshuffled from secondary lymphoid organs as HAART is instituted, or are newly primed. A thorough phenotyping analysis on the clonal level combining the presence or absence of certain markers that are indicative of the "exodus" of $CD8^+$ clones from secondary organs back into the blood during HAART, would then address their origins. The redistribution of $CD4^+$ T cells was initially reported by Bucy et al in blood and lymph nodes of HIV-1 infected individuals [263]. The results indicated that after initiation of HAART the absolute numbers of $CD4^+$ T cells decreased

per lymph node in parallel with reductions in the surface expression of VCAM-1 and ICAM-1, adhesion molecules known to mediate lymphocyte confiscation in lymphoid tissues. Interestingly, absolute numbers of $CD4^+$ T cells increased in the blood, indicating a reshuffling induced due to therapy [263]. Later on, it was shown that the reconstitution of $CD4^+$ T cell compartment occurred in a biphasic manner. The initial reconstitution involved the expansion of non-proliferating $CD4^+$ T cells and was associated with both increases in memory and naïve CD4 + T cells. The second phase was associated with proliferating $CD4^+$ T cells returning to baseline levels [264].

It is possible to speculate, given that antigen is always present even under HAART [265, 266], that the reconstitution of $CD4^+$ compartment under HAART primed additional $CD8^+$ T cell responses from the naïve pool and that new CD8+ T cells were primed to enter the HIV-specific memory $CD8^+$ T cell pool [267, 268]. Data depicted in Figure 5 A and B show that the functional changes that accompany the fluctuations in viral loads are contributed by persisting clonotypes (Chapter 3; Figure 5 A), but emerging clonotypes could also take part in the HIV-specific $CD8^+$ T cell reconstitution (Chapter 3; Figure 5 B). Robust conclusions await further investigation with several persisting and emerging clonotypes.

Finally, the priming of new $CD8^+$ T cell specificities under HAART is not surprising. As HIV-specific $CD4^+$ T helper cells are not lost, instead, their turnover increases and exhibits an effector phenotype upon viral recrudescence [269]; it is not surprising that they primed new $CD8^+$ T cell specificities.

6.4 Reconstitution of the HIV-specific $CD8^+$ T cell repertoire in the context of escape: clonal deletion or persistence?

Oligoclonal populations have been reported to dictate HIV-specific $CD8^+$ T cell responses. Previously it was reported that clonally diverse TCR repertoires to immunodominant epitopes in HIV and SIV infection are needed for optimal control, as they are able to cross-recognize emerging variants [259, 270]. Therefore, three groups investigated the correlation between TCR repertoire usage and emergence of viral escape mutants. It was shown by Price et al that the SIV-specific CD8+ TCR repertoire against CM9 (an epitope that does not escape) and TL8 (an epitope that escapes) diverge in terms

of their $CD8^+$ TCR repertoires. Particularly, TCR repertoires that have a preferential Vβ and Jβ usage along with identifiable motifs within their CDR3β regions were associated with escape (TL8), whereas no such biases were observed in the TCR repertoires of CM9 [223]. Similar observations were reported in HCV and LCMV infection [229, 232]. Therefore, long before escape becomes evident, the molecular structures such as limited diversity within the CDR3 motif, aid in facilitating its emergence. In Chapter 4, we sought to decipher whether such narrowed repertoires also dominated the TCR repertoires of HIV-specific epitopes that escape. Table 1 of Chapter 4 summarizes the TCR clonotypic repertoire data acquired for patients 1, 2, and 3. The TCR repertoire of patient 1 displayed a preference for Vβ 18 and Jβ 2.5 and a discernable RGR motif within the CDR3β region. Such preference in Vβ and Jβ and motifs has been reported within the CDR3β region in the influenza matrix protein (HLA-A*0201-MP58-66) and SIV (Mamu-A*01 TL8) epitope-specific CD8+ TCR repertoires [204, 205, 223]. Therefore, it is tempting to speculate that such structural determinants could influence the nature of the responding repertoires [195].

The TCR repertoire in patient 2 was restricted and eventually dominated by one clonotype at 21 months, coincident with the emergence of escape (Chapter 4; Table 1). Unfortunately, we did not have earlier time points that allowed us to track when this clonotype was detected. Interestingly, at 27 months, when the patient was on HAART, we observed a broadening of the epitope-specific repertoire. This was in agreement with our previous observations that HAART led to the recruitment of new CD8+ T cell specificities (Chapter 3; Table 1).

In patient 3, we observed a degree of cross-reactivity between wild-type and variant epitopes (Chapter 4; Table 1 and Figure 2). Both KKK and RKR-specific $CD8^+$ T cell repertoires were not biased and remained oligoclonal over time. We did not detect any motifs within the CDR3β regions. Thus, although there was a discrepancy between the sequencing techniques used to evaluate viral diversification between the time points, we report here that even in the context of escape, such biases in the TCR repertoires could be alleviated due to the presence of cross-reactive clonotypes. Similar results were published by Turnbull et al., where the authors reported that poor cross-recognition of variants was

associated with a bias to a particular TCR Vβ, while efficient variant cross-recognition was not associated with bias to particular Vβ [219].

Deletion of clonotypes has been reported during transition from acute to chronic phase of HIV-1 [105, 208]. Our data show clonal deletion of HIV-specific $CD8^+$ T cell clonotypes from acute to chronic phases in patients 1, 2, and 3. Interestingly in patients 1 and 3, clonotypes that persisted over time were of high avidity (Chapter 4; Figure 3 C). It is reasonable to speculate that although high avidity clonotypes are more sensitive to apoptosis and potentially die under chronic stimulation of antigen [271], in the face of escape; these clonotypes are able to survive, since they compete for limited resources of wild-type antigen concomitant with the emergence of escape. Persistence of high avidity clonotypes has been reported in other chronic viruses such as EBV and CMV, and indicates that an "optimal" level of antigen is a determinant factor for the fate of these clonotypes [231, 230].

6.5 Functional reconstitution of HIV-specific $CD8^+$ T cell repertoire in the context of HAART is accompanied by broad $CD8^+$ TCR repertoires: insights into TCR repertoire usage

Initiating HAART is recommended in the acute phase of HIV-1 infection to reduce viral dissemination and its harmful effects on the immune system [272]. The advantages of early administration of HAART in acute HIV-1 infection have been reported previously and provided insights into the mechanisms that led to the reconstitution of cellular immunity. Altfeld et al reported that early administration of HAART in individuals acutely infected with HIV-1 and who were treated before seroconversion exhibited relatively modest HIV-specific $CD8^+$ T cell responses directed against few epitopes, had strong $CD4^+$ T helper responses and showed less viral diversification when compared to individuals who were treated later in HIV-1 infection. In the former group, a negative correlation was established between the breadth of HIV-specific $CD8^+$ T cell responses and plasma viral loads. In contrast, $CD4^+$ T cell help was lost and viral diversification was broad in the latter group. These results showed that early versus late administration of HAART leads to distinct clinical outcomes [262]. Moreover, HIV-specific effector

functions that were present persisted but did not expand after initiation of HAART [262, 273].

Recently, investigators have focused their attention on the qualitative features of HIV-specific $CD8^+$ T cells such as polyfunctionality. Polyfunctional $CD8^+$ T cells have been reported in a subset of HIV-1 infected patients called LTNPs [114], in HLA-B*2705 individuals [115], in $CD8^+$ responses to Vaccinia Virus [117] , and in $CD8^+$ responses to HIV-2 infection [116]. Therefore, we and others hypothesized that the initiation of HAART would lead to the functional improvement of HIV-specific $CD8^+$ T cell responses. It was reported by Daucher et al that the functionality of HIV-specific $CD8^+$ T cells upon antigen reencounter, following structured interruption of treatment (SIT), was indicative of the emergent level of plasma viral load, with polyfunctional responses being associated with decreased levels of plasma viremia after STI. Therefore, measuring the quality of HIV-specific $CD8^+$ T cell responses could be a surrogate indicator for the effects elicited by SITs [274]. Moreover, our data in Chapter 3 along with others [118, 243] indicated that HIV-specific $CD8^+$ T cells are polyfunctional under HAART. Restoration of function under HAART was HIV-specific as CMV-specific $CD8^+$ T cells were not affected by HAART intervention and remained polyfunctional (Chapter 3; Supplementary figure 3). Restoration of HIV-specific $CD8^+$ T cell function occurred predominantly with a gain in TNF-α and to a lesser degree IL-2 under HAART. These are in line with previous studies indicating that dysfunction of virus-specific $CD8^+$ T cells occurs at a hierarchical order where IL-2 is the first function to be lost followed by TNF-α and finally IFN-γ [96].

What is unique in our analysis is the fact that our cohort of patients received HAART temporarily. Although present treatment guidelines do not favor treatment interruptions, our cohort gave us the unique opportunity to study the effect of HAART on the functional profile of HIV-specific $CD8^+$ T cells and its lack during viral rebound. Interestingly, although viral loads at pre and post HAART time points were comparable, HIV-specific $CD8^+$ T cells exhibited different functional and phenotypic profiles, indicating a HAART mediated effect. Taking into account that there has been a lack of HAART durability in

maintaining control, it is conceivable that these populations are potentially lost later during chronic infection, due to persistent antigenic drive.

Another unique feature of our present study described in Chapter 3 is our clonotypic analysis preformed at pre, during and post HAART time points and the functional changes that occur along this high turnover of HIV-$CD8^+$ T cell responses. The broadening of HIV-specific $CD8^+$ T cell repertoire under HAART could potentially reflect the redistribution of $CD8^+$ T cells from secondary lymphoid organs. The previous studies could not delineate whether the overall restoration of cytokine secretion capacity of HIV-specific $CD8^+$ T cells is in fact a functional improvement on the single cell level or the preferential survival of cytokine producing cells. Our results indicate that both above explanations underlie the mechanisms of $CD8^+$ T cell restoration under HAART. TCR clonotypic and functional data from patient 3 indicated that a single clonotype could undergo functional improvement with changes in viral load (Chapter 3; Table 1 and Figure 3). This indicates that persisting clonotypes contribute to the enhanced polyfunctionality observed under HAART. It is also possible that emerging clonotypes contribute to the functional enhancement (Chapter 3; Figure 5 A), although further analysis needs to be performed to reach more robust conclusions. Of note, 3 of the patients studied had HAART initiated in the acute phase of infection, except for patient 4, who started HAART 6 months after the estimated date of infection. This patient showed relatively different patterns of restoration of function (Chapter 3; Figure 3 B) and maturation status (Chapter 3; Figure 2 A) when compared to the other patients. Since an acute pre HAART time point was unavailable in this patient, nonetheless restoration of function was still observed albeit not as evident as it was with the other patients. This could simply be due to the initiation of HAART at a later time point when compared to the other patients. However, notwithstanding patient 4, our data clearly indicate a HAART mediated functional restoration of the HIV-specific $CD8^+$ T cell compartment and indicate that polyfunctionality reflects a reduced in vivo antigen exposure.

6.6 Functional reconstitution of HIV-specific $CD8^+$ T cell repertoire in the context of escape from original wild-type antigen is accompanied by narrow $CD8^+$ TCR repertoires: insights into TCR repertoire usage

In Chapter 4 we studied the functional profile of HIV-specific $CD8^+$ T cells against epitopes that escaped. This allowed us to study the effect of "antigenic loss" on the functional profile of residual wild-type-specific $CD8^+$ T cells. Our results in Figure 3 A of Chapter 4 indicated that HIV-specific $CD8^+$ T cells exhibited improved function concomitant with the emergence of escape. Specifically, HIV-specific $CD8^+$ T cell responses at the early time points were dictated by mono-functional responses that were mainly IFN-γ^+ cells and to lesser degree CD107a producing cells. Coincident with the emergence of escape with each patient, polyfunctional cells became evident in the functional repertoire. The 3^+ cells were mainly $CD107a^+$ IFN-γ^+ TNF-α^+ and to a lesser extent $CD107a^+$ IFN-γ^+ IL-2^+. Moreover, 4+ cells were also present in patient 1 and 4 (Chapter 4; Figure 3 A and Supplementary figure 1 D), indicating that restoration of polyfunctionality, albeit at low frequencies, was possible for some responses.

Interestingly, the enhanced function that was observed with the loss of antigenic drive due to emergence of escape was accompanied by narrow TCR repertoires for patients 1 and 2 (Chapter 4; Table 1). Previously it was shown that broader repertoires are needed for better control of variants, since they include several TCR specificities with different antigen sensitivity that could contain emerging variants [259, 270]. The focusing of the repertoire could be due to the transition from acute to chronic phase, where several clonotypes, including high avidity clonotypes are lost from the HIV-specific $CD8^+$ T cell pool [105, 208]. Moreover, if we could potentially think of the emerging escape mutants as heterologous challenge, then this could explain why the repertoires to escaping epitopes are narrow [239]. This however seems to be an unlikely explanation, since the loss of clonotypes and narrowing of repertoires against heterologous challenge has been described for acute infections where the original wild-type antigen is completely lost. In regards to chronic infections, wild-type antigens wane over time due to emergence of escape but unlikely reach extinction.

Our results indicated that the focusing of the repertoire is accompanied by increase in antigen sensitivity (Chapter 4; Figure 3 C and Supplementary figure 1 E). We observed that as the repertoire narrowed and was dictated by one clonotype as in the case of patient 1, antigen sensitivity was augmented. From a mechanistic point of view, our data in the DNA-based multiplex analysis in Supplementary figure S2 indicated that this particular clonotype persisted over time and showed varying levels of polyfunctionality. The mechanism for survival of high affinity clonotypes in the context of escape could be explained by the fact that these clonotypes persist and compete for limited sources of antigen. In fact, it was reported that high affinity clonotypes exhibit better protection of viral control than lower affinity clonotypes [275]. La Gruta et al established correlations between cytokine expression and TCR avidity in the mouse model, such that the quality of $CD8^+$ T cell responses in terms of cytokine secretion was enhanced for high avidity $CD8^+$ T cells. Moreover, Almeida et al showed that the HLA-B*2705 restricted epitope KK10 exhibited higher antigen sensitivity and were polyfunctional in contrast to other HLA alleles and this resulted in competent eradication of HIV-infected cells. Recent work suggesting a correlation between functional avidity and function in HIV-1 infection could help explain the immunodominance and superior function of HLA-B responses over other populations [276].

Furthermore, for patient 3, we observed a degree of cross-reactivity between wild-type and variant peptides. This was associated with TCR repertoires that were not as narrow and restricted as that of patient 1 (Chapter 4; Table 1). In fact, the TCR repertoires for both wild-type and variant peptides were oligoclonal and were not associated with CDR3 motifs. A likely explanation for this could be that in the face of escape, cross-reactive clonotypes dominate the TCR repertoire with broader specificities to contain variants. A study by Turbull et al reported that poor cross-recognition of variants was associated with a bias to a particular TCR Vβ, while efficient variant cross-recognition was not associated with a bias to particular Vβ. Since we did not observe any biases in the repertoire, we can conclude that this is a case of efficient-cross recognition. The authors also provided evidence that efficient cross-recognition was associated with delayed disease progression. We could not make such correlations, since we have performed our analysis with only one epitope-specific $CD8^+$ T cell response per patient. Altogether, although we observed

differences in TCR repertoire usage, the functional profile of epitope-specific $CD8^+$ T cells improved concomitant with loss of wild-type antigen due to the persistence of high affinity clonotypes.

6.7 Phenotypic dissection of polyfunctional HIV-specific $CD8^+$ T cell populations

Restoration of function under HAART and concomitant with the emergence of escape of HIV-specific $CD8^+$ T cell responses was accompanied by changes in maturation status and activation of these cells. Although we did not combine phenotypic analysis with functional profiling in the same experiment, the changes that accompany the maturation status along with the functional improvement are quite relevant.

According to previous reports and based on the surface markers CCR7 and CD45RA; we identified four T cell subsets within the $CD8^+$ T cell compartment: Naïve ($CCR7^+$ $CD45RA^+$), Central Memory (T_{CM}) ($CCR7^+CD45RA^-$), Effector Memory (T_{EM}) ($CCR7^-$ $CD45RA^-$) and Effector Memory RA+ (T_{EMRA}) ($CCR7^-CD45RA^+$) (Chapter 3; Figure 2. A) [135]. In agreement with previous results, HIV-specific $CD8^+$ T cells under high viral load, express low levels of CD127 (IL-7Rα) [277]. In our longitudinal analysis and in contrast to CMV-specific $CD8^+$ T cells, HIV-specific $CD8^+$ T cells exhibited a distinct maturation phenotype reported previously ($CCR7^-$ $CD45RA^-$). CMV-specific $CD8^+$ T cells were $CCR7^-CD45RA^+$ (Supplementary figure 2). Notwithstanding patient 4, where the initiation of HAART was at 6 months after estimated date of infection, initiation of HAART, led to a significant increase in T_{CM} $CD8^+$ T cells and T_{EMRA} cells (Chapter 3; Figure 2. A).

We observed a sharp increase in CD127 expression on HIV-specific $CD8^+$ T cells with the initiation of HAART, indicating a transition from effector to a resting memory phenotype [118]. CD127 levels immediately decreased at the first time point following termination of HAART in all four patients. In contrast, CD127 expression was stable for CMV-specific $CD8^+$ T cells (data not shown).

In Chapter 4, we observed a decrease in the less differentiated memory $CCR7^-$ $CD27^+CD45RA^-$ HIV-specific $CD8^+$ T cells coincident with the emergence of escape for all patients (Chapter 4; Figure 4. A, Figure 5. A, and Supplementary figure 1 F).

Interestingly, this decrease in frequency of less differentiated memory $CD8^+$ T cells was accompanied by an increase in the frequencies of effector populations of $CCR7^-CD27^-CD45RA^+$. Furthermore, we observed that the decrease in the less differentiated memory populations was due to a drop in PD-1 expression frequencies and an increase in CD127 expression levels. This is also in line with data indicating that escape leads to loss of PD-1 expression on the wild-type-specific $CD8^+$ T cell populations and an increase in CD127, indicating a transition from effector to memory phenotype [118, 243, 256].

6.8 Conclusions and future perspectives : Implications in immune modulation; Is there a significance of restoring HIV epitope-specific $CD8^+$ T cell responses in HIV infection?

Collectively, our results indicate that antigen load is the cause for the dysfunction of HIV-specific $CD8^+$ T cells. Although our data is hypothesis generating owing to the small number of our cohort, nonetheless, future studies with bigger sample sizes could help reach more robust conclusions.

While structured interruptions of therapy are not favored, the broadening of the HIV-specific TCR $CD8^+$ repertoire under HAART, even when HIV-specific $CD8^+$ T cell responses are of low frequency, suggests that manipulations aiming to preserve it could be promising. In fact, manipulation of the TCR repertoire in the context of persistent viral infections has been shown in the context of vaccination. An example of such manipulation has been reported and suggests that vaccination of HAART treated individuals with the MVA.HIVA that comprises HIV-1 clade A gag p24/p17 sequence fused to multiCTL epitope gene, leads to the expansion of vaccine-specific $CD8^+$ T cell responses to *Gag*, *Nef*, and *Pol.* These expansions are polyclonal in nature in sharp contrast to the oligoclonal TCR repertoires observed in vaccinees who did not elicit any response and unvaccinated controls. Moreover, although vaccination led to the transient upregulation of PD-1, which rapidly returned to base line levels, the polyclonal expansions of vaccine induced $CD8^+$ T cells displayed better proliferative potential and in vivo viral suppression activity. Thus, strategies that aim at inducing polyclonal responses under conditions of HAART appear to be promising [241].

Moreover, even in the face of escape, it appears that high avidity clonotypes persist for longer periods, as antigenic drive to wild-type antigen wanes. Therefore, defining an optimal concentration that is sufficient to preserve such clonotypes could be helpful in vaccine design, even in the face of ongoing HIV evolution. Notably, our data is concluded from single epitope responses per patient appearing at very low frequencies, and it seems unlikely that they would aid in viral containment. Further experiments that study both several dominant and even subdominant responses could help explain not only the impact of antigen load and viral sequence diversification on the fate of HIV-specific $CD8^+$ T cells, but assist in finding correlations between the functional profile of these cells and control of viremia. Finally, delineating "polyfunctionality" of HIV-specific $CD8^+$ T cells from LTNPs, HLA-B*2705 individuals, HIV-2 infection, and Vaccinia virus vaccinees, could help understand which "combination" of polyfunctionality is needed for optimal viral control.

References

1. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.
2. Gallo, R.C., et al., *Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS.* Science, 1984. **224**(4648): p. 500-3.
3. Simon, V., D.D. Ho, and Q. Abdool Karim, *HIV/AIDS epidemiology, pathogenesis, prevention, and treatment.* Lancet, 2006. **368**(9534): p. 489-504.
4. Santiago, M.L., et al., *SIVcpz in wild chimpanzees.* Science, 2002. **295**(5554): p. 465.
5. Hahn, B.H., et al., *AIDS as a zoonosis: scientific and public health implications.* Science, 2000. **287**(5453): p. 607-14.
6. Rowland-Jones, S.L. and H.C. Whittle, *Out of Africa: what can we learn from HIV-2 about protective immunity to HIV-1?* Nat Immunol, 2007. **8**(4): p. 329-31.
7. John-Stewart, G.C., et al., *Subtype C Is associated with increased vaginal shedding of HIV-1.* J Infect Dis, 2005. **192**(3): p. 492-6.
8. Cullen, B.R., *Regulation of HIV-1 gene expression.* FASEB J, 1991. **5**(10): p. 2361-8.
9. Gallo, R.C., *Tat as one key to HIV-induced immune pathogenesis and Tat (correction of Pat) toxoid as an important component of a vaccine.* Proc Natl Acad Sci U S A, 1999. **96**(15): p. 8324-6.
10. Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* Annu Rev Biochem, 1998. **67**: p. 1-25.
11. Swann, S.A., et al., *HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway.* Virology, 2001. **282**(2): p. 267-77.
12. Willey, R.L., et al., *Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4.* J Virol, 1992. **66**(12): p. 7193-200.

13. Zhang, H., et al., *Human immunodeficiency virus type 1 Vif protein is an integral component of an mRNP complex of viral RNA and could be involved in the viral RNA folding and packaging process.* J Virol, 2000. **74**(18): p. 8252-61.
14. Romani, B. and S. Engelbrecht, *Human immunodeficiency virus type 1 Vpr: functions and molecular interactions.* J Gen Virol, 2009. **90**(Pt 8): p. 1795-805.
15. Stebbing, J., B. Gazzard, and D.C. Douek, *Where does HIV live?* N Engl J Med, 2004. **350**(18): p. 1872-80.
16. Alter, G. and M. Altfeld, *NK cell function in HIV-1 infection.* Curr Mol Med, 2006. **6**(6): p. 621-9.
17. Arthos, J., et al., *HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells.* Nat Immunol, 2008. **9**(3): p. 301-9.
18. Medzhitov, R. and C.A. Janeway, Jr., *Innate immune recognition and control of adaptive immune responses.* Semin Immunol, 1998. **10**(5): p. 351-3.
19. Pasare, C. and R. Medzhitov, *Toll-like receptors: linking innate and adaptive immunity.* Microbes Infect, 2004. **6**(15): p. 1382-7.
20. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nat Immunol, 2004. **5**(10): p. 987-95.
21. Pope, M. and A.T. Haase, *Transmission, acute HIV-1 infection and the quest for strategies to prevent infection.* Nat Med, 2003. **9**(7): p. 847-52.
22. Haase, A.T., *Perils at mucosal front lines for HIV and SIV and their hosts.* Nat Rev Immunol, 2005. **5**(10): p. 783-92.
23. Lehner, T., et al., *The emerging role of innate immunity in protection against HIV-1 infection.* Vaccine, 2008. **26**(24): p. 2997-3001.
24. Pulendran, B. and R. Ahmed, *Translating innate immunity into immunological memory: implications for vaccine development.* Cell, 2006. **124**(4): p. 849-63.
25. Mavilio, D., et al., *Characterization of the defective interaction between a subset of natural killer cells and dendritic cells in HIV-1 infection.* J Exp Med, 2006. **203**(10): p. 2339-50.
26. Sheehy, A.M., et al., *Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein.* Nature, 2002. **418**(6898): p. 646-50.

27. Stremlau, M., et al., *The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys.* Nature, 2004. **427**(6977): p. 848-53.
28. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.
29. Kawamura, T., et al., *Low levels of productive HIV infection in Langerhans cell-like dendritic cells differentiated in the presence of TGF-beta1 and increased viral replication with CD40 ligand-induced maturation.* Eur J Immunol, 2001. **31**(2): p. 360-8.
30. Pion, M., et al., *APOBEC3G/3F mediates intrinsic resistance of monocyte-derived dendritic cells to HIV-1 infection.* J Exp Med, 2006. **203**(13): p. 2887-93.
31. Banchereau, J., et al., *Immunobiology of dendritic cells.* Annu Rev Immunol, 2000. **18**: p. 767-811.
32. Bonifaz, L., et al., *Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance.* J Exp Med, 2002. **196**(12): p. 1627-38.
33. Hawiger, D., et al., *Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo.* J Exp Med, 2001. **194**(6): p. 769-79.
34. Ito, T., et al., *Specialization, kinetics, and repertoire of type 1 interferon responses by human plasmacytoid predendritic cells.* Blood, 2006. **107**(6): p. 2423-31.
35. Beignon, A.S., et al., *Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions.* J Clin Invest, 2005. **115**(11): p. 3265-75.
36. Feldman, S., et al., *Decreased interferon-alpha production in HIV-infected patients correlates with numerical and functional deficiencies in circulating type 2 dendritic cell precursors.* Clin Immunol, 2001. **101**(2): p. 201-10.
37. Herbeuval, J.P., et al., *Differential expression of IFN-alpha and TRAIL/DR5 in lymphoid tissue of progressor versus nonprogressor HIV-1-infected patients.* Proc Natl Acad Sci U S A, 2006. **103**(18): p. 7000-5.
38. Boasso, A., et al., *HIV inhibits CD4+ T-cell proliferation by inducing indoleamine 2,3-dioxygenase in plasmacytoid dendritic cells.* Blood, 2007. **109**(8): p. 3351-9.

39. Giorgi, J.V., et al., *Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage.* J Infect Dis, 1999. **179**(4): p. 859-70.
40. Brenchley, J.M., et al., *Microbial translocation is a cause of systemic immune activation in chronic HIV infection.* Nat Med, 2006. **12**(12): p. 1365-71.
41. Saito, T. and M. Gale, Jr., *[RIG-I mediated hepatic innate immune signaling that controls HCV infection].* Uirusu, 2008. **58**(2): p. 105-15.
42. Stevenson, M., *HIV-1 pathogenesis.* Nat Med, 2003. **9**(7): p. 853-60.
43. Schmitz, J.E., et al., *Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes.* Science, 1999. **283**(5403): p. 857-60.
44. Koup, R.A., et al., *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.* J Virol, 1994. **68**(7): p. 4650-5.
45. Trachtenberg, E., et al., *Advantage of rare HLA supertype in HIV disease progression.* Nat Med, 2003. **9**(7): p. 928-35.
46. Yant, L.J., et al., *The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication.* J Virol, 2006. **80**(10): p. 5074-7.
47. Goulder, P.J. and D.I. Watkins, *HIV and SIV CTL escape: implications for vaccine design.* Nat Rev Immunol, 2004. **4**(8): p. 630-40.
48. Klenerman, P., Y. Wu, and R. Phillips, *HIV: current opinion in escapology.* Curr Opin Microbiol, 2002. **5**(4): p. 408-13.
49. Goulder, P.J., et al., *Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS.* Nat Med, 1997. **3**(2): p. 212-7.
50. Kelleher, A.D., et al., *Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses.* J Exp Med, 2001. **193**(3): p. 375-86.
51. Allen, T.M., et al., *Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection.* J Virol, 2004. **78**(13): p. 7069-78.

52. Leslie, A.J., et al., *HIV evolution: CTL escape mutation and reversion after transmission.* Nat Med, 2004. **10**(3): p. 282-9.
53. Casazza, J.P., et al., *Immunologic pressure within class I-restricted cognate human immunodeficiency virus epitopes during highly active antiretroviral therapy.* J Virol, 2005. **79**(6): p. 3653-63.
54. Brown, S.A., et al., *CD8+ T-cells: are they sufficient to prevent, contain or eradicate HIV-1 infection?* Curr Drug Targets Infect Disord, 2005. **5**(2): p. 113-9.
55. Kloetzel, P.M., *Antigen processing by the proteasome.* Nat Rev Mol Cell Biol, 2001. **2**(3): p. 179-87.
56. Yewdell, J.W., *Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses.* Immunity, 2006. **25**(4): p. 533-43.
57. Draenert, R., et al., *Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection.* J Exp Med, 2004. **199**(7): p. 905-15.
58. Milicic, A., et al., *CD8+ T cell epitope-flanking mutations disrupt proteasomal processing of HIV-1 Nef.* J Immunol, 2005. **175**(7): p. 4618-26.
59. Zimbwa, P., et al., *Precise identification of a human immunodeficiency virus type 1 antigen processing mutant.* J Virol, 2007. **81**(4): p. 2031-8.
60. Tenzer, S., et al., *Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance.* Nat Immunol, 2009. **10**(6): p. 636-46.
61. Choudhuri, K. and P.A. van der Merwe, *Molecular mechanisms involved in T cell receptor triggering.* Semin Immunol, 2007. **19**(4): p. 255-61.
62. Finke, J.H., et al., *Loss of T-cell receptor zeta chain and p56lck in T-cells infiltrating human renal cell carcinoma.* Cancer Res, 1993. **53**(23): p. 5613-6.
63. Mizoguchi, H., et al., *Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice.* Science, 1992. **258**(5089): p. 1795-8.
64. Trimble, L.A., et al., *Human immunodeficiency virus-specific circulating CD8 T lymphocytes have down-modulated CD3zeta and CD28, key signaling molecules for T-cell activation.* J Virol, 2000. **74**(16): p. 7320-30.
65. Wherry, E.J., et al., *Molecular signature of CD8+ T cell exhaustion during chronic viral infection.* Immunity, 2007. **27**(4): p. 670-84.

66. Lee, K.M., et al., *Molecular basis of T cell inactivation by CTLA-4.* Science, 1998. **282**(5397): p. 2263-6.

67. Parry, R.V., et al., *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Mol Cell Biol, 2005. **25**(21): p. 9543-53.

68. Mehandru, S., et al., *Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract.* J Exp Med, 2004. **200**(6): p. 761-70.

69. McCune, J.M., *The dynamics of CD4+ T-cell depletion in HIV disease.* Nature, 2001. **410**(6831): p. 974-9.

70. Pitcher, C.J., et al., *HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression.* Nat Med, 1999. **5**(5): p. 518-25.

71. Pantaleo, G. and R.A. Koup, *Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know.* Nat Med, 2004. **10**(8): p. 806-10.

72. McKay, P.F., et al., *Global dysfunction of CD4 T-lymphocyte cytokine expression in simian-human immunodeficiency virus/SIV-infected monkeys is prevented by vaccination.* J Virol, 2003. **77**(8): p. 4695-702.

73. Shedlock, D.J. and H. Shen, *Requirement for CD4 T cell help in generating functional CD8 T cell memory.* Science, 2003. **300**(5617): p. 337-9.

74. Sun, J.C. and M.J. Bevan, *Defective CD8 T cell memory following acute infection without CD4 T cell help.* Science, 2003. **300**(5617): p. 339-42.

75. Lichterfeld, M., et al., *Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells.* J Exp Med, 2004. **200**(6): p. 701-12.

76. Migueles, S.A., et al., *HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors.* Nat Immunol, 2002. **3**(11): p. 1061-8.

77. Zimmerli, S.C., et al., *HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells.* Proc Natl Acad Sci U S A, 2005. **102**(20): p. 7239-44.

78. Younes, S.A., et al., *HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity.* J Exp Med, 2003. **198**(12): p. 1909-22.

79. Ogg, G.S., et al., *Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA.* Science, 1998. **279**(5359): p. 2103-6.

80. Betts, M.R., et al., *Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection.* J Virol, 2001. **75**(24): p. 11983-91.

81. Kiepiela, P., et al., *CD8+ T-cell responses to different HIV proteins have discordant associations with viral load.* Nat Med, 2007. **13**(1): p. 46-53.

82. Dunham, R., et al., *The AIDS resistance of naturally SIV-infected sooty mangabeys is independent of cellular immunity to the virus.* Blood, 2006. **108**(1): p. 209-17.

83. Wang, Z., et al., *Th-1-type cytotoxic CD8+ T-lymphocyte responses to simian immunodeficiency virus (SIV) are a consistent feature of natural SIV infection in sooty mangabeys.* J Virol, 2006. **80**(6): p. 2771-83.

84. Walker, C.M., et al., *CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication.* Science, 1986. **234**(4783): p. 1563-6.

85. Price, D.A., et al., *Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection.* Proc Natl Acad Sci U S A, 1997. **94**(5): p. 1890-5.

86. Borrow, P., et al., *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.* J Virol, 1994. **68**(9): p. 6103-10.

87. Borrow, P., et al., *Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus.* Nat Med, 1997. **3**(2): p. 205-11.

88. Pantaleo, G., et al., *Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV.* Nature, 1994. **370**(6489): p. 463-7.

89. Zhang, D., et al., *Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic.* Blood, 2003. **101**(1): p. 226-35.

90. Appay, V., et al., *HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function.* J Exp Med, 2000. **192**(1): p. 63-75.

91. Andersson, J., et al., *Low levels of perforin expression in CD8+ T lymphocyte granules in lymphoid tissue during acute human immunodeficiency virus type 1 infection.* J Infect Dis, 2002. **185**(9): p. 1355-8.

92. Betts, M.R., et al., *The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration.* J Immunol, 2004. **172**(10): p. 6407-17.

93. Saez-Cirion, A., et al., *HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype.* Proc Natl Acad Sci U S A, 2007. **104**(16): p. 6776-81.

94. Shankar, P., et al., *Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection.* Blood, 2000. **96**(9): p. 3094-101.

95. Wherry, E.J., J.N. Blattman, and R. Ahmed, *Low CD8 T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination.* J Virol, 2005. **79**(14): p. 8960-8.

96. Wherry, E.J., et al., *Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment.* J Virol, 2003. **77**(8): p. 4911-27.

97. Vogel, T.U., et al., *Functional impairment of simian immunodeficiency virus-specific CD8+ T cells during the chronic phase of infection.* J Virol, 2001. **75**(5): p. 2458-61.

98. Xiong, Y., et al., *Simian immunodeficiency virus (SIV) infection of a rhesus macaque induces SIV-specific CD8(+) T cells with a defect in effector function that is reversible on extended interleukin-2 incubation.* J Virol, 2001. **75**(6): p. 3028-33.

99. Gruener, N.H., et al., *Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus.* J Virol, 2001. **75**(12): p. 5550-8.
100. Lee, P.P., et al., *Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients.* Nat Med, 1999. **5**(6): p. 677-85.
101. Schlaak, J.F., et al., *HBV-specific immune defect in chronic hepatitis B (CHB) is correlated with a dysregulation of pro- and anti-inflammatory cytokines.* Clin Exp Immunol, 1999. **115**(3): p. 508-14.
102. Wherry, E.J., et al., *Antigen-independent memory CD8 T cells do not develop during chronic viral infection.* Proc Natl Acad Sci U S A, 2004. **101**(45): p. 16004-9.
103. Zajac, A.J., et al., *Viral immune evasion due to persistence of activated T cells without effector function.* J Exp Med, 1998. **188**(12): p. 2205-13.
104. Kostense, S., et al., *Persistent numbers of tetramer+ CD8(+) T cells, but loss of interferon-gamma+ HIV-specific T cells during progression to AIDS.* Blood, 2002. **99**(7): p. 2505-11.
105. Lichterfeld, M., et al., *Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection.* J Virol, 2007. **81**(8): p. 4199-214.
106. Barber, D.L., et al., *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature, 2006. **439**(7077): p. 682-7.
107. Day, C.L., et al., *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.* Nature, 2006. **443**(7109): p. 350-4.
108. Petrovas, C., et al., *PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection.* J Exp Med, 2006. **203**(10): p. 2281-92.
109. Trautmann, L., et al., *Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction.* Nat Med, 2006. **12**(10): p. 1198-202.
110. Boni, C., et al., *Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection.* J Virol, 2007. **81**(8): p. 4215-25.
111. Radziewicz, H., et al., *Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression.* J Virol, 2007. **81**(6): p. 2545-53.

112. Urbani, S., et al., *PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion.* J Virol, 2006. **80**(22): p. 11398-403.

113. Darrah, P.A., et al., *Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major.* Nat Med, 2007. **13**(7): p. 843-50.

114. Betts, M.R., et al., *HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells.* Blood, 2006. **107**(12): p. 4781-9.

115. Almeida, J.R., et al., *Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover.* J Exp Med, 2007. **204**(10): p. 2473-85.

116. Duvall, M.G., et al., *Polyfunctional T cell responses are a hallmark of HIV-2 infection.* Eur J Immunol, 2008. **38**(2): p. 350-63.

117. Precopio, M.L., et al., *Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses.* J Exp Med, 2007. **204**(6): p. 1405-16.

118. Rehr, M., et al., *Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy.* J Virol, 2008. **82**(7): p. 3391-404.

119. Almeida, J.R., et al., *Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity.* Blood, 2009. **113**(25): p. 6351-60.

120. Wherry, E.J. and R. Ahmed, *Memory CD8 T-cell differentiation during viral infection.* J Virol, 2004. **78**(11): p. 5535-45.

121. Kaech, S.M., E.J. Wherry, and R. Ahmed, *Effector and memory T-cell differentiation: implications for vaccine development.* Nat Rev Immunol, 2002. **2**(4): p. 251-62.

122. Bourgeois, C., B. Rocha, and C. Tanchot, *A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory.* Science, 2002. **297**(5589): p. 2060-3.

123. Kaech, S.M., et al., *Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells.* Nat Immunol, 2003. **4**(12): p. 1191-8.

124. Madakamutil, L.T., et al., *CD8alphaalpha-mediated survival and differentiation of CD8 memory T cell precursors.* Science, 2004. **304**(5670): p. 590-3.
125. Lau, L.L., et al., *Cytotoxic T-cell memory without antigen.* Nature, 1994. **369**(6482): p. 648-52.
126. Murali-Krishna, K., et al., *Persistence of memory CD8 T cells in MHC class I-deficient mice.* Science, 1999. **286**(5443): p. 1377-81.
127. Tan, J.T., et al., *Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells.* J Exp Med, 2002. **195**(12): p. 1523-32.
128. Kennedy, M.K., et al., *Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice.* J Exp Med, 2000. **191**(5): p. 771-80.
129. Lodolce, J.P., et al., *IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation.* Immunity, 1998. **9**(5): p. 669-76.
130. Becker, T.C., et al., *Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells.* J Exp Med, 2002. **195**(12): p. 1541-8.
131. Kieper, W.C., et al., *Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells.* J Exp Med, 2002. **195**(12): p. 1533-9.
132. Maraskovsky, E., et al., *Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/- mice.* Cell, 1997. **89**(7): p. 1011-9.
133. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.
134. Appay, V., et al., *Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections.* Nat Med, 2002. **8**(4): p. 379-85.
135. Champagne, P., et al., *Skewed maturation of memory HIV-specific CD8 T lymphocytes.* Nature, 2001. **410**(6824): p. 106-11.
136. Halwani, R., et al., *Generation and maintenance of human memory cells during viral infection.* Springer Semin Immunopathol, 2006. **28**(3): p. 197-208.
137. Appay, V., et al., *Phenotype and function of human T lymphocyte subsets: consensus and issues.* Cytometry A, 2008. **73**(11): p. 975-83.

138. Wherry, E.J., et al., *Lineage relationship and protective immunity of memory CD8 T cell subsets.* Nat Immunol, 2003. **4**(3): p. 225-34.
139. Papagno, L., et al., *Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection.* PLoS Biol, 2004. **2**(2): p. E20.
140. Stemberger, C., et al., *A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets.* Immunity, 2007. **27**(6): p. 985-97.
141. Arens, R., et al., *Constitutive CD27/CD70 interaction induces expansion of effector-type T cells and results in IFNgamma-mediated B cell depletion.* Immunity, 2001. **15**(5): p. 801-12.
142. Gamadia, L.E., et al., *The size and phenotype of virus-specific T cell populations is determined by repetitive antigenic stimulation and environmental cytokines.* J Immunol, 2004. **172**(10): p. 6107-14.
143. Crotty, S., et al., *Cutting edge: long-term B cell memory in humans after smallpox vaccination.* J Immunol, 2003. **171**(10): p. 4969-73.
144. Van Epps, H.L., et al., *Long-lived memory T lymphocyte responses after hantavirus infection.* J Exp Med, 2002. **196**(5): p. 579-88.
145. Rickinson, A.B. and D.J. Moss, *Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection.* Annu Rev Immunol, 1997. **15**: p. 405-31.
146. Gillespie, G.M., et al., *Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors.* J Virol, 2000. **74**(17): p. 8140-50.
147. Chisari, F.V. and C. Ferrari, *Hepatitis B virus immunopathogenesis.* Annu Rev Immunol, 1995. **13**: p. 29-60.
148. McMichael, A.J. and S.L. Rowland-Jones, *Cellular immune responses to HIV.* Nature, 2001. **410**(6831): p. 980-7.
149. Xu, X.N., G.R. Screaton, and A.J. McMichael, *Virus infections: escape, resistance, and counterattack.* Immunity, 2001. **15**(6): p. 867-70.
150. von Herrath, M.G., et al., *Vaccination to treat persistent viral infection.* Virology, 2000. **268**(2): p. 411-9.

151. Hel, Z., et al., *Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques.* Nat Med, 2000. **6**(10): p. 1140-6.

152. Richards, C.M., et al., *Protection against recurrent ocular herpes simplex virus type 1 disease after therapeutic vaccination of latently infected mice.* J Virol, 2003. **77**(12): p. 6692-9.

153. Tryniszewska, E., et al., *Vaccination of macaques with long-standing SIVmac251 infection lowers the viral set point after cessation of antiretroviral therapy.* J Immunol, 2002. **169**(9): p. 5347-57.

154. Tussey, L.G., et al., *Antigen burden is major determinant of human immunodeficiency virus-specific CD8+ T cell maturation state: potential implications for therapeutic immunization.* J Infect Dis, 2003. **187**(3): p. 364-74.

155. Belkaid, Y., et al., *CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity.* Nature, 2002. **420**(6915): p. 502-7.

156. Zaph, C., et al., *Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites.* Nat Med, 2004. **10**(10): p. 1104-10.

157. Kaech, S.M. and E.J. Wherry, *Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection.* Immunity, 2007. **27**(3): p. 393-405.

158. Furtado, M.R., et al., *Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy.* N Engl J Med, 1999. **340**(21): p. 1614-22.

159. McCune, J.M., *Viral latency in HIV disease.* Cell, 1995. **82**(2): p. 183-8.

160. Cohen, G.B., et al., *The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells.* Immunity, 1999. **10**(6): p. 661-71.

161. Schwartz, O., et al., *Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein.* Nat Med, 1996. **2**(3): p. 338-42.

162. Seeger, M., et al., *HIV-1 tat inhibits the 20 S proteasome and its 11 S regulator-mediated activation.* J Biol Chem, 1997. **272**(13): p. 8145-8.

163. Bakhanashvili, M. and A. Hizi, *Fidelity of the RNA-dependent DNA synthesis exhibited by the reverse transcriptases of human immunodeficiency virus types 1 and 2 and of murine leukemia virus: mispair extension frequencies.* Biochemistry, 1992. **31**(39): p. 9393-8.

164. Coffin, J.M., *HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy.* Science, 1995. **267**(5197): p. 483-9.

165. Aebischer, T., et al., *In vitro selection of lymphocytic choriomeningitis virus escape mutants by cytotoxic T lymphocytes.* Proc Natl Acad Sci U S A, 1991. **88**(24): p. 11047-51.

166. Pircher, H., et al., *Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo.* Nature, 1990. **346**(6285): p. 629-33.

167. Evans, D.T., et al., *Rapid and slow progressors differ by a single MHC class I haplotype in a family of MHC-defined rhesus macaques infected with SIV.* Immunol Lett, 1999. **66**(1-3): p. 53-9.

168. Barouch, D.H., et al., *Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes.* Nature, 2002. **415**(6869): p. 335-9.

169. Goulder, P.J., et al., *Patterns of immunodominance in HIV-1-specific cytotoxic T lymphocyte responses in two human histocompatibility leukocyte antigens (HLA)-identical siblings with HLA-A*0201 are influenced by epitope mutation.* J Exp Med, 1997. **185**(8): p. 1423-33.

170. Winkler, C., et al., *Genetic restriction of AIDS pathogenesis by an SDF-1 chemokine gene variant. ALIVE Study, Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC).* Science, 1998. **279**(5349): p. 389-93.

171. Moore, C.B., et al., *Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level.* Science, 2002. **296**(5572): p. 1439-43.

172. Goulder, P.J., et al., *Evolution and transmission of stable CTL escape mutations in HIV infection.* Nature, 2001. **412**(6844): p. 334-8.

173. Friedrich, T.C., et al., *Reversion of CTL escape-variant immunodeficiency viruses in vivo.* Nat Med, 2004. **10**(3): p. 275-81.

174. Schneidewind, A., et al., *Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication.* J Virol, 2007. **81**(22): p. 12382-93.

175. Friedrich, T.C., et al., *Extraepitopic compensatory substitutions partially restore fitness to simian immunodeficiency virus variants that escape from an immunodominant cytotoxic-T-lymphocyte response.* J Virol, 2004. **78**(5): p. 2581-5.

176. Mothe, B.R., et al., *Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication.* J Virol, 2003. **77**(4): p. 2736-40.

177. Pal, R., et al., *ALVAC-SIV-gag-pol-env-based vaccination and macaque major histocompatibility complex class I (A*01) delay simian immunodeficiency virus SIVmac-induced immunodeficiency.* J Virol, 2002. **76**(1): p. 292-302.

178. Zhang, Z.Q., et al., *Mamu-A*01 allele-mediated attenuation of disease progression in simian-human immunodeficiency virus infection.* J Virol, 2002. **76**(24): p. 12845-54.

179. Altfeld, M., et al., *Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection.* AIDS, 2003. **17**(18): p. 2581-91.

180. Crawford, H., et al., *Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients.* J Exp Med, 2009. **206**(4): p. 909-21.

181. Matano, T., et al., *Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial.* J Exp Med, 2004. **199**(12): p. 1709-18.

182. O'Connor, D., et al., *Understanding cytotoxic T-lymphocyte escape during simian immunodeficiency virus infection.* Immunol Rev, 2001. **183**: p. 115-26.

183. Barouch, D.H., et al., *Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys.* J Virol, 2003. **77**(13): p. 7367-75.

184. Goulder, P.J., et al., *Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection.* J Exp Med, 2001. **193**(2): p. 181-94.

185. Brander, C., et al., *Lack of strong immune selection pressure by the immunodominant, HLA-A*0201-restricted cytotoxic T lymphocyte response in chronic human immunodeficiency virus-1 infection.* J Clin Invest, 1998. **101**(11): p. 2559-66.

186. Klenerman, P., et al., *Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants.* Nature, 1994. **369**(6479): p. 403-7.

187. Goulder, P.J. and P. Klenerman, *Cytotoxic T lymphocytes and viral adaptation in HIV infection.* Curr Opin HIV AIDS, 2006. **1**(3): p. 241-248.

188. McAdam, S., et al., *Immunogenic HIV variant peptides that bind to HLA-B8 can fail to stimulate cytotoxic T lymphocyte responses.* J Immunol, 1995. **155**(5): p. 2729-36.

189. Serwold, T., et al., *ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum.* Nature, 2002. **419**(6906): p. 480-3.

190. Lichterfeld, M., et al., *A viral CTL escape mutation leading to immunoglobulin-like transcript 4-mediated functional inhibition of myelomonocytic cells.* J Exp Med, 2007. **204**(12): p. 2813-24.

191. Leslie, A.J. and P.J. Goulder, *HIV escape and attenuation by cytotoxic T lymphocytes.* Curr Opin HIV AIDS, 2006. **1**(1): p. 34-39.

192. Friedrich, T.C., et al., *Subdominant CD8+ T-cell responses are involved in durable control of AIDS virus replication.* J Virol, 2007. **81**(7): p. 3465-76.

193. Murphy, K.P., et al., *Janeway's immunobiology*. 7th ed. 2008, New York: Garland Science. xxi, 887 p.

194. Kedzierska, K., et al., *Tracking phenotypically and functionally distinct T cell subsets via T cell repertoire diversity.* Mol Immunol, 2008. **45**(3): p. 607-18.

195. Turner, S.J., et al., *Structural determinants of T-cell receptor bias in immunity.* Nat Rev Immunol, 2006. **6**(12): p. 883-94.

196. Bousso, P., et al., *Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires.* Immunity, 1998. **9**(2): p. 169-78.

197. Arstila, T.P., et al., *A direct estimate of the human alphabeta T cell receptor diversity.* Science, 1999. **286**(5441): p. 958-61.

198. Correia-Neves, M., et al., *The shaping of the T cell repertoire.* Immunity, 2001. **14**(1): p. 21-32.

199. Maynard, J., et al., *Structure of an autoimmune T cell receptor complexed with class II peptide-MHC: insights into MHC bias and antigen specificity.* Immunity, 2005. **22**(1): p. 81-92.

200. Belz, G.T., P.G. Stevenson, and P.C. Doherty, *Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses.* J Immunol, 2000. **165**(5): p. 2404-9.

201. Turner, S.J., et al., *Analysis of clonotype distribution and persistence for an influenza virus-specific CD8+ T cell response.* Immunity, 2003. **18**(4): p. 549-59.

202. Turner, S.J., et al., *Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific CD8+ T cell populations.* Nat Immunol, 2005. **6**(4): p. 382-9.

203. Morrison, J., et al., *Identification of the nonamer peptide from influenza A matrix protein and the role of pockets of HLA-A2 in its recognition by cytotoxic T lymphocytes.* Eur J Immunol, 1992. **22**(4): p. 903-7.

204. Lehner, P.J., et al., *Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V beta 17 gene segment.* J Exp Med, 1995. **181**(1): p. 79-91.

205. Moss, P.A., et al., *Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 8987-90.

206. Stewart-Jones, G.B., et al., *A structural basis for immunodominant human T cell receptor recognition.* Nat Immunol, 2003. **4**(7): p. 657-63.

207. Rebai, N., et al., *Analysis of the T-cell receptor beta-chain variable-region (V beta) repertoire in monozygotic twins discordant for human immunodeficiency virus: evidence for perturbations of specific V beta segments in CD4+ T cells of the virus-positive twins.* Proc Natl Acad Sci U S A, 1994. **91**(4): p. 1529-33.

208. Pantaleo, G., et al., *Evidence for rapid disappearance of initially expanded HIV-specific CD8+ T cell clones during primary HIV infection.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9848-53.

209. Wilson, J.D., et al., *Oligoclonal expansions of CD8(+) T cells in chronic HIV infection are antigen specific.* J Exp Med, 1998. **188**(4): p. 785-90.

210. Soudeyns, H., et al., *Initiation of antiretroviral therapy during primary HIV-1 infection induces rapid stabilization of the T-cell receptor beta chain repertoire and reduces the level of T-cell oligoclonality.* Blood, 2000. **95**(5): p. 1743-51.

211. Gorochov, G., et al., *Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy.* Nat Med, 1998. **4**(2): p. 215-21.

212. Roglic, M., et al., *T cell receptor (TCR) BV gene repertoires and clonal expansions of CD4 cells in patients with HIV infections.* Clin Exp Immunol, 1997. **107**(1): p. 21-30.

213. Pantaleo, G., et al., *The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia.* Proc Natl Acad Sci U S A, 1997. **94**(1): p. 254-8.

214. Pantaleo, G. and A.S. Fauci, *New concepts in the immunopathogenesis of HIV infection.* Annu Rev Immunol, 1995. **13**: p. 487-512.

215. Gorochov, G., et al., *Down-regulation of CD8+ T-cell expansions in patients with human immunodeficiency virus infection receiving highly active combination therapy.* Blood, 2001. **97**(6): p. 1787-95.

216. Islam, S.A., et al., *Persistence of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte clones in a subject with rapid disease progression.* J Virol, 2001. **75**(10): p. 4907-11.

217. Dong, T., et al., *HIV-specific cytotoxic T cells from long-term survivors select a unique T cell receptor.* J Exp Med, 2004. **200**(12): p. 1547-57.

218. Meyer-Olson, D., et al., *Fluctuations of functionally distinct CD8+ T-cell clonotypes demonstrate flexibility of the HIV-specific TCR repertoire.* Blood, 2006. **107**(6): p. 2373-83.

219. Turnbull, E.L., et al., *HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently.* J Immunol, 2006. **176**(10): p. 6130-46.

220. Yu, X.G., et al., *Mutually exclusive T-cell receptor induction and differential susceptibility to human immunodeficiency virus type 1 mutational escape associated with a two-amino-acid difference between HLA class I subtypes.* J Virol, 2007. **81**(4): p. 1619-31.

221. Yu, X.G., et al., *Random T-cell receptor recruitment in human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells from genetically identical twins infected with the same HIV-1 strain.* J Virol, 2007. **81**(22): p. 12666-9.

222. Chen, Z.W., et al., *The TCR repertoire of an immunodominant CD8+ T lymphocyte population.* J Immunol, 2001. **166**(7): p. 4525-33.

223. Price, D.A., et al., *T cell receptor recognition motifs govern immune escape patterns in acute SIV infection.* Immunity, 2004. **21**(6): p. 793-803.

224. Horwitz, M.S., Y. Yanagi, and M.B. Oldstone, *T-cell receptors from virus-specific cytotoxic T lymphocytes recognizing a single immunodominant nine-amino-acid viral epitope show marked diversity.* J Virol, 1994. **68**(1): p. 352-7.

225. Whitton, J.L., et al., *Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity.* J Virol, 1989. **63**(10): p. 4303-10.

226. Sourdive, D.J., et al., *Conserved T cell receptor repertoire in primary and memory CD8 T cell responses to an acute viral infection.* J Exp Med, 1998. **188**(1): p. 71-82.

227. Lin, M.Y. and R.M. Welsh, *Stability and diversity of T cell receptor repertoire usage during lymphocytic choriomeningitis virus infection of mice.* J Exp Med, 1998. **188**(11): p. 1993-2005.

228. Blattman, J.N., et al., *Evolution of the T cell repertoire during primary, memory, and recall responses to viral infection.* J Immunol, 2000. **165**(11): p. 6081-90.

229. Cornberg, M., et al., *Narrowed TCR repertoire and viral escape as a consequence of heterologous immunity.* J Clin Invest, 2006. **116**(5): p. 1443-56.

230. Price, D.A., et al., *Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses.* J Exp Med, 2005. **202**(10): p. 1349-61.

231. Trautmann, L., et al., *Selection of T cell clones expressing high-affinity public TCRs within Human cytomegalovirus-specific CD8 T cell responses.* J Immunol, 2005. **175**(9): p. 6123-32.

232. Meyer-Olson, D., et al., *Limited T cell receptor diversity of HCV-specific T cell responses is associated with CTL escape.* J Exp Med, 2004. **200**(3): p. 307-19.

233. Callan, M.F., et al., *T cell selection during the evolution of CD8+ T cell memory in vivo.* Eur J Immunol, 1998. **28**(12): p. 4382-90.

234. Venturi, V., et al., *The molecular basis for public T-cell responses?* Nat Rev Immunol, 2008. **8**(3): p. 231-8.

235. Gillespie, G.M., et al., *Strong TCR conservation and altered T cell cross-reactivity characterize a B*57-restricted immune response in HIV-1 infection.* J Immunol, 2006. **177**(6): p. 3893-902.

236. Argaet, V.P., et al., *Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus.* J Exp Med, 1994. **180**(6): p. 2335-40.

237. La Gruta, N.L., S.J. Turner, and P.C. Doherty, *Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific CD8+ T cell responses: correlation of cytokine profile and TCR avidity.* J Immunol, 2004. **172**(9): p. 5553-60.

238. Price, D.A., et al., *Public clonotype usage identifies protective Gag-specific CD8+ T cell responses in SIV infection.* J Exp Med, 2009. **206**(4): p. 923-36.

239. Welsh, R.M., *Private specificities of heterologous immunity.* Curr Opin Immunol, 2006. **18**(3): p. 331-7.

240. Nikolich-Zugich, J., M.K. Slifka, and I. Messaoudi, *The many important facets of T-cell repertoire diversity.* Nat Rev Immunol, 2004. **4**(2): p. 123-32.

241. Yang, H., et al., *Broad TCR usage in functional HIV-1-specific CD8+ T cell expansions driven by vaccination during highly active antiretroviral therapy.* J Immunol, 2007. **179**(1): p. 597-606.

242. Kaufmann, D.E., et al., *Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction.* Nat Immunol, 2007. **8**(11): p. 1246-54.

243. Streeck, H., et al., *Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells.* PLoS Med, 2008. **5**(5): p. e100.
244. Schwartz, R.H., *T cell anergy.* Annu Rev Immunol, 2003. **21**: p. 305-34.
245. Macian, F., et al., *Transcriptional mechanisms underlying lymphocyte tolerance.* Cell, 2002. **109**(6): p. 719-31.
246. Blackburn, S.D., et al., *Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection.* Nat Immunol, 2009. **10**(1): p. 29-37.
247. Velu, V., et al., *Enhancing SIV-specific immunity in vivo by PD-1 blockade.* Nature, 2009. **458**(7235): p. 206-10.
248. Ha, S.J., et al., *Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection.* J Exp Med, 2008. **205**(3): p. 543-55.
249. Cecchinato, V., et al., *Immune activation driven by CTLA-4 blockade augments viral replication at mucosal sites in simian immunodeficiency virus infection.* J Immunol, 2008. **180**(8): p. 5439-47.
250. Blattman, J.N. and P.D. Greenberg, *PD-1 blockade: rescue from a near-death experience.* Nat Immunol, 2006. **7**(3): p. 227-8.
251. Almanzar, G., et al., *IFN-gamma production by CMV-specific CD8+ T cells is high in elderly donors.* Exp Gerontol, 2004. **39**(5): p. 863-5; author reply 867-8.
252. Makedonas, G. and M.R. Betts, *Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection.* Springer Semin Immunopathol, 2006. **28**(3): p. 209-19.
253. Blair, P.J., et al., *CTLA-4 ligation delivers a unique signal to resting human CD4 T cells that inhibits interleukin-2 secretion but allows Bcl-X(L) induction.* J Immunol, 1998. **160**(1): p. 12-5.
254. Dornadula, G., et al., *Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy.* JAMA, 1999. **282**(17): p. 1627-32.
255. Zhang, L., et al., *Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy.* N Engl J Med, 1999. **340**(21): p. 1605-13.

256. Petrovas, C., et al., *SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection.* Blood, 2007. **110**(3): p. 928-36.

257. Sauce, D., et al., *PD-1 expression on human CD8 T cells depends on both state of differentiation and activation status.* AIDS, 2007. **21**(15): p. 2005-13.

258. Estes, J.D., et al., *Early resolution of acute immune activation and induction of PD-1 in SIV-infected sooty mangabeys distinguishes nonpathogenic from pathogenic infection in rhesus macaques.* J Immunol, 2008. **180**(10): p. 6798-807.

259. Douek, D.C., et al., *A novel approach to the analysis of specificity, clonality, and frequency of HIV-specific T cell responses reveals a potential mechanism for control of viral escape.* J Immunol, 2002. **168**(6): p. 3099-104.

260. Kaufmann, D.E., et al., *Limited durability of viral control following treated acute HIV infection.* PLoS Med, 2004. **1**(2): p. e36.

261. Streeck, H., et al., *Immunological and virological impact of highly active antiretroviral therapy initiated during acute HIV-1 infection.* J Infect Dis, 2006. **194**(6): p. 734-9.

262. Altfeld, M., et al., *Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection.* J Exp Med, 2001. **193**(2): p. 169-80.

263. Bucy, R.P., et al., *Initial increase in blood CD4(+) lymphocytes after HIV antiretroviral therapy reflects redistribution from lymphoid tissues.* J Clin Invest, 1999. **103**(10): p. 1391-8.

264. Fleury, S., et al., *Long-term kinetics of T cell production in HIV-infected subjects treated with highly active antiretroviral therapy.* Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5393-8.

265. Popovic, M., et al., *Persistence of HIV-1 structural proteins and glycoproteins in lymph nodes of patients under highly active antiretroviral therapy.* Proc Natl Acad Sci U S A, 2005. **102**(41): p. 14807-12.

266. Smith, B.A., et al., *Persistence of infectious HIV on follicular dendritic cells.* J Immunol, 2001. **166**(1): p. 690-6.

267. Carcelain, G., P. Debre, and B. Autran, *Reconstitution of CD4+ T lymphocytes in HIV-infected individuals following antiretroviral therapy.* Curr Opin Immunol, 2001. **13**(4): p. 483-8.

268. Autran, B., et al., *Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease.* Science, 1997. **277**(5322): p. 112-6.

269. Scriba, T.J., et al., *HIV-1-specific CD4+ T lymphocyte turnover and activation increase upon viral rebound.* J Clin Invest, 2005. **115**(2): p. 443-50.

270. Charini, W.A., et al., *Clonally diverse CTL response to a dominant viral epitope recognizes potential epitope variants.* J Immunol, 2001. **167**(9): p. 4996-5003.

271. Snyder, J.T., et al., *Molecular mechanisms and biological significance of CTL avidity.* Curr HIV Res, 2003. **1**(3): p. 287-94.

272. Ho, D.D., *Time to hit HIV, early and hard.* N Engl J Med, 1995. **333**(7): p. 450-1.

273. Oxenius, A., et al., *Early highly active antiretroviral therapy for acute HIV-1 infection preserves immune function of CD8+ and CD4+ T lymphocytes.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3382-7.

274. Daucher, M., et al., *Virological outcome after structured interruption of antiretroviral therapy for human immunodeficiency virus infection is associated with the functional profile of virus-specific CD8+ T cells.* J Virol, 2008. **82**(8): p. 4102-14.

275. Derby, M., et al., *High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL.* J Immunol, 2001. **166**(3): p. 1690-7.

276. Bihl, F., et al., *Impact of HLA-B alleles, epitope binding affinity, functional avidity, and viral coinfection on the immunodominance of virus-specific CTL responses.* J Immunol, 2006. **176**(7): p. 4094-101.

277. Paiardini, M., et al., *Loss of CD127 expression defines an expansion of effector CD8+ T cells in HIV-infected individuals.* J Immunol, 2005. **174**(5): p. 2900-9.