Characterization of the HIV-specific repertoire of T lymphocytes and insight into CD8 T cell mediated immunologic memory

McGill University, Montréal

May 2002

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

Abstract

Persistent infections result in protracted confrontations between virus and host as immune responses curb viral dissemination, but during which immune responses are also modulated by the enduring presence of virus. Here, aspects of such interactions are first evidenced in a longitudinal study of the phenotypic and molecular evolution of the CD4 and CD8 T cell receptor (TCR) repertoires of HIV-infected pediatric subjects. Whereas Vβ-specific expansions were detected in both cellular compartments significantly more frequently in HIV-1 infected patients as compared to uninfected subjects born to infected mothers, specific TCR β-chain clonotypic over-representations and the persistence of expanded clones were only evidenced in the CD8 compartment, consistent with patterns of antigen-driven immune responses. Whereas a critical role for HIV-specific CD8 T cell-mediated immune responses in the control of HIV-1 infection has been established, this control is incomplete and gradually lost as disease progresses. Reports detailing altered function of HIV-specific CD8 T cells during the course of infection suggest that the evolution of these responses are differently modulated as compared to that of other chronic viral infections. The recent identification of distinct memory T cell subsets differing in functional properties and propensity to homeostatically migrate to secondary lymphoid organs, together with knowledge of the dual role of these organs in the orchestration of immune responses and as primary sites of HIV-1 replication, led us to study the differentiation and distribution of HIV-specific CD8 T cells within blood and lymph nodes in the context of chronic HIV-1 infection. Characterization of the phenotypic, functional and proliferative properties of antigen-specific CD8 T cell subsets allowed us to delineate a differentiation pathway based on the expression of CD45 isotype and CC-chemokine receptor 7 (CCR7), and undergone by T cells following encounter with cognate antigen. Comparative assessment of HIV- and cytomegalovirus-specific responses exhibited by dually-infected patients evidenced a skewed maturation of HIV-specific memory CD8 T lymphocytes and their altered distribution in blood and lymph nodes. These findings contribute to our understanding of immunologic memory, may be relevant to HIV-1 pathology and are pertinent to the design, as well as evaluation of therapeutic regimens and vaccination strategies.

Résumé

Lors d'une infection chronique, la confrontation soutenue entre le virus et le système immunitaire de l'hôte mène au contrôle de la dissémination virale d'une part, et à la modulation de la réponse immunitaire d'autre part. Un premier aspect d'une telle interaction entre virus et hôte est ici mis en évidence dans une étude longitudinale des caractéristiques phénotypiques et moléculaires du répertoire de récepteurs de cellules T (RcT) de lymphocytes CD4 et CD8 d'enfants infectés par le virus d'immunodéficience humaine (VIH). Bien que des expansions Vβ-spécifiques soient observées dans chaque compartiment, les amplifications persistantes et spécifiquement clonotypiques, caractéristiques d'expansions antigènes-spécifiques, ne sont observées que parmi les cellules CD8. En dépit d'un rôle important attribué à la réponse T CD8 pour contrôler l'infection, ce contrôle est incomplet et diminue graduellement au cours de la progression de la maladie. Des évidences suggérant que la fonction des cellules T CD8 spécifiques du VIH soit altérée suggère que l'évolution d'une telle réponse soit modulée différemment de celle caractéristique d'autres infections chroniques. L'identification de sous-populations distinctes de cellules T mémoires différenciées par leurs propriétés fonctionnelles et par leur ciblage différentiel aux organes lymphoïdes secondaires nous a incité à étudier la différentiation et la distribution de cellules T CD8 spécifiques chez des patients en infection chronique. Ainsi, l'étude des propriétés phénotypiques, fonctionnelles et de la capacité à proliférer des cellules T CD8 spécifiques d'antigènes viraux nous a permis d'établir un cheminement de différentiation de cellules T mémoires enclenché suite à une rencontre avec leur antigène et basé sur l'expression d'isotypes CD45 et du récepteur de chémokines CCR7. L'étude de la distribution de cellules T CD8 spécifiques du VIH et du cytomégalovirus chez les patients co-infectés a de plus permis de mettre en évidence un biais de maturation des cellules T CD8 mémoire spécifiques du VIH dans le sang, ainsi que d'observer que la distribution de cellules mémoires VIH-spécifiques des ganglions lymphoïdes est similaire à celle du sang. Ces travaux contribuent à améliorer la compréhension de la mémoire immunitaire et pourraient être pertinents à l'étude de la pathogénese du VIH, ainsi qu'au développement de thérapies et de vaccins.

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Contributors

Chapter 2: Transient T cell receptor beta-chain variable region-specific expansions of $CD4^+$ and $CD8^+$ T cells during the early phase of pediatric human immunodeficiency virus infection: characterization of expanded cell populations by T cell receptor phenotyping. Soudeyns,H., Champagne,P., Holloway,C.L., Silvestri,G.U., Ringuette,N., Samson,J., Lapointe,N., and Sekaly,R.P. J. Infect. Dis. *181*, 107-120.

R.P. Sékaly and H. Soudeyns originally conceived this project. J. Samson and N. Lapointe initiated and coordinated the cohort of pediatric subjects characterized in this study. N. Ringuette and G. Silvestri performed the flow-cytometry analyses therein. Along with H. Soudeyns and C.L. Holloway, P. Champagne performed the molecular cloning of Vβ chains from Child 6 and Child 9. H. Soudeyns, C.L. Holloway and P. Champagne performed the original analysis of data. H. Soudeyns, R.P. Sékaly, and P. Champagne primarily contributed to the interpretation of the results. H. Soudeyns, R.P-Sékaly, and P. Champagne wrote the article and made the figures.

Chapter 3: Skewed maturation of memory HIV-specific CD8 T lymphocytes. Champagne,P., Ogg,G.S., King,A.S., Knabenhans,C., Ellefsen,K., Nobile,M., Appay,V., Rizzardi,G.P., Fleury,S., Lipp,M., Forster,R., Rowland-Jones,S., Sekaly,R.P., McMichael,A.J., and Pantaleo,G. Nature *410*, 106-111.

The cohort of patients studied studied was instigated and coordinated by G. Pantaleo and G.P. Rizzardi. G. Pantaleo, G.S. Ogg and A.J. McMichael were originally involved in a collaboration for the characterization of HIV-specific CD8 T cells in the cohort of patients that was eventually characterized, in-depth by P. Champagne in this publication. In the context of this original collaboration, G.S. Ogg and A.S. King had previously screened the patients of the cohorts characterized herein by ELISpot for

HIV-specific reactivities; thus, *de facto*, they identified the pool of patients on which the ensuing characterization was performed.

G.S. Ogg and A.J. McMichael provided the plasmids encoding for HLA-B7 and -B8 used by K. Ellefsen and P. Champagne for tetramer production. Tetramer production was originally set up and optimized in our laboratory by P. Champagne, on the basis of an original protocol and HLA-A2-encoding plasmid kindly provided by M.M. Davis, and β2m-encoding plasmid provided by T. Jardetzky. G.S. Ogg and A.S. King provided an original aliquot of HIV-specific tetramers used in preliminary experiments; K. Ellefsen and P. Champagne produced all other tetrameric molecules used. R. Förster and M. Lipp provided the anti-CCR7 antibody.

A.S. King and P. Champagne performed the original cell surface phenotyping of HIV-specific CD8 T cells. M. Nobile and S. Fleury performed preliminary experiments that provided optimal conditions for CFSE-tracking experiments, as well as the *in vitro* stimulation of sorted cells and Ki-67 analyses, respectively. P. Champagne used these conditions for the experiments published herein. C. Knabenhans performed the cell sorting and trained P. Champagne on flow cytometry. V. Appay and S. Rowland-Jones had previously optimized intracellular cytokine measurement (ICM) assays: V. Appay and P. Champagne performed the ICM experiments published herein.

G. Pantaleo and P. Champagne made the original set of observations from which this publication stemmed. G. Pantaleo and P. Champagne originally designed the experimental course followed, with advice from R.P. Sékaly, G.S. Ogg and A.J. McMichael. All data in this publication were originally analyzed by P. Champagne, and interpreted by G. Pantaleo and P. Champagne. G.P. Rizzardi and P. Champagne performed statistical analyses. R.P. Sékaly, G.S. Ogg and A.J. McMichael contributed to refining aspects of data interpretation. G. Pantaleo and P. Champagne conceived the figures and wrote the article.

Chapter 4: Distribution and functional analysis of memory antiviral CD8 T cell responses in HIV-1 and cytomegalovirus infections. Kim Ellefsen, Alexandre Harari, Patrick Champagne, Pierre-Alexandre Bart, Rafick-Pierre Sékaly, Giuseppe Pantaleo. European Journal of Immunology *32*: 3756-3764.

The study was supervised by G. Pantaleo and instigated following observations by P. Champagne. The cohort studied is the same as in Chapter 3, now followed by P.-A. Bart. K. Ellefsen and P. Champagne produced the tetrameric molecules used therein. K. Ellefsen and P. Champagne performed the cell surface stainings, flow-cytometric analysis, phenotypic characterizations and distribution analyses. K. Ellefsen and A. Harari performed the functional assays. The data presented were analyzed by K. Ellefsen, A. Harari, G. Pantaleo and P. Champagne.

Used, in part, throughout the introduction and discussion: Learning to remember: generation and maintenance of T-cell memory. Champagne, P., Dumont, A. R., and Sekaly, R. P. DNA Cell Biol. 20[12].

Although this review article is not integrally incorporated in this thesis, passages from it were used to develop the thesis introduction, as well as part of the discussion. This review article is the result of extensive brainstorming between the three authors, hypotheses and models having been developed through consensus and contribution of all involved. A. Dumont and P. Champagne made the figures and the article was jointly written.

Editing of the thesis: I wish to acknowledge and thank Sylwia Wasiak, Carmen Estrela, Gaëlle Breton, Alain Dumont, Rémi Cheynier, and Rafick-Pierre Sékaly for editorial help and comments.

Acknowledgements

I am indebted to many individuals who have helped, influenced and guided me throughout my years of doctoral studies. Without them, this thesis would not be.

I first wish to express my most sincere gratitude to my mentors, Rafick-Pierre Sékaly and Giuseppe Pantaleo. Rafick: thank you for teaching me scientific rigor and critical thinking, for pushing me to surpass myself and for your constant support. Gepi: thank you foremost for teaching me focus and drive. Thank you both for your trust and for the countless opportunities you have offered me for achievement and success. The two of you have done more than just supervise my training: you have taught me about science and taught me about research, but most of all, you have taught me about life.

I am also grateful to many colleagues, now friends, who have accompanied me during my training at the Laboratoire d'Immunologie, in Montréal and at the Laboratoire d'Immunopathologie du Sida, in Lausanne. In a relay throughout the years, you have each taught me by your strengths and example. In particular, and chronologically as our paths crossed, I wish to express my most sincere thanks to Eric Rhéaume, Pascal Lavoie, Hugo Soudeyns, Alain Dumont, Kim Ellefsen, and Alexandre Harari. In particular, Eric: for welcoming me into research and teaching me much. Kim and Pascal: for many stimulating discussions, for being there in time of need, and for inspiration. Hugo, Alain and Alex: for many thoughtful conversations and many a laugh had together. To my other lab-mates and collaborators: thanks for the discussions, for what you have taught me and for all you have contributed to my doctoral training experience, both in and out of the lab.

On a personal note, I wish to thank those dearest to me. Sylwia, je te savais toujours à mes cotés; tu m'as inspiré: grace à toi, j'ai réussi. Lynn, tu m'as toujours encouragé, sans compromis; c'est aussi grace à toi. Finalement, à mes parents, Monique et Pierre: ma réussite sera aussi toujours la vôtre. Merci pour tout ce que vous avez fait et pour tout ce que vous m'avez donné: mes efforts vous sont dédiés.

1. Introduction

1.1. The immune system

1.1.1. General consideration of health and disease: on the immune system, native and acquired immunity

Health results from an organism's ability to adapt and maintain the internal homeostatic balance it requires for life when challenged by the various endogenous and exogenous stimuli it encounters. A delicate balance between health and disease results from the ongoing, complex and dynamic interactions taking place between pathogens and host organism. Classically in this context, immunity refers to the host's ability to protect itself from colonization by foreign entities, such as microorganisms, and to preserve its integrity. In order to achieve this purpose, the human body has developed multiple lines of defense in the face of its environment.

The human body's first lines of defense against microorganisms are the properties of its surfaces exposed to the external environment[1]. These properties include the integrity of skin tissue, as well as desquamation. Exocrine secretions such as those of sweat, sebaceous, lacrymal and pyloric glands further protect against microorganisms, in part due to proteins that they contain (ex. lysozyme, lactoferrin) and/or to their extreme acidity or alkalinity. Secretion of viscous mucus by mucosal surfaces, as well as its removal by ciliary action and/or peristalsis further protects internal body surfaces such as those of the respiratory and gastrointestinal tracts. Furthermore, the body's normal flora of commensal microorganisms limits the body surface available for colonization by pathogenic organisms[2].

Beyond these first barriers, the concerted action of various molecules, as well as tissue-resident, blood and lymph-circulating cells complete the mechanisms of immunity through what are referred to as the native (or innate) and acquired (or specific) branches of the immune system[1]. The key characteristics of natural immunity are that it is present prior to any assault and that its response is not qualitatively or quantitatively enhanced by

previous exposure to the stimulating agent[1]. Molecular mediators of the innate immune system include histamine, leukotrienes, chemokines and the class I interferons, as well as the components of the alternative complement cascade[1]. Cells contributing to the innate branch of the immune system include eosinophils that respond primarily to parasitic infections, basophils that contribute to hypersensitivity reactions; and the phagocytic neutrophils (polymorphonuclear leukocytes): together, these cell types are known as the granulocytes[3]. Natural Killer (NK) cells are also cellular mediators of native immunity. NK cells' cytocidal activity against some tumour or virally-infected cells that have down-regulated surface class I major histocompatibility complex (MHC) expression is due to the breach of a regulatory negative-signal otherwise provided by NKR/KIR receptors when normally engaged by constant determinants of the MHC[4-6].

Many of the molecular determinants stimulating the native immune response are known as pathogen-associated molecular patterns (PAMPs)[7]. Because they serve essential physiological functions for the microorganism, PAMPs are often conserved and it is now becoming clear that the native immune system has in fact evolved mechanisms to detect them through the expression of a diversity of invariant receptors. The specificity with which these often unrelated determinants are recognized is remarkable. PAMPs include the double stranded RNA which characterizes some viral life cycle-intermediates, the lipopolysaccharide (LPS) component of the cell wall of Gram-negative bacteria, flagellin, as well as unmethylated bacterial DNA motifs; which have respectively been demonstrated to be specifically bound by Toll-like receptor (TLR)–3, TLR-4, TLR-5, and TLR-9[7,8].

The effectiveness of the immune system hinges on the coordinated interaction between its native and acquired branches[1,9]. Macrophages and dendritic cells form the key cellular interface to this purpose. These two cellular types express TLRs and are activated by PAMPs. Furthermore, they are critical components of the native immune system because they phagocytose foreign particles and mediate the intracellular destruction of invading microorganisms. Additionally, macrophages and dendritic cells are specialized in order to efficiently induce acquired immunity[3,10-13].

Acquired immunity results mainly from the action of B-cells and of T- lymphocytes, which respectively mediate what are historically referred to as the humoural and cellular immune responses[1,9]. B-lymphocytes are activated by encounter with particulate antigens that can be found in solution, in their native form, and in their natural context within proteins. In contrast, in the majority of cases, T-cells recognize protein-derived antigenic determinants that have undergone variable degrees of antigen-processing and that are presented in the context of major histocompatibility complexes (MHC)[9].

1.1.2. T cell subsets: division of labour and functional complementarity

B lymphocyte-mediated effector functions target exclusively extracellular antigen, and a first subtype of T cells, which expresses the γδ T cell receptor (TCR), recognizes a largely unspecified variety of determinants, including carbohydrates and proteins[14,15]. The T cells specifically characterized in the studies detailed herein express αβ TCRs and exclusively recognize endogenously derived peptides and exogenous protein derivatives presented in the context of MHC[16].

Canonically, αβ T lymphocytes express either of a CD4 or CD8 coreceptor on their surface and specialize for discrete function. CD4-expressing T lymphocytes mainly orchestrate T-dependent B-cell responses and provide auxiliary coordination of the CD8 response: they primarily act through providing regulatory signals by secreting cytokine mediators and by regulating their expression of membrane-bound co-stimulatory molecules. Accordingly, they are referred to as the "helper" subtype, although some of the cytokines they secrete have direct effector properties[3,17]. Distinctly, "cytotoxic" CD8 T lymphocytes act as direct effectors targeting and destroying cells that are infected by intracellular pathogen or differ significantly from the normal host cells. The CD8 T cell's arsenal for action also includes cytokines and membrane-bound effector molecules, but also lytic enzymes, which they produce and secrete in a polarized manner upon stimulation by infected, tumour, or foreign cells[3].

A common approach has been adopted in order to define what is perceived as normal and host-derived (self), as opposed to what is recognized as modified, foreign (or non-self), by αβ T lymphocytes of either subset. The strategy is bi-partite. First, for both cell types, antigen is presented in the context of a selection of specialized cell-surface molecules, which are highly polymorphic in the human population and which serve to operationally distinguish "self" from "foreign": these are the major histocompatibility molecules (MHC) molecules of class I and class II[18,19]. Second, a selection of all αβ T cells takes place in the thymus, whereby T lymphocytes are positively selected to recognize self-MHC and negatively selected in order not to respond to normal host-derived peptides which self-MHC present[20-22]. This selection leads to the elimination of T cells that would have otherwise engendered autoimmunity, and provides a mechanism for T cells to reject allogeneic tissue grafts that express non-self MHC, recognize tumour cells that present a range of proteins not normally expressed by self-MHC, and target pathogen-infected cells.

1.1.3. Antigen processing and presentation

Distinct mature CD4 and CD8 T lymphocyte compartments have specialized as sentinels of discrete extracellular and intracellular environments, respectively. Consequently, independent strategies have been adopted in order to allow T lymphocytes to separately sample both of these compartments within the human body. CD4 T cells recognize their antigenic determinants in the context of class II MHC, whereas CD8 T lymphocytes recognize antigen presented in the context of class I MHC[23-25].

The metabolic pathway leading to class II MHC cell-surface expression is designed to select peptide fragments of antigen that is predominantly exogenous to the presenting cell for display to CD4 T lymphocytes[26,27]. The class II MHC consists of a heterodimer of α and β polypeptide chains that are stabilized by interaction with processed peptides, which they affix in the membrane-distal groove they combine to form[28-32]. In humans, MHC class II molecules are encoded within the human leukocyte antigen (HLA) region on chromosome 6p21.3, at three distinct loci: DR, DP and DQ. Each gene locus consists of

functional α and β chains, the latter of which exhibits the most highly intra-species degree of polymorphism[33]. For each individual, both alleles of each locus are expressed in a co-dominant manner on a subset of cells that includes monocytes, macrophages, B-lymphocytes, dendritic cells and some thymic endothelial cells. Furthermore, class II MHC expression can be upregulated on T lymphocytes and somatic cells following activation or exposure to certain cytokines[34].

Class II MHC are synthesized within the endoplasmic reticulum (ER) where they form heterodimers and are found in association with a trimer consisting of any of a number of splice-variants of the type-II membrane-bound and non-polymorphic invariant chain (Ii) protein[35]. Particularly, a segment of the Ii chaperone known as the Class II-associated invariant chain peptide (CLIP) binds into the peptide-binding groove of the class II MHC[36-45]. This results in the effective exclusion of the myriad of peptides otherwise derived from endogenous protein synthesis and cytoplasmic degradation, from being affixed to class II MHC as the αβIi complex traffics through the ER, trans-Golgi network and into the endosomal compartment[46-49]. Transit of class II MHC through the early and late endosomal compartments is associated with the gradual carboxy-terminal cleavage of the Ii that eventually only leaves CLIP. CLIP is eventually catalytically removed with the contribution of HLA-DM, and replaced by peptides derived from proteins that were ingested into the endosomal compartment by internalization or phagocytosis, and subsequently partially processed by various lysosomal proteases[50-56]. Class II MHC binds peptides of variable length on the basis of specific interactions between the pockets formed within its groove and the peptide's amino acid carbon backbone[57-59,59-63]. The peptide-loaded class II MHC then traffic to the cell surface where they are free to interact with T cells. The interaction of class II with T lymphocytes takes place through contact with the T cell receptor and binding of the lymphocytes' CD4 co-receptor to both class II MHC chains[23,24,64-67].

The presentation of endogenously derived epitopes by class I MHC is the result of a distinct processing pathway. Class I MHC is a heterodimeric cell surface molecule, constituted by the non-covalent interaction of the monomorphic β2-microglobulin (β2m) chain with a highly polymorphic membrane-bound class I MHC α "heavy" chain and

complexed with a short peptide, which generally does not exceed 9-10 amino acids in length[68];[69];[70];[59]. In humans, the class I α chains are also encoded on the short arm of chromosome 6 at three distinct loci: HLA-A, -B, and –C[33]. As for class II MHC, both class I MHC α chain alleles of each locus are co-dominantly expressed. Class I MHC is present on the surface of virtually all nucleated somatic cells and its expression is further regulated by cytokines[34].

The nascent class I heavy chain expressed in the lumen of the ER, is first found bound to calnexin, a chaperone[71-73]. Upon association of the heavy and β2m chains, calnexin is released and the heterodimeric class I MHC is bound by other chaperones, namely calreticulin and most importantly, tapasin[74,75]. Tapasin catalyses the loading of peptides into the class I MHC groove by promoting the MHC's interaction with the transporter associated with antigen processing (TAP)-1 and TAP-2 proteins at the ER membrane[76,77]. TAP-1 and 2 belong to the family of ATP-binding cassette (ABC) transporters and form a heterodimer that translocates peptides from the cytoplasm to the ER lumen[78-82].

The peptides that are ultimately presented by class I MHC at the cell surface are the result of extensive processing by the intracellular machinery: their strict selection reflects the discriminating molecular bias introduced by a variety of intervening elements. The following are notable:

i. The assortment of intracytoplasmic proteins. Peptides may be loaded onto class I MHC by alternative routes than the one described above, as is probably the case when soluble antigenic peptides are exogenously added to cells in many *in vitro* stimulation protocols[83-85]. Nevertheless, the major physiological mechanism producing class I epitopes is the ubiquitinylation-targeted degradation of recycled and *de novo*-synthesized host- or intracellular pathogen-derived proteins[86]. The variety of these proteins thus constitutes the diversity of the recruitment pool from which the selection of class I MHC peptides begins[87].

ii. Proteosomal processing and the immunomodulation of preoteosomal activity. The ubiquitinylation of proteins targets them for degradation by the 26S proteosome[88,89]. The 26S proteosome consists of regulatory subunits associated with the 20S catalytic proteosome, a cylindrical structure of four stacked rings composed of multiple α-type and β-type subunits, which define proteosomal function, processivity and specificity[90]. Altogether, the proteolytic activities, which are conducted by β-subunits, exhibit trypsin-like, chymotrypsin-like, as well as peptidyl-glutamylpeptide-hydrolysing (PGPH) specificities; and is a carboxy-terminal protease[90]. In most cells, immune activation, and more specifically the action of interferon-γ, results in the production of three inducible subunits that replace the constitutive catalytic β-type proteosome components low molecular weight protein (LMP)-9, -17, and –19 in the newly synthesized proteosomes[87]. Two of these immune response-induced units, namely LMP-2 and –7, are encoded by the HLA locus while the third, multicatalytic endopeptidase complex-like (MECL)-1, is not[33]. The resulting modified proteosome is called the immunoproteosome. Furthermore, immune activation also results in modifying the induction of new regulatory subunits, the proteosome-associated activator complex (PA)-28[90]. The end result is presently thought to be an enhanced productivity, and perhaps a change in the catalytic specificity, though this is an area of ongoing debate[91-100]. Interestingly, it has recently been demonstrated that mature dendritic cells express the immunoproteosome and may thus constitutively present a divergent set of antigen[94,101].

iii. Other protease activities. Other cytosolic protease activities are relevant to peptide presentation by MHC, especially to the amino-terminal trimming of peptides[102]. Furthermore, intra-lumenal ER resident proteases also contribute to the N-terminal trimming of peptides prior to and following their binding to the MHC peptide-binding groove[103-105].

iv.TAP polymorphism. TAP exhibits preferences with regards to the size of the peptides it transports, and in some instances, with regards to the sequence of the peptides it translocates[106-113]. Given the confirmed polymorphism of TAP within the human population, a role in peptide selection has been postulated[114-116]. Furthermore, TAP processivity is enhanced in the context of immune activation[117].

v.MHC polymorphism. Class I MHC is highly polymorphic within the human population[118-125]. While individuals who are homozygous at all class I loci nevertheless express three different MHCs, fully heterozygous individuals have six. Class I polymorphism is evident throughout the sequence of the heavy chain, including some of the amino acid residues known to contact the T cell receptor, but it is particularly salient at residue positions that contact the peptide. The affinity of a peptide for a class I molecule mostly results from interactions taking place between the side-chains of one or two key peptide residues, referred to as the anchor residues, and the MHC atom pockets that accommodate them[121]. The peptide sequence is therefore critical to its binding to a receptor, and MHCs exhibit charted preferences for particular amino acids at fixed positions[59,126-129]. For example, class I HLA-A0201 is known to preferably bind peptide nine-mers with the sequence X[LM]XXXXXX[LV], where no restraints are placed for the X-marked positions[59]. The properties and position of anchor residues vary extensively between alleles.

vi.Structure of the MHC peptide-binding groove. Several class I MHC structures have been defined by X-ray crystallography and confirm a tight binding of the complex with its associated peptides. The class I α chain is characterized by three globular domains resulting from intra-chain disulfide bridges. β2m non-covalently associates with the membrane-proximal α3 domain and with the floor of the α1 domain[119,130]. The peptide-binding groove is formed through the contribution of both α1 and α2 domains of the heavy chain and physical

constraints for peptide size are imposed by the groove, which is bordered laterally by α-helices of each of the α1 and α2 domains. [120,131].

Class I MHC molecules to which an appropriate peptide has bound with sufficient affinity is stabilized by the interaction. It then transits through the Golgi apparatus and to the cell membrane by the default secretory pathway[87]. As for class II MHC, the key interaction of the peptide-class I MHC is with the T cell receptor of circulating lymphocytes. Distinctly, class I MHC interacts with the CD8 co-receptor molecule, which binds conserved amino acids of the α3 region near residue positions 222-229 of the heavy chain[132,133].

Thus, a broad diversity of potentially antigenic determinants is presented to T cells by both class II and class I MHC. As will be further discussed, the involvement of the CD4 and CD8 molecules to the process of T cell engagement is critical, in part for the affinity which they contribute to the interaction of MHC with TCR, but also because of their contribution to the initiation of the TCR- induced signal through their association with p56Lck, a protein tyrosine kinase of the src family[134]. Nevertheless, the T cell receptor is the central element mediating the recognition of the plethora of peptide-MHC combinations, and discriminating which will be tolerated from which will be targeted by an antigen-specific immune response.

1.1.4. Genetics of the T cell receptor

The T cell receptor (TCR) is a heterodimeric cell surface molecule that mediates the recognition of antigen-MHC complexes by T lymphocytes. Two distinct types of TCRs have been identified; thus TCRs either consist of an assembly of γ and δ, or of α and β TCR chains[135]. As a result of the molecular processes that give rise to each individual TCR chain, the expression of a γδ or αβ TCR by T lymphocytes is mutually exclusive. TCR chains result from the somatic rearrangement of multiple germ-line gene segments within each of three distinct chromosomal TCR loci: α/δ, β, and γ. In the human genome, the TCR loci are located at 14q11 (TCR α/δ) and on either arm of chromosome 7, at 7q35

(TCRβ) and 7p15 (TCRγ): the genomic organization of these loci is illustrated in figure 1[136]. Analysis of the nucleotide sequence of the genome regions encompassing the TCR loci reveals that the TCR's constituting elements are spread over hundreds of kilobases of DNA. Moreover, the genomic organization of the combined α/δ locus is of particular interest as some of the constituting elements of α and δ chains are shared, and because much of the δ polypeptide-encoding germ-line segments are deleted from the chromosome upon rearrangement of a αTCR chain.

Within each TCR locus, several distinct elements are thus rearranged and joined during T cell differentiation. These elements consist of the multiple variable (V), diversity (D), joining (J) and constant (C) β regions that can be assembled and encode for mature TCRβ chains, and of the Vα and Jα regions that can be joined to determine the rearranged TCR α chains. The multiple non-contiguous segments encoding for each subfamily member of the V, (D), J, and C TCR segments are clustered in arrays and interspersed with sequences representing pseudogenes and gene relics, as well as sequences encoding for proteins unrelated to the TCR (figure 1)[136,137].

The TCRβ sub-genome is spread over 685-kilobases of DNA and its analysis has revealed 65 different Vβ gene segments, as wells as 2 Dβ, 13 Jβ and 2 Cβ segments[137]. All but one of these 82 gene segments is oriented in the same direction: 64 of the Vβ elements are encoded throughout near 600 kilobases of the 5' portion of the TCRβ locus, the 65th is found in reversed orientation at the 3' extremity of the locus. 19 of the Vβ segments represent pseudogenes, while 46 are functional Vβ segments which can be divided into 26 Vβ families that share more than 75% sequence homology. The Dβ-, Jβ-, and Cβ-encoding gene segments are organized in separate clusters consisting respectively of Dβ1, Jβ1.1-1.6, and Cβ1, followed by Dβ2, Jβ2.1-2.7, and Cβ2. Genes encoding for the TCRα polypeptide include more than 50 Vα and some 60 Jα segments, followed by a single Cα gene fragment[136,138].

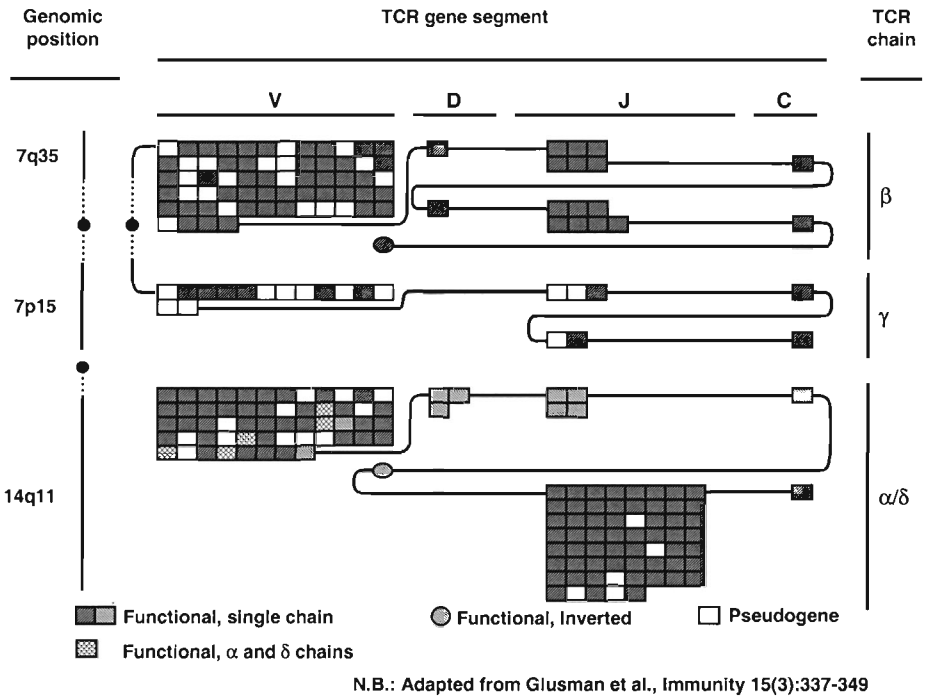

Figure 1. The TCR gene loci. *The TCRβ and γ loci are located on chromosome 7, respectively at q35 and p15. The δ-chain encoding segments are located within the TCRα chain locus, hence this locus is denoted as the α/δ locus. The gene segments are depicted as classified into V, (D), J and C families and in the sequence in which they are encoded on their respective loci. Pseudogenes are illustrated by white boxes and interspersed between functional gene segments, illustrated by barred boxes. Several V gene segments of the α/δ locus are used for rearrangements of both α and δ chains, and are illustrated with crossed boxes. Single functional Vβ and Vδ gene segments are encoded after their respective C gene segments, in an inversed orientation as compared to the remainder of their respective loci.*

Many TCR segments are hypothesized to be the result of the duplication of ancestral genes, followed by the diversification of the gene segments. Indeed, the gene segments present within the TCR loci are highly polymorphic. Nevertheless, stringent selection appears to preserve the integrity of TCR loci, as is evident from the significantly lower frequency of pseudogene insertions observed therein as compared to that observed in adjacent regions of the chromosome: the density within the human and mouse TCRα loci is of 0.67 pseudogenes per 100kb, as compared to 2.8 in the olfactory receptors gene locus that precedes the TCRα[136].

1.1.5. Recombination of the TCR loci

The TCR somatic recombination process is analogous to that which characterizes the immunoglobulin heavy and light chain loci of B lymphocytes, the end-result of which is the generation of a diverse repertoire of recognition units possessing specificity for a broad diversity of determinants[139-141]. Issued from myeloid precursors, immature T lymphocytes rearrange their TCR loci as they undergo maturation in the thymus[142-146].

A first recombination event at the β gene locus results in the fusion of a single D with a selected J segment[142]. This is followed by the recombination of a Vβ region-encoding segment with the fused DJ, resulting in a mature VDJ βTCR gene rearrangement. Each genomic Vβ segment encompasses sequences for its promoter region and leader peptide in a first exon, followed by the actual Vβ-encoding sequence in a second exon[136]. D and J segments are encoded by single exons while the TCRβ C regions are the result of the eventual RNA splicing to join five exons. A functional βTCR rearrangement only results if the V-D-J recombination events allow for the transcritption of a messenger RNA encoding for the expression of an in-frame VDJ-C βTCR gene product. Similarly, expression of a functional VJ-C αTCR polypeptide chain depends on the appropriate recombination of a Vα to a Jα, an event that leads to the removal of δTCR-encoding DNA regions and resulting in the aforementioned exclusive expression of an αβ TCR by the majority of circulating mature T lymphocytes. TCR gene recombination may take place on either or both alleles of each of an individual's TCR chains. Furthermore, recombination is an ordered process whereby a first set of rearrangements must lead to the production of a functional βTCR polypetide chain before any rearrangement of the αTCR locus is allowed to take place[142,147,148].

Somatic recombination of the TCR germ line sequences is targeted by specific recombination signal sequences (RSS)[135]. These highly conserved signals flank each of the TCR V, (D), J gene segments to be rearranged and consist of two consensus

sequences, a heptamer and a nonaner (αβTCR consensus sequence "CACAGTG" and ACACAAACC", respectively), separated by a non-conserved spacer of 12 or 23 nucleotides in length[136,149-151]. Rearrangement takes place between an RSS with a 12-nucleotide spacer and one with a 23-nucleotide spacer[152]. Thus, each V gene segment may recombine with any (D) or J segment of the same locus, and recombination of two segments of the same family does not occur.

The recombination event between two TCR gene segments is first characterized by cleavage of the genomic DNA immediately adjacent to the segments' respective RSS heptamers. This is followed by the transient formation of a DNA intra-strand hairpin structure at the extremity of each of the TCR-coding segments, and by the elimination of the intervening DNA sequence from the chromosome. These hairpins are then resolved when enzymatically nicked by an endonuclease activity before the coding ends are joined and recombination at the site is complete. Several essential enzymatic activities intervene in TCR locus recombination. They include:

i. The recombination activation genes (RAG)-1 and RAG-2 proteins, which are absolutely required for recombination of the TCR loci to take place[152]. These gene products play a predominant role in the early stages of recombination as they are involved in the recognition of the RSS sequences and have been shown *in vitro* to be sufficient for the enzymatic cleavage at RSS sites and hairpin formation[153-155].

ii. DNA-remodelling activities. Indeed, locus accessibility appears to be a key regulator of recombination activity. Chromatin-remodelling activities such as the chromatin-associated proteins high-mobility group (HMG)-1 or HMG-2 have been shown to enhance recombination, as have histone acetylation and the hSWI/SNF complex [156-159]

iii. Components of the ubiquitous DNA double-strand break repair and non-homologuous end joining machinery. The link between V(D)J recombination and double-strand break repair was established through some mutational complementation groups, including SCID-mice, and gene knockout strategies[152]. Thus, components demonstrated to be

involved include the large catalytic subunit of the DNA-dependent protein kinase ($DNA\text{-}PK_{CS}$), as well as the DNA-stabilizing Ku70/86 heterodimer. DNA ligase IV and XRCC4 are also essential for recombination[160-164].

The successful end result of TCR loci recombination processes is the expression of a functional TCR at the surface of immature thymocytes that then complete their maturation in the thymus before exiting to the immunologic periphery. The recombination process also results in the formation of loops of DNA that are excised from the chromosome and are known as T cell recombination excision circles, or TRECs[165-168]. TRECs are extrachromosomal and do not replicate; as such, they are diluted with every cell division of an original T cell clone. TRECs are thought to stably persist as episomes within T cells and are therefore most enriched within cells that have not undergone extensive division, such as thymocytes expressing a TCR, recent thymic emigrants and peripheral naïve cells[165-168].

1.1.6. Generation of TCR diversity: shaping the TCR repertoire

αβTCRs are faced with the daunting task of sampling the myriad of host and foreign peptides that are presented in the context of one's MHC. Thus, the immune system has evolved to make use of several molecular strategies in order to generate a broad assortment of TCRs capable of recognizing a wide variety of antigenic determinants. These strategies are evidenced in the genetic properties of the immature TCR loci as well as in the intricacies of the processes of recombination and chain assortment that characterize TCR maturation. The mechanisms of αβTCR diversity are the following:

i. The polymorphism of the variety of genetically encoded V, (D), and J TCR segments, constitutes a first mechanism contributing to the diversity of TCRs that can be created and allowed to patrol the human host.

ii. The second mechanism results from the genetic recombination process, which shuffles different combinations of these germline-encoded polymorphic segments in order to generate different TCR polypeptide chains.

iii. The resolution of the hairpin structure intermediates, which transiently characterize each of the coding joints contributing to the Vβ-Dβ, Dβ-Jβ, and Vα-Jα rearrangements, results in further diversity. Indeed, short palindromic sequences, known as P-regions, frequently result from the concerted activity of the endonuclease that indiscriminately cleaves the hairpin, of the polymerase that then synthesizes the required complementary DNA strand, and of an exonuclease that may then trim each of the recombination interfaces as outlined in the previous section.

iv. The N-regions observed at the recombination junctions of the TCR are the predominantly the result of the untemplated addition of nucleotides by the terminal deoxynucleotidal transferase (TdT) enzyme, as was most tangibly demonstrated using TdT knockout mice[169,170]. The absence of functional TdT activity appeared neither to have gross effects on lymphocyte differentiation, nor to result in immune deficiencies as would have been apparent from evident susceptibilities to infections[169,170]. However, the analysis of TCR sequences in TdT knockout mice revealed a virtually complete absence of N-nucleotide regions, while templated P-regions were present with a similar frequency as that observed in wild type mice[169,170]. N-additions actually represent the mechanism through which the greatest amount of diversity is generated within the TCR loci: the diversity of TCRs observed in the periphery of TdT knockout mice has been estimated to be between 5-10% of that present in wild type counterparts[171]. Expectedly, analysis of the length of the TCRβ CDR3 regions, which encompass all recombination junctions, revealed shorter average length in TdT knockout mice as compared to wild type mice[171]. Though the reduced peripheral TCR diversity may have been hypothesized to be the result of a subsequent selection on the basis of structural constraints imposed by the shorter CDR3β length, this does not appear to be the case as the

distribution of CDR3β lengths would then have been expected to be skewed: in fact, they remain normally distributed. Furthermore, it is also interesting to note that TdT deletion does not result in a narrowing of the spectrum of CDR3β lengths, proof that the observed tendency for CDR3β lengths to follow a gaussian distribution is not pre-eminently due to this enzymatic activity, but rather is intrinsic to the rest of the recombination and selection processes that guide T cell maturation.

v. Further TCR diversity results from the fact that the receptor is heterodimeric and that distinct TCRs result from each αβ assortment. Indeed, a unique β chains may be paired with several different α chains. Analysis of the diversity of TCRs present in the mouse has revealed that a single β chain may be paired with at least two α chains[172]. Estimates of the TCR diversity present in the blood of humans suggest that a single βTCR chain may be associated, on average, with at least 25 different Vα chains[173].

These various mechanisms can result in the generation of some $10^{14\text{-}16}$ theoretically unique αβTCRs and therefore support the generation of a broad diversity of potential TCRs[135]. The theoretical diversity of T cell clones as mathematically estimated on the basis of the diversity-generating mechanisms outlined above is then partially curbed by the processes of positive and negative selection that take place during thymic T cell maturation. Finally, as will be discussed, the diversity of T cell clones present in an individual is modulated by this individual's immune experience. Each T cell having rearranged a given, unique αβTCR is the precursor of the lineage of clones that may ensue from its mitotic proliferation; each daughter-cell expresses the clonal αβTCR, which may then effectively be perceived as the T cell clone's signature. The sum of all the different TCRs expressed by an individual's pool of T cells is referred to as her/his molecular TCR repertoire. For an estimated 10^{12} systemic T cells, the human peripheral molecular TCR repertoire has been estimated to encompass 10^{6} distinct TCR β-chains[173].

1.1.7. Structure of the TCR

The αβ TCR is a cytoplasmic membrane-bound heterodimer having a molecular weight of approximately 90,000 daltons[174]. Structurally, the TCR was first inferred to be akin to immunoglobulins (Ig) due to the tremendous amount of polymorphism observed in peptide mapping analyses[175-177]. This working model was then reinforced when these molecules' similar genetic disposition and their shared diversity-generating strategies were demonstrated: while the TCR chain's variable domains are encoded by the rearranged germline V(D)J segments, their constant domains are encoded by the C gene segment[178-180].

Further substantiation of the TCR's close relationship with Ig came from their respective deduced primary amino acid sequences. Discernably, the primary protein structure of the TCR is characterized by sections of remarkably high sequence diversity interspersed amongst "framework" regions of relative homology. By homology with Ig, the TCR's regions of high sequence diversity were labelled complementarity-determining regions (CDR) and were predicted to form the TCR's surface of interaction with MHC-peptide ligands[181,182]. CDR1, 2, and 3 have been identified for both α and β chain, while the HV4 or CDR4 is a fourth, somewhat less variable region of the β chain [135,183-186]. The diversity of the CDR1, 2, and 4 solely results from the germline-encoded polymorphism of the TCR loci, as these hypervariable regions are integrally encoded by the TCR V gene segments. The hypervariable CDR3α and β are outstanding, as their sequence is the direct result of the somatic recombination of the TCR loci and of the tremendous diversity generated at the V(D)J junction.

The structure of the extracellular portion of TCRs has been determined by X-ray crystallography[187-194]. Together with the information gained from site-directed mutagenesis studies, much insight has been gained into the mechanisms through which αβTCR function is achieved[195,196]. As predicted, both TCR chains belong to the superfamily of immunoglobulin (Ig)-like proteins. As such, the TCR variable and constant regions are each characterized by Ig-like domains formed by separate sets of

intra-chain disulfide bond-linked β-barrel structures. The α and β TCR chains are associated through a disulfide bond, which is extracellularly-located near the plasma membrane and formed between conserved cysteine residues of their respective constant regions[174]. The αβTCR is further stabilized by the glycosylation of multiple sites on each of its constituent chains[174]. The configuration of the TCR is such that the constant domains of the α and β chains form the base of the receptor, while the TCR variable domains are oriented away from the T lymphocyte such that its hypervariable CDR regions are solvent-exposed and free to engage ligands [191,192].

1.1.8. Antigen-specific engagement of the T cell receptor

The interaction between the TCR and a cognate ligand is the *sine qua non* of an antigen-specific immune response. While γδ TCRs can recognize free proteins and non-peptides, αβ T lymphocytes are activated upon engagement of their TCRs by antigenic MHC-peptide complexes for which they have exquisite specificity[135,174]. X-ray crystal structures of several murine and human, class I and class II MHC have revealed that the antigenic peptides, which they present, are positioned within membrane-distal α-helix-delineated grooves[29,31,124,125,128,131,197-208]. Due to the diversity of peptides and to MHC polymorphism, the combination of a peptide and of the protruding helices of the MHC allele that presents it thus constitutes an atomic surface of unique complexity, which serves as the distinctive molecular signature to be scrutinized by TCRs.

The ternary complexes of αβTCRs in association with the peptide and MHC, which they recognize, have now also been crystallized[196]. Figure 2 illustrates this interaction. As hypothesized by extension of studies having determined the antibody regions contributing to antigen-specificity, by comparison with the crystal structure of antigen-antibody complexes and by deduction from the crystal structures of TCR alone, these ternary crystal structures have confirmed that the TCR interface for the TCR-MHC-peptide interaction is entirely contributed by the variable domains of its α and β chains, with hypervariable CDRs and framework regions contacting the MHC. The TCR Vα and Vβ

domains contact the MHC α-helices that border the peptide groove and meet over the peptide. The emerging consensus is that the TCR binds the peptide-MHC surface diagonally, with the axis of the peptide and the longer axis of the TCR at an angle varying between 45 and 80 degrees [209]. First proposed by functional mutagenesis studies, this orientation was further supported by the crystallized complex of the human A6 αβTCR and HLA-A0201 class I MHC loaded with the human T-cell leukemia virus-1 (HTLV-1) Tax peptide and by the crystal of the ternary complex of the murine 2C TCR and its peptide-loaded H-2K^b complex[191,192,210]. The diagonal orientation of the TCR over peptide-loaded class II MHC was also put forth by functional mutagenesis studies and confirmed by crystallography of the complexes[205,211,212].

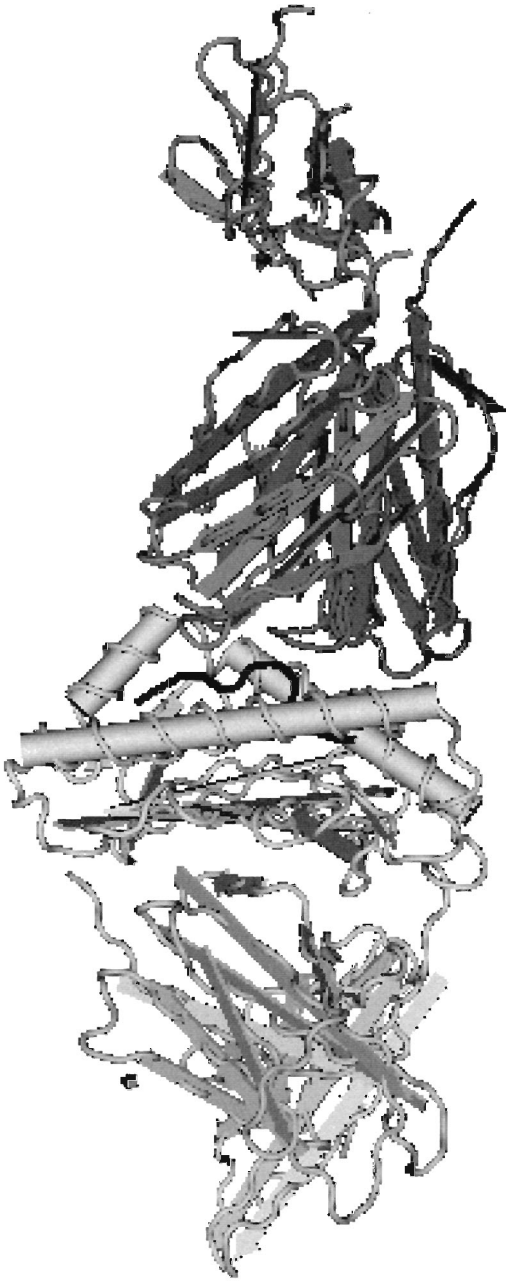

Figure 2. Illustration of the crystal structure of the ternary complex of the human A6 TCR with its cognate HTLV-1 Tax 11-19 (LLFGYPVYV) peptide in the context of HLA-A0201. *This illustration was derived using Cn3D v.3 software from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and crystal coordinates from D.N. Garboczi et al.* [191] *(NCBI identification number: 1AO7). The TCRβ chain is in blue, the TCRαchain (with incomplete TCR α) is in red. The peptide (in yellow) is in the magenta HLA-A*O201 heavy chain. β2m is in green.*

An interesting observation made by Garboczi and colleagues is that, of the twenty hydrogen bonds formed between the A6 TCR and HLA-A0201-Tax complex, eight are to conserved and three to polymorphic residues of the MHC, while nine are to the Tax peptide[191]. Further analysis reveals that eleven of the twenty hydrogen bonds are formed with the contribution of the hypervariable CDR3 loops that have resulted from recombination of the V(D)J junction and the untemplated modifications they have undergone. The other nine hydrogen bonds are mediated by germline-encoded hypervariability of the Vβ segments. These observations point to the molecular constraints resulting in a productive TCR-MHC-peptide interaction, but underline the incredible plasticity of the TCR which results in the generation of a pool of T cells capable of recognizing the myriad of prospective peptides presented within the diversity of the tremendously polymorphic species-specific MHC gene pool. In the human population, the diversity of TCR segments can thus be hypothesized to have co-evolved with the pool of MHC alleles encoded by the human genome, whereas it is restricted by selection mechanisms within an individual.

Early attempts to substitute variable region loops onto unrelated TCR chains in order to transfer antigenic specificity had revealed the complexity of the recognition mechanism[182]. Crystallized ternary complexes have now revealed that paramount interactions are mediated by each of the CDRs. In the structure obtained for the interaction of the human A6 αβTCR with the HLA-A0201-Tax complex, the CDR1, 2 and 3 of the αTCR and CDR3β loop contact conserved and variable residues of the MHC. It is the CDR1 and CDR3 segments of both α and β chains that contribute predominantly to the interaction with the peptide. The CDR1α extends over the N-terminus of the peptide, while the CDR1β contacts its second to last C-terminal residue. Meanwhile, the hypervariable CDR3α is positioned over the centre of the peptide and the topologically adjacent CDR3β loop contributes hydrogen bonding and/or van der Waals contacts to peptide residues 5-8[191].

As such, the TCR CDR3β contributes over 50% of the contact surface at the interface with the MHC-loaded peptide, while CDR1α and CDR1β each provide approximately 25%[191]. As protein function is guided by protein structure, TCR specificity is highly influenced by the molecular determinants of the Vβ region, which contribute the receptor's surface of interaction with MHC-peptide. The predominant role of the hypervariable CDR3 is especially salient. In fact, several studies have demonstrated that TCRs with common specificity for a given epitope share molecular characteristics; including comparable length of the contacting loops, same electric charge or similar bulkiness of side-chain residues at fixed loop positions, and sometimes even conserved amino acid sequence[213-221]. These findings are the rationale of studies that seek to establish the features of immune responses through the molecular characterization of TCR chain sequences, as does the first study presented herein in Chapter 2.

Experimental approaches used to dissect the characteristics of receptor chains include cytofluorometry-based approaches using Vβ-specific antibodies, as well as Southern-blot or PCR-based detection of TCR sequences[222-225]. TCR spectratyping (immunoscope), DNA heteroduplex tracking assay (HTA), as well as the molecular sub-cloning and sequencing of TCR chains are alternate means to dissect this complexity and such approaches have been used in several disease contexts, including HIV-1[225-228].

The interaction between TCR and peptide-MHC is thus mediated by complex atomic surfaces to which multiple residues of the TCR, on the one hand, and peptide-MHC, on the other, contribute. Each interface may thus be perceived as a matrix with a precise three-dimensional topology: involved amino acid residues contribute either indirectly, by imposing structural constraint, or directly, by contributing atoms for hydrogen bonding or van der Waals forces. The dynamics of interaction between an MHC-peptide matrix and that of a TCR thus provide a molecular basis for discrimination resulting in tolerance or activation of the T cell.

1.1.9. The T cell signalling machinery

The intracellular tails of the human TCR chains are no more than 3-4 amino acids in length[229]. As such they do not possess intrinsic enzymatic activity, nor do they exhibit sequence motifs associated with intracellular signalling capacity. In fact, the functional TCR complex results from the association of two operational units: first, the highly diverse, mostly extracellular, heterodimeric γδ or αβ TCR "recognition" unit described above, and second, a highly conserved signal-transducing unit consisting of an assembly of the CD3 γ, δ, and ε, as well as the ζ chain[229-232]. The signalling pathways initiated by TCR engagement are illustrated in figure 3.

Association of the TCR with the ζ chain and CD3 signaling complex was first demonstrated by αβTCR immunoprecipitation experiments performed with conditions making use of gentle detergents, such as Triton X-100 or digitonin [174,229]. A divalent model of the TCR-CD3 complex is presently proposed. According to this model, each of two disulfide-bond linked CD3ε proteins are thought to interact primarily with the respective β chains of two TCRs, while two disulfide bond-linked ζ chains associate with the TCRs' α chains. Single CD3γ and CD3δ chains complete the proposed multimeric complex. The interactions leading to this ultrastructure are primarily thought to be mediated by complementarily charged residues within the transmembrane portion of the proteins and have been suggested by site-directed mutagenesis studies and immunoprecipitaion experiments[229].

Unlike the TCR heterodimer, the CD3 proteins and ζ-chain possess significant intracytoplasmic tails presenting the conserved [YxxL/I (7-8 amino acids) YxxI/L] immunoreceptor tyrosine-based activation motifs (ITAMs)[233-235]. Each CD3δ, ε, γ chain possesses one ITAM, while the ζ chain has three[236-241]. It is the tyrosine residues within the signalling complex ITAMs that are phosphorylated by *src*-family kinases $p59^{Fyn}$ and

p56^{Lck} upon cognate engagement of the TCR, and thus initiate intracellular signalling in the T cell [242-244].

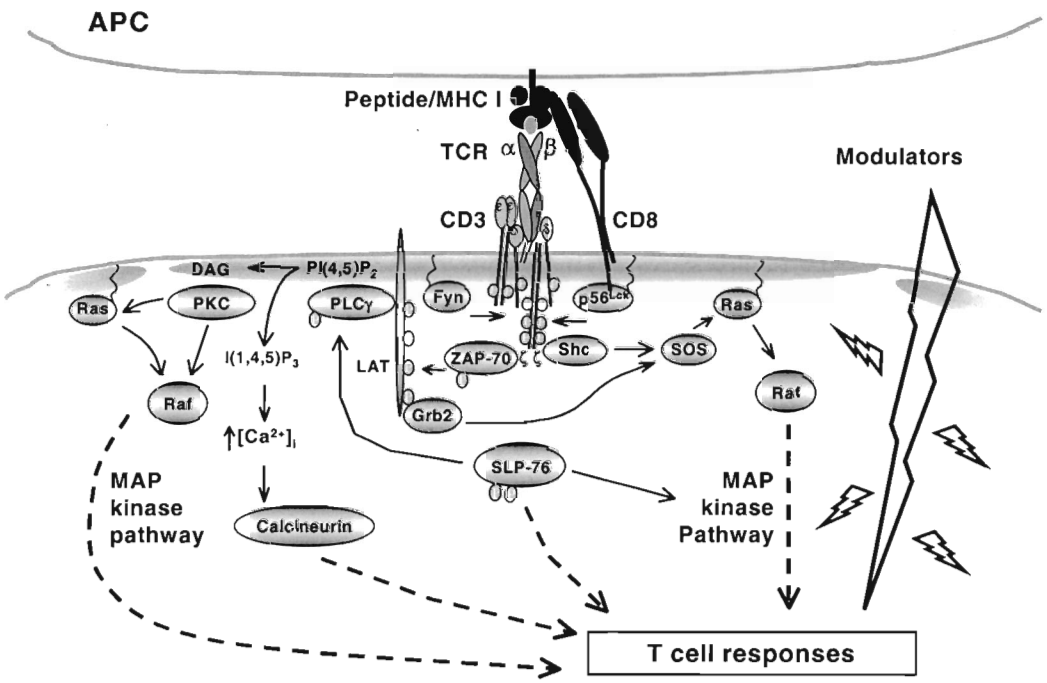

Figure 3. TCR signalling. *TCR engagement by cognate peptide-MHC class I complex results in the initiation of the intracellular signalling cascade. First, the src-family protein-tyrosine kinases p59^{Fyn} and p56^{Lck} are activated, leading to the phosphorylation of the CD3 ITAMs and the Syk-family protein tyrosine kinase, Zap-70. In turn, SLP-76 and LAT are phosphorylated, leading to the recruitment PLCγ and the resulting activation of the MAP kinase pathway through the action of PKC, as well as an increase in the intracellular calcium concentration. Alternatively, through the action of various adaptor proteins and the Sos guanine-nucleotide exchange factor, Ras is activated and engages the MAP kinase cascade. Beyond the direct TCR-induced signals, the engagement of other cell-surface receptors by their appropriate membrane-bound or soluble ligands further modulate T cell responses. The net outcome for the T cell is a broad range of responses that include the up- or down-regulation of surface molecules, the secretion of soluble mediators, proliferation and differentiation.*

Several signalling pathways are induced by antigen-specific stimulation of T cells. Through interaction with src-homology 2 domains, the specific phosphorylation of ITAMs results in the recruitment of *syk*-family kinases Syk and ZAP-70, which respectively auto-phosphorylate or are phosphorylated[245]. The signal is then perpetrated through intermediates, such as linker for activation with T cells (LAT), and the involvement of multiple signalling molecules, including protein kinase C (PKC), phosphotidyl inositol-3 kinase (PI3K), and phospholipase C-γ (PLC-γ). The ensuing signalling cascades result in the intracellular release of calcium, the induction of the Ras pathway through the MAP-family serine-threonine kinases, and the activation or recruitment to the nucleus of multiple gene transcription activators such as nuclear factor of activated T cell (NFAT), AP1 and nuclear factor NF-κB[241,246,247].

Beyond the direct induction of signalling pathways by engagement of the TCR, the signal and cellular response is further directed by the engagement of other cell-surface molecules, such as CD28, CTLA-4 and Fas, and by immediate cytokine environment of the cell, including interleukins and chemokines[248]. As will be further discussed, these molecular events modulate the activation of T cells, the induction of their effector capacities, their proliferation, and differentiation.

1.1.10. Current concepts underlying the TCR-mediated activation of T cells

The concepts thought to be at play in specifically regulating the induction of TCR signalling and the activation of T cells through the TCR are based on the following original models:

1) Ligand-binding induced TCR conformational change model. This first model proposes that signalling is initiated as a result of a conformational change of the TCR molecule following its engagement[249]. Evidence for such an induction of signal was originally put forth by experiments using monovalent antibodies to stimulate T cells and was later supported by reports that single-chain soluble peptide-class I MHCs

could induce activation of CD8-expressing T cells [250,251]. Nevertheless, direct demonstration of a conformational change-induced activation is lacking. Furthermore, X-ray crystal structures of TCR and ternary TCR-peptide-MHC complexes strongly suggest that, while significant conformational flexibility at the contact interface argues for an induced-fit mode of interaction, this has no influence on the overall configuration of the rest of the TCR, and thus is not suggestive of conformation change-induced activation of the intracellular signalling cascade downstream of the TCR[191,252]. Nevertheless by definition, experiments based on engineered TCR ectodomains do not allow for the study of the subtle changes that might be induced in the trans-membrane and intra-cytoplasmic molecular domains, which rule the interaction with signalling partners as well as the cell-surface distribution of the receptors, and thus may yet reveal a role for TCR conformational modulation in signal induction.

2) The peptide-MHC/TCR affinity-based model. Early studies suggested that the induction of a T cell response was directly correlated to the affinity of the T lymphocyte's TCR for the recognized antigen-MHC. As such, T cell signalling would not result from a conformational change of the TCR, but rather from a peptide-MHC complex's propensity to engage and recruit TCR with sufficient efficiency. The concept of avidity further expands this model as it accounts not only for the affinity of a peptide-MHC ligand for a TCR, but also introduces the notion of the density of these ligands on a given cell [253-258].

These original models have since been critically refined by the following concepts:

i. The characterization of altered peptide ligands (APL) has contributed much to our understanding of the induction of T cell responses[213,259-261]. Essentially, an APL is a modified peptide antigen that differs from a defined native T cell epitope only by one or a few of the amino acid residues, which interact with an identified cognate TCR. Thus, in a given antigen-specific system, APLs may be classified as agonists, which stimulate the full range of cellular responses; partial agonists, which stimulate some, but not all

cellular functions, and with lesser efficiency than the native agonist epitopes; or antagonists, that not only do not stimulate a functional response, but also attenuate the efficiency of subsequent stimulation attempts with agonist peptide and may even anergize the T cell[262-264].

ii. Interaction half-life, or the concept of TCR-peptide-MHC dwell-time. The functional properties of various peptide-MHC ligands were first ascribed to their differing ranges of affinity for TCR. A more thorough dissection of the kinetics of the interaction has revealed that the key factor defining the peptide-MHC properties is the rate at which it dissociates from the TCR, mathematically, this "off-rate" is directly related to the half-life of the interaction[265-269]. As discussed earlier, the engagement of the TCR directly results from the recognition of a cognate binding topology, or three-dimensional atomic matrix, offered by peptide-MHC. As such, the functional differences observed between various APLs are the result of the modification of the surface of interaction. Given the significant contribution of the MHC to this interface, a logical extension of the APL phenomeneon is that the mutation of relevant TCR-contact amino-acid residues of the MHC would also modulate the affinity of the complex, as well as its half-life and the ensuing T cell responses. Furthermore, in an elegant study, Kalergis and colleagues proceeded to demonstrate that mutation of TCR residues that modified kinetic properties of TCR interaction with a given peptide-MHC also resulted in the induction of a spectrum of responses ranging from no stimulation to activation of T cell function[270].

iii. The TCR serial triggering hypothesis. MHC molecules loaded with a given antigenic peptide are few on the surface of antigen-presenting cells. Furthermore, they are presented amongst a majority of MHCs loaded with endogenous antigen. For these reasons, it was postulated that a mechanisms must exist in order to amplify the "signal" provided by the antigen over the "noise" of endogenous peptides. In 1995, a seminal study by Valitutti and colleagues demonstrated that peptide-MHC complexes could serially engage multiple TCRs[271]. Discovery of this mechanism offered the first explanation of how the few antigenic epitopes on a given antigen-presenting cell or

virally-infected cell could suffice to induce a T cell to respond: as few as 100-200 MHC molecules have in fact been shown to be sufficient[272].

iv. The kinetic proofreading model of TCR signalling. Currently, this is the leading model proposed to conceptualize the TCR's ability to discriminate between ligands. It postulates that because the engagement of the TCR by various peptide-MHC varies with respect to affinity and association/dissociation kinetics, different TCR ligands induce signals that differ in their quality. According to this hypothesis, complete signal induction occurs as a result of a TCR engagement that is characterized by what is then considered an optimal interaction with peptide-MHC. Too low an affinity due to a rapid off-rate would therefore result in an incomplete signal, just as too long a half-life of interaction might reduce the efficiency of the stimulation due to hindrance with TCR-signal amplification mechanisms, such as the serial-triggering process. This model is supported by biochemical evidence gathered in experiments where T cells were stimulated by ligands of variable affinity and kinetic properties, such as various agonist, partial agonist and antagonist peptides. Such experiments showed that TCR signalling could be characterized by alternative patterns of ITAM phosphorylation, as well as by a variability in the intracellular acidification and calcium flux induced, and in the gene transcription stimulated; all of which underlie the effector and proliferative functions exhibited at the cellular level[261,269,273-284]. According to this model, TCR engagement by low-affinity ligands would have too short a half-life to sustain the complete induction of early signalling events, and only higher affinity ligands could then stimulate the full range of T cell responses [241,285,286]. Recently published observations by Rosette and colleagues call for a further refinement of the kinetic proofreading model. Indeed, they have shown that some low-affinity agonist ligands may yet stimulate T cells fully, but with much delay and with no detection of TCR-proximal signal induction. Of the various parameters studied, the only evidence for the accumulation of a signalling effector was the extremely distal phosphorylation of the c-Jun gene transcription element[287].

1.1.11. Molecular events underlying the antigen-specific activation of T cells

Whether as a result of induced conformational changes in the shape of the TCR or through cognate ligand-induced multimerization, the engagement of multiple TCRs by peptide-MHC complexes leads to their clustering at the cell surface, and the gathering of the receptors in close proximity of co-receptors and signalling partners ultimately results in the induction of the intracellular signalling cascades. Mechanistically, the ability of peptide-MHC ligands to engage the TCR and the duration of their interaction are thought to be the driving forces for the effective induction of TCR signals.

Clustering has been suggested to result from TCR-TCR dimerization, but there is little supporting evidence for such direct interaction. Although dimers were originally suggested by the publication of the crystallographic structure of a dimer of human class II HLA-DR I, subsequently-derived structures of murine and human, class I and class II MHCs have failed to show a propensity for dimerization[28,288]. Mathematical extrapolations put forth following TCR downregulation experiments have also suggested TCR dimerization, but in the absence of direct proof. Furthermore, serious questions have been raised about a 1999 published report that had put forth an allosteric TCR-TCR dimerization on the basis of bi-phasic profiles obtained in surface plasmon resonance (SPR) analyses of TCR-MHC interactions: rectification of the purported technical oversights indicates that SPR profiles do not suggest TCR dimerization[288,291]. Finally, evidence for the oligomerization of the murine 2B4 TCR with its moth cytochrome C (MCC) class II I-E^k ligand has also been suggested by light scattering studies of soluble complexes, but similar study of other murine and human class I and class II complexes have failed to reproduce the phenomenon[288,292]. Nevertheless, all of the above-mentioned studies which have attempted to directly demonstrate TCR dimers are undermined by the fact that the experimental setups make use of engineered soluble ectodomains which were either produced *in vitro*, or secreted by various cell types: one therefore has to be careful when projecting the observations to *bona fide* TCRs that are membrane bound, possess intra-cytoplasmic domains, and are naturally associated with protein partners.

Current perspective is that the most probable mechanism for antigen-induced T cell signalling involves the re-organisation of macromolecular complexes of proteins at the surface of the T cell, and the assembly, within close proximity, of the intracellular instigators of the signalling cascade. Capping of TCRs in this fashion has classically been performed using bivalent antibodies; antigen-specific approaches to stimulate T cells with peptide-MHC multimers also suggest that TCR clustering, but not necessarily a directly mediated TCR-TCR interaction, leads to T cell activation[293-295].

Thus, several lines of evidence suggest that the activation of T cells results from a reorganization of several molecules, including the TCR and its signalling partners, at the surface of the cell. Critically, the recent and ongoing characterization of the immunological synapse, which is also known as the supramolecular activation cluster (SMAC), has provided substantiation for a physiological framework for the molecular events surrounding TCR engagement, clustering, and signalling[296-303].

The immunological synapse is a site of intensely dynamic inter-cellular communication that is established as a T cell contacts an antigen-presenting cell, which displays its cognate ligand. For the T cell, the formation of the immunological synapse is an active process that relies on the rearrangement of the actin cytoskeleton. This rearrangement is induced, following TCR-mediated T cell activation, through the engagement of the T cell's co-stimulatory molecules CD28 and lymphocyte function-associated antigen 1 (LFA-1) by the antigen-presenting cell's CD80/CD86 and intercellular adhesion molecule 1 (ICAM-1), respectively[304]. Though a calcium flux occurs following TCR engagement, intracellular calcium signalling is not essential to the cytoskeletal rearrangement[305]. The polarization of various cell-surface receptors is TCR-signal independent, but the end result of the inter-molecular engagements is their capping, along with TCR, at the cell-cell interface[304]. Interestingly, the mirror assembly of the synapse on the antigen-presenting cell takes place passively, with co-stimulatory ligands and MHC bearing both agonist and endogenous (null) peptides contributing to this interface[306]. The stabilization of the synapse within a relatively compact area may thus serve as a mechanism to amplify the T cell response[300,306].

The migration of the various receptors recruited to the synapse is essentially bi-phasic. On the T cell, an original ultra-structure consisting of central LFA-1 molecules surrounded by a concentric rim of TCRs is first formed within 30 seconds, and is observed simultaneously with TCR signalling. The TCRs then migrate towards the centre of the circle, while the adhesion molecules are excluded to the outer rim[300]. This conformation is identical to that originally described for the stable SMAC, consisting of central cSMAC to which the θ-isoform of protein kinase C (PKC) localizes, and the associated concentric peripheral pSMAC of LFA-1 adhesion molecules[299,307].

Several groups investigating the liquid-ordered phase of cholesterol and sphingolipid-rich membrane microdomains referred to as "lipid rafts" are also currently evidencing similar ultra-structural organization[308]. Several lines of evidence suggest that lipd rafts and the formation of the immunological synapse may be intrinsically coupled. First, TCRs and molecules associated with the signalling cascade they instigate are enriched in the lipid rafts of activated cells[309-313]. Furthermore, several key players in T cell activation possess the glycosyl phosphatidyl inositol (GPI)-anchored tails required for inclusion in the lipid rafts and/or have been shown to be associated with them; these include Lck, Fyn, PKC-θ and CD8[308-315]. Finally, as with the immunological synapse, lipid raft reorganization is also induced by the engagement of co-stimulation following T cell activation[316,317]. The relation between the immunological synapse and lipid rafts is not completely clear yet, but it is interesting to suggest that lipid rafts may play a critical role in orchestrating the molecular distribution that is characteristic of the immunologcal synapse.

It is important to note, however, that observations until now indicate that the consolidation of a mature immunological synapse takes several minutes[300]. For this reason, it is hypothesized that the induction of immediate T cell effector functions does not rely on its formation. The synapse may nevertheless play a functional role for the T cell, as its formation has been shown to parallel the polarization of the microtubule organizing center, golgi apparatus, and lytic granules and to include a secretory domain[296,318,319]. Other events such as proliferation are thought to require sustained

signalling such as that which is provided by the synapse[300]. A role for the immunological synapse in the induction of T cell differentiation is therefore also foreseeable.

1.1.12. Specific-antigen induced T cell responses and their modulation, T cell effector mechanisms

Beyond the TCR, a variety of molecules have been shown either to participate in the immunological synapse, or to be actively excluded from it. Critically, the TCR and these accessory molecules only instigate some of the intricate sets of commands that are integrated by the cell and modulate the TCR signal. Soluble cytokine mediators further contribute, resulting in the complex pattern of response exhibited by T cells upon stimulation.

During a T cell-mediated immune response, T cells bearing TCRs of appropriate specificity for an antigenic foreign peptide/self-HLA complex are activated and recruited into the response. The individual contributions of the various cells involved in the response then shape the overall characteristics at the level of the entire immune cell population. The complex interactions result in a plethora of amplifying and inhibitory feedback loops, as well as in cascades of cellular responses. Activated T cells may display a broad array of effector functions that contribute to the immune response. These include:

i. Recruited cells are induced to undergo multiple rounds of mitosis through the process of clonal expansion[320]. T cell clones that have encountered their specific antigen are thus driven to proliferate into populations of like-clones that then patrol the organism. Overwhelming expansions of antiviral CD8 T cells have been demonstrated in murine models of influenza and LCMV infection, in macaque SIV infection, in humans experiencing acute infectious mononucleosis, as well as in adult HIV-1 infection[321-327]. This selective expansion of specific TCR clones therefore results in the skewing

of the T cell receptor repertoire due to selective amplification of the relevant effector T cells.

ii. The production and secretion of various cytokines. The various soluble effectors secreted by T cells include interleukins such as IL-2 and IL-10, effector molecules such as Tumour Necrosis Factor-α (TNF-α) and IFN-γ, as well as chemokines such as CCL4 and CCL5 (MIP-1β and RANTES)[328-335]. As illustrated by the classical Th1/Th2 paradigm, following stimulation, populations of CD4 T cells may be induced to preferentially secrete distinct sets of cytokines, a phenomenon echoed in CD8 T lymphocytes as Tc1 and Tc2[331,336-339].

iii. The formation and polarized release of lytic granules. Critically, following engagement, CD8 T cells are induced to produce granzymes A and B as well as perforin[340-344]. These molecules are then released in a polarized manner to induce the apoptotic death of target cells that are specifically recognized as presenting modified or foreign antigenic epitopes within their class I MHC, or presenting allogeneic MHC[319,345,346].

iv. The upregulation or downregulation of effector cell-surface molecules that contribute to various aspects of the immune response. Key effector molecules up-regulated by activated T lymphocytes include cell surface apoptosis-inducing proteins such as FasL and membrane (TNF-α) receptor, as well as apoptosis-sensitizing proteins such as Fas and the Death Receptors (DR)[343,344,347-349]. Significantly, upon activation, antigen-specific T cells also modulate their expression of various chemokine receptors that then dictate the expressing-cell's migratory pattern. The polarization of T cells with regards to the effector functions they develop are also associated with the selective acquisition of distinct chemokine receptors and trafficking patterns[350-356].

1.1.13. Guiding encounters: immune cell trafficking and the orchestration of immune responses

Originally derived from hematopoeitic stem cells located in the bone marrow, each of the specialized cellular components of the immune system results from differentiation induced by summations of complex maturation signals[357]. As pertinently illustrated by the ontogeny of T cells having first undergone differentiation and selection in the thymus prior to their peripheral dissemination to secondary lymphoid organs, their timely migration to discrete functional environments is tributary to the the *in vivo* maturation of immune cells[358-360]. Correspondingly, the effectiveness of T cell-mediated immune responses is also a consequence of the exquisite coordination of interactions between various cell types[361-364]. Thus, the organization of the immune system relies on the maintenance of specialized anatomic structures, but also on the ability of immune cells to appropriately traffic through tissue and precisely home to sites of encounter[361,365-371]. To a great extent, immune cell trafficking is guided by the chemokine-chemokine receptor system.

Chemokines are small, positively charged, soluble (with the exception of the membrane-anchored fractalkine) molecules that serve as chemoattractants to cells of the immune system[372,373]. More than forty chemokines have been identified to date and are summarized in Table 1. In accordance with their characteristic N-terminal motifs, chemokines are molecularly classified as belonging to the cysteine (C), CX_3C, CC, or CXC chemokine families, with most known chemokines belonging to the latter two families[372-374]. Various cell types produce mixtures of chemokines in response to different stimuli. Following their secretion, chemokines may or may not associate with the extracellular matrix, yet effective concentration gradients of the chemokines are established[375-379].

Table 1. Nomenclature for human chemokines.

Systemic name	Human ligand	Other names or reference
CXCL	CXCL1	Groα/MGSA-α
	CXCL2	Groß/MGSA-ß
	CXCL3	Groγ
	CXCL4	PF4
	CXCL5	ENA-78
	CXCL6	GCP-2
	CXCL7	NAP-2
	CXCL8	IL-8
	CXCL9	Mig
	CXCL10	IP-10
	CXCL11	I-TAC
	CXCL12	SDF-1/PBSF
	CXCL13	BLC/BCA-1
	CXCL14	BRAK/Bolekine
	CXCL15	Lungkine
	CXCL16*	[380,381]
XCL	XCL1	Lymphotactin/SCM-1α/ATAC
	XCL2	SCM-1ß
CX3CL	CX3CL1	Fractalkine/Neurotactin
CCL	CCL1	I-309
	CCL2	MCP-1
	CCL3	MIP-1α
	CCL4	MIP-1ß
	CCL5	RANTES
	CCL7	MCP-3
	CCL8	MCP-2
	CCL11	Eotaxin
	CCL13	MCP-4
	CCL14	HCC-1/HCC-3
	CCL15	HCC-2/Leukotactin
	CCL16	HCC-4/LEC
	CCL17	TARC
	CCL18	DC-CK1/PARC/AMAC-1
	CCL19	MIP-3ß/ELC/Exodus-3
	CCL20	MIP-3α/LARC/Exodus-1
	CCL21	6Ckine/SLC/Exodus-2
	CCL22	MDC/STCP-1/ABCD-1
	CCL23	MPIF-1
	CCL24	MPIF-2/Eotaxin-2
	CCL25	TECK
	CCL26	SCYA26/Eotaxin-3
	CCL27	(MCC)/ALP/CTACK/Eskine
	CCL28*	[382]

Adapted from [373]

*Recently characterized and proposed in their respectively cited references

Chemokine receptors belong to the family of seven-transmembrane G_i protein-coupled receptors (GPCRs). Engagement of chemokine receptors by their appropriate ligands has been shown to result in the induction of distinct intracellular signalling cascades[383]. Whether dimerization of the GPCRs is physiologically relevant to their function remains a matter of debate[372,378,383]. Nevertheless, various components of the Janus kinases/signal transducers and activators of transcritpion (Jak/STAT) system are involved following Jak recruitment to, and phosphorylation of, the chemokine receptors[383]. Several signalling pathways are engaged by chemokine receptors through trimeric G-protein signalling: the seclusion of the associated pertussis-toxin sensitive $G\alpha_i$ subunit by GTP-binding results in the release of the $\beta\gamma$ subunits that are then free to interact with signalling partners[372,383]. These include tyrosine kinases and PLC, which lead to intracellular Ca^{++} flux and the activation of PKC isoforms and ensuing cascades; as well as the PI3K and MAPK cascades[383-386]. Furthermore, activation of members of the GPCR serine threonine kinases (GRK) provides a link with clathrin-mediated endocytic machinery[385]. The end result of chemokine receptor engagement is therefore three-fold: 1) adhesion, and cytoskeletal movements resulting in polarization and chemotaxis, 2) the induction/modulation of gene transcription, and 3) internalization and recycling/degradation of the receptor[361,363,383,387-390]. The induction of signalling pathways that is also recruited by other receptors, including the TCR, is of particular interest: this convergence of signals has not been thoroughly investigated until now, but a modulatory role for chemokines on TCR signalling may henceforth be postulated as CCL21 has been shown to influence the homeostatic proliferation of T cells in mice[391]. Finally, chemokine receptor downregulation following engagement provides indication of a negative regulatory loop whereby immune cell migration can be postulated to be the result of a relay of chemotactic signals[392].

Interestingly, the majority of chemokines interact with multiple different chemokine receptors, and different receptors are bound by various chemokines as illustrated in figure 4[372,373,393]. In fact, this apparent promiscuity between receptors and ligands most probably dissimulates a mechanism of intricate specificity where cells are guided by the assortment of nearby chemokines and the sequence of chemokine combinations, which they

encounter and are responsive to. Furthermore recent work, which showed that different chemokines that bind a same receptor induce different responses, has added further evidence purporting that the complex patterns of receptor-ligand interactions are not simply redundant: indeed, while CCL21 results in the downregulation of CCR7, CCL19 does not[394].

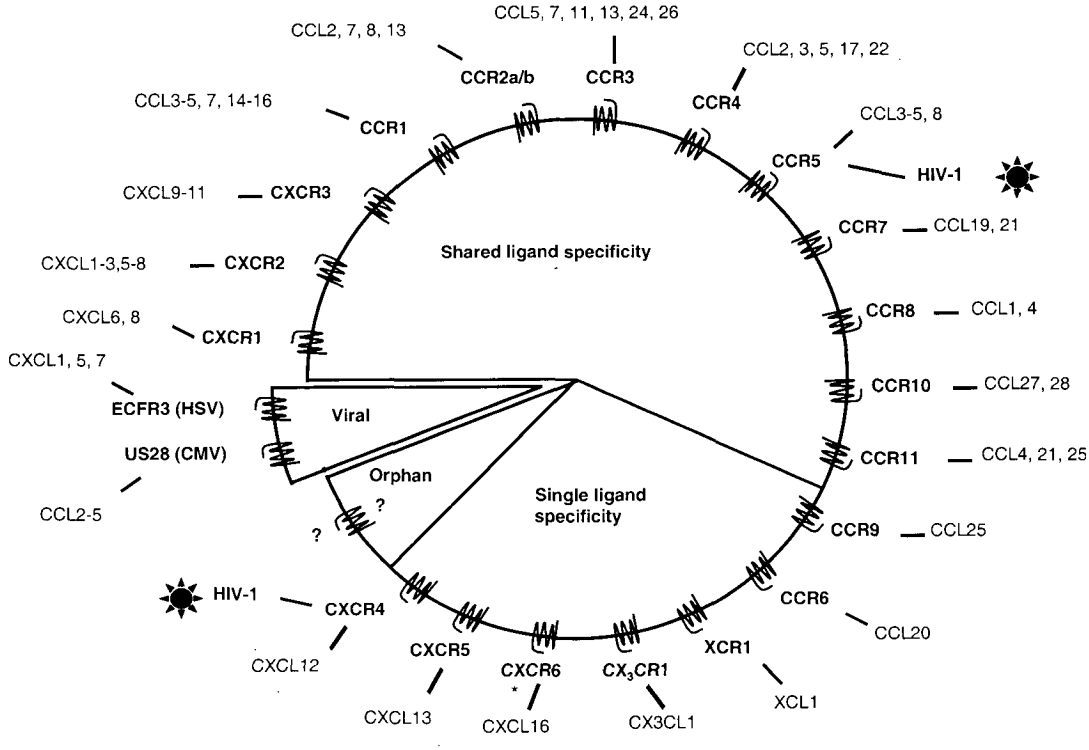

Figure 4. Chemokine receptors and their natural ligands. *Nineteen different chemokine receptors encoded by the human genome have thus far been identified and associated with known ligands. While a few receptors have until now been shown to be specific for single chemokines, the majority bind a variety of soluble ligands. Furthermore, the natural ligand to numerous orphan receptors have yet to be identified and the receptors to several chemokines have not been cloned. Viruses, notably the herpesviridae CMV and and HSV, have also been shown to encode for functional viral chemokine receptors that may play a role in viral pathology. Importantly, HIV-1 mainly uses the CCR5 and CXCR4 chemokine receptors as co-receptors for viral entry into CD4-expressing T cells. Modified from Proudfoot, A.E.I. et al. Immunol. Rev. 177: 246-256. * Recently proposed by Matloubian, M. et al. Nat. Immunol. 1: (4)298-304 and Willbanks, A. et al. J. Immunol. 166: (8)5145-5154*

The action of the chemokine-chemokine receptor system is two-fold. First, it is directly involved in specifically targeting and mediating the extravasation of circulating immune cells into tissue[361,395-397]. Immune cell behaviour has been observed by targetting fluorescently-labelled immune cells to a chosen site with a selected chemokine and by then tracking their migration, including under flow conditions mimicking *in vivo* conditions, and directly *in vivo* by microscopy[398-401]. Thus, an established sequence of key events characterises the extravasation of lymphocytes into targeted tissue. The selectin-mediated low-affinity adhesion of the cells to the vascular endothelium first results in the rolling of lymphocytes against the blood vessel wall[400,402]. This allows for the engagement of chemokine receptors by their cell-surface associated ligands, which in turn leads to the upregulation of integrin molecules that trigger the immobilization of the cells against the vessel wall[368,397,403,404]. Only then do immune cells extravasate into the tissue through diapedesis, in a process mediated by shear forces within the lumenal space of vessels[405].

The second recognized role of the chemokine-chemokine receptor lies in its action within tissue[361]. There, immune cells bearing appropriate receptors actively migrate up gradients of increasing chemokine concentrations to eventually localize to sites of recruitment[406]. The repulsion of T cells by a chemokine has also been observed: indeed, CXCL12 (SDF-1) has been shown to either attract or repulse T cells in a dose-dependent, CXCR4-mediated manner[407]. In all cases, it is the expression of an appropriate receptor, which confers upon a cell its reactivity to a given chemokine.

In a newly arising paradigm, chemokines can be ascribed to either of two functional subsets: while chemokines that are secreted in the context of immune activation can be classified as "inflammatory", "homeostatic" chemokines are regulators of the normal, resting-state dynamics of the immune system [363,372]. The corollary is that the receptors, which confer a cell with reactivity to such ligands, will also contribute to the functional profile of the cell that bears it. As such, "inflammatory" chemokines such as CCL2, CCL3 and CCL5 (MIP-1α, MIP-1β, and RANTES, respectively) are secreted in the context of immune responses and are bound by CCR5, which is expressed on memory

and activated T cells[408,409]. Activated T cells are thus attracted to sites of inflammation. Conversely, homeostatic chemokines such as CCL19 and 21 (ELC and SLC, respectively), which are respectively secreted by dendritic cells prior to transcytosis to the lumen of high endothelial venules and secreted by endothelial cells of the secondary lymphoid organs, bind CCR7, which is involved in the homing of immune cells to the lymph node[403,409-418]. In fact, CCR7 is essential to the proper formation of functional lymph nodes and the elaboration of T cell-mediated immune responses[366,419,420].

A caveat to the formulation of the paradigm resides in that one cell's inflammatory chemotactic signal is sometimes another's homeostatic regulator: thus, naïve CCR7-expressing T cells use CCL19 and CCL21 in order to homeostatically target lymph nodes, while dendritic cells use the same chemokine receptor/chemokine combination once they have encountered peripheric antigen, have matured, and are trafficking back to secondary lymphoid organs in order to orchestrate immune responses[362,363,421-425]. A reformulation of the paradigm might thus be that of "central" vs. "peripheral" homing chemokine receptors.

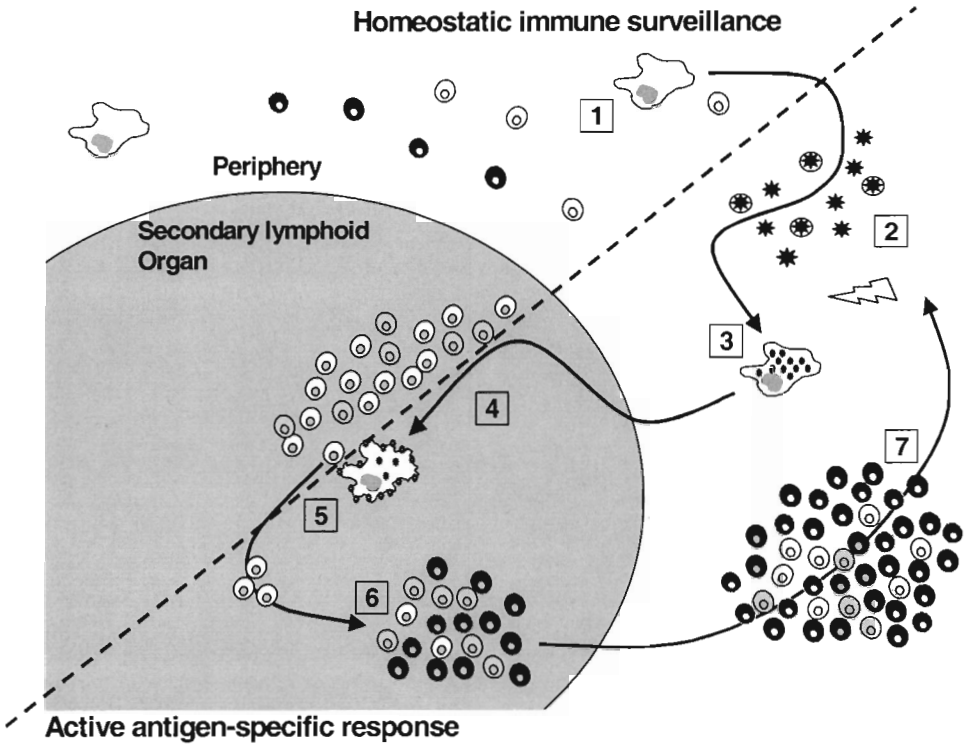

Figure 5. Immune cell trafficking and the orchestration of CD8 T cell responses. *Immune cells, including dendritic cells and T cells, homeostatically circulate throughout the body (1). Upon encounter with infected cells or antigen in the presence of inflammatory stimuli (2), immature dendritic cells endowed with superior antigen uptake capacity are induced to mature (3), thereby increasing their efficiency for antigen-presentation, and up-regulating co-stimulatory molecules. Simultaneously, their chemokine receptor expression is modulated such that activated dendritic cells are preferentially targeted to migrate towards secondary lymphoid organs where they are screened by multiple T cells with distinct TCR specificities (4). Specific CD8 T cells that are engaged by their appropriate peptide-MHC ligand with appropriate co-stimulatory conditions are clonally selected (5). Activated T cells are then induced to undergo clonal expansion as individual cells within the responding pool functionally differentiate (6) and disseminate to the periphery where they home to sites of inflammation, guided by inflammatory chemokines (7).*

A dynamic perspective of immune response has evolved as follows, and as illustrated in figure 5. Typically, first exposure to a foreign antigen takes place at

the peripheral site where a pathogen has entered the body. There, a rapid response is expected from mediators of native immunity and results in the recruitment, through the action of "inflammatory" chemokines, of various cells of the immune system, including the phagocytic macrophages and particularly immature dendritic cells[362,363]. At the site of inflammation, these immature dendritic cells endowed with superior antigen-uptake capacity are subjected to maturation stimuli[426]. The result is 1) their reduced capacity to up-take antigen, 2) their increased ability to present antigen, 3) their upregulation of costimulatory molecules and 4) a coordinated switch in chemokine receptor expression from peripheral homing receptors to central homing receptors[10,362,363,422,426]. In particular, their upregulation of CCR7 directs them to migrate towards secondary lymphoid organs, where they will encounter T cells within an environment that is appropriate to their efficient activation and recruitment[362,363,414,422,424,425,427,428]. This in turn leads to dissemination of activated effectors for helper CD4 T cells to support B-cell follicles as well as T cell responses, and activated CD8 T cells to migrate to the peripheral sites of antigenic invasion[350-352,354,367,429]. Altogether, the observed cellular immune response results from the summation of the properties exhibited by various antigen-specific T cell subsets that are then disseminated. Furthermore, various T cells expressing a range of complementary functions is then tributary to the effectiveness of the response.

1.1.14. T cell differentiation subsets

T cells are classified into subsets on the basis of their history and functional properties, and not because they express a specific set of phenotypic markers. As T cells are constantly re-modelled by the various stimuli they meet, the resulting dynamic nature of their differentiation precludes their categorization into statically defined subsets. In fact, at any given time and anatomical site, combinations of commonly used markers serve at best to restrict focus to pools of T cells within which subset-specific properties are enriched [430,430,430]. Table 2 provides an overlook of some of the molecules commonly used to dissect T cell subsets.

Table 2: Enrichment markers for T cell subsets[248]

	Marker	Expression enriched in subsets of	Protein superfamily	Reference
Cluster of differentiation molecules	**CD11b***	E	Integrin	431
	CD25 (IL-2Rα)	E		432
	CD27	N, M	TNF-Receptor	431
	CD28	N, M	Immunoglobulin-like	431
	CD44*	E, M	Cartilage link protein	432,433
	CD45RA[T]	N, E	PTP Receptor	431,432
	CD45RO[T]	E, M	PTP Receptor	432
	CD49d*	E, M	Integrin	431
	CD56	E, M	Immunoglobulin-like	434
	CD57	E		431
	CD62L	N, M	C-type lectin	432,433
	CD69	E	C-type lectin	432
	CD95 (Fas)	E, M	TNF-Receptor	431
	CD95L (FasL)	E, M	TNF	431
	CD137 (4-1BB)	E	TNF-Receptor	435
	CD154 (CD40L)	E	TNF	436
	CD134 (OX-40)	E	TNF-Receptor	437
	CD152 (CTLA-4)	E	Immunoglobulin-like	438
Chemokine receptors	**CCR5**	E, M	G-protein-coupled receptor	432
	CCR6	E, M	G-protein-coupled receptor	432
	CCR7	N, M	G-protein-coupled receptor	432
	CXCR3	E, M	G-protein-coupled receptor	432
	CXCR4	N	G-protein-coupled receptor	432
Others	**ICOS**	E	Immunoglobulin-like	439
	Ly6c	M		432
	PNA ligand*	E, M	O-glycan	440
	1B11 ligand*	E	O-glycan	441

N: naïve, E: effector, M: memory
PTP: Protein Tyrosine Phosphatase
TNF: Tumour Necrosis Factor
* Denotes markers established for murine T cells only
[T] CD45RA and RO are isoforms of the CD45 molecule

Mature peripheral T cells may nevertheless be subdivided into two categories: naïve cells and antigen-experienced cells. Naïve cells are those that have not been stimulated by their specific ligand. They are functionally relatively quiescent, although they undergo low-

level homeostatic proliferation [442,443,443,443]. Activation of naïve T cells will only occur if they encounter their specific peptide/MHC complexes at the surface of professional antigen-presenting cells (APC) able to provide them with adequate costimulatory signals [444-450].

Antigen-experienced cells may be subdivided into several functional subsets. Effector T lymphocytes are specific to an antigen which is present and induces them to proliferate (i.e. clonal expansion) as well as to exert effector function through the secretion of cytokines and/or the polarized release of cytotoxic granules [451]. The second subset of antigen-experienced T cells consists of memory T lymphocytes that are the key protractors of immunological memory [432,452]. *In vivo* studies have shown that memory CD8 T cells can migrate to inflammatory sites and acquire effector properties much faster than naïve cells [453-455]. Nevertheless, classically, memory T lymphocytes differ from activated effectors in that they are characterized by the lack of some major ready-effector functions [456]. These include the polarized secretion of cytotoxic granules and the production of certain cytokines (IFN-γ): the induction and maintenance of these functions require sustained TCR engagement by cognate peptide/MHC [438,457]. Furthermore, both types of antigen-experienced T cells differ from naive T cells in their enhanced sensitivity for antigen [447,458-461]. This is in part due to the increased expression of signalling components such as p56Lck and to a developmentally regulated rearrangement of the topology of their signalling machinery [462-464,464,465].

Phenotypic differences between T cell subsets not only result from differential protein expression, but also from differential post-translation modification of some of these proteins. In mice, activated and memory cells have been shown to bind peanut agglutinin (PNA) to a greater extent than naïve cells, suggesting a reduced number of sialic acid residues on the cell surface O-glycans of antigen-experienced cells [440]. Furthermore, differential O-glycan modification of mucin-type glycoproteins such as CD43 has been shown to discriminate between effector and memory cells [441].

A key characteristic of acquired immune responses is the establishment of immunological memory; defined as a more rapid and more effective secondary immune response directed against a previously encountered pathogen (figure 6). Immunological memory results from a carefully coordinated interplay between cells of the native and acquired immune system and is first established in the context of a primary encounter with an antigen.

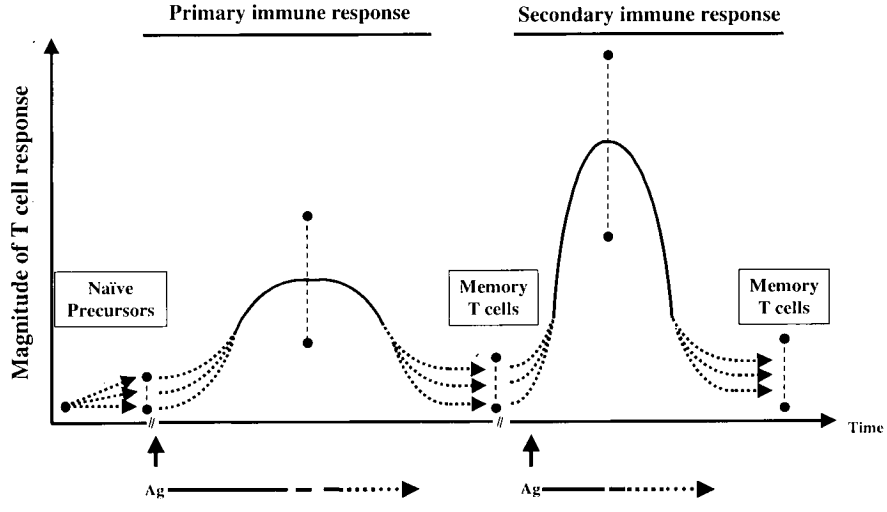

Figure 6. Antigen-specific T cell immune responses. *Recent thymic T cell migrants found in the periphery are derived from single T cell clone precursors having undergone low-level homeostatic expansion. Prior to any antigen-driven expansion, specific peptide-MHC combinations are recognized by variable numbers of T cell clones. Upon encounter with their specific antigen, T lymphocytes undergo clonal expansion and constitute the primary immune response. Resolution of the antigenemia results in the death phase of the primary response, during which effector responses become undetectable. Memory is seeded during the primary immune response and persists in absence of detectable antigen. Re-encounter with antigen results in the secondary response characterized by a rapid expansion of memory T cells. Altogether, memory responses are recruited more rapidly, display stonger effector activity, and are resolve antigenemia more rapidly than primary responses.*

As will be discussed in the light of our findings, the mechanisms underlying the induction and maintenance of T cell immunologic memory are only beginning to be unveiled. They reside in complex events influencing the properties of single cells as well as that of entire

cell populations. Recent technological developments have yielded analytical tools allowing for the multi-parametric real-time analysis of single T cells both *in vivo* and directly *ex-vivo*. These have been instrumental to our investigations. They include tetrameric MHC reagents, which serve to exquisitely identify T cells specific for an antigen of interest and dyes, such as carboxyfluorescein succidimidyl ester (CFSE), which label live cells that can then be tracked through multiple rounds of division [466,467]. In conjunction with flow cytometric measurement of various intra-cellular and extra-cellular markers, we have used approaches allowing for the simultaneous multi-parametric analysis of cell phenotypes and function.

1.2. The Human Immunodeficiency Virus type-1

1.2.1. Viral etiology of human AIDS

The Human Immunodeficiency Virus is the etiologic agent of the Acquired Immunodeficiency Syndrome (AIDS). HIV-1 was first isolated in 1983 by three independent groups and was then referred to as Lymphadenopathy-Associated Virus (LAV), Human T-Lymphotropic Virus type-3 (HTLV-III), and AIDS-Associated Virus type-2 (ARV-2) [468-470]. Subsequent molecular cloning and sequencing of the different viral isolates revealed that they shared several characteristics, and they were later classified as isolates of the HIV-1 species [471-477]. Three years following the discovery of HIV-1, a genetically distinct yet distantly related human primate virus, HIV-2, was identified and molecularly cloned [478-481]. HIV-1 and HIV-2 are closely related to distinct families of the Simian Immunodeficiency Virus (SIV). In fact, evidence strongly suggests that HIV species are the result of multiple distinct zoonotic infections with SIV[482]. While SIV species phylogenetically related to HIV-2 have been identified in sooty mangabey (*Cercocebus atys*) monkeys from western Africa, HIV-1 is phylogenetically more closely related to strains of SIVcpz whose natural host is the *Pan troglodytes troglodytes* chimpanzee[483-485]. Experimental models based on the infection of nonhuman primates with SIV now constitute key systems used to investigate HIV disease. In humans, HIV-1 infections are the predominant cause of AIDS: in spite of a relative stabilization of the number of new cases of HIV-1 infection in most high-income nations during the 1990's, a renewed trend for an increased number of new infections in these countries has now been reported; furthermore, the number of newly infected individuals still increases world-wide[486]. In 2001, it was estimated that 40 million individuals were HIV-infected, 2.7 million of which were children under 15 years of age[486].

1.2.2. Genetic diversity of HIV-1

HIV-1, the retroviral agent studied herein, is a viral species belonging to the family *Retroviridae*, genus *Lentivirus* [487]. The main characteristics of the *Retroviridae* lies in that their genome consists of single-stranded RNA bearing positive polarity with respect to translation, and in that their life cycle is contingent on a virally-encoded RNA-dependent DNA polymerase enzyme, the reverse transcriptase (RT). The salient characteristic of HIV-1 RT is that it is highly processive, yet markedly errkdor-prone because it does not possess a 3'exonuclease proofreading activity[488]. *In vitro* measurements have shown an error rate of one error per 6000 nucleotides transcribed, while the rate of mutation *in vivo* is estimated at one base pair per viral genome per round of replication[488,489]. These errors consist of deletions, insertions and frameshift mutations; and they can take place anywhere in the viral genome[488-492]. The majority of these errors are deleterious and even lethal to the virus. However, particular mutations introduced at specific sites can confer a survival advantage to a virion facing certain stress from its host environment [493,494]. Furthermore, beyond the virus' propensity to mutate, recombination occurs between different viral genomes[495].

The mechanistic properties of RT underlie the tremendous genetic diversity of HIV-1 generated within an individual. A subject infected by HIV-1 is thus rapidly colonized by a broad diversity of viral quasispecies whose individual genotypes ascribe the virions with distinct phenotypic and functional properties. Essentially, the viral population within an individual is in constant evolution, each new mutation conferring potential advantage or disadvantage to a viral progeny[488,494,496-499]. This results in a significant immunologic challenge as each virion then represents a potentially novel pathogen to be countered by the immune system; essentially, each new transmission of the virus between cells (or between individuals) results in an evolutionary "bottle-neck" effect that seeds the founders upon which viral diversity is furthered.

Beyond the heterogeneity of an individual's quasispecies, the genetic diversity of HIV-1 is therefore evident at the inter-subject level: this outlines a potential challenge to the

development of broadly applicable vaccine strategies. On a worldwide scale, and on the basis of phylogenetic relationships, HIV-1 isolates identified to date have been classified into three distinct clades: groups M, N and O[500]. Clade "M" represents the main clade comprising HIV-1 subtypes A-H and J-K, all of which significantly differ from HIV-1 strains classified as clade "O" (historically named "outlier" strains) and the newly defined clade "N" of "non-M, non-O" strains[501]. Recombination occurs within and between HIV-1 clades, and has resulted in "mosaïc" viruses that are also known as HIV Circulating Recombinant Forms[501]. Interestingly, when comparing the sequence of various HIV-1 clades to that of SIVcpz, the three groups of HIV-1 are not closest to each other, but rather they are most closely related to the SIVcpz sequence, thus supporting the hypothesis for multiple cross-species transmission events[482].

1.2.3. Genomic organization of HIV-1

Characteristically of retroviruses, the genome of HIV-1 is diploid and consists of two single strands of RNA[487]. RT is present within the enveloped viral capsid, in close proximity of the viral RNA, and functions to reverse transcribe the RNA genome into linear, double-stranded DNA for its subsequent non site-specific integration into host-cell DNA. Viral reverse transcription mechanistically involves two inter-strand transfers by RT, events that underlie the recombination of HIV-1 strains[487]. It is the host genome-integrated form of viral DNA, referred to as "proviral" DNA, which can then be transcribed by the host cell machinery and gives rise to viral progeny.

The HIV-1 genome is illustrated in figure 7. The proviral sequence encodes for the classical structural retroviral gene products Gag, Pol and Env, flanked by two U3-R-U5 proviral long terminal repeats (LTRs)[502]. The U3 region of the 5' proviral LTR possesses the core promoter, enhancer and modulatory transcriptional domains necessary for HIV-1 replication, whereas the R and U5 regions of the LTR encode mainly for RNA regulatory sequences[487]. As such, HIV-1 transcription relies on Sp1 and TATAA-binding protein (TBP), is enhanced by NF-κB, and modulated by other cellular factors including AP-1 and NFAT[487]. Together, these cellular factors thus direct viral replication, determining in

part whether HIV-1 will rapidly replicate or lay in a relatively quiescent state akin to viral latency.

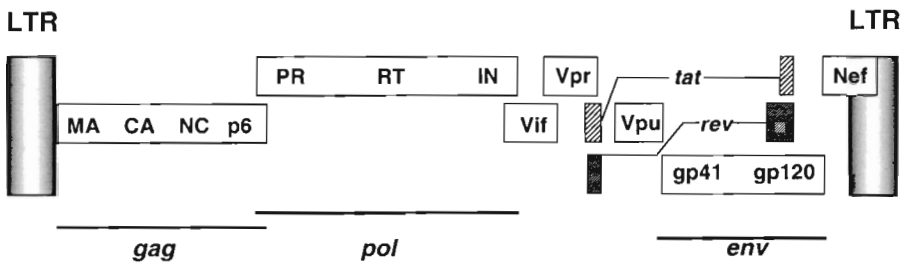

Figure 7. Illustration of the genomic organization of HIV-1. *Once integrated into host DNA, the coding sequences of HIV-1 are niched between two LTRs, each consisting of U3-R-U5 regions. The 5'LTR encodes the promoter enhancer regions. Open reading frames are translated from all three reading frames. The structural gag, pol and env genes encode for their multiple gene-products, which are eventually proteolytically cleaved during virion maturation. Vif, Vpr, Vpu, Tat, Rev and Nef are the accessory proteins of HIV-1.*

In all, HIV-1 has nine ORFs[502]. The *gag* gene encodes for a single open reading frame (ORF), which results in the p55 polyprotein that is then proteolytically processed into the matrix (MA, p17), capsid (CA, p24), p2, nucleocapsid (NC, p7), p1 and P6 proteins that structurally constitute the virion and associate with the contents it carries, including genomic viral RNA. The single *pol* ORF encodes for RT and its RNAse H activity that are required for reverse-transcription, for the integrase that catalyses viral DNA insertion into host DNA, as well as the protease that processes the viral polyproteins. Finally, *env* yields the envelope surface gp120 that confers the virus with its cell-specific tropism, and the trans-membrane gp41 that serves in viral fusion to the host cell.

HIV-1 also possesses an assortment of six non-structural gene products, two of which are essential to its replication. First, Tat is a virally-encoded trans-activating element that enhances viral RNA transcription and promotes the proper elongation of full-length viral RNA, in part through binding the trans-activating response (TAR) element encoded by the R subdomain of the 5'LTR[503]. Second, Rev serves as a post-transcriptional transactivator by binding Rev-responsive elements and contributing to the proper splicing, shuttling and translation of viral mRNA[504-506]. Although Vif, Vpr, Vpu and Nef are dispensible for replication, their contribution to the evolutionary fitness of HIV-1 is nevertheless highlighted by the fact that they have been preserved by other primate lentiviruses[495]. These small proteins serve multiple purposes to the virus, the majority of which are probably still unresolved. Of these, Vif and Nef are noteworthy because they have been shown to significantly enhance the infectivity of viruses. In particular, Nef will be further discussed for its role in immune evasion of HIV-1 and its demonstrated impact on disease progression in humans.

1.2.4. The life cycle of HIV-1

HIV-1 entry into permissive cells is first directed by the interaction of gp120 with CD4[66,507,508]. The ensuing conformational change allows for gp120 to directly contact the cellular seven-transmembrane proteins that serve as its co-receptors[509,510]. Finally, the interaction between gp120 and an appropriate CD4 and co-receptor pair results in the exposure of the hydrophobic portions of gp41, which then mediates fusion of the viral particle with the cellular membrane and the intracellular release of the viral capsid[511]. Once inside the cell, the viral RNA is reverse transcribed by the virally encoded RT and the resulting double-stranded DNA is transported to the nucleus where it is integrated into chromosomal DNA[487]. Integrated proviral DNA is then transcribed by the cellular machinery, resulting in the production of viral genomes and proteins that are assembled as new viral particles in the cellular cytoplasm[487]. Viral progeny then buds through the plasma membrane, in a process that requires organized lipid raft microdomains and

results in the acquisition of host cell-derived membrane-bound proteins by the virion[512-515].

Critically, HIV-1 replicates rapidly. Following the productive infection of a cell, it is estimated that viral progeny may be produced within hours of infection and that an average lifecycle of HIV-1 is approximately 1.2 days in length[516,517]. The total HIV-1 production has been estimated to be approximately 10^{10} virions per day, with plasma virions having a mean lifespan of 0.3 days in the host[516]. Many of these HIV-1 virions are non-infectious due to the high number of damaging mutations introduced by the RT. Nevertheless, the viral burden that ensues infection is sizeable and contributes to the rapid propagation of the infection.

1.2.5. Cellular tropism of HIV-1

It is the highly glycosylated viral envelope surface gp120 protein that confers HIV-1 with its ability to infect cells. While most HIV-1 strains require that a cell express CD4, CD4-independent HIV-1 strains have been isolated[518-520]. *In vitro*, HIV-1 has been reported to infect a broad range of human cells, whether or not they expressed the primary viral receptor CD4[489]. *In vivo*, however, it is $CD4^+$ T lymphocytes and cells of the monocytic lineage that have been unequivocally shown to be primarily targeted by HIV-1[521].

Traditionally, laboratory HIV-1 isolates have been classified on the basis of their ability to infect T cell lines (T-tropic strains), macrophages (M-tropic strains) or both (dual-tropic strains)[522]. Although clinical isolates do not exhibit the clear dichotomy seen with laboratory strains, these *in vitro* characteristics are extremes of an analogous preference of clinical isolates for certain cell types. Furthermore, the historical observation of the differential ability of various HIV-1 isolates to form syncytia when passaged through cells *in vitro* has also been shown to largely segregate with the isolate's tropic properties[523]. Beyond its interaction with the primary CD4 receptor, it is gp120's ability to interact with selected seven-transmembrane co-receptors of HIV-1 that ultimately refine its tropism and underlie the preference exhibited by different isolates[524]. A strain's

tropism and phenotypic properties are thus dictated by the sequence of a virus' gp120 and are thus influenced by mutations occurring therein. Interestingly, an evolution of HIV-1 gp120 from M-tropic to T-tropic preference has been shown to take place throughout disease progression[525-529].

The co-receptors of HIV-1 are endogenous cellular chemokine receptors that include CCR1, CCR2b, CCR3, and most importantly CCR5 and CXCR4[509]. Significantly, and as predicted from the expression spectra of these chemokine receptors, primary clinical HIV-1 isolates predominantly using CXCR4 are T-cell tropic, isolates that use CCR5 infect a broader variety of cells, including T cells and macrophages[530-540]. Although its significance to HIV-1 infection is still unclear, a virally-encoded chemokine receptor homologue, US28 of CMV, has been demonstrated to be useable by HIV-1 in lieu of its usual host cell co-receptors[541]. As CMV naturally infects endothelial cells and cells of the myeloid lineage, HIV-1 and CMV co-infection may thus further influence the natural course of disease.

Recently, gp120 has also been shown to interact with dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) in a process that may well be critically important in enabling establishment of HIV-1 infection[542]. Although DC-SIGN is not a receptor allowing viral entry, it has been demonstrated to enhance *trans*-infection of T cells by HIV-1[542,543]. DC-SIGN, which is expressed by macrophages and dendritic cells, was originally demonstrated to mediate interaction between the APC and T cells expressing ICAM-3[544,545]. Beyond its function as an adhesion molecule, DC-SIGN has been shown to mediate the internalization of antigen for eventual presentation, and could thus theoretically be of use in eventual vaccination strategies that seek efficient antigen presentation by dendritic cells[546]. Interestingly, HIV-1 Nef is reported to increase DC-SIGN surface expression on infected dendritic cells, a process that may enhance infectivity[547].

Thus upon establishing an original infection, HIV-1 associates with various cell types whose distinct migratory properties further contribute to the virus' broad dissemination

throughout the body. Furthermore, intrinsic differences between the various cell-types targeted by HIV-1 result in the establishment of viral reservoirs with different replication kinetics and susceptibilities to immune responses and therapeutic intervention. Together with other viral properties, the tropism of HIV-1 is therefore key to the establishment of the productive and persistent infection that characterizes AIDS and the natural course of HIV-disease progression.

1.3. The Acquired Immunodeficiency Syndrome

1.3.1. Natural course of HIV-1 infection

The natural course of HIV-1 infection, illustrated in figure 8, is classically divided into three phases, which are characterized by distinct clinical, virologic, and immunologic parameters[548]. Clinically, a syndrome of variable severity and duration characterizes primary HIV-1 infection. In many cases, however, primary HIV-1 infection is not immediately diagnosed. This is in part because its disease manifestations are similar to that of other viral infections, such as acute infectious mononucleosis[489,549,550]. Nevertheless retrospectively, it has been estimated that some 70% of HIV-1 infected individuals experienced such symptoms upon primary infection[548].

The second stage of disease progression is the chronic phase of HIV-1 infection. During chronic HIV-1 infection the patient does not exhibit particular clinical symptoms of disease. As will be further discussed, this clinical latency phase is observed in spite of the fact that virologic and immunologic parameters clearly demonstrate that an intense and protracted confrontation is taking place between the virus and host. In fact, although the chronic phase of HIV-1 disease is of variable length, with few exceptions HIV-1 infection inexorably progresses towards the third and final stage of AIDS-defining illness. AIDS is characterized by multiple opportunistic infections and the occurrence of rare cancer types that lead to the eventual demise of the patient[551,552].

The clinical course of a typical progression of HIV-1 infection is characterized by a chronic infection phase that lasts for eight to ten years[550]. In contrast, rapid progressors may experience AIDS-defining illness within months of primary infection while long-term nonprogressors (LTNP) remain asymptomatic for more than fifteen years[550]. The complex viral and host factors that contribute to disease progression and the variable length during which a patient sustains chronic infection and resists AIDS are only beginning to be understood.

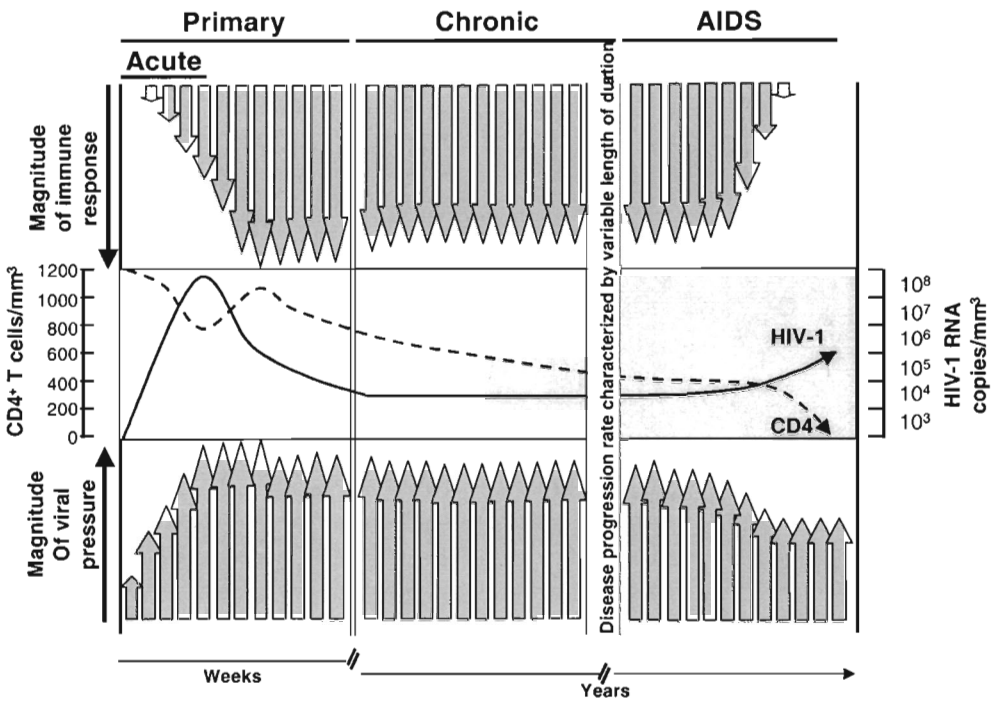

Figure 8. The natural course of HIV-1 infection. *HIV-1 infection is classically divided into three phases. The primary phase of infection is characterized by rapid viral replication and systemic dissemination, as well as by the characteristic loss of CD4 T cells. Gradually, the immune system begins to counter the pathogen with an increasingly potent response that is mainly mediated by T cells and that curbs the plasma viral load until an apparent equilibrium is reached. The recent insight into distinct characteristics of the CD4 and CD8 T cell responses orchestrated during the original days of infection suggest that a critical phase of infection, which is herein referred to as the acute phase of infection, may be hypothesized although it is not yet accurately defined and circumscribed. The establishment of a viral set point and the appearance of measurable titers of anti-HIV-1 antibody signal the beginning of the chronic phase of infection. Chronic HIV-1 infection is the asymptomatic phase of infection that may last for a variable length of time, with an average of 8-10 years. Patients who experience a rapid progression to disease may never have reached a stable viral set point and may progress to the final stages of disease within months, while LTNP may remain asymptomatic for more than 15 years. As indicatied by persistent plasma viremia and the gradual loss of CD4 T cells, an intense and protracted struggle between the virus and the host's immune system is taking place throughout chronic HIV-1 infection. The occurrence of characteristic opportunistic infections and neoplasms signals the onset of AIDS. During this*

final stage of disease, all immune responses are lost; and CD4 T cell counts continue to fall until the inexorable demise of the patient.

1.3.1.1. Virologic parameters

Virologically, the primary HIV-1 infection (PI) phase is characterized by an intense plasma viremia that peaks 3 to 6 weeks post-infection[549,553]. The virus can then be demonstrated through *in vitro* virus culture methods, by the detectable presence of plasma HIV-1 Gag p24 protein (p24 antigenemia), as well as by more sensitive molecular means that quantify plasma viral RNA and cell-associated viral RNA and DNA. The partial resolution of the extremely high plasma viremia, which often peaks at more than 10^7 viral RNA copies per ml of blood during primary HIV-1 infection, is temporally associated with transition to the chronic phase of infection[549]. A relatively stable level of plasma viremia, referred to as the viral set-point, is then established at the onset of the chronic phase of infection and has been shown to be of strong predictive value for the rate of disease progression[554]. Finally, the onset of AIDS is associated with an increase of all virologic parameters.

Upon establishing an original infection, HIV-1 rapidly disseminates and seeds itself throughout the body: within hours or days, it is detectable in body fluids including blood, body secretions and cerebrospinal fluid, as well as in various solid tissues and organs[528,553,555]. Notably, these tissues infected by immunodeficiency virus include skin as well as intestines, and critically, thymus, spleen and lymph nodes[556-567]. The infection within these latter lymphoid organs is most stunningly observed by the pathology it induces, as evidenced by the gradual destruction of their anatomic architecture and their ensuing functional attrition[558,567-570]. Specifically once infected, the secondary lymphoid organs rapidly become primary foyers of viral replication that sustain the infection throughout the course of disease[564-567,571,572].

1.3.1.2. Immunologic parameters

Characteristic immunologic parameters also define the natural course of HIV-1 infection. CD4 T lymphocyte dysfunction is a hallmark of HIV-1 disease, as immediately obvious from the charting of blood CD4 T cell counts throughout disease progression (figure 8) [568]. Primary HIV-1 infection is characterized by a sharp decrease in the number of circulating $CD4^+$ T cells, a clinical parameter that may somewhat normalize in subjects following the resolution of the extreme primary viremia, but steadily decreases through chronic infection. The low CD4 T cell count eventually attained by patients with progressive disease is associated with AIDS-defining illness and the final stages of disease[489]. Some patients with extreme primary infection syndrome and transiently low CD4 T cell counts similar to those reached in the AIDS phase may also temporarily experience AIDS-like symptoms[549,573]. Furthermore, in patients with chronic HIV-1 infection, the ratio of circulating CD4 to CD8 T cells is markedly altered, and often inversed[574,575]. Both $CD4^+$ T cell depletion and the increased proliferation of CD8 T cells involved in immune responses underlie the alteration of this immunologic clinical parameter.

HIV-1 infected individuals who progress to AIDS retain antigen-specific T cell responses against recall antigen and innocuous latent viral infections for a variable period of time during chronic infection. In fact, it is the progressive loss of these responses that is evidenced by the appearance of opportunistic disease, which first clearly signals late chronic infection and the onset of AIDS[550]. Thus, chronic and latent infections by *herpesviridae* family virus such as Epstein-Barr virus (EBV), Herpes Simplex virus (HSV-1 and -2) and Cytomegalovirus (CMV) remain under control throughout asymptomatic chronic HIV-1 infection, much as they do in non-immunocompromised, HIV-1 uninfected subjects[576-580]. Particularly, CMV containment is known to be the result of antigen-specific CD8 T cell responses as suggested by the correlation between recovery of protection from CMV disease and the appearance of CMV specific CD8 T cells in bone marrow transplant patients, and as demonstrated by the seminal report that an asymptomatic CMV carrier state could be re-instated and maintained in bone-marrow

transplant patients who received CMV specific CD8 T cells that had been expanded *in vitro*[581,582]. Specifically, functional antigen-specific CD8 T cell-mediated immune responses, directed amongst others at the pp65 495-503 (NLVPMVATV) epitope, are known to contribute to the immune control of CMV[580,583-586]. Critically, T cell immune responses to CMV are present during asymptomatic chronic HIV-1 infection[587-589]; and it is highly probable that it is the effective loss of these response that results in the onset of CMV-disease symptoms that are clinical markers of HIV-1 disease progression and contribute to define AIDS[552,579,590-592].

T cell-mediated immune responses directed against HIV-1 are evident from the onset of infection[593]. Critically, during primary HIV-1 infection, the appearance of functional CD8 T cell responses is temporally associated with the original curbing of viral replication and the onset of a 10^{1-4} copies reduction in viral load. Significantly, CD8 T cell responses are detected even prior to circulating titers of anti-HIV antibodies. Nevertheless, the systemic viremia is only reduced and the virus is not eradicated by the immune response alone: HIV-1 specific immune responses will be discussed in greater detail.

1.3.2. Highly active antiretroviral therapy (HAART)

Medical interventions by administration of chemotherapeutic regimens that consist of cocktails of antiretroviral compounds significantly alter the course of HIV-1 infection. The various agents currently used include nucleoside inhibitors of RT such as AZT (zidovudine), d4T (stavudine), ddI (didanosine), 3TC (lamivudine) and Abacavir, as well as non-nucleoside analog inhibitors of the RT, such as Efavirenz and Nevirapine, and protease inhibitors, including Amprenavir, Indinavir, Nelfinavir and Saquinavir. Next-generation compounds targeting viral entry into the susceptible cells, such as T-20 that targets gp41, are not yet in wide usage[594]. HAART reduces the viral load within all body compartments and contributes to the normalization of clinical and immunologic parameters that are disturbed by HIV-1 infection. These latter include the replenishment of the CD4 T cell numbers to normal or near-normal levels, first evident through the re-distribution of existing T cell pools and supported by *de novo* CD4 T cell synthesis, as

well as a normalization of lymphoid and thymic architecture and a tempering effect on the general state of immune activation, with normal CD4 and CD8 T cell dynamics being reached[595-599]. Furthermore, immune responses directed at recall antigen and normally innocuous viral infections, including CMV, which might have been lost by some HIV-1 infected individuals, convalesce following effective treatment with HAART.

Individually, all antiretroviral therapies currently in use are plagued because they select viral isolates whose mutations result in drug resistance; nevertheless, their combination in highly effective cocktails reduces efficient viral replication in such a way as to also slow-down the appearance of drug-resistant strains[600]. Moreover, the number of approved compounds presently in use and our knowledge of the molecular mechanisms of viral drug-resistance in HIV-1 do allow for the assembly of salvage therapies upon resistance breakthrough[601-603]. Nevertheless, potential combinations are limited, compliance issues are recurring, costs are prohibiting and the toxicity of HAART is significant. Furthermore, although viremia may be stably controlled by HAART to levels where it is barely detectable in lymph nodes or tissue using the most sensitive assays presently developed, HIV-1 establishes pools of productively infected cells that sustain the infection for prolonged periods of time[604-611]. Notably, one such pool is predicted to have a long enough half-life to support lifelong (>60 years) infection with HIV-1, should resistance breakthrough not occur and should patients remain strictly compliant with regimens that already prove toxic to a significant proportion of them within a few years[612]. Elaborate strategies based on a broad spectrum of rationales are presently being attempted, some using immunosuppressors to limit the original size of the latently infected T cell pool, while others try immunomodulation with cytokines to purge it from virus[589,613-616].

With some success, investigators are also experimenting with various formulae of scheduled therapy interruptions (STI), attempting to reduce the significant secondary effects of HAART while keeping the virus in check[617-619]. Although HAART and pharmaceutical strategies currently show clear clinical benefit, new avenues need to be explored to broaden the arsenal available to counter HIV-1. Vaccine and other immune-

based strategies may therefore prove key, yet their potential is mostly unexploited because the complexity of the phenomena that rule them remains largely unexplored. A better comprehension of the pathogenesis of HIV-1 infection, the identification of immune correlates of protection and most importantly, a better understanding of the immune responses directed at the virus are tributary to the development of such immune-based strategies.

1.3.3. Pathogenesis of HIV-1 infection

As the main cellular targets of the virus, CD4-expressing cells are those primarily affected by infection with HIV-1. Mechanistically, the net loss of CD4 cells is thought to be the result of both virally mediated cytopathology and, most importantly, of immunopathogenic consequences of the intense anti-viral immune response and ensuing immune dysregulation[489,548,550,620]. The net result of HIV-1 infection is the massive destruction of immune system cells, both those that are infected and bystander cells eliminated in the ensuing general immune dysregulation. While the homeostasis of other cell-types may be perturbed, including macrophages and dendritic cells, current data best documents the decimation of CD4 T lymphocytes.

Original studies by Wei and colleagues and D.D. Ho and colleagues, who characterized the replenishment of peripheral $CD4^+$ T cells following chemotherapeutic treatment of HIV-1 viremia with a protease inhibitor, led to the formulation of the "tap and sink" model to illustrate the production and destruction dynamics of CD4 T cells during infection[621,622]. Subsequent estimates suggested that the pool of HIV-1 infected CD4 T-cells have an average half-life of 1.6 days for a mean lifespan of 2.2 days[516]. By tracking the incorporation of ^2H glucose *in vivo* in cycling cell DNA in pulse-chase experiments, Hellerstein and colleagues later estimated that CD4 T cells that normally have an average half-life of 87 day turnover three times as fast, at a rate of $0.029^{\pm}0.005$ cells per day representing a half-life of 24 days, during chronic HIV-1 infection[623]. *Ex vivo* study of the Ki-67 antigen, which is an intra-nuclear marker of ongoing T cell proliferation because it is expressed at all phases of the cell cycle except G0, in the blood and lymph nodes

corroborate the finding that CD4 T cell turnover is accelerated in HIV-1 infected patients[596,624-626].

The pathogenesis of HIV-1 is also evident within secondary lymphoid organs, including lymph node and spleen, as well as within bone marrow and thymus[558,560,561,564,565,570-572,627,628]. As such, the apparent normalization of CD4 counts in the quasi-steady state with virus that takes place after primary infection is the result of the depletion of mature CD4 T cell pools and of interference with the replenishment of CD4 T cell stocks, countered by a persistent ongoing immune proliferative response and the engagement of homeostatic maintenance mechanisms that include the redistribution of cells into the blood compartment, their proliferation and an input of *de novo* produced T cells[560,623,625,629-635]. The continued progression to disease is evidence that the balance of these factors still favours depletion and that maintenance and replenishment mechanims eventually fail.

Beyond the high level of viral replication that takes place within an infected activated cell, viral pathogenesis of HIV-1 infection results directly from the action of viral gene products within infected cells and of bystander cells affected by viral gene products[636-639]. Although the formation of syncytia, which are non-functional multi-nucleated cells resulting from the fusion of infected cells, has been demonstrated *in vivo*, their reportedly low frequency suggests that this is not a primary mechanism of pathology[489,640]. Several HIV-1 accessory proteins have been shown to interact with cellular partners, thus diverting the normal cellular processes and altering cellular function. Vpr has been shown to alter progress into the cell cycle and protect from apoptosis while Vpu has been shown to induce apoptosis[641-651]. HIV-1 Nef induces cytopathy when transgenically expressed in mice[652]. Furthermore in human cells, HIV-1 Nef has been shown to interact with the TCR ζ-chain, to initiate a cellular program similar to that of cell activation, and to induce the upregulation of FasL on the surface of infected cells, thus underlying the potentially aberrant induction of apoptosis in cells within its vicinity[653-656]. HIV-1 Tat has been shown to enhance activation-induced apoptosis[636,657,658]. Finally, soluble and cell surface

gp120 have been shown to signal through the chemokine co-receptor they bind, and gp120 has been shown to induce apoptosis through a CD4-mediated signal[636,659-661].

Immunopathogenic mechanisms also underlie CD4 T cell depletion: in fact, it is arguably the premier cause of CD4 T cell loss. Infected cells are thus targeted for destruction through antibody-dependant cell cytotoxicity (ADCC) antibodies specific for cell-surface gp120[662-666]. Most importantly, immunopathogenesis results from the HIV-specific antiviral immune response that is 1) supported by HIV-specific CD4 T cells, and 2) directly mediated by HIV-specific CD8 T lymphocytes that eliminate cells harbouring the virus through cytolysis and the induction of apoptosis. As such, a unique characteristic of HIV-1 infection is therefore that $CD4^+$ T cells represent both the hunters and hunted.

1.3.4. CD4 T-cell mediated immune responses against HIV-1

There is strong evidence that T cell-mediated immune responses against HIV-1 are being orchestrated upon primary infection. Nevertheless, immune dysfunctions can soon be observed and the inexorable demise of HIV-1 infected patients who progress to AIDS is the result of the net loss of their ability to mount effective immune responses against the variety of challenges that one normally encounters, whether from foreign organisms or altered-self.

Much data suggest that "helper" CD4 T cells play a critical role in coordinating antiviral immune responses and in the maintenance of CD8 T cell activity during persistent viral infection in animal models[667-669,669-673]. A similar contribution of CD4 T cells to antiviral immune responses is also supported in humans[581,674]. Particularly, in HIV-1 infection, the progressive loss of functional antigen-specific CD4 T cells, most evident during the later stages of disease, are associated with the appearance of opportunistic disease and with the reactivation of chronic CMV and herpes simpex lesions[489,675,676].

Significantly, HIV-specific CD4 T cell responses, including proliferation as well as the secretion of IFN-γ and chemokines, are preserved in HIV-1 infected individuals who

control vireamia without recourse to treatment[677]. As such, the preservation of HIV-specific CD4 T cell responses is associated with the maintenance of health. While individuals with progressive disease also display such responses, progression through chronic HIV-1 infection is associated with the loss of HIV-specific CD4 T cell responses[678-680]. Interestingly, HIV- specific CD4 T cells lacking proliferative capacity but able to secrete IFN-γ have also been described: this dichotomy is proposed to explain, in part, the difficulty had with identifying HIV-specific CD4 T cell responses with classical proliferative assays[681]. Critically, early treatment during acute primary HIV-1 infection leads to the preservation of HIV- specific CD4 T cell responses[677,682]. While CD4 T cells are known to orchestrate B-cell responses, they are also direct effectors of antiviral immunity through the action of the cytokines and chemokines they secrete. Most importantly, their action is critical to the elaboration of effective CD8 T cell responses.

1.3.5. CD8 T cell immune responses against HIV-1 and the immunopathogenesis of HIV-1 infection

Substantial evidence supports a role for virus-specific CD8 T cell activity in the control of immunodeficiency virus infection but, to date, the direct formal demonstration of this fact in humans infected with HIV-1 has not been possible because it would require experimental approaches that cannot ethically be performed. Simian immunodeficiency virus (SIV) infection of macaques are the best animal models currently available for the study of immunodeficiency virus-specific responses apparenting HIV-1-specific responses in humans: these models have provided direct confirmation of the role of antiviral CD8 T cells in the control of immunodeficiency virus. SIV-specific CD8 T cells have been observed in infected simians, throughout the primary and chronic phases of disease progression[683-687]. Moreover, the emergence of SIV-specific cytolytic CD8 T cells in macaques coincides with control of the viremia and SIV-specific CD8 T cells have been shown to migrate to sites of viral replication[556,688]. Activated SIV-specific CD8 T cells are present both within the blood and lymph nodes of infected macaques[689,690]. Furthermore, SIV-specific CD8 T cells have been shown to contribute to the establishment of the viral set point that follows primary infection and characterizes

chronic infection: significantly during both of these stages of infection, an antibody-mediated depletion of CD8 T cells results in a marked increase of systemic viremia that is resorbed once CD8 T cell pools are replenished[691-694].

Altogether, CD8 T lymphocytes may contribute to counter HIV-1 infection through separate mechanisms: first, they do so through the secretion of virus-inhibiting factors, including but not limited to chemokines such as CCL3, 4, and 5 (MIP-1α, MIP-1β and RANTES, respetively) that compete with HIV-1 for its CCR5 receptor and thus protect cells from being infected[393,695-703]. Second, *in vitro*, HIV-1 specific CD8 T cells have been shown to directly induce the death of artificial targets and genuine, infected primary $CD4^+$ T cells[704,705].

Notably, strong virus-specific CD8 T cell responses have been repeatedly observed throughout primary and chronic HIV-1 infection, both in adults and pediatric patients[466,577,706-719]. Clonotypic expansions of CD8 T cells and TCR Vβ-specific amplifications have been shown during the course of HIV-1 infection, to frequencies of a magnitude that strongly suggests antigen-specific CD8 T cell responses[222,223,720]. Furthermore in seminal work published in 1994, the functional characterization of CD8 T cells isolated from a patient experiencing primary HIV-1 infection and using a Vβ TCR segment which was overly represented within the individual's repertoire, revealed that this Vβ-specific clonotypic amplification was enriched in CD8 T cells that mediated HIV-specific activity[326]. Accordingly, HIV-specific CD8 T cell responses have been shown to consist of largely oligoclonal expansions that could persist through time[225,323,710,721]. Importantly, the qualitative nature of the βTCR-chain repertoire has been shown to be a good prognosticator of disease progression: while monotypic βTCR-chain amplifications are associated with rapid progression to disease, perturbations of the TCR repertoire that involve amplifications within multiple different Vβ families are associated with slower progression to disease[722].

In vivo, HIV-specific CD8 T cells have been confirmed at anatomic sites of infection and viral replication, including the spleen and tonsils, during chronic HIV-1 infection[561,563]. Although, a given HIV-specific CD8 T cell clonotype may quantitatively accumulate preferentially away from lymph nodes during the primary phase of infection, HIV-specific CD8 T cell function directed at a broad array of viral epitopes has been observed within chronically infected lymph nodes[723,724]. Furthemore, autologuous HIV-specific CD8 T cells that have been manipulated *ex vivo* have been shown to home in to sites of immune replication upon re-infusion into the HIV-1 infected subjects and display effector function[725,726].

In infected patients, a temporal correlation has been observed between the appearance of HIV-specific CD8 T cell activity and the curbing of peripheral blood viremia during primary HIV-1 infection[727,728]. CD8 T cell mediated-immune responses are thought to contribute to infected subjects' ability to delay progression to disease, although such responses are also present in progressors to disease[489,548,568,593,722,729-732]. HIV-specific CD8 T cell responses have also been observed in individuals infected with an attenuated variant of HIV-1 that is characterized by a defective *nef* gene and who progress more slowly to disease[733]. Furthermore, CD8 T lymphocytes responding cytotoxically, or not, to various HIV-1 derived epitopes have also been observed in HIV-1 exposed, yet persistently seronegative subjects[698,701,734-739]. Interestingly, the immunologic characterization of such subjects subsequent to their infection and seroconversion revealed that previously identified reactivities were now absent, a circumstantial observation that is nevertheless suggestive of the hypothesis that a protective role provided by these cells was lost[740].

A broad array of immunodominant and less dominant epitopes derived from all HIV-1 structural and accessory proteins have been identified for their ability to induce antigen-specific cytotoxic T cell responses, as well as the production and secretion of various effector cytokines at various stages of infection with HIV-1[741-746]. In spite of the fact that the immunodominant CD8 T cell responses to Gag (77-85) and Pol (476-484) have been identified in a majority of patients during chronic infection, responses to these epitopes

represent but a fraction of the broad scope of parallel responses that are directed at HIV-1[713,747].

Interestingly, during chronic HIV-1 infection, an inverse correlation between viral load and the frequency of CD8 T cells specific for known immunodominant HIV-1 Gag (77-85) and Pol (476-484) epitopes has been reported although there is accumulating evidence that such a correlation is not always observed[327,748]. Considering the complexity of immune responses directed at HIV-1, perhaps is this not surprising? HIV-specific CD8 T cell mediated antiviral responses are dynamic; as such they have an impact on viral disseminantion but they are also modulated by presence of the virus. The protracted interaction between virus and host is expected to result in strong evolutionary pressures on both systems.

1.3.6. HIV-1 escape from the immune system

The persistence of the HIV-1 infection depends on the virus' ability to avoid recognition by the immune system. Beyond HIV-1's high rate of replication, rapid systemic dissemination and extended tropism to a broad variety of cells, some of which are less accessible to surveillance by the immune system, several viral properties further promote HIV-1's ability to establish persistent infection. Viral escape from antibodies and effective humoural immunity may result from epitope mutation, but particularly from the extensive glycosylation of the single viral surface protein complex accessible to antibodies: the gp120 portion of its receptor unit[749]. Viral escape from CD8 T cells is thought critical and viral strategies include:

i. HIV-1's ability to establish relatively quiescent infection, akin to viral latency, in resting CD4 T cells[608]. The low ensuing levels of viral replication in these cells are thus expected to result in a low frequency of antigenic epitopes being presented at the cellular surface, effectively dissimulating the virus from detection by CD8 T lymphocytes. Furthermore, infectious viral particles may stealthily associate with

dendritic cells for prolonged periods of time[542,566,567]. Nevertheless, in these circumstances, viral antigen may be presented without viral replication[750].

ii. Virus-induced protection of the cells it infects by interference with molecules that would normally be recruited when an effector CD8 T cell attempts to induce the apoptosis of the infected host cell. HIV-1 Nef has been shown to interact with and inhibit apoptosis signal-regulating kinase 1 (ASK-1), thereby protecting the cell from Fas and TNFα-induced apoptosis, two effector pathways used by cytotoxic CD8 T cells[751]. Nevertheless, the anti-apoptotic effect of HIV-1 infection remains an area of debate, as Vpu has been demonstrated to promote the death of infected cells by apoptosis[650,651]. Furthermore, HIV-1 Vpr has been proposed by some to protect from cellular apoptosis, while other groups have unveiled that it actually enhances it[641,642,646-649,752].

iii. Viral-induced upregulation of effector molecules on the surface of infected cells that may induce the death of encountered CD8 T lymphocytes. HIV-1 Nef has been shown to induce the upregulation of FasL on the surface of the cells it infects, these infected cells thereby acquiring the capacity to induce the apoptosis of immune cells, including CD4 T cells and effector CD8 T lymphocytes engaged to counter the infection[638,656,753-756]. Critically, HIV-specific CD8 T cells have been shown to be particularly prone to apoptosis[756].

iv. Viral intereference with epitope presentation within the context of class I MHC. HIV-1 Vpr has been shown to interfere with an early step in class I MHC biosynthesis[757]. Furthermore, HIV-1 Nef has induces the down-regulation of cell surface class I MHC, thereby protecting infected cells from destruction by HIV-specific CTLs[646,758-760]. Strikingly, Nef selectively down-regulates HLA-A and B complexes, ensuring that cell surface expression of other class I molecules remains unaffected so as to protect the infected cell from destruction by NK cells[761].

v. Viral escape by the mutation of previously recognized immunodominant epitopes of HIV-1. The high rate of nucleotide substitution taking place during viral replication may result in mutations that preclude proper processing of a viral epitopes by the cellular machinery, their presentation by MHC, or their recognition by previously recruited effector CD8 T cell. In the SIV model, such viral escape has been shown to result in the selection of variant viral quasispecies, lost immune control of the virus during primary infection and in the failure of a vaccine to induce the immunologic control of HIV-1[762-764]. Viral escape has also been observed in humans infected with HIV-1 and is similarly associated with viral breakthrough[765-768]. Interestingly, the persistence advantage conferred by viral escape mutations is further supported by the recent demonstration that these variant quasispecies are preferentially transmitted in the context of vertical HIV-1 infection to children expressing the matched HLA[769].

vi. Immune deviation by HIV-1. Due to the fast replication rate and its error-prone RT, the HIV-1 genome rapidly accumulates mutations as the virus disseminates throughout the body, forcing the host immune system to constantly redirect its aim. Even at the onset of infection, the innoculate that infects an individual consists of a relatively heterogenous mixture of viral genomes: as such, the virus that is disseminated throughout an individual is a population of closely related, yet diverse genomes that often bear distinct antigenic epitopes[562,770,771]. It is interesting to hypothesize that epitope variants of HIV-1 may eventually display altered peptide ligand properties with regards to antigen-specific CD8 T cells having been recruited by a given immunodominant epitope, and that this may have an influence on the function and persistence of this reactivity[772,773]. An interesting caveat of current approaches to the identification of the diversity of responses to HIV-1 lies in the fact that a limited number of effector function readouts are being used. In particular, it is reasonable to assume that IFN-γ production precedes a standard program of effector function recruitment[329]. But does it assuredly do so? In light of potential defects in IFN-γ production by HIV-specific T cells at various stages of infection with HIV-1 and with respect for the diversity of potential functions that could play a role in immune function, it will be of interest to assess function with a broader array of

parameters. Although new epitopes are being evidenced that induce HIV-specific immune responses, could variant epitopes elicit different responses on the basis of parameters that are not yet defined for them, such as MHC-peptide/TCR affinity?

1.3.7. Modulation of HIV-specific immune responses throughout HIV-1 infection

CD8 T-cell mediated immune responses are instigated as a response to the presence of foreign antigen. The evolution of such antiviral responses is complex and, most importantly, they are constantly modulated in response to changes in the dynamics of virus-host interaction. Subjected to the protracted insult of high antigenic titers and viral persistence, HIV-specific T cells are constantly subject to modulation. Furthermore, the functional and physical deletion of antigen-specific CD4 T cells in the course of disease progression effectively results in HIV-1 applying, on the immune system, a unique pressure that essentially expands HIV-1's arsenal for successful evasion from immune irradication.

The evolution of HIV-specific CD8 T cell responses through time is indicated by the observed variations in the frequency of antigen-specific precursors throughout disease progression, and by the demonstration that the preferred targets of HIV- specific CD8 T cell responses change between the time of primary infection and progression to the chronic phase of infection[774-776]. Interestingly, during primary HIV-1 infection, correlations between the time elapsed since infection and the magnitude and breadth of HIV-specific CD8 T cell responses have also been described[719]. In fact, the recent insight gained by analysis of the fine antigenic specificity of HIV- specific CD8 T cell immune responses argues for a reconsideration of the standard subdivision of the natural course of HIV-1 infection and the recognition of an acute infection phase that follows the onset of infection and may perhaps last until the peak of HIV-1 viremia is attained during primary HIV-1 infection. The relevance of this period of natural disease progression is that it may in fact precede the irremediable alteration of the infected patient's HIV-specific immune response capability. Clinically, this stage would be delimited by the period of time during

which an individual experiences a rising HIV-1 viremia and displays HIV-specific CD4 and CD8 T cell responses, but has not seroconverted to the virus. Strictly, this proposed phase is distinct from the primary infection phase, which extends to seroconversion and the establishment of a viral set point (figure 8). Although this acute phase of infection is only recently coming under distinct scrutiny, it will be interesting to assess whether viral epitopes then recognized may differ even from those targetted later during primary HIV-1 infection or during the chronic phase and have until now been the focus of attention[763,777]. Furthermore, successful resolution of viremia with instigation of HAART early during acute primary HIV-1 infection and prior to seroconversion has been shown to preserve CD4 and CD8 HIV-specific T cell responses that would otherwise possibly remain amplified into chronic infection, but would nevertheless then be lost should HAART be later instated[682,778]. This suggests a critical window of opportunity for treatment early after infection, but prior to an irreversible alteration of the immune response during the peak of HIV-1 viremia; interventions during the acute phase of infection may allow for the preservation of CD4 and CD8 T cell immune responses and the establishment of immunologic memory that could then be called upon by scheduled treatment interruption or by therapeutic vaccination strategies.

Clonal exhaustion of HIV-specific CD8 T lymphocytes is a premier mechanism of possibly irreversible immune modulation by high antigenic titer and persistent infection that has been studied in the context of primary HIV-1 infection[779]. Originally described in murine models of persistent infection with lympho-choriomeningitis virus (LCMV), clonal exhaustion results in the effective deletion of clonal populations of T cells, thus raising the spectre of a functional deletion of T cells that target a selected viral epitope[780-783]. Clonal exhaustion could therefore allow definitive viral evasion from immune recognition. Potentially contributing mechanisms include 1) affinity of the specific MHC-peptide/TCR interaction 2) the resulting avidity of this interaction, 3) the persisting antigenic stimulus that results in senescence of the CD8 T cell and, critically, clonal exhaustion is thought to be supported by 4) the loss of CD4 T cell help.

Beyond clonal exhaustion, the loss of CD4 T cell help is thought to underlie other immune dysfunctions during HIV-infection. Particularly, in the absence of HAART, a positive correlation has been established between CD4 T cell proliferative responses to HIV-1 p24 antigen and the frequency of HIV-specific CTL precursors[679]. In the mouse, it has been shown that the lack of $CD4^+$ T cell help could result in a loss of CD8 T cell effector function, with potential viral immune escape occurring[784]. Similarly, in humans, HIV-1 and CMV specific $CD8^+$ T cells have been shown to persist in the absence of $CD4^+$ T cells, but to nevertheless be functionally impaired[592]. This impairment was reversible, as *in vitro* culture in the presence of IL-2 restored their effector capacity[592]. Several lines of evidence suggest that the functional deletion of antigen-specific $CD4^+$ T cells precedes their physical deletion. Indeed, a similarly reversible impairment of CD8 T cell function has been described in HIV-1 infected patients that still have significant numbers of peripheral CD4 T cells, a lack of antigen responsiveness that has been linked to defective expression of the TCR ζ chain[785-787].

1.4. Project Rationale and Research Objectives

Infection with HIV-1 results in the unique modulation of the host immune system as a result of viral tropism and of the action of viral proteins, but also due to the intensity of viral replication and the virus' propensity to rapidly mutate. Specifically, protracted viral persistence and escape from the immune system is postulated to result in a unique pressure that modulates virus-specific immune responses. The work presented in this thesis was undertaken with the intent to evidence the dynamics of HIV-specific CD8 T cell immune responses, as well as to identify and study particular characteristics of HIV-specific T cell-mediated immune responses that could eventually serve as immunologic correlates of protection, both as prognostic indicators of disease progression and for the evaluation of therapeutic and vaccination strategies.

The specific research objectives of the work detailed in this thesis were:

i. Through a longitudinal, flow-cytometry based characterization of the CD4 and CD8 TCR repertoires of HIV-1 infected pediatric patients, to assess whether pediatric subjects exhibit Vβ-specific expansions akin to those displayed by adults and having been shown to be prognosticators of disease progression.

ii. Through the molecular cloning and sequencing of TCR β-chains within observed Vβ-specific expansions in the CD4 and CD8 T cell pools, to identify and track TCR clonotypes in order to assess their presence, relative representation and persistence through time, thereby gaining insight into the dynamics of antigen-specific CD8 T cell responses throughout the course of HIV-1 infection.

iii. Having observed the dynamic evolution of antigen-specific CD8 T cells in the context of HIV-1 infection, to assess the properties of HIV-specific CD8 T cells

with regards to the newly established phenotypic and functional subsets of effector and central memory T lymphocytes.

iv. Having phenotypically identified four subsets of HIV-specific CD8 T lymphocytes in the blood of chronically infected subjects, to assess their proper functional characteristics.

v. To establish whether the four subsets of antigen-specific memory CD8 T lymphocytes observed amongst HIV-specific CD8 T cells are also observeable in chronic CMV infection and to establish whether target virus-specific differences exist.

vi. Having confirmed the presence of phenotypically and functionally distinct memory T lymphocyte subsets within the pools of HIV-1 and CMV-specific CD8 T cells, to expose whether and how these memory subsets are related with regards to differentiation.

vii. Having established the skewed distribution of HIV-specific memory CD8 T lymphocytes in the blood of chronically infected patients, to confirm the presence of HIV-specific CD8 T cells in the lymph nodes of infected patients and to assess their distribution between the established maturation subsets.

viii. To assess differences between the distributions of HIV-specific memory CD8 T cells in blood as compared to lymph nodes of chronically HIV-infected patients.

ix. To assess the effect of HAART and the successful resolution of HIV-1 viremia to below 50 copies per ml of blood on the quantity and quality of HIV-specific memory CD8 T lymphocytes.

2. Transient T cell receptor β-chain variable region-specific expansions of $CD4^+$ and $CD8^+$ T cells during the early phase of pediatric human immunodeficiency virus infection: characterization of expanded cell populations by T cell receptor phenotyping

Overwhelming clonal expansions of antiviral CD8 T cells have been demonstrated in human adults experiencing acute HIV-1 infection and patterns of Vβ-specific T cell over-representation have been shown to be of prognostic value for HIV-1 disease progression in adults[722]. The extent to which pediatric HIV-1 infected patients also evolve such CD8 T cell expansions had yet to be determined. Furthermore, CD4 T cell clonotypic expansions had yet to be molecularly characterized in humans. The phenotypic and molecular characterization of a longitudinal pediatric cohort provided for a unique opportunity to perform these observations and, critically, to witness the dynamic evolution of CD8 T cell responses through time.

Transient T Cell Receptor β-Chain Variable Region-Specific Expansions of $CD4^+$ and $CD8^+$ T Cells during the Early Phase of Pediatric Human Immunodeficiency Virus Infection: Characterization of Expanded Cell Populations by T Cell Receptor Phenotyping

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Published in The Journal of Infectious Diseases

2000 (181): 107-120

Notes

This study protocol was approved by the Ethics Committee of the Centre Hospitalier Universitaire Mère-Enfant/Hôpital Sainte-Justine, Montréal, where the study was conducted. In all cases, informed consent was obtained from the parents or legal guardians. Counseling was provided.

Financial support: National Health Research Development Program of Canada (6605-4040-AIDS), Medical Research Council (MRC) of Canada, Fonds de la Recherche en Santé du Québec, the Canadian Foundation for AIDS Research, the National Institutes of Health (AI-235516), and the Pediatric AIDS Foundation (50549-17-PG to R.-P.S.). H.S. and P.C. are supported by the Fonds de la Recherche en Santé du Québec; G.U.S. is a recipient of a fellowship from the Istituto Superiore di Sanità, Rome, Italy; R.-P.S. is a senior MRC scientist.

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Abstract

T cell receptor (TCR) repertoire perturbations are commonly detected in $CD8^+$ T cells during adult primary human immunodeficiency virus (HIV) infection and have been associated with HIV-specific cytotoxic T cell responses. By use of flow cytometry, transient high-level TCR β-chain variable region-specific expansions of $CD4^+$ and $CD8^+$ T cells were observed more frequently in HIV-infected children than in children exposed to HIV who remained uninfected. TCR β-chain diversity analysis and diversity-specific polymerase chain reaction were used to study the clonality of expanded $CD4^+$ and $CD8^+$ subsets. In $CD8^+$ T cells, structural features of the complement-determining regions 3 were altered during the course of the expansion, and persistent TCR clonotypes were observed, consistent with antigen-driven selection. In contrast, TCR β-chain variable region-specific expansions without clonotypic overrepresentation or persistence were observed in $CD4^+$ T cells, possibly related to HIV-specific helper T cell responses or to the progressive destruction of the $CD4^+$ cell compartment.

Introduction

Vertical transmission is the predominant mode by which children and infants acquire human immunodeficiency virus type 1 (HIV-1) infection. Viral infection can take place in utero, perinatally, and through breast-feeding [1, 2]. The time course and clinical latency period of HIV-associated disease for vertically infected children and infants are significantly shorter than for HIV-infected adults [3, 4]. Nevertheless, children experience a form of primary HIV infection that is clearly similar to that observed in adults. Phenotypes analogous to the rapid progressors and long-term survivors described among adults are also found among HIV-infected pediatric patients [5]. Therefore, as is the case for adults, the different rates of progression of pediatric HIV disease could be linked to host factors involved in the individual's ability to mount antigen-specific immune responses [6].

The T cell receptor (TCR) is the principal effector molecule in the antigen-specific cellular immune response, because it directly mediates recognition of antigen associated with major histocompatibility complex (MHC) molecules at the surface of antigen-presenting cells [7]. TCRs are heterodimeric cell-surface molecules encoded by genes produced through somatic recombination of noncontiguous variable (V), diversity (D), and joining (J) germline segments [8]. The rearrangement of αβTCR genes takes place during thymic development, and idiotypic expression of mature TCR genes gives rise to a peripheral T cell pool composed of highly varied antigenic specificities. TCR diversity is mainly focused within its complement-determining regions 3 (CDR3), located at the V(D)J junctions, consistent with the fact that these short, hypervariable segments are directly involved in the recognition of peptide-MHC complexes [9]. The combined sum of all of the different TCRs and specificities in an individual forms his or her TCR repertoire, which can be analyzed by various methods, including semiquantitative polymerase chain reaction (PCR). For measurements of the TCR β-chain repertoire, the most reliable results are obtained by flow cytometry, for which a large number of reagents are available [10-12].

The strong association between the TCR β-chain repertoire and the HLA type of the subject complicates the design of study groups from which meaningful comparisons of repertoire data can be drawn [13]. This limits the significance of cross-sectional studies on TCR repertoire among HIV-infected subjects [14, 15] and underlines the need to perform longitudinal studies. In HIV-discordant monozygotic twins, HIV infection induces multiple macroscopic alterations of the TCR repertoire in the $CD4^+$ T cell compartment during all clinical phases of the disease [10]. Significant repertoire differences can also be detected in $CD4^+$ T cells collected from lymph nodes, compared with those from peripheral blood of HIV-infected patients [16, 17]. Notably, a significant proportion of adults undergoing primary HIV infection exhibit transient high-level oligoclonal expansions of $CD8^+$ T cells bearing specific TCR β-chain variable region (TCRBV) determinants [18]. These expanded cells display MHC-restricted HIV-specific cytotoxicity, making them part of the initial antigen-specific immune response to HIV-1 [18]. Most important, qualitative differences in TCRBV-specific expansion patterns during primary HIV infection were associated with faster rates of HIV disease progression [19].

TCRBV-specific expansions of $CD8^+$ T cells have been observed in a number of HIV-infected children and infants, as well as in some HIV-negative children born to HIV-infected mothers [16, 20, 21]. To confirm the incidence and the dynamics of these transient expansions in children, and to characterize the phenotype and clonality of the expanded cells, a detailed longitudinal study of the TCR repertoires of 8 children with vertically acquired HIV infection and 4 HIV-negative children born to HIV-infected mothers was undertaken.

Materials and Methods

Subjects. Eight children, vertically infected with HIV-1, were included in this study. HIV-1 infection was confirmed by virus isolation and/or DNA PCR done at 2 different time points. At age 12 months, 3 of the 8 HIV-infected children were symptomatic, according to the classification system of the Centers for Disease Control and Prevention [22] (Table 1). At age 24 months, 6 of the 8 were symptomatic: 1 child had experienced mild symptoms (A1), 1 had moderate symptoms (B2), and 4 had severe symptoms (C3; Tables 1). Two children had opportunistic infections: 1 had esophageal candidiasis at age 24 months and 1 had Pneumocystis carinii pneumonia at age 28 months. Five children experienced progressive neurologic disease, manifested by the loss of developmental milestones, impaired intellectual ability, brain atrophy, progressive symmetric motor deficits, paresis, abnormal muscle tone, and/or ataxia. Four of the 5 HIV-infected children with AIDS-defining illnesses died, at 9, 12, 31, and 36 months (mean ± SD, 21.5 ± 14.2 months). Six of the infants were treated with ≥1 antiretroviral agents, starting at various times after birth (mean ± SD, 7.17 ± 3.50 months). Longitudinal examination of T cell counts revealed that children 7, 8, and 11 exhibited precipitous declines in their $CD4^+$ T cell populations, consistent with rapid HIV disease progression [5] (figure 1).

A control group composed of 4 uninfected children born to HIV-infected mothers was also included in this study. All of their mothers were asymptomatic and had been treated with zidovudine during pregnancy (starting at a mean of 23.0 ± 8.44 weeks of pregnancy). These 4 infants received zidovudine during 6 weeks after birth and were confirmed to be HIV-uninfected at 6 months of life, after 5 consecutive cultures negative for HIV.

Cell isolation and flow cytometric analysis. Total peripheral blood samples (~2 mL) were collected at birth (cord blood), at 2-week intervals for the first 2 months when possible, and then every 2-3 months. Peripheral blood mononuclear cells were purified on Ficoll-Paque gradients (Pharmacia, Uppsala, Sweden). Cell samples were then frozen in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) and 7% dimethyl sulfoxide. On thawing, cells were gently washed with warm RPMI medium containing

10% FBS, and viability was assessed by trypan blue exclusion. Aliquots of 50,000 cells were then distributed in round-bottom 96-well plates, where they were washed in PBS containing 2% FBS and 0.01% sodium azide. Direct staining was done with fluorescein isothiocyanate-labeled anti-TCRBV monoclonal antibodies (MAbs). Anti-TCRBV2 (E22E7.2), anti-TCRBV3 (LE.89), anti-TCRBV5S2 (36213), anti-TCRBV8 (56C5), anti-TCRBV13S1 (IMMU222), anti-TCRBV13S6 (JU74.3), anti-TCRBV14 (CAS1.1.3), anti-TCRBV16 (TAMAYA1.2), anti-TCRBV17 (E17.5F3.15.13), anti-TCRBV20 (ELL1.4), anti-TCRBV21S3 (IG.125), and anti-TCRBV22 (IMMU546) were obtained from Immunotech (Marseille, France). Anti-TCRBV6S7 (OT145) and anti-TCRBV12 (S511) were purchased from T Cell Diagnostics (Cambridge, MA). Unlabeled anti-TCR MAbs were also used in indirect staining. These included anti-TCRBV5S2-3 (MH3-2), anti-TCRBV9 (MKB1), anti-TCRBV13S2 (13.2), anti-TCRBV19, and anti-TCRBV23S1 (HUT78). In these cases, TCR staining was revealed with fluorescein isothiocyanate-conjugated human-adsorbed goat anti-mouse IgG (Life Technologies, Bethesda, MD). Cells were then counterstained with either anti-CD4 or anti-CD8 MAbs, conjugated to phycoerythrin (anti-Leu-2a and anti-Leu-3a; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Analysis was done on 10,000 acquired events, gated according to forward and side scatter, on a FACScan driven by the CellQuest software package (Becton Dickinson). The significance threshold for longitudinal variations in TCRBV expression levels was set at a 2-fold difference and >5% overall representation for any specific TCRBV determinant, as described elsewhere [10, 11, 16, 18].

TCR β-chain cloning and diversity analysis. Total RNA was extracted from 10^6 live cells from each time point by use of the RNAzol method (Cinna-Biotecx, Houston, TX). cDNA was synthesized according to protocols published elsewhere [11], by use of poly(dT)16 and avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). The TCR β-chain was amplified with a 3' constant region primer (3'Cβ *Sac*II) and 5' TCRBV-specific primers (Table 2). Amplification conditions were 27 cycles (94°C for 30 s; 55°C for 45 s; 72°C for 1 min) in a PCR system 9600 thermal cycler (Perkin-Elmer Cetus, Emeryville, CA). PCR products were digested with *Sal*I and *Sac*II and cloned into the polylinker of pBSKS+ (Stratagene Cloning Systems, La Jolla, CA). Insert-positive

recombinants were sequenced unidirectionally, by use of the dideoxy-termination method (United States Biochemicals, Saranac Lake, NY). DNA sequences were aligned to the published sequences of TCRBV2 [23], TCRBV6S7 [24], TCRBV14 [25], TCRBV20 [26], and the TCR β-chain joining segment (TCRBJ) subloci TCRBJ1-TCRBJ2 [27] and have been submitted to GenBank (accession numbers AF189371-AF189589). Nomenclature of TCRBV gene segments is according to [28].

Diversity-specific (DS) PCR. Oligonucleotide primers complementary to the CDR3 regions of specific clonotypes were designed and used in semiquantitative PCR reactions with cognate [α-^{32}P]-labeled 5' TCRBV-specific primers. PCR conditions were as above. A 200-bp fragment of the TCR α-chain constant region (TCRAC) gene was coamplified in each reaction, to serve as an internal amplification control, as described elsewhere [10, 11]. Particular care was taken to maintain the control PCR reaction within the linear amplification range [10, 11, 16, 18]. Primers used were 9.47Udiv ($CD8^+$ clonotype G), 9.143Udiv ($CD8^+$ clonotype E), 9.138Rdiv ($CD8^+$ clonotype F), B4Udiv ($CD4^+$ clonotype SHPSTVPF), B124Udiv ($CD4^+$ clonotype VGDK), and B545Udiv ($CD4^+$ clonotype APLGP; Table 2). PCR products were separated on 10% polyacrylamide gels containing 7 M urea. Gels were exposed to film without drying. Quantitation of radioactive signals was done with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values are expressed as a percentage of the TCRAC control in each lane. This technique has been shown to have a sensitivity threshold of 1 in 2×10^5 cells with most combinations of primers [29].

Results

Transient TCRBV-specific expansions of T cells in children from HIV-infected mothers

Two-color flow cytometric analysis with a large panel of anti-TCRBV MAbs, capable of recognizing ~60% of the TCR β-chain repertoire, revealed that transient TCRBV-specific expansions of T cells could be observed in all of the 8 HIV-infected children studied. These expansions were detected in both the $CD4^+$ and the $CD8^+$ T cell subsets (figure 2). The number of TCRBV-specific expansions observed in individual children during the course of the study period was variable, ranging from 1 (child 8) to 8 (child 4) in the $CD4^+$ cell subset and from 1 (child 8) to 11 (child 6) in the $CD8^+$ cell subset (figure 2). The mean number of TCRBV-specific expansions in HIV-infected children was not significantly different between the $CD4^+$ and $CD8^+$ T cell subsets ($P = .28$, unpaired t test). There was also no correlation between the number of expanded TCRBV subsets in $CD4^+$ T cells and $CD8^+$ T cells within individual children ($R^2 = .21$), as determined by use of linear regression analysis. The magnitude of TCRBV-specific expansions was variable but sometimes spectacular. In several children, some TCRBV subsets reached levels up to 15% within the $CD4^+$ T cell compartment, levels that are rarely observed in uninfected persons (figure 2*A*). As for $CD8^+$ T cells, in the case of child 9, $TCRBV14^+$ T cells constituted >30% of the peripheral $CD8^+$ T cell compartment (figure 2*B*). Moreover, unlike what is usually seen in superantigen-induced responses [30], there was little or no significant overlap between the identity of the TCRBV subsets that were amplified within the $CD4^+$ T cell compartment and those amplified in the $CD8^+$ cell compartment. Interestingly, however, $TCRBV17^+$ T cells were amplified within the $CD4^+$ T cell subset in 5 of 8 children analyzed.

With respect to timing, TCR repertoire perturbations were detected as early as 1 week after birth (child 8) and could be observed in most children across the entire study period-that is, throughout the first year of life. Results show that 67.7% of all TCRBV-specific expansions of $CD4^+$ T cells occurred before age 2 months, whereas only 42.9% of $CD8^+$

cell expansions took place during that same period (figure 2). This difference suggests that, in these children, TCRBV-specific $CD4^+$ T cell expansions had a tendency to occur at an earlier age than did $CD8^+$ cell expansions.

Transient TCRBV-specific expansions were also noted in all 4 uninfected children born to HIV-infected mothers. The frequency of these expansions in the $CD4^+$ cell subset ranged from 0 (child 236) to 1 (children 214, 232, and 254) and in the $CD8^+$ cell subset from 1 (children 214 and 254) to 4 (child 236; figure 3). In uninfected children born to HIV-infected mothers, expansions occurred significantly more frequently in the $CD8^+$ T cell subset than in the $CD4^+$ T cell subset ($P = .05$, unpaired t test). Most important, uninfected children appeared to experience much less frequent TCRBV-specific expansions of $CD4^+$ T cells ($P = .022$, unpaired t test) and $CD8^+$ T cells ($P = .05$, unpaired t test) than did their HIV-infected counterparts. Finally, the magnitude of these expansions was, in general, smaller than those observed in HIV-infected children in both the $CD4^+$ and $CD8^+$ T cell compartments (figures 2 and 3).

These results indicate that the TCR repertoire is heavily affected during primary pediatric HIV infection. The significant difference in the number of TCRBV-specific expansions between HIV-infected and -uninfected children suggests that these are the result of the primary immune response to HIV or represent the product of HIV-induced immunopathology.

To more fully characterize the clonality of expanded cells, we focused our attention on 2 HIV-infected children, child 6 and child 9. Child 6 exhibited a transient expansion of cells expressing the TCRBV6S7 and TCRBV20 segments, with relative representation values reaching 12.6% and 17.4%, respectively (figure 2*A*). These 2 expansions comprised well-defined increase and decline components, in keeping with the TCRBV-specific expansions observed in adult patients undergoing primary HIV infection [18]. The 2 expansions were slightly asynchronous: the proportion of TCRBV6S7-expressing cells peaked at 6 months of age, whereas that of TCRBV20-expressing cells had reached maximal levels 4.5 months earlier. Most interestingly, these 2 expansions exclusively

comprised cells expressing CD4, and the representation of $CD8^+$ T cells in the TCRBV6S7 and TCRBV20 compartments was low and did not fluctuate correspondingly during the study period (figure 2). High-level TCRBV-specific expansions were also seen in child 9, in whom cells expressing TCRBV14 reached as much as 30% of $CD8^+$ T cells at 12 months of age (figure 2*B*). Once again, no corresponding fluctuations in the levels of $TCRBV14^+$ $CD4^+$ T cells were seen in that patient across the duration of the study (figure 2).

Presence of multiple clonotypic expansions of $CD8^+$ T cells in child 9

To establish whether the large progressive expansion of cells expressing TCRBV14 was due to the amplification of a single clone (monoclonal expansion) or of a few $TCRBV14^+$ clones exhibiting structural similarity within the CDR3 loop (oligoclonal expansion), PCR-amplified $TCRBV14^+$ TCR β-chains were subcloned into plasmid vectors. Nucleotide sequence analysis of the CDR3 regions showed that overall 67 (43.5%) of 154 molecular clones were composed of 8 multiply represented sequence prototypes (clonotypes A-H), strongly suggesting that cells expressing these TCR clonotypes were highly overrepresented in the starting $CD8^+$ $TCRBV14^+$ T cell population (figure 4*A*). The global proportion of oligoclonally expanded clonotypes relative to unique sequences at any given time point varied from 4.5% soon after birth (17 days) to >80% of clones at 4 months of age. The distribution of these 8 clonotypes through time was composed of well-defined expansion and decline components (figure 4*B*).

With regard to CDR3 structural conservation, TCRBJ usage varied throughout the study period, except for a relatively high frequency of rearrangements using TCRBJ2.2 at 1, 6 and 9 months of age, due in the last 2 cases to the predominance of TCRBJ2.2-using clonotypes E and G (figure 4*A*). The length of the CDR3 loop in these TCR β-chains was also restricted; CDR3 length distribution shifted from being initially unimodal (40.9% and 78.9% of clones with a length of 8 amino acid residues at 17 days and 1 month, respectively) to being bimodal (peaks at 9 and 13 amino acid residues between 2.5 months and 6 months; figure 4*A*). This distribution further evolved; by 9 months, the bimodal bias

in CDR3 length was no longer present, and, instead, a preferred size of 8 amino acids was again observed (50.0% and 42.1% of clones with a length of 8 amino acid residues at 9 and 12 months, respectively; figure 4*A*). Finally, except for the predominant clonotypes, no significant CDR3 consensus sequence conservation could be derived throughout the course of the study period. Taken together, these results suggest that a time-dependent selection process for T cell specificities was taking place in child 9, acting on T cells expressing TCRBV14. This process appeared to hinge partly on the selection of a preferred TCRBJ segment (TCRBJ2.2) and CDR3 length (9 or 13 residues). V-region bias and focusing of these structural features, even in the absence of conservation in the primary amino acid sequence of CDR3, is indicative of ongoing antigen-specific selection processes [31, 32].

Persistence of amplified $CD8^+$ clonotypes in child 9

DS-PCR methodology, which takes advantage of the uniqueness of the CDR3 loop expressed by individual T cell clones to establish their relative representation within the T cell pool, was used to validate and improve the resolution of the relative representation data obtained through cloning and sequencing protocols. This method was shown to have a sensitivity threshold of 1 in 2×10^5 cells with most combinations of primers [29]. As seen in figure 5*A*, the presence of clonotype E was observed in peripheral blood mononuclear cell samples throughout the study period, indicating that in this child the T cell clone bearing this TCR was present with a detectable frequency very soon after birth. In contrast, clonotype F first became detectable at age 2.5 months, suggesting that the expansion of this T cell clone may have been initiated by an antigen-specific stimulation occurring shortly before this time point (figure 5*B*). Relative homogeneity in the intensity of TCRAC controls indicated that this effect was not due to variant mRNA levels, cDNA synthesis, or amplification efficiency. The temporal distribution of both clonotypes was roughly Gaussian (figure 5, *graphs*), consistent with results obtained in longitudinal studies of HIV-infected adults during primary HIV infection [29]. Interestingly, both of these clonotypes were most represented at 9 months, a time at which the TCRBV14 expansion had not yet peaked (figure 2*B*). This indicates that oligotypic expansion was not

the only factor driving the expansion of the TCRBV14 subset and that other structural features of the TCRBV14 TCR may have been involved in determining antigen-specific responsiveness of this TCRBV subset. Data obtained by DS-PCR closely match those independently obtained in cloning and sequencing (diversity analysis) experiments (figure 4), and the consistency between these experimental approaches reinforces the credence of the results obtained.

Diversity analysis of $CD4^+$ T cell expansions in child 6

The 2 transient TCRBV-specific expansions of $CD4^+$ T cells observed in child 6 were also tested for clonality. Results show that neither of the expanded TCRBV6S7 and TCRBV20 subsets was composed of highly represented sequences, but that they were made up of numerous unique, singly represented clonotypes (figure 6). This was also the case at the peak of the expansion phase (6 months for TCRBV6S7; 1.5 months for TCRBV20). With respect to conservation of the CDR3 layout, no consensus amino acid sequence could be derived, whereas CDR3 length was normally distributed, peaking at 9 or 10 amino-acid residues in all cases (figure 6). With TCRBV20, there was, however, an unusual bias toward usage of TCRBJ1 segments, which are collectively used only 30% of the time in the general population [33]; at age 2 days, TCRBJ1 usage was 71.4%, and by age 6 months, it was 72% (figure 6*B*). This biased TCRBJ usage was markedly reduced at age 1.5 months, which corresponds to the peak expansion of TCRBV20 (figure 6*B*), suggesting that expanded cells preferentially expressed TCRBJ2-using TCRs.

Three clonotypes expressed by $CD4^+$ T cells (SHPSTVPF, VGDK, and APLGP) were also selected for DS-PCR analysis on the basis of optimal primer length, homology, and G/C composition. None of these clonotypes was detected at any of the time points, suggesting that their relative representation level within the total T cell population was <1 in 10^5 cells, even at the time at which they were themselves cloned from the cDNA pool (data not shown). These results underscore fundamental differences in clonality between expansions of $CD4^+$ and $CD8^+$ T cell subsets in HIV infection.

Discussion

On the basis of the results presented above, a number of statements can be made regarding perturbations in the TCR β-chain repertoire during pediatric HIV infection. First, as reported elsewhere [20, 21], transient high-level expansions of $CD8^+$ T cells expressing specific TCRBV determinants were readily detected in all 8 children vertically infected with HIV, despite the fact that the anti-TCRBV MAb panel that we used covers only ~60% of the expressed TCRBV repertoire. This frequency closely parallels the reported rate of occurrence of these expansions in acutely infected adults, suggesting that TCRBV-specific expansions are quite common during primary HIV infection [18]. TCRBV-specific expansions of $CD8^+$ T cells were also identified in HIV-negative children born to HIV-infected mothers, but they were of a lesser magnitude and occurred with a significantly reduced frequency. These results are consistent with those of another recent study [21] and with the existence of potent and early HIV-specific cytotoxic T lymphocyte responses in both infected and uninfected children from HIV-positive mothers [34, 35].

Furthermore, molecular analysis revealed the presence of several persistent and highly represented TCR clonotypes in the $CD8^+$ T cell compartment, all of which exhibited well-defined expansion and decline phases. Sequence analysis showed progressive alteration of the structural features of the CDR3 regions, including bimodalization of CDR3 length and focusing of TCRBJ usage. Restriction in TCR β-chain CDR3 length has been shown to be the first structural characteristic to be selected during the course of the maturation of antigen-specific immune responses [31].

It is important to mention that in the absence of functional data our results do not provide direct proof of the functional role of these expanded cells in pediatric HIV patients. However, similar expansions in acutely infected adults have been consistently associated with HIV-specific cytotoxic T lymphocyte activity [18, 29], which is temporally correlated with down-regulation of initial HIV viremia [36, 37]. Stable expansions of largely nonfunctional $CD8^+$ T cells have been described among older adults [38]. However, expansions seen in children were transient and were probably the result of a

recent antigen-driven process, possibly involving HIV. Accordingly, anti-HIV cytotoxic T lymphocyte responses have been detected in HIV-infected and -uninfected children from HIV-infected mothers [34, 35, 39]. Therefore, these results suggest that oligoclonally expanded $CD8^+$ T cells are part of the primary cytotoxic T lymphocyte response to HIV-1 in infected children. As seen among acutely infected adults, most, if not all, high-level TCRBV-specific expansions of $CD8^+$ T cells are transient to a variable degree [18, 29]. A decline in the peripheral representation of these cells might be caused by cytotoxic T lymphocyte escape mutation [40] or by clonal exhaustion of cytotoxic T lymphocyte effectors [29, 41].

Significant TCRBV-specific expansions of $CD4^+$ T cells were also detected in HIV-infected children. This is, to our knowledge, the first report of $CD4^+$ T cell expansions in pediatric HIV patients. Similar to the situation seen with the $CD8^+$ T cell subset, significantly less TCRBV-specific $CD4^+$ T cell expansions were observed in HIV-negative children born to HIV-positive mothers. Stable mono- and oligoclonal populations of $CD4^+$ T cells have been recently described in HIV-infected adults [42, 43]. However, in contrast with these reports, the expanded $CD4^+$ T cell populations observed in HIV-infected children were transient and did not exhibit clonotypic overrepresentation or temporal persistence. The lack of clonotypic persistence of $CD4^+$ T lymphocytes may be explained by the continuous killing of these cells by HIV, perhaps coincident with their antigen-specific activation [44, 45].

A comparison of the TCRs expressed by the expanded $CD4^+$ T cells revealed little of the CDR3 layout conservation classically seen in MHC class II-restricted responses [9], with the exception of a transient bias in TCRBJ usage. Thus, a large component of the selecting element seems to reside within the TCRBV region itself, a situation reminiscent of superantigen stimulation [46] but also consistent with some antigen-specific immune responses and with proposed mechanisms of antigen recognition by the TCR [32, 47]. Therefore, an alternative hypothesis to explain the origins of TCRBV-specific expansions of $CD4^+$ T cells is that these cells were activated by a superantigen, potentially associated with HIV [48] or another coinfecting pathogen [49]. Because superantigen stimulation

results in T cell activation, it could lead to enhanced HIV replication and concomitant destruction of $CD4^+$ T cells, should it take place within an HIV-infected subject [50]. However, in spite of meticulous investigation, the existence of a putative HIV-encoded superantigen has never been directly substantiated [14-17, 48, 51]. Epstein-Barr virus was also postulated to encode a molecule with superantigen properties that would specifically cause the expansion of T cells expressing TCRBV6S1 and TCRBV6S3 [52]. In this case, however, clinical evidence failed to demonstrate that child 6 was infected with Epstein-Barr virus at or around the TCRBV6S7 expansion phase (data not shown). Superantigen activity that stimulated T cells expressing TCRBV12 was also associated with cytomegalovirus, another pathogen that commonly infects newborns [49, 53]. The molecular nature of these putative herpesvirus superantigens has not been elucidated. Because of the multiplicity of ubiquitous human pathogens, it is not possible to exclude that the TCRBV-specific expansions of $CD4^+$ T cells seen in child 6 were caused by a yet-uncharacterized superantigen. Regardless, the fact that expanded $CD4^+$ T cells express different TCRBV segments in different children and the lack of TCRBV identity between expanded $CD4^+$ and $CD8^+$ T cell subsets in individual subjects argue strongly against the involvement of a superantigen in the process of transient $CD4^+$ T cell expansion.

Although we have no direct evidence of the function of the $CD4^+$ cells within the TCRBV-specific expansions described, HIV-specific $CD4^+$ helper T cell responses have been recently identified in primary HIV infection, and their magnitude was inversely correlated with viral load [54]. Alternatively, these cells could represent $CD4^+$ class II-restricted cytotoxic T lymphocytes [55, 56]. Because of CD4 expression, these 2 cell types would be directly susceptible to HIV-mediated cytopathology and thus disappear early during primary infection. This could explain why, despite extensive investigation, TCRBV-specific expansions of $CD4^+$ T cells have only recently been observed among HIV-infected adults [42, 43] and why transient expansions of $CD4^+$ T cells appear to take place earlier in life than do $CD8^+$ cell expansions in pediatric HIV infection.

In summary, transient high-level TCRBV-specific expansions were observed in HIV-infected and -uninfected children born to HIV-infected mothers and involved both $CD8^+$

and $CD4^+$ T cells. Molecular phenotyping of TCRs expressed by the expanded cell subsets revealed that, while the $CD8^+$ T cell expansions were clearly oligoclonal and comprised multiple persistent T cell clonotypes, $CD4^+$ T cell expansions were largely polyclonal, and clonotypic persistence was not observed. Pediatric HIV-1 infection is therefore associated with an important remodeling of the TCR repertoire that, in adults, is associated with primary anti-HIV cytotoxic T lymphocyte responses. Transient, TCRBV-specific expansions of $CD4^+$ T cells may represent another arm of cell-mediated immunity (Th cells, $CD4^+$ cytotoxic T lymphocytes) and/or may reflect ongoing destruction of the $CD4^+$ T cell pool by HIV.

Acknowledgments

Part of this work was performed by H.S. in partial fulfillment of the requirements of the Ph.D. degree, Department of Microbiology and Immunology, McGill University. We thank G. P. Rizzardi for useful discussions.

Table 1. Clinical and immunological characteristics of 8 human immunodeficiency virus-infected children.

Child	Survival (months)	Clinical status	CDC classification[a]
4	-	Lymphadenopathy, hepatosplenomegaly	A1
5	-	Esophageal candidiasis, pneumococcal endocarditis, *Candida* septicemia	B2
6	31	*Pneumocystis carinii* pneumonia (at 28 months)	N1
7	12	Failure to thrive, pneumonia, *Candida* septicemia, encephalopathy	C3
8	9	Failure to thrive, encephalopathy, chronic diarrhea	C3
9	36	Encephalopathy	C3
10	-	-	N1
11	-	Failure to thrive, encephalopathy	C3

Table 2. Sequences of T cell receptor β-chain variable region (TCRBV) and clonotype-specific oligonucleotides.

Oligonucleotide	Specificity	Sequence
3′Cβ *Sac*II	TCRBC	AGATCTCCGCGGCTGATGGCTCAAAC
Vβ6ex	TCRBV6	GGCCGTCGACAGGTGCTGGAGTCTCCC AGACCCCCAGTA
Vβ14 *Sal*I	TCRBV14	GGCCAGGTCGACAGTTAACAGTGACTT GTTCTCAGAA
HUT102 *Sal*I	TCRBV20	GGCCAGGTCGACTGCACTGTGGAGGG AACATCAAACCCCAACC
Vβ2 *Sal*I	TCRBV2	GGCCAGGTCGACGAGCTGGGTTATCT GTAAGAG
9.143Udiv	Clonotype E	TCCTAGTCCCGGGGACCCTAAA
9.138Rdiv	Clonotype F	ATCCGACATCCCGCTAGATCGA
9.47Udiv	Clonotype G	CTCCCCGGTAGATCCCGAAGC
B4Udiv	SHPSTVPF	AAAAGGCACTGTCGAAGGGTGG
B124Udiv	VGDK	GGGCTGATTTTTGTCCCCTAC
B545Udiv	APLGP	CTCATTGGGGCCTAGAGGGGC

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Figure 1. $CD4^+$ and $CD8^+$ T cell counts in human immunodeficiency virus-infected children. Total $CD4^+$ (□) and $CD8^+$ (◊) T cell counts were directly determined in total blood samples by use of flow cytometry.

Figure 2. Transient T cell receptor β-chain variable region (TCRBV)-specific expansions of T cells in 8 children vertically infected with human immunodeficiency virus. TCRBV repertoire was typed by 2-color flow cytometric analysis using an extensive panel of TCRBV-specific monoclonal antibodies. $CD4^+$ *(A)* and $CD8^+$ *(B)* T cell subsets were analyzed in separate stainings. Arrowheads highlight significant expansions, as defined by a 2-fold difference and >5% overall representation. Nomenclature of TCR β-chain segments is according to [28]. Stainings not done were as follows: child 4, TCRBV13S1, 2 months; child 7, TCRBV3, 2 weeks, and TCRBV14, 9 months; child 9, TCRBV8, 2 weeks; child 11, TCRBV13S1 and TCRBV13S6, 2 weeks.

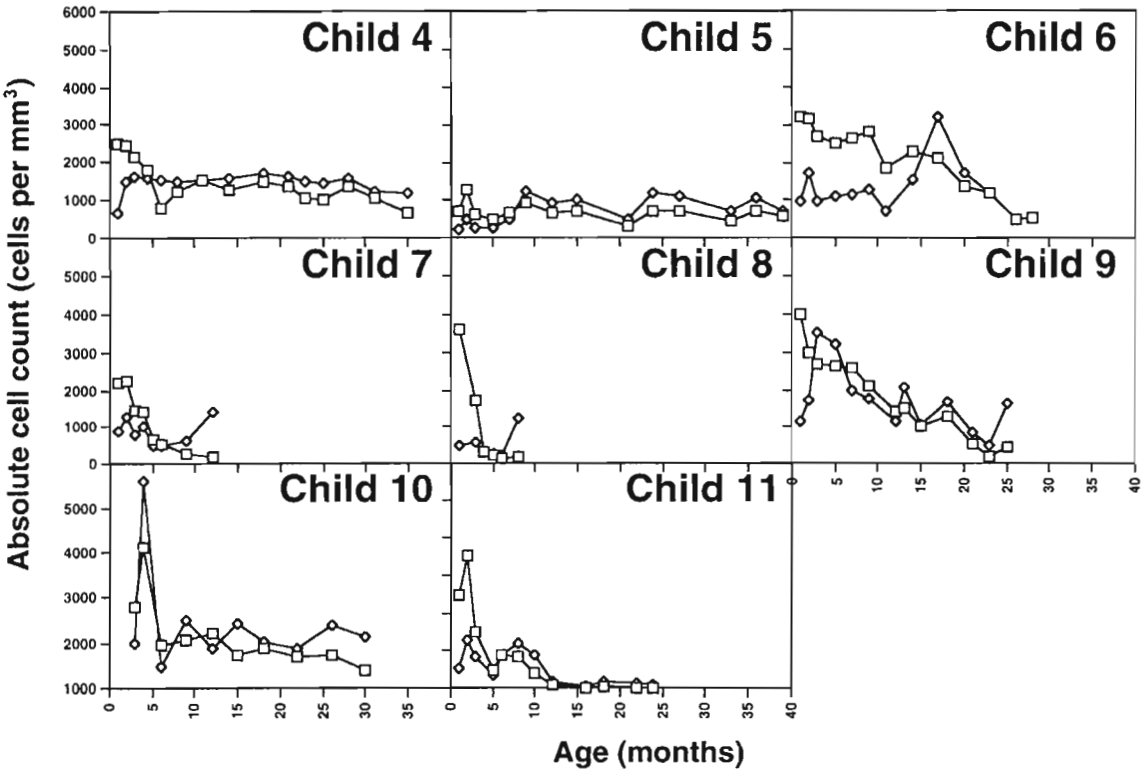
Figure 3. Transient T cell receptor β-chain variable region (TCRBV)-specific expansions of T cells in 4 uninfected children born to human immunodeficiency virus-infected mothers. TCRBV repertoire was typed by flow cytometric analysis using a panel of TCRBV-specific monoclonal antibodies. $CD4^+$ *(A)* and $CD8^+$ *(B)* T cell subsets were analyzed in separate stainings. Arrowheads highlight significant expansions, as defined by a 2-fold difference and >5% overall representation. TCR β-chain segment nomenclature is according to [28]. Stainings not done were as follows: child 214, TCRBV3, 6 months; child 232, TCRBV2, TCRBV3, TCRBV8, TCRBV13S6, TCRBV21, and TCRBV22, 4 months; child 236, TCRBV19, 4 months; child 254, TCRBV5S2-3, TCRBV13S2, and TCRBV19, 4 months.

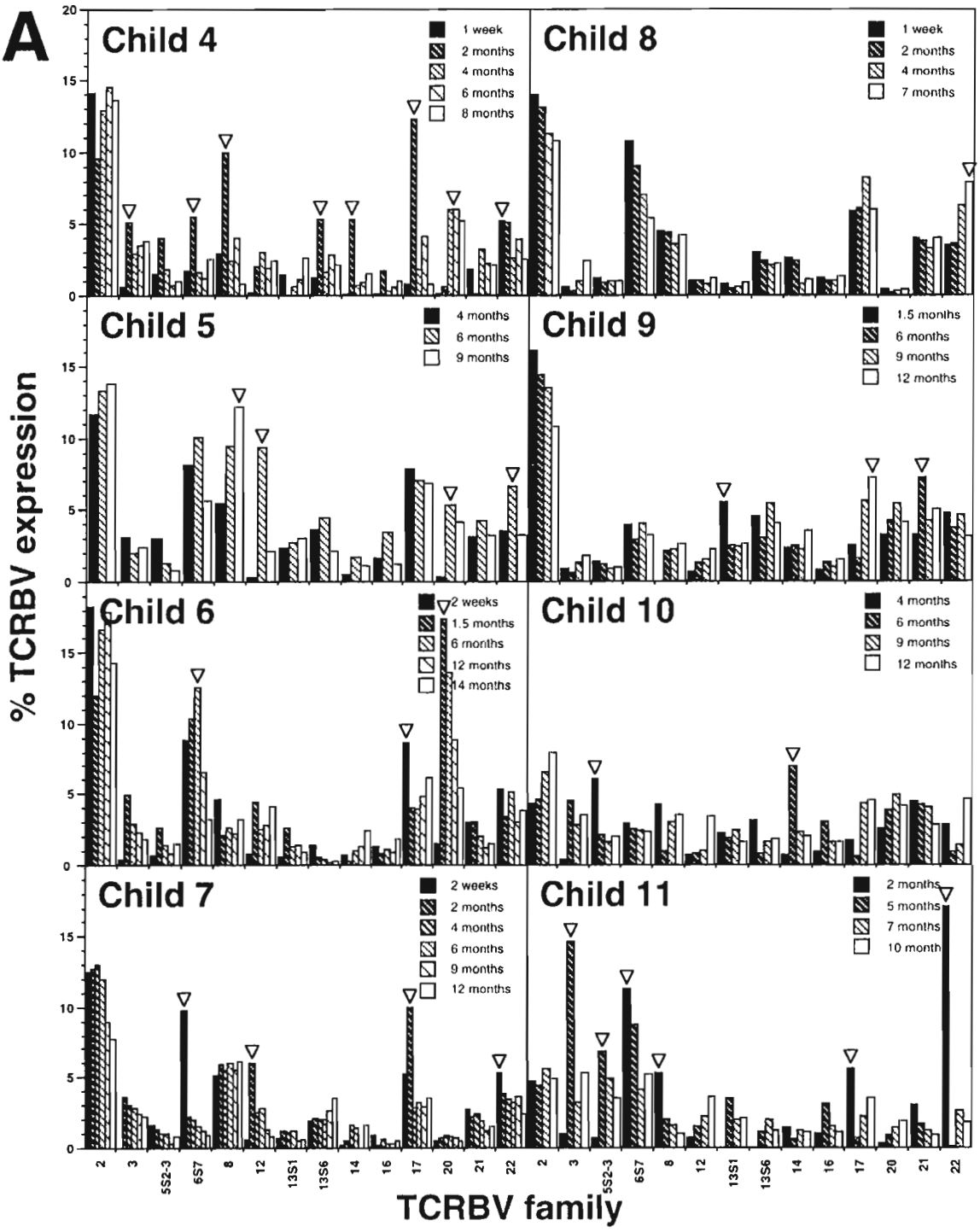
Figure 4. Diversity analysis of T cell receptor β-chain variable region (TCRBV) subset TCRBV14 in child 9, revealing high-level $CD8^+$ clonotypic expansions. *A,* TCRBV14 β-chain clones were amplified from cDNA samples taken from child 9 at various times during first year of life (17 days and 1, 2.5, 4, 5, 6, 9, and 12 months). V, TCRBV region residues; N1(D)N2, TCR β-chain diversity region and nontemplated residues; J, TCR β-

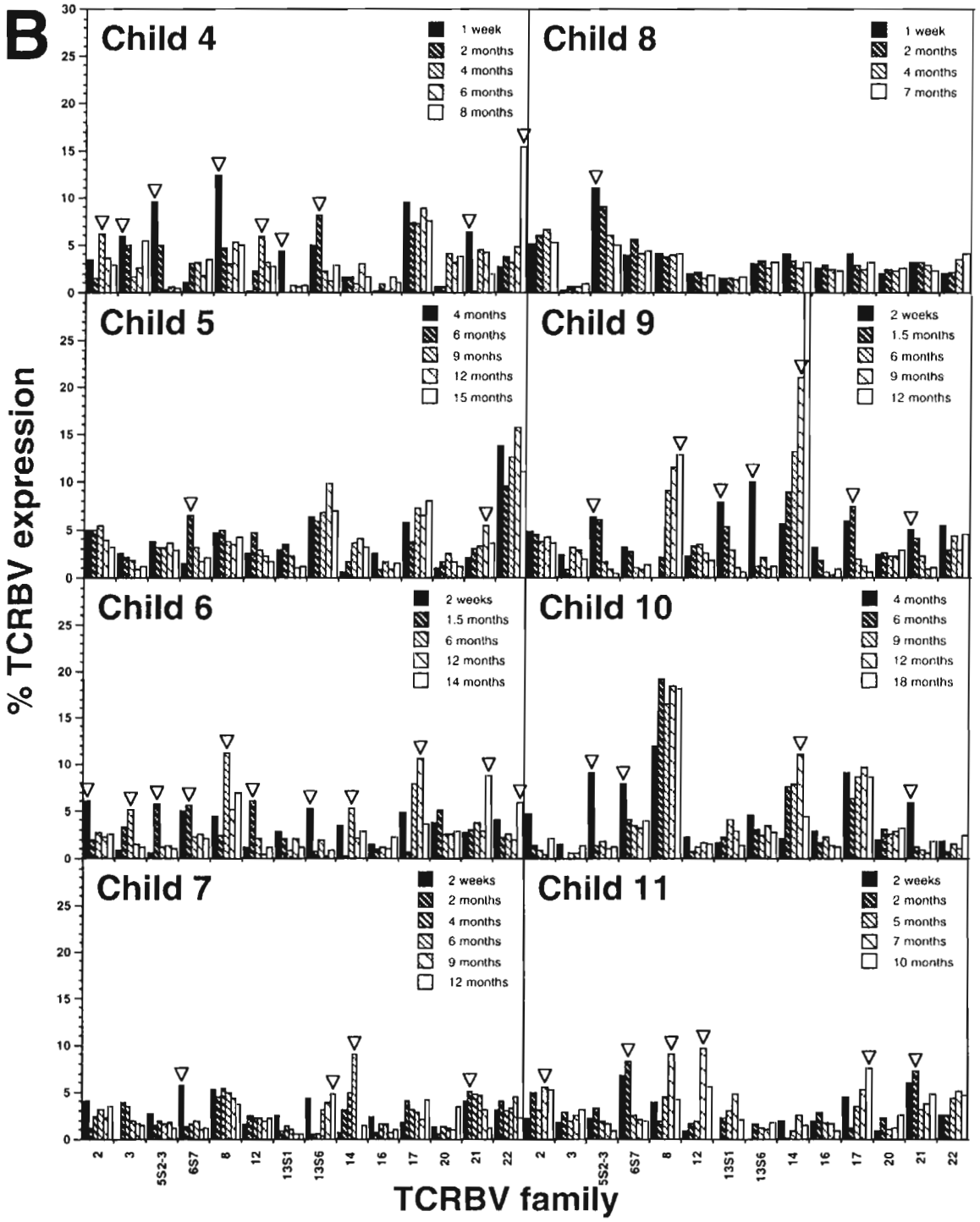
chain joining region (TCRBJ) residues; id, TCRBJ gene identity; L, CDR3 length, determined according to [31]. Letter codes on the right refer to persistently overrepresented TCR clonotypes tracked through multiple time points. *B,* Relative representation of 8 amplified clonotypes (A-H), as % of total no. of TCR β-chain clones analyzed at each time point. Nomenclature of TCRBV segments is according to [28], and that of TCRBJ segments is according to [27]. ND, not determined.

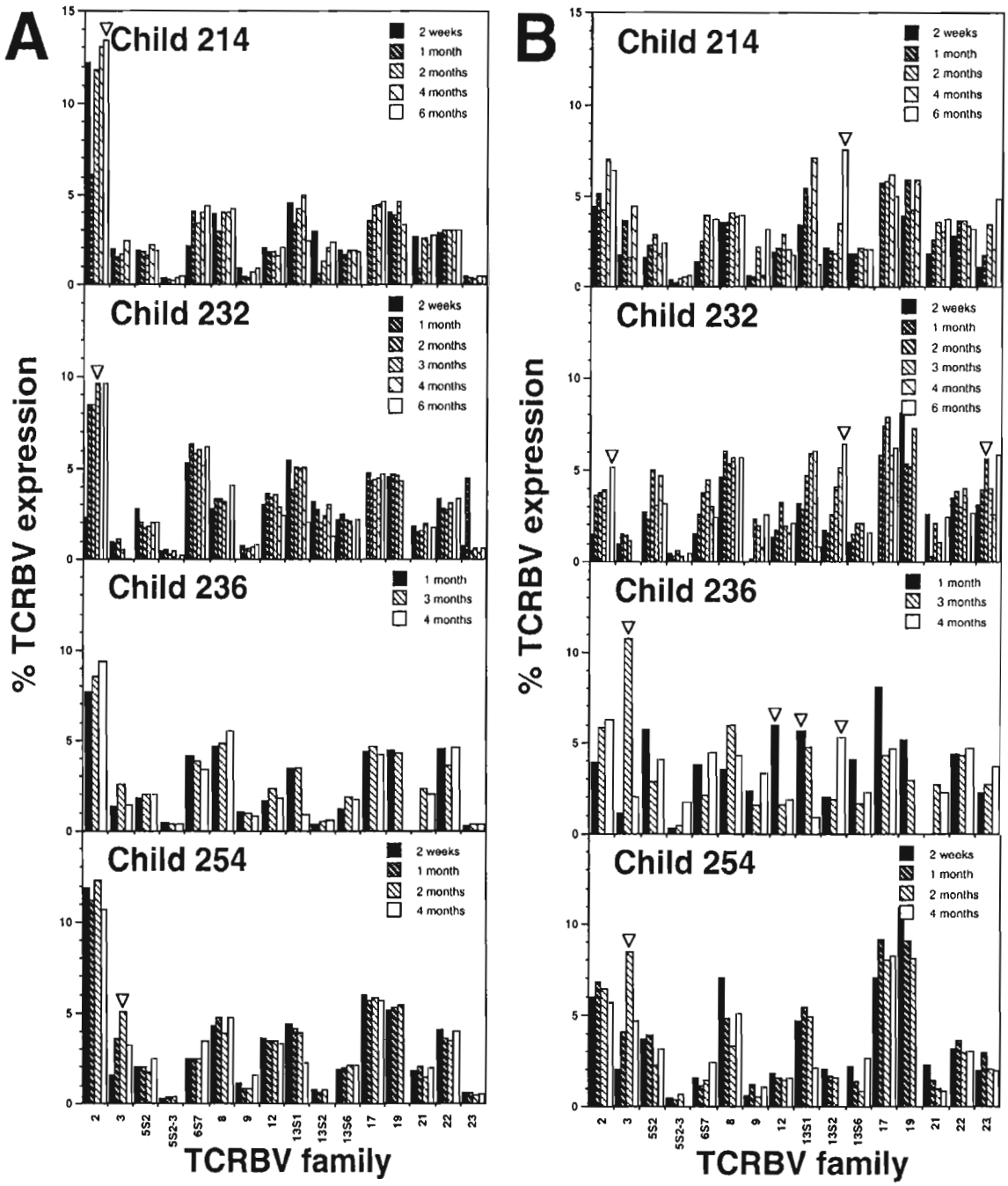
Figure 5. Relative representation of specific T cell receptor (TCR) clonotypes in child 9. Semiquantitative diversity-specific polymerase chain reaction (PCR) was done with primers complementary to CDR3 regions of selected TCR β-chain clonotypes. Two predominant clonotypes (E and F) were tracked throughout first year of life of child 9. 200-bp bands correspond to TCR α-chain constant region (TCRAC) internal control; 250-bp bands correspond to clonotype-specific PCR product. Histograms below represent values of radioactive signals from clonotype-specific bands, quantitated by use of a PhosphorImager and normalized to the intensity of the TCRAC band [29]. MW, molecular weight.

Figure 6. Diversity analysis of expanded T cell receptor β-chain variable region (TCRBV)6S7 *(A)* and TCRBV20 *(B)* subsets in child 6, revealing absence of clonotypic overrepresentation and persistence in $CD4^+$ T cell compartment. TCRβ-chain clones were amplified from cDNA samples taken from child 6 at age 2 days, 1.5 months, and 6 months, with TCRBV-specific and constant region primers. V, TCRBV region residues; N1(D)N2, TCR β-chain diversity region and nontemplated residues; J, TCR β-chain joining region (TCRBJ) residues; id, TCRBJ gene identity; L, CDR3 length, determined according to [31]. Nomenclature of TCRBV segments is according to [28], and that of TCRBJ segments is according to [27].


Child 4
Child 5
Child 6
Child 7
Child 8
Child 9
Child 10
Child 11
Absolute cell count (cells per mm3)
Age (months)


A
Child 4
1 week
2 months
4 months
6 months
8 months
Child 8
1 week
2 months
4 months
7 months
Child 5
4 months
6 months
9 months
Child 9
1.5 months
6 months
9 months
12 months
Child 6
2 weeks
1.5 months
6 months
12 months
14 months
Child 10
4 months
6 months
9 months
12 months
Child 7
2 weeks
2 months
4 months
6 months
9 months
12 months
Child 11
2 months
5 months
7 months
10 month
% TCRBV expression
0
5
10
15
20
2
3
5S2-3
6S7
8
12
13S1
13S6
14
16
17
20
21
22
TCRBV family


B
Child 4
1 week
2 months
4 months
6 months
8 months
Child 8
1 week
2 months
4 months
7 months
Child 5
4 months
6 months
9 months
12 months
15 months
Child 9
2 weeks
1.5 months
6 months
9 months
12 months
Child 6
2 weeks
1.5 months
6 months
12 months
14 months
Child 10
4 months
6 months
9 months
12 months
18 months
Child 7
2 weeks
2 months
4 months
6 months
9 months
12 months
Child 11
2 weeks
2 months
5 months
7 months
10 months
% TCRBV expression
0
5
10
15
20
25
30
2
3
5S2-3
6S7
8
12
13S1
13S6
14
16
17
20
21
22
TCRBV family


A
B
Child 214
Child 232
Child 236
Child 254
2 weeks
1 month
2 months
3 months
4 months
6 months
% TCRBV expression
TCRBV family
2
3
5S2
5S2-3
6S7
8
9
12
13S1
13S2
13S6
17
19
21
22
23

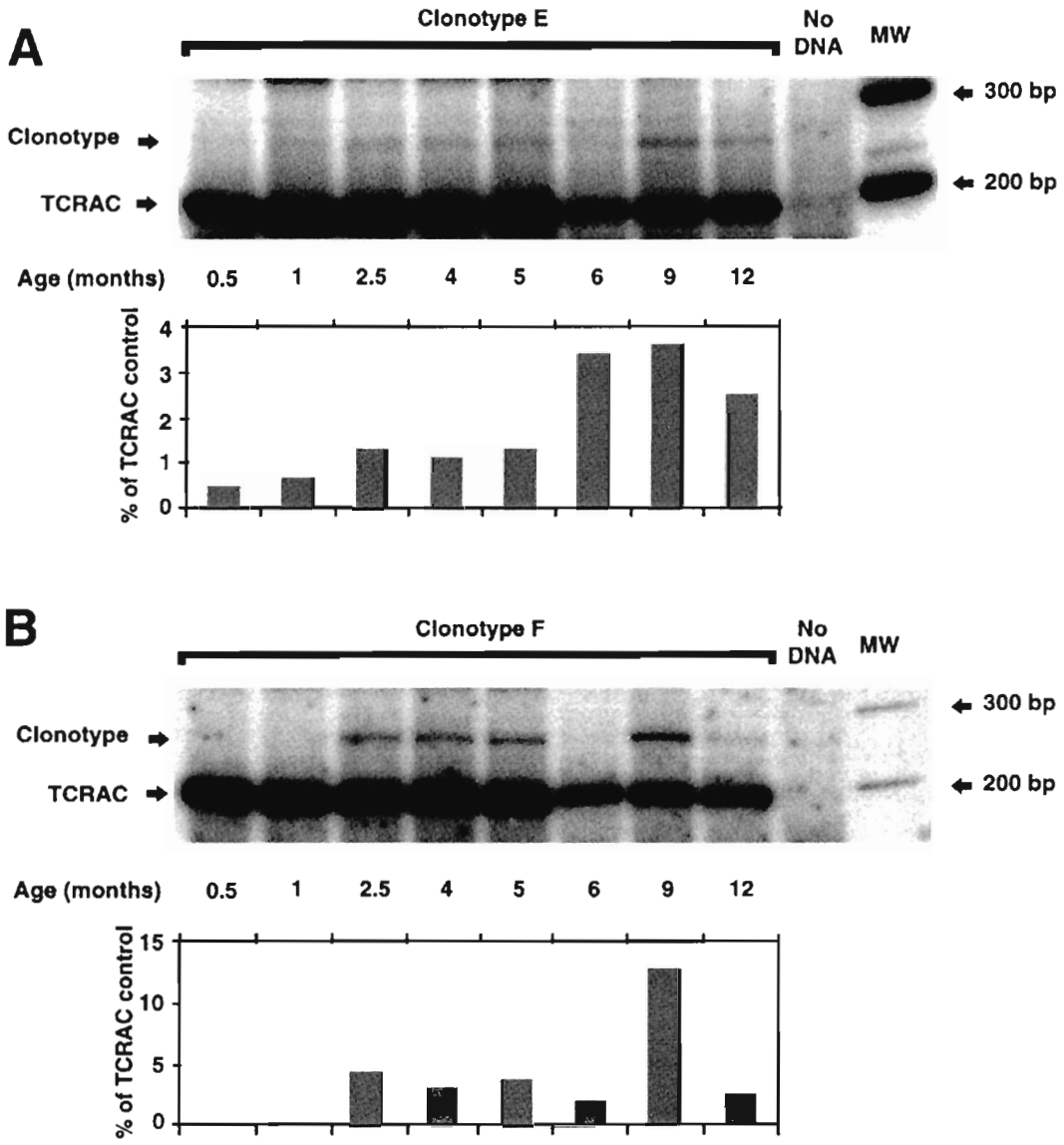
A

	V	N1(D)N2	J id	L		n
17 days						
	CAS	SWTSE	ETQYF[2.5]	8	A	1
	CAS	SPSWGS	TEAFF[1.1]	9		
	CASS	LTGQGH	TEAFF[1.1]	10		
	CASS	ILQGRG	TEAFF[1.1]	10		
	CASS	LTGEGH	TEAFF[1.1]	10		
	CASS	SRPLGA	AFF[1.2]	8		
	CAS	SQQGPL	YNSPLHF[1.6]	11		
	CASS	FSIGN	NYGYTF[1.6]	10		
	CAS	SQQGPL	YNSPLHF[1.6]	11		
	CASS	LSAGG	YNEQFF[2.1]	10		
	CASS	LSMGGPLG	NEQFF[2.1]	12		
	CASS	FTGA	GELFF[2.2]	8		
	CAS	SISGSG	TGELFF[2.2]	10		
	CASS	LIGD	TGELFF[2.2]	9		
	CASS	YTGE	DTQYF[2.3]	8		
	CASS	WTA	QETQYF[2.5]	8		
	CASS	WTSE	ETQYF[2.5]	8		
	CASS	PRDYT	SGANVLTF[2.6]	12		
	CASS	STVEG	EQYF[2.7]	8		
	CAS	TPLPGSS	SYEQYF[2.7]	11		
	CASS	FSASH	EQYF[2.7]	8		
	CASS	STVEG	EQYF[2.7]	8		
1 month						
	CAS	SWTSE	ETQYF[2.5]	8	A	1
	CASS	WTGG	YEQYF[2.7]	8	B	2
	CASS	YGIGR	YEQYF[2.7]	9	C	1
	CAS	RTGL	TEAFF[1.1]	7		
	CASS	LTPQAMA	TF[1.2]	8		
	CASS	FQPRQGP	NSPLHF[1.6]	12		
	CASS	WGGM	NEQFF[2.1]	8		
	CASS	FGGS	GELFF[2.2]	8		
	CASS	FTSA	GELFF[2.2]	8		
	CAS	SFGGS	GELFF[2.2]	8		
	CASS	FTSA	GELFF[2.2]	8		
	CASS	FTSA	GELFF[2.2]	8		
	CAS	SYTSE	GELFF[2.2]	8		
	CASS	ST	STDTQYF[2.3]	8		
	CASS	ST	STDTQYF[2.3]	8		
	CASS	WTSE	ETQYF[2.5]	8		
	CASS	LSTGGG	ETQYF[2.5]	10		
	CASS	STGED	EQYF[2.7]	8		
2.5 months						
	CASS	YGIGR	YEQYF[2.7]	9	C	2
	CAS	SRGGLAGF	TDTQYF[2.3]	12	D	2
	CASS	LGSPGLG	NTGELFF[2.2]	13	E	5
	CAS	RSSGMSD	EQFF[2.1]	9	F	2
	CASS	ITTGQG	YGYTF[1.2]	10		
	CASS	SGG	TNEKLFF[1.4]	9		
	CASS	FSGGG	ETQYF[2.5]	9		
	CASS	PTSE	YEQYF[2.7]	8		
	CASS	PTTN	YEQYF[2.7]	8		
	CASS	VAGGA	YEQYF[2.7]	9		
4 months						
	CASS	YGIGR	YEQYF[2.7]	9	C	3
	CAS	SRGGLAGF	TDTQYF[2.3]	12	D	3
	CASS	LGSPGLG	NTGELFF[2.2]	13	E	6
	CAS	RSSGMSD	EQFF[2.1]	9	F	3
	CAS	ASGS	TGELFF[2.2]	8	G	1
	CASS	FTGG	GYTF[1.2]	7		
	CASS	LGSV	NEQFF[2.1]	8		
	CASS	LGT	YNEQFF[2.1]	8		
	CAS	SRF	ANVLTF[2.6]	7		

	V	N1(D)N2	J id	L		n
5 months						
	CASS	YGIGR	YEQYF[2.7]	9	C	1
	CAS	SRGGLAGF	TDTQYF[2.3]	12	D	2
	CASS	LGSPGLG	NTGELFF[2.2]	13	E	4
	CAS	RSSGMSD	EQFF[2.1]	9	F	4
	CAS	ASGS	TGELFF[2.2]	8	G	2
	CASS	EFLGG	NSPLHF[1.6]	10		
	CASS	KTSGGLGIL	NEQFF[2.1]	13		
	CASS	LPGAYGPMA	TF[2.1]	10		
	CAS	SSTSA	GELFF[2.2]	8		
	CASS	STSEG	TQYF[2.5]	8		
	CASS	FTVVRLAGVR	ETQYF[2.5]	14		
	CASS	STSEG	TQYF[2.5]	8		
	CAS	RHTG	SGANVLTF[2.6]	10		
	CAS	RTQGH	EQYF[2.7]	7		
	CASS	FRGLMP	YEQYF[2.7]	10		
	CAS	TLRDRP	YEQYF[2.7]	9		
	CASS	FRGLMP	YEQYF[2.7]	10		
	CASS	HGIGR	YEQYF[2.7]	9		
6 months						
	CASS	LGSPGLG	NTGELFF[2.2]	13	E	4
	CAS	RSSGMSD	EQFF[2.1]	9	F	1
	CAS	ASGS	TGELFF[2.2]	8	G	1
	CASS	LQGP	YGYTF[1.2]	8		
	CASS	PGTGGG	YGYTF[1.2]	10		
	CASS	PGRGRPF	SGNTIYF[1.3]	13		
	CASS	LTGAG	QPQHF[1.5]	9		
	CASS	LYMDSD	SPLHF[1.6]	10		
	CASS	FRASY	ETQYF[2.5]	9		
	CASS	SQGLG	ETQYF[2.5]	9		
	CASS	LNRRV	SGANVLTF[2.6]	12		
	CASS	LFTGGP	SYEQYF[2.7]	11		
9 months						
	CASS	LGSPGLG	NTGELFF[2.2]	13	E	3
	CAS	RSSGMSD	EQFF[2.1]	9	F	2
	CAS	ASGS	TGELFF[2.2]	8	G	5
	CASS	PTVED	EQFF[2.1]	8	H	1
	CASS	PGVAGRY	YNEQFF[2.1]	12		
	CASS	LAGGPW	EQFF[2.1]	9		
	CASS	PLAGG	TGELFF[2.2]	10		
	CASS	PTVE	DTQYF[2.3]	8		
	CASS	STSEG	TQYF[2.5]	8		
12 months						
	CAS	RSSGMSD	EQFF[2.1]	9	F	2
	CAS	ASGS	TGELFF[2.2]	8	G	2
	CASS	PTVED	EQFF[2.1]	8	H	1
	CASS	PRQSV	NTEAFF[1.1]	10		
	CASS	HL	NTEAFF[1.1]	7		
	CASS	LRGAGD	GYTF[1.2]	9		
	CASS	LRGG	YGYTF[1.2]	8		
	CASS	FSSAGD	GYTF[1.2]	9		
	CASS	EGSNSG	LHF[1.6]	8		
	CASS	FGGG	SYNSPLHF[1.6]	11		
	CASS	LHPSGGG	NEQFF[2.1]	11		
	CAS	TRDGRF	TGELFF[2.2]	10		
	CASS	LLGTEI	TDTQYF[2.3]	11		
	CAS	RLQGG	DTQYF[2.3]	8		
	CASS	LF	STDTQYF[2.3]	8		
	CASS	PTSE	YEQYF[2.7]	8		
	CA	GSPTTN	YEQYF[2.7]	9		

B

clonotype	17 days	1 month	2.5 months	4 months	5 months	6 months	9 months	12 months
A	**4.55**	**5.26**	nd	nd	nd	nd	nd	nd
B	nd	**10.5**	nd	nd	nd	nd	nd	nd
C	nd	**5.26**	**11.8**	**15.0**	**3.85**	nd	nd	nd
D	nd	nd	**11.8**	**15.0**	**7.69**	nd	nd	nd
E	nd	nd	**29.4**	**30.0**	**15.4**	**26.7**	**18.8**	nd
F	nd	nd	**11.8**	**15.0**	**15.4**	**6.67**	**12.5**	**10.5**
G	nd	nd	nd	**5.00**	**7.69**	**6.67**	**31.3**	**10.5**
H	nd	nd	nd	nd	nd	nd	**6.25**	**5.26**


A
Clonotype E
No DNA
MW
300 bp
200 bp
Clonotype
TCRAC
Age (months) 0.5 1 2.5 4 5 6 9 12
% of TCRAC control
0 1 2 3 4
B
Clonotype F
No DNA
MW
300 bp
200 bp
Clonotype
TCRAC
Age (months) 0.5 1 2.5 4 5 6 9 12
% of TCRAC control
0 5 10 15

A

V	N1(D)N2	J	id	L
2 days				
CAS	RGTTG	NTEAFF	1.1	9
CASS	LATGG	TEAFF	1.1	9
CASS	FDWT	DGYTF	1.2	8
CAS	GLDRG	YGYTF	1.2	8
CASS	PQGRI	YNSPLHF	1.6	11
CASS	LGDRRP	NEQFF	2.1	10
CAS	RGGGAWG	NVLTF	2.6	10
CASS	LLAG	YEQYF	2.7	8
CASS	LDRA	YEQYF	2.7	8
CASS	FGRGGSG	EQYF	2.7	10
CASS	LGQGA	YEQYF	2.7	9
CASS	FS	YEQYF	2.7	6
CASS	LQRGP	SYEQYF	2.7	10
CAS	GPGVERGA	YEQYF	2.7	11
1.5 months				
CASS	SFVG	YGYTF	1.2	8
CASS	LRTGG	DQPQHF	1.5	10
CASS	PSAGN	NQPQHF	1.5	10
CASS	LGRMPVYS	SPLHF	1.6	12
CASS	LAGTP	NSPLHF	1.6	10
CASS	SGQGF	YNSPLHF	1.6	11
CASS	LRDTGGAFY	EQFF	2.1	12
CASS	PLLY	EQFF	2.1	7
CASS	VGLAGGL	YNEQFF	2.1	12
CASS	LVRGTTN	TDTQYF	2.3	12
CASS	RGPPF	TDTQYF	2.3	10
CASS	FPAGETA	STDTQYF	2.3	13
CASS	LWGGP	STDTQYF	2.3	11
CASS	AVLAGGL	ETQYF	2.5	11
CASS	QGAGR	TYEQYF	2.7	10
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CASS	LVTRAA	YEQYF	2.7	10
CASS	HQAG	YEQYF	2.7	8
CASS	LVG	YEQYF	2.7	7
CASS	SG	YEQYF	2.7	6
6 months				
CAS	RLGDLVG	TEAFF	1.1	10
CAS	RLDP	NTEAFF	1.1	8
CASS	PTVG	NTEAFF	1.1	9
CASS	FPSFMVTGQT	NTEAFF	1.1	15
CASS	SRDRESA	EAFF	1.1	10
CASS	LAGDRRE	YGYTF	1.2	11
CASS	RPP	QPQHF	1.5	7
CASS	LGG	YNSPLHF	1.6	9
CASS	WDRS	NSPLHF	1.6	9
CASS	LWTS	SYNEQFF	2.1	10
CASS	LVGTDF	TGELFF	2.2	11
CASS	LANLAGGP	DTQYF	2.3	12
CASS	LGIV	QETQYF	2.5	9
CASS	ILAGAT	QETQYF	2.5	11
CASS	GDRV	QETQYF	2.5	9
CASS	WASGE	ETQYF	2.5	9
CASS	SEPQGLAGLS	YEQYF	2.7	13
CASS	LPGSP	YEQYF	2.7	9
CASS	LSSQGA	YEQYF	2.7	10

B

V	N1(D)N2	J	id	L
2 days				
CAW	TGG	NYGYTF	1.2	7
CAW	SATGS	YTF	1.2	6
CAW	RQTGPL	YGYTF	1.2	9
CA	FYY	YGYTF	1.2	6
CAWS	SHPGQGG	GYTF	1.2	10
CAWS	PQDV	NEKLFF	1.4	9
CAWS	VGDK	NQPQHF	1.5	9
CAWS	VGPPDG	QPQHF	1.5	10
CAW	SHPSTVPF	NSPLHF	1.6	12
CAWS	VSLGP	SYNSPLHF	1.6	11
CAWS	RGGH	EQFF	2.1	7
CAWS	APLGP	NEQFF	2.1	9
CAW	KLAGK	NTGELFF	2.2	10
CAW	SVRGS	SYEQYF	2.7	9
1.5 months				
CA	LEYQSM	TEAFF	1.1	9
CAWS	VTR	NTEAFF	1.1	8
CAW	SARG	NTEAFF	1.1	8
CAW	TRD	TEAFF	1.1	6
CAWS	PLQG	NTEAFF	1.1	9
CAW	SPNR	NYGYTF	1.2	8
CAWS	VGSQD	GYTF	1.2	8
CAWS	LR	NYGYTF	1.2	7
CAW	TDS	NYGYTF	1.2	7
CAW	KPGGFA	GYTF	1.2	8
CAWS	LTYGQTSS	GNTIYF	1.3	13
CAWS	SFLGA	NEKLFF	1.4	10
CAWS	VRG	NQPQHF	1.5	8
CAWS	VGDRG	NQPQHF	1.5	10
CAWS	VPQGAG	YNSPLHF	1.6	12
CAWS	VSRTD	YNSPLHF	1.6	11
CA	CAPGLVL	NEQFF	2.1	10
CAWS	DGRA	YNEQFF	2.1	9
CAWS	GGGTAN	NEQFF	2.1	10
CAW	TRND	EQFF	2.1	6
CAWS	VKQG	QFF	2.1	6
CAW	TKQGAH	TGELFF	2.2	10
CAWS	PSYRGRF	ELFF	2.2	10
CAWS	IVGV	TGELFF	2.2	9
CAW	SSVLKRGP	TDTQYF	2.3	12
CAWS	VGGT	AKNIQYF	2.4	10
CAWS	DR	AKNIQYF	2.4	8
CAW	NEGQGD	EQYF	2.7	9
CAWS	TSGRT	YEQYF	2.7	9
CAWS	VRQTT	YEQYF	2.7	9
CAWS	VGGGT	YEQYF	2.7	9
6 months				
CAWS	VYGVKTGM	NTEAFF	1.1	13
CAWS	LWGVV	NTEAFF	1.1	10
CAWS	VTLQQGA	NTEAFF	1.1	12
CAWS	VRGRV	NTEAFF	1.1	10
CAWS	LQA	NTEAFF	1.1	8
CAWS	GRGLG	YGYTF	1.2	9
CAWS	PAPGFK	YGYTF	1.2	10
CAWS	VRGTGL	YGYTF	1.2	10
CAW	GDRGRH	GYTF	1.2	8
CAWS	VFGD	NYGYTF	1.2	9
CAWS	VQTS	YGYTF	1.2	8
CAWS	RDKVD	YGYTF	1.2	9
CAWS	SLQGVD	EKLFF	1.4	10
CAWS	VDG	QPQHF	1.5	7
CAWS	VSG	NQPQHF	1.5	8
CAWS	ADS	NSPLHF	1.6	8
CAW	TTGTG	SPLHF	1.6	8
CAWS	IYGQYR	NSPLHF	1.6	11
CAWS	VYL	NTGELFF	2.2	9
CAWS	ERAK	GELFF	2.2	8
CAWS	TRPTSLWY	TDTQYF	2.3	13
CAWS	LHGRI	STDTQYF	2.3	11
CAWS	AHTWDTRSLPKR	NIQYF	2.4	16
CA	CTISSLT	EQNF	2.7	9
CA	CPPGQG	SYEQYF	2.7	10

3. Skewed maturation of memory HIV-specific CD8 T lymphocytes

The study presented in Chapter 2 is a characterization of the phenotypic and molecular TCR repertoire of pediatric subjects with HIV-1 infection. This study reveals that, in the context of HIV-1 infection, the TCR repertoires of CD4 and CD8 T cells appear to be differentially modulated. Most significantly, this study reveals that, according to the parameters under scrutiny, the properties and pattern of TCR repertoire modulation in the context of pediatric HIV-1 infection are suggestive of a dynamic antigen-driven $CD8^+$ T cell-mediated immune response that is akin to that evidenced in HIV-1 infected adults.

HIV-specific $CD8^+$ T cells have clearly been evidenced throughout the acute, primary and chronic stages of pediatric and adult HIV-1 infection: in spite of the demonstrated critical role of these cells in the repression of viral replication, this control is incomplete and gradually lost as disease progresses. This has led to the hypothesis that the HIV-specific CD8 T cell-mediated immune response may be only partially effective as a result of viral properties, including the propensity for viral escape from the immune system, and due to host immunopathology, including the clonal exhaustion of HIV-specific CD8 T cells.

Recent reports have also suggested that one key mechanism potentially explaining the partial effectiveness of CD8 T cell-mediated antiviral immune responses may be an impaired or defective effector function of HIV-1 specific CD8 T cells. This was originally suggested by immunohistological studies demonstrating that, in the lymph nodes of HIV-1 infected patients, exocytic granules of $CD8^+$ T lymphocytes frequently expressed granzyme A in the absence of perforin[788]. Subsequently, a defect of HIV-1 specific CD8 T cells' ability to secrete IFN-γ following *in vitro* stimulation by HIV-1 infected CD4 T cells was observed by Shankar *et al.*[787]. This defect was corrected when exogenous IL-2 was provided, suggesting that T cell help could correct the dysfunction[787]. Similar functional defects were subsequently also observed in rhesus macaques chronically infected with SIV, one study also demonstrating an IL-2-enhaced restoration of responses[789,790]. Importantly, an impairment of HIV-specific CD8 T lymphocyte cytolytic function was evidenced by Appay *et al.* who also showed that circulating HIV-specific CD8 T cells had reduced perforin content[791]. Their phenotypic characterization of the HIV-specific $CD8^+$ T cells revealed that they persistently

expressed CD27, but lacked CD28, a phenotype that differed from that of CMV-specific cells[791]. Significantly, according to a phenotypic characterization of CD8 T lymphocytes by Hamman *et al.*, the HIV-specific cells exhibited a phenotype typical of memory cells while CMV-specific cells expressed that of genuine effector cells[431]. Altogether, these studies led us to postulate that an altered differentiation state of HIV-specific CD8 T cell could possibly underlie the various observations.

Recent work performed by Sallusto and colleagues evidenced that the expression of C-C chemokine receptor 7 (CCR7), which confers a cell with chemotactic response to the "homeostatic" chemokines CCL19 and CCL21, distinguishes two subsets of memory T lymphocytes that possess differential tissue homing characteristics and effector functions [792]. First, "central memory T cells" (Tcm) home preferentially to lymphoid organs through expression of CCR7 and CD62L; furthermore, they display little immediate effector function. In contrast, "effector memory T cells" (Tem) express tissue homing receptors associated with inflammation, do not express CCR7, and display more ready-effector function [792].

With this in mind, we set out to establish the distribution of HIV-specific $CD8^+$ T lymphocytes within the different memory phenotypic subpopulations so as to trace the evolution of these subsets during an ongoing immune response. Antigen-specific CD8 T cells were tracked using tetrameric-MHC molecules and a thorough phenotypic and functional study of the circulating CD8 T lymphocytes of chronically HIV-1 infected patients was undertaken. The characterization of the phenotypic, functional and proliferative properties of antigen-specific CD8 T cell subsets allowed us to delineate a differentiation pathway undergone by recruited $CD8^+$ T cells following encounter with their cognate antigen. Furthermore, a comparative assessment of the HIV-1 and CMV-specific $CD8^+$ T cell responses exhibited by dually infected subjects allowed us to evidence an altered peripheral distribution of HIV-specific memory CD8 T lymphocytes during chronic HIV-1 infection.

Skewed maturation of memory HIV-specific CD8 T lymphocytes

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Published in Nature 410 (6824): 106-111

Abstract

Understanding the lineage differentiation of memory T cells is a central question in immunology. We investigated this issue by analysing the expression of the chemokine receptor CCR7, which defines distinct subsets of naive and memory T lymphocytes with different homing and effector capacities[1-3] and antiviral immune responses to HIV and cytomegalovirus. *Ex vivo* analysis of the expression of CD45RA and CCR7 antigens, together with *in vitro* analysis of the cell-division capacity of different memory $CD8^+$ T-cell populations, identified four subsets of HIV- and CMV-specific $CD8^+$ T lymphocytes, and indicated the following lineage differentiation pattern: $CD45RA^+CCR7^+ \rightarrow CD45RA^-CCR7^+ \rightarrow CD45RA^-CCR7^- \rightarrow CD45RA^+CCR7^-$. Here we demonstrate through analysis of cell division (predominantly restricted to the $CCR7^+CD8^+$ T-cell subsets) that the differentiation of antigen-specific $CD8^+$ T cells is a two-step process characterized initially by a phase of proliferation largely restricted to the $CCR7^+CD8^+$ cell subsets, followed by a phase of functional maturation encompassing the $CCR7^-CD8^+$ cell subsets. The distribution of these populations in HIV- and CMV-specific $CD8^+$ T cells showed that the HIV-specific cell pool was predominantly (70%) composed of pre-terminally differentiated $CD45RA^-CCR7^-$ cells, whereas the CMV-specific cell pool consisted mainly (50%) of the terminally differentiated $CD45RA^+CCR7^-$ cells. These results demonstrate a skewed maturation of HIV-specific memory $CD8^+$ T cells during HIV infection.

A vigorous HIV-specific $CD8^+$ T-cell immune response is readily detected during primary infection[4],and the role of $CD8^+$ T cells in the control of virus replication[5] is well demonstrated; however, this control is incomplete and progressively lost as disease progresses. This has led to the hypothesis that the HIV-specific cytotoxic response is only partially effective[6].

We analysed blood mononuclear cells obtained from 18 HIV-infected subjects with no previous anti-retroviral therapy with monoclonal antibodies to CD8 and CCR7, and with HLA tetrameric complexes loaded with the relevant peptides that allow direct evaluation of antigen-specific memory $CD8^+$ T cells[7, 8]. Most (70–80%) of the $CD8^+$ T cells were $CCR7^-$ in patients 1,020 and 2,021 (Fig. 1a), and these results were confirmed in a further 16 patients (mean 76 ± 13.5%). The immunodominant epitope SLYNTVATL[9] in the gag protein, which is restricted by HLA-A2, was recognized by patients 1,020 and 2,121 (0.77 and 0.46% of blood mononuclear cells, respectively) (Fig. 1a). Most of the HIV-specific $tetramer^+(Tet^+)$ cells (~70–80%) were contained in the $CCR7^-$ population (Fig. 1a). The prevalence of HIV-specific Tet^+ $CD8^+$ cells within the $CCR7^-$ cell population was confirmed in a larger number (n = 16) of patients. The $CCR7^-$ $Tet^+CD8^+/CCR7^+Tet^+CD8^+$ cell ratio was 2.4/1.

Although most of the antigen-primed memory $CD8^+$ T lymphocytes are phenotypically characterized by the expression of the CD45RO isoform[10], a fraction of memory $CD8^+$ T cells express the CD45RA isoform[11, 12]. We then determined whether the distribution of CCR7 could further identify, *in vivo,* other subsets of $CD8^+$ T cells. In 3 (out of 5) representative patients most (70–80%) of the HIV-specific $CD8^+Tet^+$ cells were contained within the $CD45RA^-$ cell subset (Fig. 1b). More than 90% of the $Tet^+CD45RA^-$ cells were $CCR7^-$, whereas only a small percentage (5–10%) were $CCR7^+$ (Fig. 1b). About 70–80% of $Tet^+CD45RA^+$ cells expressed CCR7, whereas 10–20% of cells were $CCR7^-$ (Fig. 1b). Four cell populations of HIV-specific $CD8^+$ T cells have been identified: 1) $CD45RA^+CCR7^+$; 2) $CD45RA^-CCR7^+$; 3) $CD45RA^-CCR7^-$ and 4) $CD45RA^+CCR7^-$. On the basis of their high frequency, it is clear that the $CD45RA^+Tet^+CD8^+$ T cells are antigen-primed and expanded cells. Furthermore, the $CD45RA^+CCR7^+$ cells represented 15 ± 5.2% (mean ± s.d.) of the total HIV-specific Tet^+ cells, the $CD45RA^-CCR7^+$ cells

represented 4.1 ± 3.4%, the $CD45RA^-CCR7^-$ cells represented 71.8 ± 5.8% and the $CD45RA^+CCR7^-$ cells represented 8.9 ± 9.2%.

To determine the lineage differentiation pattern of these four subsets of $CD8^+$ T lymphocytes, we first analysed the capacity of cell division for the four cell subsets on *in vitro* stimulation, and then the differentiation patterns of purified $CD8^+CCR7^-CD45RA^-$ and $CD8^+CCR7^-CD45RA^+$ cell populations after stimulation with the specific peptide. Unfractionated mononuclear cells isolated from HIV-negative and HIV-infected subjects were stimulated with anti-CD3 plus anti-CD28 monoclonal antibody, and cell division was analysed by 5-(and 6-)carboxyl-fluorescein diacetate, succinimidyl ester (CFSE) labelling[13] and flow cytometry in different subsets of $CD8^+$ T cells characterized by the expression of CD45RA and CCR7. As shown for one (out of three) representative HIV-negative and three HIV-infected subjects, cell division was mostly confined to the populations of $CD8^+$ T lymphocytes that express CCR7 (Fig. 2a). Furthermore, the appearance of cell division was more rapid in $CD8^+CD45RA^+CCR7^+$ cells (48 h after stimulation) compared with $CD8^+CD45RA^-CCR7^+$ cells (60 h after stimulation) (Fig. 2a). No evidence for significant cell division was observed in $CD8^+CD45RA^-CCR7^-$ or in $CD8^+CD45RA^+CCR7^-$ cells during the time of observation (Fig. 2a). No cell division was observed in any $CD8^+$ T-cell subset in the unstimulated controls throughout the period of observation (data not shown).

We then determined *ex vivo* the cell division capacity of HIV-specific $CD8^+$ T-cell populations by intracellular staining for Ki67, a nuclear antigen that is expressed in all phases of the cell cycle except for the G0 phase[14]. About 10% of Tet^+ cells were $Ki67^+$ (Fig. 2b) in patients 1,004 and 1,020. Notably, only a small percentage (1–5%) of the Tet^+CCR7^- cells expressed Ki67 compared with about 30% of Tet^+CCR7^+ cells (Fig. 2b). Tet^+Ki67^+ were present in both $CD45RA^+$ and $CD45RA^-$ cell subsets (Fig. 2b). Similar results were obtained in a further three patients. These results indicate that the cell division capacity is predominantly restricted to the $CD8^+$ T-cell compartment that expresses CCR7. The low percentage of $Ki67^+$ cells in the $CCR7^-$ cell subset, which contains most of the Tet^+ cells, is consistent with a reduced proliferative capacity of the HIV-specific cytotoxic T lymphocytes[15, 16]. Taken together, these results indicate that

$CCR7^+CD8^+$ T cells—in particular $CD8^+CD45RA^+CCR7^+$ cells—function as precursors of the $CCR7^-CD8^+$ T cells.

To assess further the lineage differentiation pattern of antigen-specific cells, we isolated $CD45RA^-CCR7^-CD8^+$ cells by cell sorting, and cell cultures were phenotypically characterized at different time points after stimulation with the specific peptide and in the presence of interleukin-2 (IL-2). In patient 2,077, the percentage of Tet^+ cells within the $CD8^+CD45RA^-CCR7^-$ cell subset was 1.8% before stimulation (data not shown). After 36 h of peptide-specific stimulation there was no evidence for expansion of Tet^+ cells (1.7%) within $CD45RA^-CCR7^-CD8^+$ cells (Fig. 2c), and no substantial changes were observed at 60 h after stimulation (Fig. 2c). The lack of expansion of Tet^+ cells within the stimulated $CD45RA^-CCR7^-CD8^+$ cell population is consistent with the CFSE and Ki67 results, thus providing further evidence that this cell population cannot be efficiently expanded. These results further confirm the dichotomy with regard to the cell division and proliferative capacities among the $CCR7^+$ and $CCR7^-CD8^+$ T-cell compartments: high cell division capacity/expansion of the $CCR7^+$ versus limited capacity of cell division/expansion of the $CCR7^-CD8^+$ T-cell populations.

We next analysed the changes occurring in the expression of CD45RA and CCR7 upon *in vitro* antigen-specific stimulation of $CD45RA^-CCR7^-CD8^+$ cells. A substantial proportion (26%) of Tet^+ cells became $CD45RA^-CCR7^+$ (the upregulation of CCR7 after stimulation is consistent with previous studies[17]) whereas most (56%) acquired the $CD45RA^+CCR7^+$ phenotype at 36 h after stimulation (Fig. 2c). By 60 h after stimulation, a significant percentage (25%) of Tet^+ cells exhibited the $CD45RA^+CCR7^-$ phenotype (Fig. 2c). No phenotypic changes were observed in the Tet^- cell populations from the stimulated cultures (data not shown).

These results strongly suggest that $CD45RA^+CCR7^+CD8^+$ T cells function as precursors for $CD45RA^-CCR7^+CD8^+$, $CD45RA^-CCR7^-CD8^+$ and $CD45RA^+CCR7^-CD8^+$ cells. The results obtained from stimulated $CD45RA^-CCR7^-$ cells indicate that antigen-specific $CD8^+$ T cells at an advanced stage of differentiation may also revert to a phenotype, such as $CCR7^+$, which is typical of cells at earlier stages of cell differentiation, although they

do not acquire the functional features, such as the proliferative capacity, of precursor cells.

Patients 1,013 and 2,121, in addition to the HLA-A2-restricted SLYNTVATL gag epitope (Fig. 1a and b), recognized the NLVPMVATV epitope[18] in the pp65 protein of Cytomegalovirus (CMV) that is also restricted by HLA-A2. This provided an opportunity to determine the composition of the CMV-specific $CD8^+$ T-cell pool. The percentage of CMV-specific Tet^+CD8^+ T cells was 1.15% and 0.24% in patients 1,013 and 2,121, respectively (Fig. 3a). About 70% of CMV-specific $CD8^+Tet^+$ cells were $CCR7^-$ and 30% $CCR7^+$ (Fig. 3a), whereas most of the CMV-specific Tet^+ cells in both patients were $CD45RA^+CCR7^-$ (Fig. 3b). Similar analysis performed in a further six patients indicated that the $CD45RA^+CCR7^+$ cell subset represented about 5% ($6 \pm 1.5\%$; mean ± s.d.) of the total CMV-specific Tet^+ cells, the $CD45RA^-CCR7^+$ represented $3.8 \pm 1\%$, the $CD45RA^-CCR7^-$ represented $38.9 \pm 6.4\%$, and the $CD45RA^+CCR7^-$ represented $51 \pm 6.9\%$ of the total Tet^+ cells (Fig. 3b). Therefore, the $CD45RA^+CCR7^+$ (precursor cells) and the $CD45RA^-CCR7^-CD8^+$ T-cell populations were expanded (up to 20% and 70% of Tet^+ cells, respectively) in the HIV-specific but not in the CMV-specific cell pool. The $CD45RA^+CCR7^-CD8^+$ T-cell subset was largely represented (50% of Tet^+ cells) within the CMV-specific cell pool and poorly represented within the HIV-specific $CD8^+$ T-cell pool (5% of Tet^+ cells). Furthermore, Ki67 nuclear antigen was expressed in only 2% of the CMV-specific Tet^+ cells as compared with 10% of HIV-specific $CD8^+$ T cells (Fig. 3c). As the proportion of $Ki67^+$cells can be used as a measure of T-cell production and turnover[19, 20], these results indicate that there is at least a fivefold increase in the production of HIV-specific compared with CMV-specific $CD8^+$ T cells, and suggest that there is a constant recruitment of T cells within the HIV-specific cell pool.

It has been proposed[3] that the $CD45RA^+CCR7^-$ antigen-specific $CD8^+$ cell population, which secretes interferon-γ (IFN-γ) and expresses high perforin levels, is composed of terminally differentiated $CD8^+$ T cells. To address further this issue the $CD45RA^+CCR7^-$ CMV-specific $CD8^+$ cell population was isolated by cell sorting, and cell cultures were analysed upon stimulation with the specific peptide. The percentage of Tet^+ cells within the $CD8^+CD45RA^+CCR7^-$ cell subset was 1.4% before stimulation (data not shown). No

evidence for substantial expansion of CMV-specific Tet^+ cells was observed at 36 (1.8%) and 60 h (2%) after peptide-specific stimulation, and there was no evidence for phenotypic changes (Fig. 3d). These results further support the hypothesis[3] that the $CD45RA^+CCR7^-CD8^+$ cells are at a terminal stage of differentiation. This, together with the different cell-division capacity of the different subsets of $CD8^+$ T lymphocytes and the changes observed upon antigen-specific stimulation of $CD45RA^-CCR7^-CD8^+$ cells, favours the following lineage differentiation pattern for antigen-specific T lymphocytes: $CD45RA^+CCR7^+ \rightarrow CD45RA^-CCR7^+ \rightarrow CD45RA^-CCR7^- \rightarrow CD45RA^+CCR7^-$.

The HIV- and CMV-specific $CD8^+$ T cells were then functionally characterized for the production of IFN-γ and the expression of perforin. Patient 2,123 recognized the FLKEKGGL epitope in the Nef protein restricted by HLA-B8 (Fig. 4a). Upon peptide-specific stimulation, a large percentage (50–75%) of $CCR7^+Tet^+$ and $CCR7^-Tet^+$ cells expressed CD69, a marker of activation, and stained positive for IFN-γ (Fig. 4a). Similar results were obtained in a further three patients (data not shown). These results indicate that not only $CCR7^-$ memory $CD8^+$ T cells, but also a large percentage of $CCR7^+$ cells may secrete IFN-γ after antigen-specific stimulation. We then compared the secretion of IFN-γ and the expression of perforin in two patients with HIV- and CMV-specific $CD8^+$ T cells. We observed no differences in the secretion of IFN-γ (Fig. 4b). Perforin expression was reduced in HIV-specific $CD8^+$ T cells of patient 1,013 and was predominantly confined to the $CCR7^-$ cell subset (Fig. 4c). A defective expression of perforin[21] within the HIV-specific Tet^+CD8^+ T cells compared with CMV-specific Tet^+ cells[6] has been shown. Our results suggest that this defective perforin expression could be related to the accumulation of T cells at a specific stage of differentiation rather than a defect in perforin production.

On the basis of the expression of CD45RA and CCR7, four subsets of memory $CD8^+$ T lymphocytes have been identified, and their lineage differentiation pattern has been proposed (Fig. 5a). We have also observed large differences in the representation of the $CD8^+$ T-cell subsets within the HIV- and CMV-specific memory $CD8^+$ T-cell pools (Fig. 5b). The HIV-specific memory $CD8^+$ T-cell pool is largely composed (70%) of the pre-terminally differentiated $CD45RA^-CCR7^-$ cells, whereas the terminally differentiated

$CD45RA^+CCR7^-$ cell subset is poorly (less than 5%) represented. The latter, however, is the well-represented (50%) cell subset within the CMV-specific memory $CD8^+$ T-cell pool. The finding that most of the CMV-specific memory $CD8^+$ T cells are terminally differentiated raises the issue of how it will be possible to generate a recall response to CMV if these cells are not able to proliferate upon antigen simulation. In principle, the generation of a recall antigen response is thought to be associated with the proliferation of memory cells and their conversion to effector cells. Our results indicate a new model. In fact, the population of memory CMV-specific $CD8^+$ T cells is not only composed of terminally differentiated effector ($CD45RA^+CCR7^-$) cells, but also of precursor ($CD45RA^+CCR7^+$) memory cells. It is therefore probable that the terminally differentiated effector cells are ready to rapidly intervene on antigen re-encounter, while precursor cells will expand and ensure continuous replenishment of the effector cell pool.

Taken together, our results demonstrate a skewed maturation of HIV-specific memory $CD8^+$ T lymphocytes with the accumulation of a pre-terminally differentiated subset of memory cells. The mechanisms responsible for these findings may include rapid consumption of terminally differentiated $CD8^+$ T cells as a result of increased turnover[22], high dose antigen-induced tolerance[23, 24], and lack of antigen-specific CD4 helper activity[25, 26]. Based on our results, the high levels of antigen stimulation together with the lack of adequate HIV-specific CD4 helper activity[25, 26] may have kept HIV-specific memory $CD8^+$ T lymphocytes at the stage of $CD45RA^-CCR7^-$ cells, and prevented their further differentiation to $CD45RA^+CCR7^-$cells. These results provide new insights to the delineation of the memory $CD8^+$ antiviral immune response, and for the evaluation of the immune response induced by new vaccine candidates.

Methods

Patients All patients in this study have been enrolled in two clinical therapeutic trials (CNAB2006 study and AVIB study). These trials are open-label, observational, non-randomized prospective studies carried out at a single site (Lausanne, Switzerland). Subjects included were HIV-1-infected therapy-naive adults with a CD4 T-cell count ≥250 cells per μl and plasma viraemia ≥5,000 HIV-1 RNA copies per ml. These studies were approved by the local Institutional Review Board, and all subjects gave written informed consent.

Tetrameric HLA molecules We produced tetrameric HLA class I molecules as described[7, 8]. Prokaryotic expression vectors encoding for the extracellular portion of HLA-A2, -B7 and -B8, each tagged to a carboxy terminal BirA enzyme recognition sequence and β2-microglobulin were separately transformed into *Escherichia coli*. Protein expression was induced with 1 mM isopropyl-thio-β-D-galactopyranoside and chains were extracted from inclusion bodies. Heavy and β2m chains were refolded by dilution in the presence of the appropriate peptides. For HLA-A2, HIV Gag p17 77–85 (SLYNTVATL) and Pol 476–484 (ILKEPVHGV), CMV pp65 495–503 (NLVPMVATV); HLA-B7, HIV Nef 128–137 (TPGPGVRYPL), gp120 296–305 (RPNNNTRKSI) and Gag p24 (ATPQDLNTM); and HLA-B8, HIV Nef 89–97 (FLKEKGGL), Gag p17 24–31 (GGKKKYKL) and Gag p24 259–267 (GEIYKRWII). Refolded complexes were enzymatically biotinylated with BirA (Avidity) and fast protein liquid chromatography purified by ion exchange on a MonoQ column or by size exclusion on a Sepharose 75 column (Pharmacia Biotech). Biotinylation efficiency was assessed by band shift of biotinylated monomer with streptavidin on an SDS-PAGE protein gel and routinely evaluated to 80–95%. Extravidin-phycoerythrin (PE) or Extravidin (Sigma) coupled to Cy5 (Amersham) were then mixed with biotinylated monomeric major histocompatibility complex to a 1/4 molar ratio.

FACS analysis and sorting Cells cryo-preserved in liquid nitrogen were thawed for staining and analysis. Cells were either stained with PE-labelled tetramers for 15 min at 37 °C before 30 min incubation at 4 °C with other cell-surface antibodies, or with Cy5-coupled tetramers for 1 h at 4 °C with other surface-staining antibodies. Rat anti-human CCR7 antibody (3D12, rat IgG2a) staining was followed by goat anti-rat IgG (H+L)-fluorescein isothiocyanate (FITC), phycoerythrin (PE) (Southern Biotechnologies) or allophycocyanin (APC) (Caltag Laboratories). We used the following mouse anti-human antibodies in different combinations for cell-surface staining and sorting: anti-CD8 (IgG1, Leu-2a) coupled to FITC, PE, PerCp or APC and anti-CD45RO APC (IgG2a, UCHL-1) (Becton Dickinson), anti-CD45RA CyChrome (IgG2a, HI100) (PharMingen). For intracellular perforin and Ki67 analyses, after surface-marker labelling, cells were fixed and permeabilized with ORTHO PermeaFix (Ortho Diagnostic Systems) as per the manufacturer's instructions before intracellular staining with anti-perforin FITC (δG9, IgG2b) (PharMingen) or anti-Ki67 FITC (IgG1, MIB-1) (Immunotech), respectively. We used isotype-matched controls for intracellular stainings. Data was acquired on a FACSCalibur system, and analysed using CellQuest software (Becton Dickinson systems). We performed cell sorting on a FACSVantage (Becton Dickinson systems).

***In vitro* stimulation and CFSE tracking of cell division** Peripheral blood monocular cells (PBMCs) were labelled with CFSE (Molecular Probes)[13] by incubation with 0.6 μM CFSE for 10 min at 37 °C in PBS. Labelling was quenched with FCS and cells were washed in RPMI 10% FCS. CFSE-labelled cells were seeded at 10^6 cells per well in 48-well cell culture plates: for stimulated cultures, wells had been pre-coated overnight with 1 μg ml^{-1} purified anti-CD3 (OKT3) in PBS and washed before plating of the cells. Wells for unstimulated control cultures had been pre-incubated with PBS alone. Co-stimulation was provided by soluble, purified anti-CD28 added at a final concentration of 1μg ml^{-1}. FACS analysis of the cells was performed before culture and after 36, 48 and 60 h of *in vitro* culture.

Peptide-specific stimulation PBMCs were thawed and incubated at 37 °C in RPMI 1640 Gutamax-1 medium (Gibco BRL, Life Technologies) containing 10% inactivated AB human serum (Sigma) (Rh 10). Autologous APCs were prepared from PBMCs that were washed, irradiated (3,000 rads) and plated at 2 x 10^5 cells per well in flat-bottom 96-well cell culture plates (Costar). These APCs were incubated at 37 °C in RPMI 1640 Glutamax-1 medium for 3 h in the presence of 10 μM (final concentration) of the appropriate peptide. FACS sorted $CD8^+CD45RA^-CCR7^-$ and $CD8^+CD\ 45RA^+CCR7^-$ cells were then seeded over the pulsed-irradiated autologous APCs at 1–2 x 10^5 cells per well in culture medium containing 10% human serum, and human IL-2 (Boehringer Mannheim, Germany) was added to 4 U ml^{-1} (final concentration). The T-cell populations were harvested and stained for FACS analysis at different time points after stimulation.

Cytokine detection Intracellular cytokine production was assessed as described[19]. PBMCs were stimulated with 10 μM (final concentration) of specific peptide (or PBS for unstimulated controls) for 6h at 37 °C, in the presence of 3 μg ml^{-1} purified anti-CD28 antibody (Becton Dickinson) and, as of the second hour, with 10 μg ml^{-1} Brefeldin A (Sigma). Cells to be activated were stained with tetramer-PE for 15 min at 37 °C before activation. Cell-surface staining was completed as described after the 6h *in vitro* activation. Cells were then permeabilized with Orthopermeafix and labelled with anti-human IFN-γ APC (IgG1, B27)(PharMingen). Alternatively, activation was assessed by staining with anti-CD69 APC (Becton Dickinson).

Acknowledgements. We thank A. Wilson for providing the Extravidin-Cy5 conjugate. This work was supported by an SNF grant (Tandem project), by the EuroVac project (G.P.) and by the Leenards Foundation (G.P.), and by an NIH grant (Acute Infection, G.P.; R.P.S.). P.C. is supported by a Doctoral Award of the Medical Research Council of Canada. R.P.S. is a Canadian Institutes of Health Research senior scientist.

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Figure Legends

Figure 1 Distribution of CCR7 in different subsets of HIV-specific $CD8^+Tet^+$ cells. **a**, Blood mononuclear cells stained with anti-CD8 and anti-CCR7 monoclonal antibodies and with the A2-SLYNTVATL tetramer. Most $CD8^+$ T cells as well as most HIV-specific Tet^+ cells are contained within the $CCR7^-$ cell subset. **b**, Blood mononuclear cells stained with anti-CD8, anti-CD45 and anti-CCR7 monoclonal antibodies and with the A2-SLYNTVATL tetramer. Upon analysis on gated $CD45RA^-$ and $CD45RA^+$ T-cell populations, four subsets of HIV-specific $CD8^+$ cells were identified. Most $CD8^+Tet^+$ cells are $CCR7^-$ within the $CD45RA^-$ cell subset, whereas they are $CCR7^+$ within the $CD45RA^+$ cell subset. $CD8^+Tet^+$ cells are predominantly contained within the $CD45RA^-$ cell subset. For all the flow cytometric analyses (Figs 1b, 2b, 3a–c and 4a–c) 1.5×10^6 events were accumulated.

Figure 2 Proliferative capacity and differentiation pattern of different subsets of $CD8^+$ T lymphocytes. **a**, Unfractionated blood mononuclear cells were labelled with CFSE, stimulated with anti-CD3 plus anti-CD28 monoclonal antibody. The distribution of CCR7 and CD45RA on gated $CD8^+$ T cells is shown. Cell division was determined in different subsets of $CD8^+$T lymphocytes identified by the expression of CD45RA and CCR7 at different time points. Peaks correspond to each division cycle. Peaks at baseline (blue), 36 h (green), 48 h (red) and 60 h (black) are shown. **b**, *Ex vivo* analysis of cell division in Tet^+CCR7^- and Tet^+CCR7^+ cell populations. Blood mononuclear cells were first stained for expression of surface markers and then for intracellular expression of Ki67 nuclear antigen. On analysis of gated Tet^+CCR7^- and Tet^+CCR7^+ cell populations, expression of Ki67 was mostly restricted to the Tet^+CCR7^+ cell subset. **c**, Analysis of the differentiation pattern of different $CD8^+$ cell subsets after peptide HIV-specific stimulation *in vitro*. Blood mononuclear cells were stained with anti-CD8, anti-CD45RA and anti-CCR7 monoclonal antibodies, and sorted for $CD8^+CD45RA^-CCR7^-$ cells. The sorted cell population was then stimulated with the relevant peptide, cultured in the presence of IL-2, and analysed at 36 and 60 h for the expression of CD45RA, CCR7, and for the presence

of Tet^+ cells. The purity of the sorted populations was greater than 96%. The experiments shown in **c** are representative of two separate experiments.

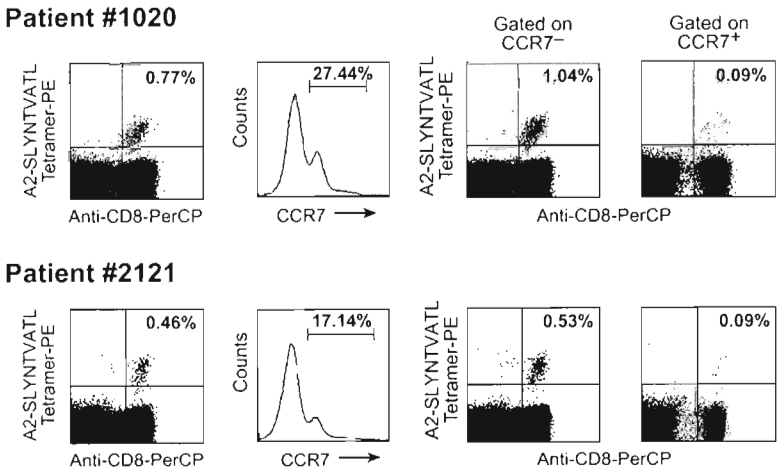
Figure 3 Distribution of CCR7 in different subsets of CMV-specific $CD8^+Tet^+$ cells. **a**, Blood mononuclear cells were stained with anti-CD8 and anti-CCR7 monoclonal antibodies and with the A2-NLVPMVATV tetramer. Most CMV-specific Tet^+ cells are contained within the $CCR7^-$ cell subset. **b**, Blood mononuclear cells were stained with anti-CD8, anti-CD45 and anti-CCR7 monoclonal antibodies and with the A2-NLVPMVATV tetramer. On analysis within $CD45RA^-$ and $CD45RA^+$ T-cell subsets, four subsets of CMV-specific $CD8^+$ cells were identified. Most $CD8^+Tet^+$ cells are $CCR7^-$ within both the $CD45RA^-$ and $CD45RA^+$ cell subsets, and are predominantly contained within the $CD45RA^+$ cell subset. **c**, Expression of Ki67 on gated CMV-specific Tet^+ cells. **d**, Analysis of phenotypic changes in the advanced differentiated $CD8^+CD45RA^+CCR7^-$ cell population. The sorted $CD8^+CD45RA^+CCR7^-$ cell population was stimulated by the CMV-specific peptide (see Methods), and the phenotypic changes were analysed at different time points. The purity of the sorted populations was greater than 98%. The experiments shown in **d** are representative of two separate experiments.

Figure 4 Functional analyses of HIV- and CMV-specific $CD8^+$ T lymphocytes. **a**, Analysis of IFN-γ secretion in HIV-specific Tet^+CCR7^- and Tet^+CCR7^- cell subsets. On stimulation with the specific peptide, blood mononuclear cells were stained with the relevant tetramer and for the surface expression of CCR7, CD69, followed by intracellular staining for the detection of IFN-γ. A large percentage of cells expressed CD69 and stained positive for IFN-γ within Tet^+CCR7^- and Tet^+CCR7^+ cell populations. **b**, Comparative analysis of IFN-γ secretion from HIV-specific and CMV-specific $CD8^+Tet^+$cells after peptide-specific stimulation. **c**, Perforin expression in different subsets of HIV-specific and CMV-specific Tet^+ cells. Most HIV-specific and CMV-specific Tet^+ cells expressing perforin were contained within the $CCR7^-$ cell subset.

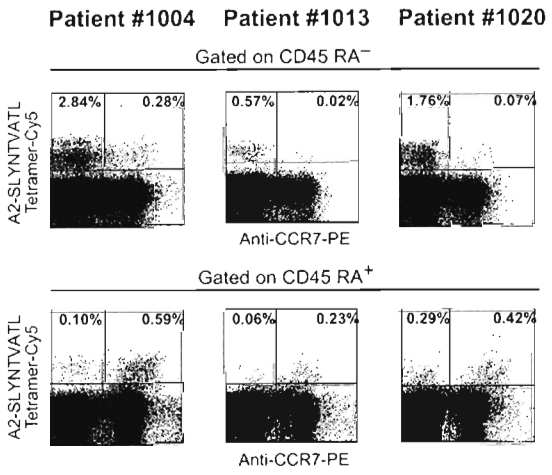
Figure 5 Lineage differentiation pattern of memory $CD8^+$ T lymphocytes. **a**, The model is based on the identification of four subsets of memory $CD8^+$ T lymphocytes

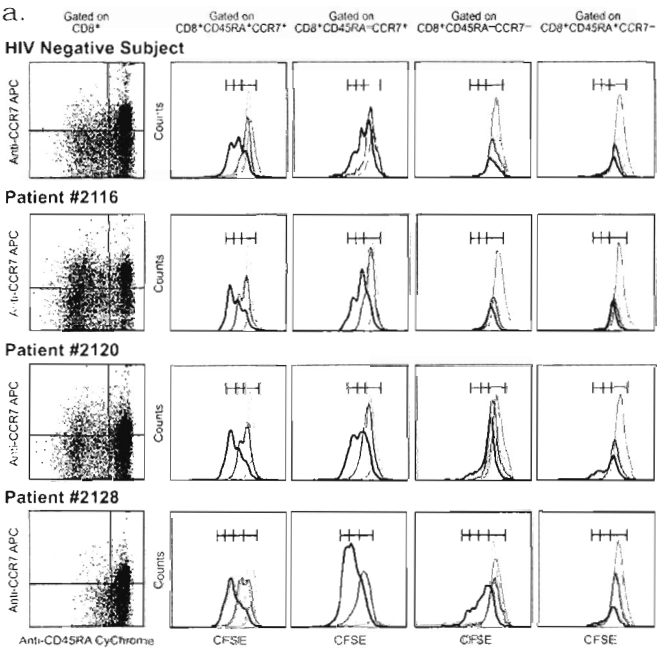
characterized by the surface expression of CD8, CD45RA, CCR7 and by their proliferative capacity, and by the differentiation patterns of the different populations of memory $CD8^+$ T lymphocytes observed after stimulation *in vitro*. $CD8^+CD45RA^+CCR7^+$ cells function as precursors for the other subsets of memory cells. The $CD8^+CD45RA^+CCR7^-$ cell population is the subset of memory $CD8^+$ T cells at the most advanced stage of differentiation. Dashed arrows indicate that $CD8^+CD45RA^-CCR7^-$ cells may revert to the phenotype of immediate precursor cells, that is, $CD45RA^-CCR7^+$, or even to $CD45RA^+CCR7^+$ cells upon antigen stimulation. These phenotypic changes are not associated with the acquisition of functions typical of cells at early stages of differentiation. Functional characterization of the above subsets in HIV-specific and CMV-specific $CD8^+$ T lymphocytes has shown that expression of perforin and IFN-γ secretion are associated with the late stages of differentiation, that is, predominantly to the $CCR7^-$ cell subsets. **b**, Differences in the composition of the HIV- and CMV-specific memory $CD8^+$ T-cell subsets. Each white cell corresponds to 10% of the corresponding $CD8^+$ T-cell subset, whereas grey cells define cell subsets below 10%.

a.

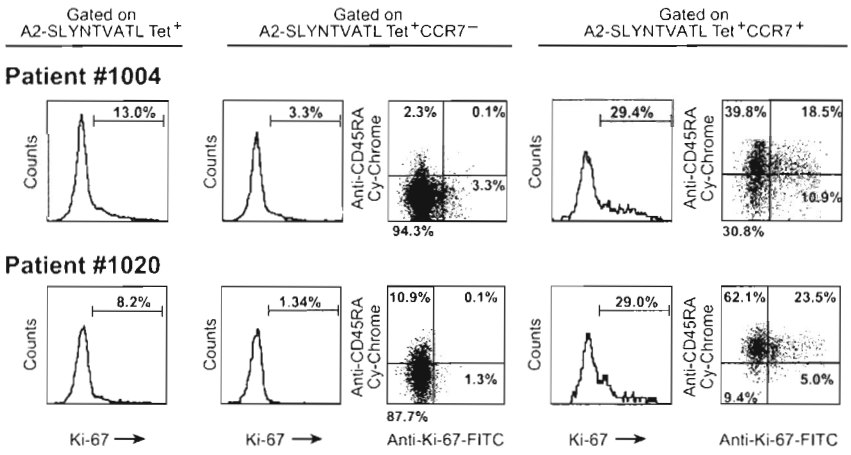

Patient #1020
Gated on CCR7−
Gated on CCR7+
A2-SLYNTVATL Tetramer-PE
0.77%
Counts
27.44%
1.04%
0.09%
Anti-CD8-PerCP
CCR7
Patient #2121
0.46%
17.14%
0.53%
0.09%

b.


Patient #1004
Patient #1013
Patient #1020
Gated on CD45 RA−
A2-SLYNTVATL Tetramer-Cy5
2.84%
0.28%
0.57%
0.02%
1.76%
0.07%
Anti-CCR7-PE
Gated on CD45 RA+
0.10%
0.59%
0.06%
0.23%
0.29%
0.42%


a.
Gated on CD8+
Gated on CD8+CD45RA+CCR7+
Gated on CD8+CD45RA-CCR7+
Gated on CD8+CD45RA-CCR7-
Gated on CD8+CD45RA+CCR7-
HIV Negative Subject
Patient #2116
Patient #2120
Patient #2128
Anti-CCR7 APC
Counts
Anti-CD45RA CyChrome
CFSE

b.

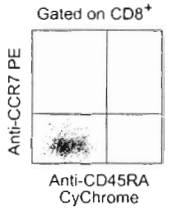

Gated on A2-SLYNTVATL Tet+
Gated on A2-SLYNTVATL Tet+CCR7−
Gated on A2-SLYNTVATL Tet+CCR7+
Patient #1004
Patient #1020
Counts
13.0%
3.3%
2.3%
0.1%
3.3%
94.3%
29.4%
39.8%
18.5%
10.9%
30.8%
8.2%
1.34%
10.9%
0.1%
1.3%
87.7%
29.0%
62.1%
23.5%
5.0%
9.4%
Anti-CD45RA Cy-Chrome
Ki-67 →
Anti-Ki-67-FITC

C.

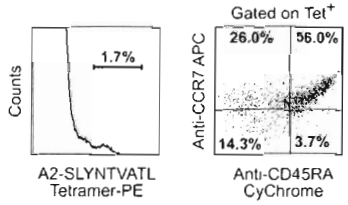
Patient 2077

Sorted $CD8^+$ $CD45RA^-$ $CCR7^-$

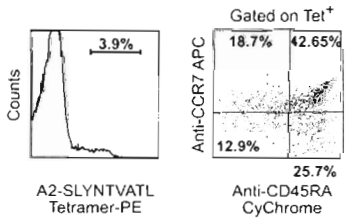
Pre-Stimulation

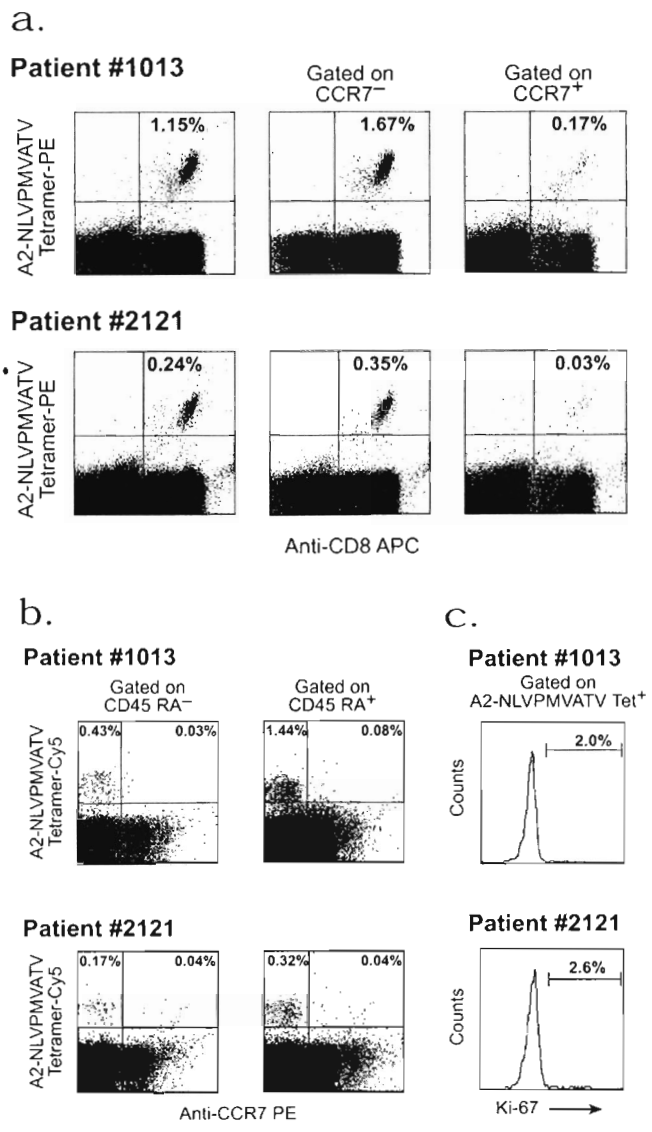


36 Hours Post-Stimulation



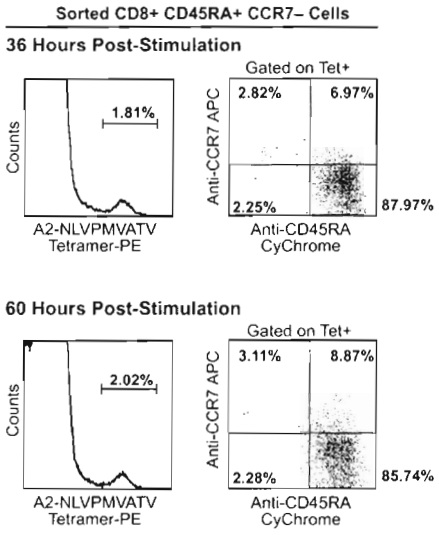
60 Hours Post-Stimulation



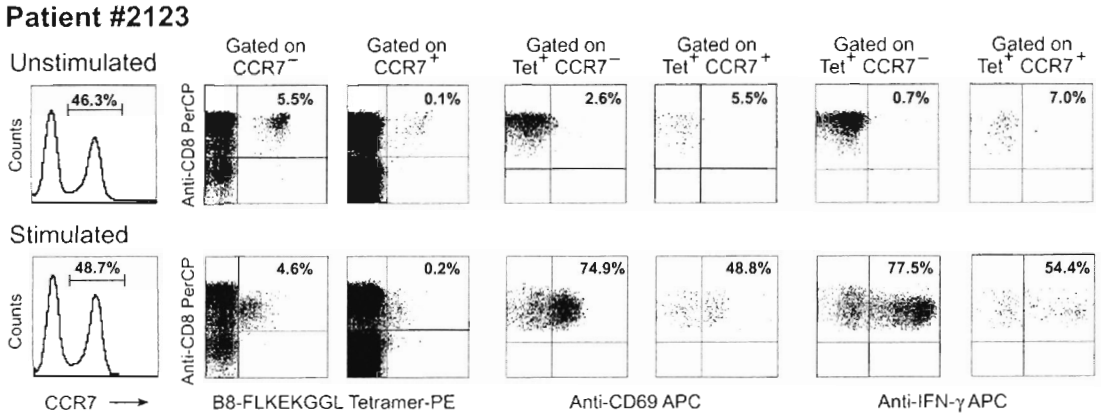

a.
Patient #1013
Gated on CCR7−
Gated on CCR7+
A2-NLVPMVATV Tetramer-PE
1.15%
1.67%
0.17%
Patient #2121
0.24%
0.35%
0.03%
Anti-CD8 APC
b.
Patient #1013
Gated on CD45 RA−
Gated on CD45 RA+
A2-NLVPMVATV Tetramer-Cy5
0.43%
0.03%
1.44%
0.08%
Patient #2121
0.17%
0.04%
0.32%
0.04%
Anti-CCR7 PE
c.
Patient #1013
Gated on A2-NLVPMVATV Tet+
Counts
2.0%
Patient #2121
2.6%
Ki-67

d.

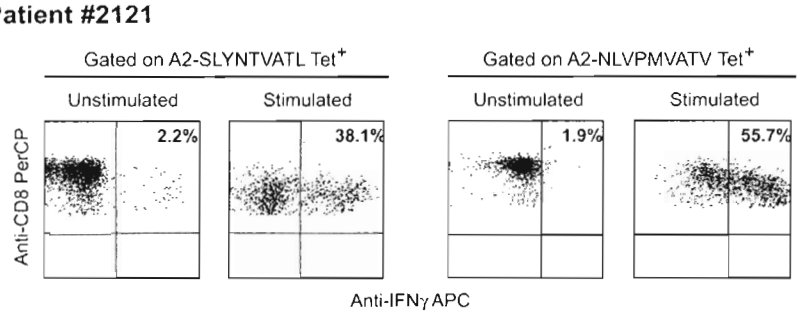
Patient 2121



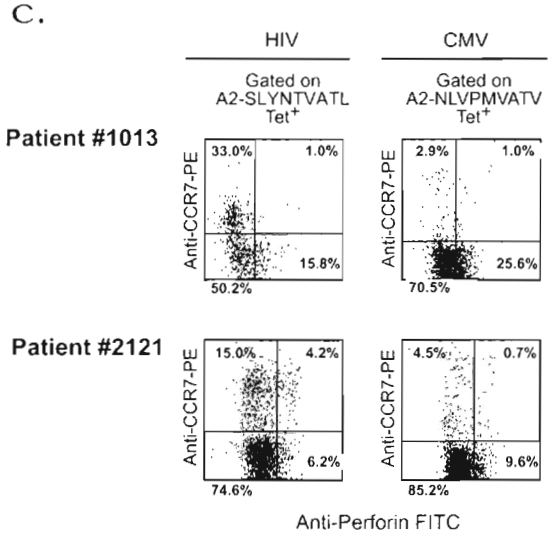
a.


Patient #2123
Unstimulated
Gated on CCR7−
Gated on CCR7+
Gated on Tet+ CCR7−
Gated on Tet+ CCR7+
Gated on Tet+ CCR7−
Gated on Tet+ CCR7+
Counts
46.3%
Anti-CD8 PerCP
5.5%
0.1%
2.6%
5.5%
0.7%
7.0%
Stimulated
48.7%
4.6%
0.2%
74.9%
48.8%
77.5%
54.4%
CCR7 →
B8-FLKEKGGL Tetramer-PE
Anti-CD69 APC
Anti-IFN-γ APC

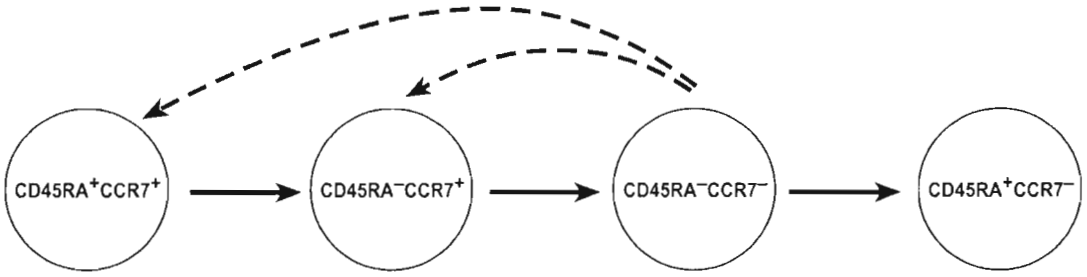
b.


Patient #2121
Gated on A2-SLYNTVATL Tet+
Gated on A2-NLVPMVATV Tet+
Unstimulated
Stimulated
Unstimulated
Stimulated
Anti-CD8 PerCP
2.2%
38.1%
1.9%
55.7%
Anti-IFNγ APC

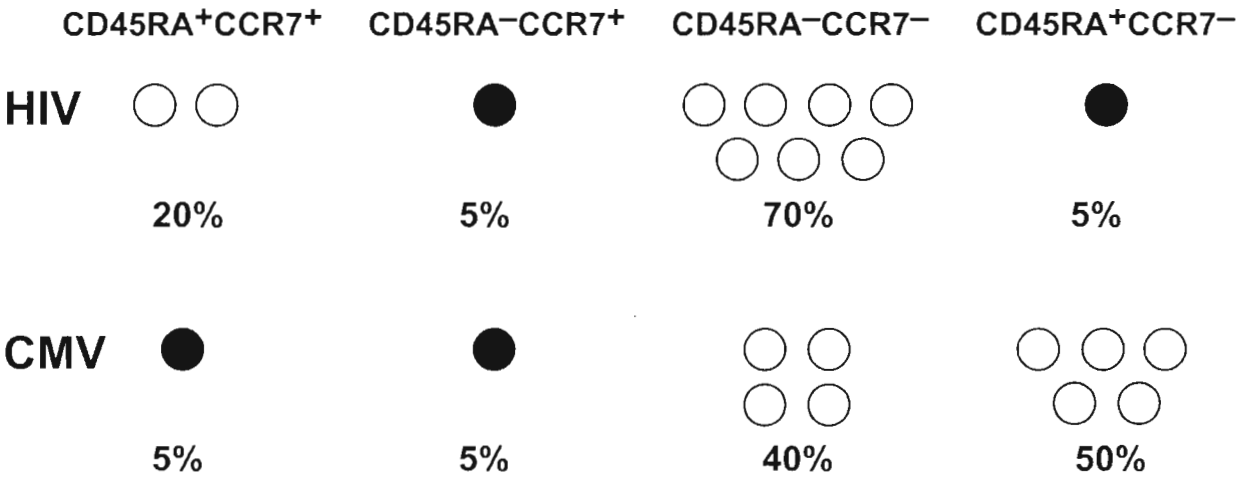
c.


HIV
CMV
Gated on A2-SLYNTVATL Tet+
Gated on A2-NLVPMVATV Tet+
Patient #1013
Anti-CCR7-PE
33.0%
1.0%
15.8%
50.2%
2.9%
1.0%
25.6%
70.5%
Patient #2121
15.0%
4.2%
6.2%
74.6%
4.5%
0.7%
9.6%
85.2%
Anti-Perforin FITC

a.


CD45RA+CCR7+
CD45RA−CCR7+
CD45RA−CCR7−
CD45RA+CCR7−

b.


CD45RA+CCR7+
CD45RA−CCR7+
CD45RA−CCR7−
CD45RA+CCR7−
HIV
20%
5%
70%
5%
CMV
5%
5%
40%
50%

4. Distribution and functional analysis of memory antiviral CD8 T cell responses in HIV-1 and cytomegalovirus infection

Following from the pattern of memory CD8 T cell lineage differentiation put forth in Chapter 3 and the observation that the evolution of HIV-specific CD8 memory T cells in the context of chronic HIV-1 infection yields a skewed distribution of these cells in the blood of patients, several questions arose. Critically, what are the mechanisms underlying the skewed maturation of HIV-specific memory CD8 T cells?

The memory CD8 T cell maturation pathway delineated in Chapter 3 rests predominantly on the characterization of the expression spectrum of CCR7 on T lymphocytes. Within context of the systemic surveillance for foreign antigen by the immune system, CCR7 functionally endows cells with the ability to traffic towards lymph nodes[366]. Critically, as outlined in section 1.1.13 and 1.3.4, lymph nodes are primary sites of immune cell recruitment and orchestration in general; but in the context of HIV-1 infection, lymph nodes are also a central foyer of viral replication and therefore, a prime site for immune response. Considering that recent studies performed in murine experimental systems and healthy humans have unveiled an association between the trafficking properties of subsets of T cells and their function in guiding the generation and maintenance of antigen-specific immune responses, an interesting hypothesis is that the skewed maturation of HIV-specific memory CD8 T lymphocytes in blood in Chapter 3 might be a result of unique a alteration of the lymph node environment of chronically infected patients[792-794]. As a basis for further studies, we proceeded to characterize the distribution of HIV- and CMV-specific memory CD8 T cells in the blood and lymph nodes of chronically co-infected patients.

Distribution and functional analysis of memory antiviral CD8 T cell responses in HIV-1 and cytomegalovirus infections

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Published in the European Journal of Immunology

2002 (32): 3756-3764

Abstract

In the present study, we have investigated the anatomic distribution and the function of different populations of HIV-1- and cytomegalovirus (CMV)-specific memory CD8 T cells. The different populations of virus-specific memory CD8 T cells were distinguished on the basis of the expression of CD45RA and CCR7, and the composition of HIV-1- and CMV-specific memory CD8 T cell pools were compared in subjects with chronic HIV-1 and CMV co-infection. The distribution of HIV-1-specific CD8 T cells was similar between blood and lymph node. However, CMV-specific CD8 T cells were accumulated predominantly in the blood away from the lymphoid tissue. The majority (>70%) of HIV-1- and CMV-specific CD8 T cells in both blood and lymph node had a phenotype, e.g. CCR7-, typical of effector T cells. HIV-1-specific memory CD8 T cells were mostly (>80%) pre-terminally differentiated cells, e.g. $CD45RA^{-}CCR7^{-}$, in both blood and lymph node while 30-50% of CMV-specific CD8 T cells were terminally differentiated, e.g. $CD45RA^{+}CCR7^{-}$. Therefore, consistently with studies in mice, antigen-specific effector memory CD8 T cells accumulate predominantly in the target organ of the pathogen in humans, and the differences in the composition of HIV-1- and CMV-specific CD8 T cell pools were also present in the lymphoid tissue. A substantial proportion (30-40%) of virus-specific $CD8^{+}CCR7^{+}$ T cells produced IFN-γ. Thus, indicating that the expression of CCR7 does not provide a clear-cut separation of memory CD8 T cells with distinct functional capacities. Taken together, these results provide further advances in the characterization of human memory CD8 T cells.

Keywords: Memory CD8 T cell • HIV-1 • Cytomegalovirus • Antiviral response

Introduction

Major advances have been recently made in the characterization of memory T cell responses in mice and humans. With regard to human memory T cells, four populations of memory T cells have been identified on the basis of the expression of CD45RA and CCR7 antigens [1]. In particular, it has been proposed that the absence and/or the presence of CCR7 expression distinguish among populations of memory T cells with different functional and homing capacities [1]. $CCR7^+$ T cells comprise the precursors of memory T cells with effector function, retain the ability to migrate from the peripheral blood to the secondary lymphoid organs, and are characterized functionally by the secretion of IL-2 and by the inability to secrete IFN-γ [1]. Memory $CCR7^+$ T cells have been termed central memory cells. $CCR7^-$ T cells contain cells with effector function, have lost the ability to home to the secondary lymphoid organs, and are characterized functionally by the secretion of IFN-γ[1]. These latter memory T cells have been termed immediate effector. Recent studies performed in mice have shed light on the distribution of virus-specific memory CD4 and CD8 T cells in different anatomic compartments [2], [3]. It has been demonstrated that memory T cells with effector function accumulate predominantly in the target organ of the pathogen [2], [3].

The composition of the pool of memory HIV-1- and cytomegalovirus (CMV)-specific CD8 T cell responses has been recently characterized in blood on the basis of CD45RA and CCR7 expression, and a model of lineage differentiation of memory CD8 T cells has been proposed [4]. It has been demonstrated that the HIV-1-specific CD8 T cells are mostly composed of pre-terminally differentiated memory T cells while CMV-specific CD8 T cells mostly consist of terminally differentiated memory T cells [4].

The distribution of the different populations of memory CD8 T cells in different anatomic compartments is not known in humans. In the present study, we have characterized the distribution and performed functional analysis of memory HIV-1- and CMV-specific CD8 T cell populations in blood and lymph nodes from the same individuals. HIV-1 and CMV infections represent ideal in vivo models to investigate the issues mentioned above.

HIV-1 and CMV have different target organs [5], some subjects are co-infected with HIV-1 and CMV, thus, providing the opportunity to compare the memory responses in the same subjects. Optimal memory CD8 T cells responses have been shown to be crucial for the control of both virus infections [6]-[10]. The results reported in the present study provide substantial contribution to the characterization of memory T cell responses in humans and new insights in the immune mechanisms of antiviral control.

Materials and methods

Patients. All patients included in this study have been enrolled in two clinical therapeutic trials (CNAB2006 study and AVIB study) [11], [12]. For the majority of subjects, matched blood and lymph node mononuclear cells were available for analysis prior to initiation of highly active antiretroviral therapy (HAART). These trials were open-label, observational, non-randomized prospective studies carried out at a single site (Lausanne, Switzerland). Subjects included were HIV-1-infected therapy-naive adults with CD4 T cell count 250 cells/μl and plasma viremia 5,000 HIV-1 RNA copies/ml. These studies were approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois, and all subjects gave written informed consent.

Tetrameric HLA molecules. Class I-peptide tetramers were produced as previously described [4], [28]. HLA-A2, -B7, -B8 and β2-microglobulin (β2m) were cloned in prokaryotic expression vectors and expressed separately in Escherichia coli stains. The selected heavy chain, β2m and the appropriate peptides were refolded by dilution to yield soluble monomeric class IHLA complexes. The peptide used for HLA-A2 were HIV-1 Gag p17 77-85 (SLYNTVATL), Pol 476-484 (ILKEPVHGV) and CMV pp65 495-503 (NLVPMVATV). For HLA-B7: HIV-1 Nef 128-137 (TPGPGVRYPL) and CMV pp65 417-426 (TPRVTGGGAM). For HLA-B8: HIV-1 Nef 89-97 (FLKEKGGL). Class I HLA-peptide monomers were biotinylated with BirA enzyme (Avidity, Denver, CO.) and purified by gel filtration on an FPLC (AmershamPharmacia biotech). Biotinylated monomers were then mixed with Extravidin-PE (Sigma-Aldrich) at a 4:1 molar ratio to form the tetramers.

Cell surface and intracellular staining. Excisional lymph node (Inguinal) biopsies and isolation of mononuclear cells from blood and lymph nodes were performed as previously described [29]. Viable cryo-preserved cells in liquid nitrogen were thawed for staining and analysis. Cells were stained with PE-labeled tetramers for 15 min at 37°C followed by addition of the cell surface antibodies and incubation for 30 min at 4°C. Rat anti-human CCR7 antibody (3D12, rat IgG2a) staining was followed by goat anti-rat IgG

(H+L)-FITC. The following mouse anti-human antibodies were used in different combinations for cell surface staining: anti-CD8 (IgG1, Leu-2a) coupled to PerCP, or APC and anti-CD45RA CyChromeTM (IgG2a, HI100). Data were acquired on a FACSCaliburTM system and analyzed using CellQuestTM software (Becton Dickinson systems).

Intracellular cytokine production was performed as previously described [4], [13]. IFN-γ production was assessed by stimulating blood and lymph node mononuclear cells with 10 μM (final concentration) of the relevant peptide during 6 h at 37°C in the presence of 3 μg/ml purified anti-CD28 antibody (Becton Dickinson) and, as of the second hour, with 10 μg/ml Brefeldin A (Sigma-Aldrich). Cells were stained with tetramer-PE for 15 min at 37°C prior to activation. Cell surface staining was completed, as described above, following the 6 h in vitro activation. Cells were then permeabilized with DAKO intrastain solution (DAKO, Denmark) and labeled with anti-human IFN-γ APC (IgG1, B27) (PharMingen). Staining with anti-CD69 APC was performed to assess cell activation. Data were acquired and analyzed as described above.

Results

Distribution of $CCR7^+$ and $CCR7^-$ CD8 T cells in blood and lymph nodes of HIV-1-negative and HIV-1-infected subjects

In preliminary analyses, we have compared the distribution of $CCR7^+$ and $CCR7^-$ CD8 T cells in blood and lymph nodes of eight HIV-1-negative and HIV-1-infected subjects. Inguinal lymph nodes were obtained from the two groups of subjects investigated. Lymph nodes from healthy HIV-1-negative subjects undergoing vascular surgery were obtained following written consent. Therefore, lymph nodes of HIV-1-negative subjects were neither neoplastic nor inflammatory lymph nodes. Flow cytometry analyses of representative HIV-1-negative and HIV-1-infected subjects and cumulative data are shown in Fig. 1. In HIV-1-negative subjects, about 50% (mean 50±19%; n=8) of blood CD8 T cells and the large majority (mean 78±4%; n=8) of lymph node CD8 T cells were $CCR7^+$. On the basis of the data above, the $CCR7^+CD8^+/CCR7^-CD8^+$ ratio was 1.0 in the blood and 3.5 in the lymph nodes. In contrast, the large majority of blood (85±8%) and lymph nodes (72±14%) CD8 T cells were $CCR7^-$ in HIV-1-infected subjects (Fig. 1). The $CCR7^+CD8^+/ CCR7^-CD8^+$ ratio was 0.18 in blood and 0.39 in lymph nodes. The differences observed in the distribution of $CCR7^+$ and $CCR7^-$ CD8 T cells in blood and lymph nodes between HIV-1-negative and HIV-1-infected subjects were statistically significant ($p<0.001$ in both blood and lymph nodes). These results provide experimental evidence for a large accumulation of memory CD8 T cells with potential effector function in lymph nodes of subjects with chronic HIV-1 infection.

Distribution of HIV-1-specific and CMV-specific CD8 T cells in blood and lymph nodes of HIV-1-infected subjects

The distribution of HIV-1-specific CD8 T cells was analyzed in 15 HIV-1-infected subjects at early stage of disease (the mean CD4 T cell count was 550 cells/μl), who were enrolled in therapeutic clinical trials [11], [12]. Lymph node biopsies were executed prior to the initiation of antiretroviral therapy and the distribution of HIV-1-specific CD8 T

cells was assessed in blood and lymph node samples obtained at the same time point. Eight out of 15 HIV-1-infected subjects were co-infected with CMV, thus, providing the opportunity to compare the distribution of virus-specific CD8 T cells in the same subjects. Blood and lymph node mononuclear cells collected at the same time points were stained simultaneously with anti-CD8 and anti-CCR7 mAb and with the relevant peptide-MHC tetramer (Tet) complex. Flow cytometry analysis representative of the distribution of HIV-1- and CMV-specific CD8 T cells in blood and lymph node are shown in Fig. 2A and C. CD8 T cells of Patient 1001 recognized the FLKEKGGL epitope restricted by HLA-B8 within the nef protein of HIV-1. The percentage of HIV-1-specific $CD8^+Tet^+$ cells was similar in the blood (2.23%) and in the lymph node (2.05%) (Fig. 2A). According to previous studies [4], the majority (about 80%) of HIV-1-specific CD8 T cells were $CCR7^-$ in both blood and lymph nodes (Fig. 2A). As for the distribution of the total HIV-1-specific CD8 T cells, the percentage of CCR7- and $CCR7^+CD8^+Tet^+$ cells was similar in the blood and in the lymph nodes (Fig. 2A). The results above were confirmed by the analysis of the cumulative data of 15 HIV-1-infected subjects (Fig. 2B). The mean percentage of HIV-1-specific $CD8^+Tet^+$ cells was 0.75±0.61% in the blood and 0.54±0.53% in the lymph nodes. The mean percentage of $CCR7^-CD8^+Tet^+$ cells was 1.62±1.19% in the blood and 1.90±1.74% in the lymph nodes. The mean percentage of HIV-1-specific $CCR7^+CD8^+Tet^+$ cells was 0.63±0.51% and 1.0±1.01% $CCR7^+$ in the blood and in the lymph nodes, respectively (Fig. 2B). The slight differences observed in distribution of HIV-1-specific CD8 T cells between blood and lymph nodes were not statistically significant (p=0.15).

We performed similar analysis to assess the distribution of CMV-specific CD8 T cells in the blood and in the lymph nodes of HIV-1 and CMV co-infected subjects. CD8 T cells of subject 2083 recognized the NLVPMVATV epitope restricted by HLA-A2 within the pp65 protein of CMV. The analysis of the distribution of CMV-specific $CD8^+$ T cells showed that the percentage of $CD8^+Tet^+$ cells was 2.02 in the blood compared to 0.20 in the lymph nodes (Fig. 2C). Similar differences in the distribution of CMV-specific CD8 T cells between blood and lymph nodes were observed in the $CCR7^-$ and $CCR7^+$ CMV-specific populations and confirmed by the analysis of the cumulative data of the eight

subjects investigated (Fig. 2D). The mean percentage of CMV-specific $CD8^+Tet^+$ cells was 1.18±0.81; the CMV-specific Tet^+ cells were enriched within the $CCR7^-$ (3.09±2.21%), while $CCR7^+CD8^+Tet^+$ were 0.79±1.10%. The mean percentage of $CD8^+Tet^+$, $CCR7^-CD8^+Tet^+$ and $CCR7^+CD8^+Tet^+$ cell populations in the lymph nodes was 0.07±0.05, 0.25±0.30 and 0.22±0.21, respectively. The differences observed in the distribution of CMV-specific CD8 T cells in the blood and in the lymph nodes were highly statistically significant (p=0.002). These results demonstrate that there are major differences in the distribution of HIV-1-specific and CMV-specific CD8 T cells in different anatomic compartments.

Memory HIV-1- and CMV-specific CD8 T cell populations in lymph nodes

Four populations of memory HIV-1- and CMV-specific CD8 T cells have been identified based on the expression of CD45RA and CCR7 antigens [4]. The lineage differentiation pattern demonstrated indicated that the $CD45RA^+CCR7^+$ virus-specific CD8 T cells serve as precursors for the other populations of memory T cells, e.g. $CD45RA^-CCR7^+$, $CD45RA^-CCR7^-$ and $CD45RA^+CCR7^-$ [4]. The $CD45RA^+CCR7^-CD8^+$ T cells corresponded to terminally differentiated memory cells. Interestingly, as previously shown [4], a large proportion (50%) of blood CMV-specific memory CD8 T cells were mostly composed of terminally differentiated cells, e.g. $CD45RA^+CCR7^-$ [4]. Pre-terminally differentiated $CD45RA^-CCR7^-$ cells accounted for 40% and the $CD45RA^+$/$CD45RA^-CCR7^+$ cells for less than 10% of CMV-specific memory CD8 T cells. Pre-terminally differentiated $CD45RA^-CCR7^-$ cells represented the large majority (70%) of HIV-1-specific memory CD8 T cells. Terminally differentiated $CD45RA^+CCR7^-$ accounted for 5% and the other $CCR7^+$ cell populations for about 25% of HIV-specific memory CD8 T cells [4]. In the present study, we analyzed the distribution of the different populations of virus-specific memory CD8 T cells in lymph nodes of six subjects with HIV-1/CMV co-infection. Lymph nodes were obtained prior to the initiation of anti-retroviral therapy. As shown for the blood [4], the pre-terminally differentiated $CD45RA^-CCR7^-CD8^+$ T cell population was the dominant cell population

(76.0±11.5%) within lymph node HIV-1-specific CD8 T cells (Fig. 3). Only a minority (7.7±7.7%) of HIV-1-specific CD8 T cells were terminally differentiated, e.g. $CD45RA^{+}CCR7^{-}$, and memory $CCR7^{+}$ CD8 T cells were 15.3±9.8% (Fig. 3). With regard to the CMV-specific memory CD8 T cell populations, the percentage of pre-terminally differentiated $CD45RA^{-}CCR7^{-}$ cells (44.8±18.8%) in the lymph nodes was similar to that observed in blood. The percentage of terminally differentiated $CD45RA^{+}CCR7^{-}$ T cells was slightly decreased (27.3±15.0%), while that of $CCR7^{+}$ CD8 T cells substantially increased ($CD45RA^{+}CCR7^{+}$ cells 14.4±9.2% and $CD45RA^{-}CCR7^{+}$ cells 13.6±17.0%). These results indicate that the composition of the pool of HIV-1- and CMV-specific memory CD8 T cells was similar in the blood and in the lymph nodes.

Functional analysis of virus-specific memory CD8 T cell populations in blood and lymph nodes

Previous studies have shown that the secretion of IFN-γ is exclusive of the memory CD8 T cells lacking CCR7 [1]. To further address the functional differences between $CCR7^{-}$ and $CCR7^{+}$ memory virus-specific CD8 T cells, blood and lymph node mononuclear cells of seven HIV-1-infected individuals were stimulated with the relevant peptide and assessed for the ability to secrete IFN-γ. Flow cytometry profiles of a representative (out of seven) subjects are shown in Fig. 4A. These results clearly indicated that a substantial proportion of HIV-1-specific $CCR7^{+}$ CD8 T cells have the ability to secrete IFN-γ(mean percentage in blood: 41±23.2 in the $CCR7^{+}$ vs. 54.5±30.7 in the $CCR7^{-}$ cell populations; in lymph nodes: 20.6±15.4 in the $CCR7^{+}$ vs. 27.2±17 in the $CCR7^{-}$ cell populations). In order to exclude the possibility that this was typical of $CCR7^{+}$ CD8 T cells during an ongoing chronic immune response as it the case in HIV-1 infection, similar functional analysis was performed for CMV-specific $CCR7^{+}$ CD8 T cells. With regard to CMV, the analysis was limited to the blood since the low percentage of virus-specific cells in lymph node prevented to analyze a number of events statistically significant. As it is shown in Fig. 4B, a substantial proportion of CMV-specific $CCR7^{+}$ CD8 T cells was able to secrete

IFN-γ (mean percentage in blood: 44.4±14 in the $CCR7^+$ vs. 63.5±15 in the $CCR7^-$ cell populations; n=7).

Discussion

The development of immunological memory is crucial for the generation of effective immune responses against viruses. Both memory CD8 and CD4 T cell responses have been shown to play an important role in the mechanisms of control of a series of virus infections including Herpes Simplex virus, Epstein Barr virus, CMV and also HIV-1 [6]-[10], [13]-[19]. Therefore, the characterization of memory T cell responses may help to delineate the protective components of the immune response, to design immune-based interventions that may render more effective the antiviral immune responses and, more importantly, to understand the type of immune response that vaccine candidates should stimulate to achieve protection against the pathogen encountered.

The last 2-3 years have been marked by a number of fundamental contributions in the characterization of memory T cell response. The mouse model has been extremely instrumental to demonstrate that a) the persistence of memory T cells is not dependent upon the presence of the antigen [20], [21]; and b) memory T cells with effector function accumulate in the target organ of the pathogen away from the lymphoid tissue [2], [3]. The availability of a series of cell surface markers has allowed to identify different populations of memory T cells with different homing and functional capacities in humans, and to propose coherent models of differentiation of memory T cells [1], [4], [22].

The information regarding the anatomic distribution of the different populations of memory T cells are very limited. Most of the studies on the characterization of HIV-1- and CMV-specific immune responses have been performed in blood mononuclear cells. However, recent studies performed in SIV-infected monkeys [23] and in chronically infected HIV-1-infected subjects [24] have shown that the SIV/HIV-1-specific CD8 T cell immune response present in the blood is very similar to that observed in the lymph nodes from both quantitative and qualitative standpoints.

In the present study, we have addressed a series of relevant issues including a) the anatomic distribution of the different populations of virus-specific (HIV-1 and CMV) memory CD8 T cells, b)the qualitative composition of the pool of HIV-1- and CMV-specific memory CD8 T cells in the blood and in the lymph nodes, and c) the ability of the different populations of memory CD8 T cells to secrete IFN-γ, a cytokine that should be secreted only by cells with a certain phenotype that defines effector function.

The finding that most (80%) of lymph node CD8 T cells from HIV-negative subjects were $CCR7^+$, is consistent with the hypothesis that memory $CD8^+CCR7^+$ T cells, e.g. central memory cells, home preferentially to the lymphoid tissue and serve as precursors for memory effector cells [1], [4]. In fact, immune responses are generated within the lymphoid tissue and the effector cells once generated may leave eventually the tissue and enter the blood. Therefore, a higher proportion of cells with effector function, e.g. $CCR7^-$, should be contained in the blood compared to the lymph nodes. Indeed, this is what we have observed in HIV-negative subjects. However, the proportion of $CD8^+CCR7^-$ cells was significantly increased in both blood and lymph nodes of HIV-1-infected subjects (about 70% $CD8^+CCR7^-$ compared to about 30% in HIV-negative subjects). The increase in the lymph node $CD8^+CCR7^-$ T cells in HIV-1-infected subjects is not only due to HIV-1-specific CD8 T cells, but is also the result of the bystander, e.g. cytokine driven, T cell activation associated with virus infection [25]. The chronic accumulation of memory $CD8^+CCR7^-$ T cells, which are able to secrete inflammatory cytokines such as IFN-γ and TNF-α, may contribute substantially to support the chronic inflammatory reaction that is one of the major responsible for the histopathological abnormalities observed in the lymph nodes of HIV-1-infected subjects [[26].

As mentioned above, the proportion of SIV/HIV-1-specific CD8 T cells is similar between blood and lymph nodes [23], [24]. We have analyzed the anatomic distribution, e.g. blood and lymph nodes, of CD8 T cells specific to a series of epitopes within the gag, pol, p24 and nef HIV-1 proteins by the tetramer technology, in a group of subjects with chronic HIV-1-infection. According to previous studies [23], [24], the percentage of HIV-1-specific CD8 T cells was not significantly different between blood and lymph nodes. In

addition, the proportion of HIV-1-specific $CCR7^+$ and $CCR7^-$ CD8 T cells was similar within the two anatomic compartments. Interestingly, an unequal distribution of CMV-specific CD8 T cells was observed between blood and lymph nodes. Memory CMV-specific $CCR7^+$ and $CCR7^-$ CD8 T cells were accumulated mostly (tenfold higher frequency) in the blood as compared to the lymph nodes. The explanation for the different anatomic distribution between HIV-1- and CMV-specific CD8 T cells results from the fact that HIV-1 and CMV have different target organs: the lymphoid tissue for HIV-1 and the lung, the cervix and the retina for CMV. Since the subjects included in the present study were at early stage of HIV infection with no signs of active CMV disease, there were no clinical indications for executing bronchial alveolar lavage to assess the localization of CMV-specific CD8 T cells in the lung. However, the finding that they accumulate in the blood away from the lymphoid tissue is consistent with the previous observations in mice [2], [3]. Therefore, we provide evidence that the anatomic distribution of memory antigen-specific CD8 T cells is dependent upon the target organ of the pathogen also in humans.

Major differences have been recently demonstrated in the composition of the pool of memory HIV-1- and CMV-specific CD8 T cells in the blood [4], [22]. HIV-1-specific CD8 T cells are mostly composed of pre-terminally differentiated, e.g. $CD45RA^-CCR7^-$ cells while a substantial proportion of CMV-specific CD8 T cells are terminally differentiated, e.g. $CD45RA^+CCR7^-$, memory cells. These differences may translate in a different ability to control virus replication since although both populations of $CCR7^-$ effector CD8 T cells secrete IFN-γ, the terminally differentiated $CD45RA^+CCR7^-$ CD8 T cells are the effector cells that express high levels of perforin [1]. Therefore, it was important to determine whether these differences in the composition of the pool of circulation HIV-1- and CMV-specific CD8 T cells were present in the lymphoid tissue. The results reported in the present study demonstrate that the quantitative differences in the representation of the different population of blood HIV-1- and CMV-specific memory CD8 T cells where also observed in the lymph node. Therefore, these results further support the hypothesis for a skewed maturation of HIV-1-specific CD8 T cells and

demonstrate that the alterations observed do not result from a redistribution of $CD45RA^-$ $CCR7^-$ cells from the lymphoid tissue to the blood [27].

The expression of CCR7 has been proposed to distinguish between memory CD8 T cells with effector function and different homing capacities [1]. In particular, the lack of CCR7 expression defines a population of memory CD8 T cells, e.g. $CCR7^-$, with effector function as measured by the ability to secrete IFN-γ and to mediate cytotoxic activity. The findings that the majority of virus-specific memory CD8 T cells are $CCR7^-$ and that a substantial proportion of these cells secrete IFN-γ and express perforin supports the model viewing $CCR7^-$ CD8 T cells as the population composed of cells with effector function [1]. However, in the present study, we provide clear evidence that a substantial proportion (40-50%) of blood and lymph nodes HIV-1- and CMV-specific $CCR7^+$ CD8 T cells did secrete IFN-γ. Therefore, the expression of CCR7 does not allow a clear-cut distinction among memory CD8 T cells that are or are not able to secrete IFN-γ in the context of chronic viral infections. These findings are in agreement with recent studies that have investigated the relationships between certain functions and phenotypes of memory CD8 T cells [4], [22].

The observations reported in the present study provide new insights in the distribution and the functional characterization of virus-specific memory CD8 T cells in different anatomic compartment in humans.

Acknowledgements

This work was supported by research grants of the Swiss national Fund (3139-58913.99) and the European Commission (QLK2-CT-1999-01321).

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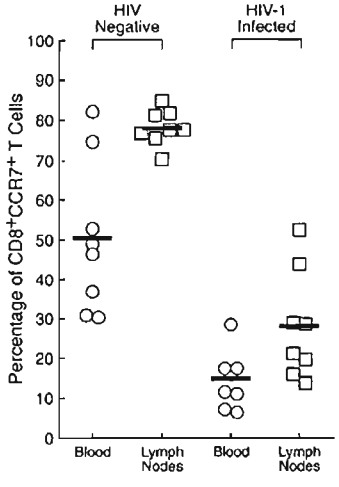
Figure 1. Comparative analysis of the expression of CCR7 in CD8 T cells isolated from blood and lymph nodes of HIV-negative and HIV-1-infected subjects. Cumulative data on the analysis of eight HIV-negative and HIV-1-infected subjects are shown. Blood and lymph node mononuclear cells were obtained prior to the initiation of antiviral therapy in HIV-1-infected subjects and from HIV-negative subjects undergoing vascular surgery. Isolation of mononuclear cells from blood and lymph node were performed as described in Material and Methods.

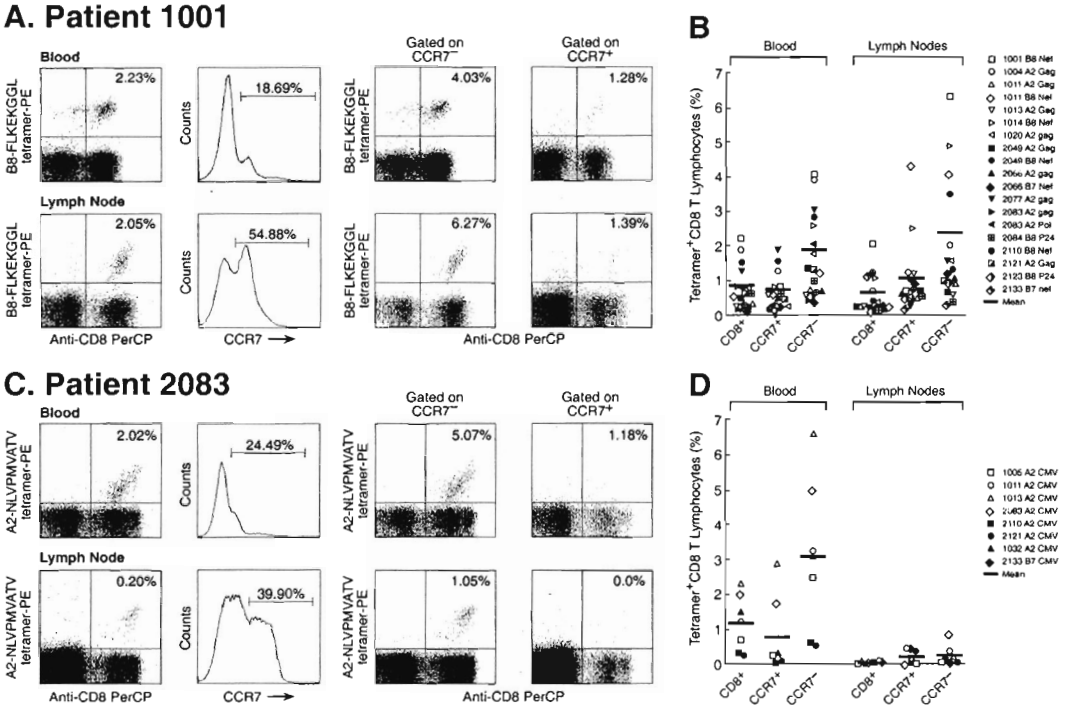
Figure 2. Analysis of the distribution of HIV-1- and CMV-specific CD8 T cells in the blood and in the lymph node. A. Blood and lymph node mononuclear cells of Patient 1001 were stained with anti-CD8 and anti-CCR7 antibodies, and with a tetramer folded with the nef FLKEKGGL epitope restricted by HLA-B8. The distribution of HIV-1-specific $CCR7^+$ and $CCR7^-$ CD8 T cells in blood and lymph node are also shown. B. Cumulative data on the distribution of HIV-1-specific $CD8^+$, $CD8^+CCR7^+$ and $CD8^+CCR7^-$ T cells in blood and lymph node of 15 HIV-1-infected subjects. C. Blood and lymph node mononuclear cells of Patient 2083 were stained with anti-CD8 and anti-CCR7 antibodies, and with a tetramer folded with the pp65 NLVPMVATV epitope restricted by HLA-A2. The distribution of CMV-specific $CCR7^+$ and $CCR7^-$ CD8 T cells in both anatomic compartments are also shown. D. Cumulative data on the distribution of CMV-specific $CD8^+$, $CD8^+CCR7^+$ and $CD8^+CCR7^-$ T cells in blood and lymph node of eight subjects with HIV-1/CMV co-infection. In the flow analyses shown, at least 10^6 events were acquired and gated on lymphocytes.

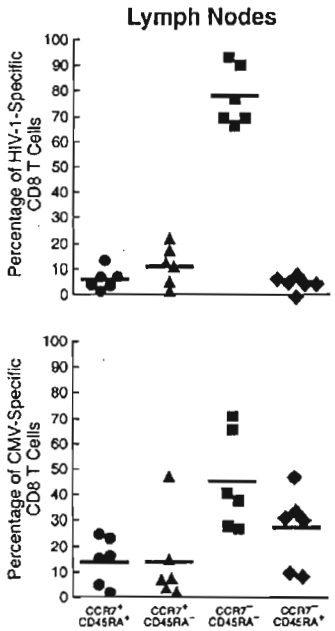
Figure 3. Analysis of different populations of memory HIV-1- and CMV-specific CD8 T cells. Mononuclear cells isolated from lymph nodes of six subjects with HIV-1/CMV co-infection were stained with the relevant tetramer complex and with anti-CD8, anti-CCR7 and anti CD45RA antibodies. Cumulative data on the relative percentage of $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, $CD45RA^-CCR7^-$, and $CD45RA^+CCR7^-$ HIV-1- and CMV-specific CD8 T cell populations are shown. The horizontal continued line indicates the mean percentage of the different cell populations. Isolation of mononuclear cells from

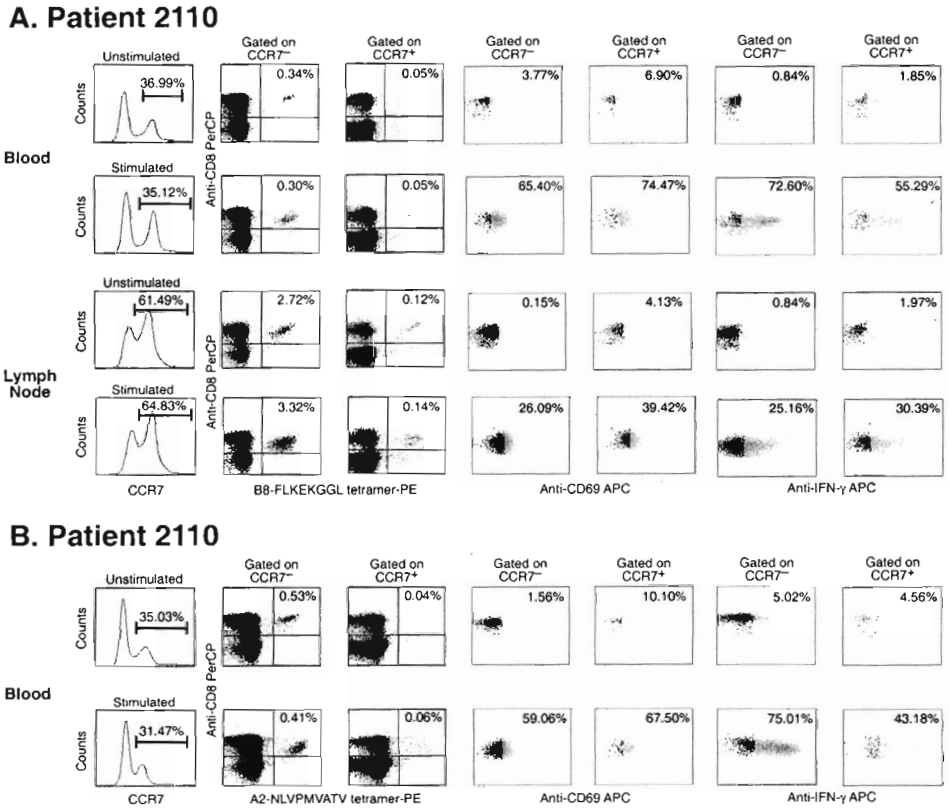
lymph node and flow cytometry analysis were performed as described in the Methods. In the flow analyses shown, at least 10^6 events were acquired and gated on lymphocytes.

Figure 4. Functional analysis of HIV-1- and CMV-specific CD8 T cells within different populations of memory cells defined by the expression of CCR7. One representative subject out of seven (patient 2110) are shown. Blood and lymph node mononuclear cells were stimulated with the relevant HIV-1- and CMV-specific peptides and analyzed for the expression of CD8, CCR7, CD69, IFN-γ (intracellular expression) and for the binding with the relevant peptide-tetramer complex. The data show the expression of CD69 and IFN-γ within $CD8^+CCR7^-$ and $CD8^+CCR7^+$ T cell populations. Negative control: unstimulated blood mononuclear cells. The cluster of events shown in red corresponds to the responder CD8 T cells, i.e. expressing CD69 and IFN-γ, while the cluster of events in black corresponds to the non-responder CD8 T cells. The data are expressed as the percentage of cells expressing CD69 and IFN-γ within $CD8^+CCR7^-$ and $CD8^+CCR7^+$ T cell populations. In vitro antigen-specific stimulation and flow cytometry analysis of blood and lymph node mononuclear cells were performed as described in Material and Methods. In the flow analyses shown, at least 10^6 events were acquired and gated on lymphocytes.


HIV Negative
HIV-1 Infected
Percentage of CD8+CCR7+ T Cells
100
90
80
70
60
50
40
30
20
10
0
Blood
Lymph Nodes
Blood
Lymph Nodes


A. Patient 1001
Blood
Lymph Node
B8-FLKEKGGL tetramer-PE
2.23%
18.69%
4.03%
1.28%
2.05%
54.88%
6.27%
1.39%
Counts
Gated on CCR7⁻
Gated on CCR7⁺
Anti-CD8 PerCP
CCR7
B
Blood
Lymph Nodes
Tetramer⁺CD8 T Lymphocytes (%)
CD8⁺
CCR7⁺
CCR7⁻
Mean
C. Patient 2083
A2-NLVPMVATV tetramer-PE
2.02%
24.49%
5.07%
1.18%
0.20%
39.90%
1.05%
0.0%
D


Lymph Nodes
Percentage of HIV-1-Specific CD8 T Cells
Percentage of CMV-Specific CD8 T Cells
100
90
80
70
60
50
40
30
20
10
0
CCR7+ CD45RA+
CCR7+ CD45RA−
CCR7− CD45RA−
CCR7− CD45RA+


A. Patient 2110
Gated on CCR7−
Gated on CCR7+
Gated on CCR7−
Gated on CCR7+
Gated on CCR7−
Gated on CCR7+
Unstimulated
Stimulated
Blood
Lymph Node
Counts
Anti-CD8 PerCP
36.99%
0.34%
0.05%
3.77%
6.90%
0.84%
1.85%
35.12%
0.30%
0.05%
65.40%
74.47%
72.60%
55.29%
61.49%
2.72%
0.12%
0.15%
4.13%
0.84%
1.97%
64.83%
3.32%
0.14%
26.09%
39.42%
25.16%
30.39%
CCR7
B8-FLKEKGGL tetramer-PE
Anti-CD69 APC
Anti-IFN-γ APC
B. Patient 2110
Gated on CCR7−
Gated on CCR7+
Gated on CCR7−
Gated on CCR7+
Gated on CCR7−
Gated on CCR7+
Unstimulated
Stimulated
Blood
Counts
Anti-CD8 PerCP
35.03%
0.53%
0.04%
1.56%
10.10%
5.02%
4.56%
31.47%
0.41%
0.06%
59.06%
67.50%
75.01%
43.18%
CCR7
A2-NLVPMVATV tetramer-PE
Anti-CD69 APC
Anti-IFN-γ APC

5. Effect of anti-retroviral therapy on the distribution of HIV-specific CD8 T lymphocytes

Altogether, with respect to the distribution of CD8 T cells directed at the immunodominant HIV-1-derived epitopes, the data presented in Chapters 3 and 4 suggest that HIV-specific CD8 T cell responses are broadly disseminated throughout blood and lymph nodes during the chronic phase of infection. This evidence is in agreement with the independent observation that individual HIV-specific CD8 T cell clones may preferentially accumulate away from the predominant sites of viral replication[723], while the majority of HIV-specific CD8 T cell responses are often enriched within the lymph nodes of chronically HIV-1 infected patients[723,724]. Although HIV-specific, Tet^+ cells present amongst blood and lymph node CD8 T lymphocytes, the studies presented in Chapter 4 clearly show that these populations within either anatomic compartment consist of heterogeneous pools of cells, with both $CCR7^+$ and $CCR7^-$ CD8 T cells present.

As these CD8 T cell subsets may present with different properties beyond homing and function[792,795], it would be of interest to establish whether their expansion and distribution across memory subsets equally rely on the persistence of antigen. In order to begin investigation of this question, we assessed the effect of anti-retroviral therapy on the antigen-specific subset of memory CD8 T lymphocytes in both blood and lymph node compartments. In design, however, the study herein presented only allowed for a sufficient number of cells to be characterized by FACS within $CCR7^{+/-}$ CD8 T cell subsets without finer analysis. Hence, although the observations presented herein are of interest, they remain preliminary to ongoing studies and are further discussed only in the larger context of this thesis.

Materials and methods

Subjects. All patients included in this study were enrolled in two clinical therapeutic trials (CNAB2006 study and AVIB study). These trials are open-label, observational, non-randomized prospective studies carried out at a single site (Lausanne, Switzerland). Subjects included were HIV-1-infected therapy-naive adults with CD4 T cell count ≥250 cells/μL and plasma viremia ≥5000 HIV-1 RNA copies/ml. The samples characterized in this study were obtained prior to anti-retroviral therapy, and 63 or 72 weeks (AVIB or CNAB trials, respectively) following onset of therapy, at a time when viremia was below 50 HIV-1 RNA copies/ml. Blood and excisional inguinal lymph node biopsies were obtained at both time points. These trials were approved by the local Institutional Review Board, and all subjects gave written informed consent.

Tetrameric HLA molecules. Tetrameric HLA class I molecules were produced as previously described[6,7] and as presented in Chapter 3. Prokaryotic expression vectors encoding for the extracellular portion of HLA-A2, -B7, and -B8, each tagged to a carboxy-terminal BirA enzyme recognition sequence and β2-microglobulin were separately transformed into *E.coli*. Protein expression was induced with 1mM IPTG (final concentration) and chains were extracted from inclusion bodies. Heavy and β2m chains were refolded by dilution in the presence of the appropriate peptides. For HLA-A2: HIV-1 Gag p17 77-85 (SLYNTVATL) and Pol 476-484 (ILKEPVHGV). For HLA-B7: HIV-1 Nef 128-137 (TPGPGVRYPL), gp120 296-305 (RPNNNTRKSI), and Gag p24 (ATPQDLNTM). For HLA-B8: HIV-1 Nef 89-97 (FLKEKGGL), Gag p17 24-31 (GGKKKYKL), and Gag p24 259-267 (GEIYKRWII). Refolded complexes were enzymatically biotinylated with BirA (Avidity) and fast protein liquid chromatography purified by ion exchange on a MonoQ column or by size exclusion on an S-75 column (Pharmacia Biotech). Biotinylation efficiency was assessed by band shift of biotinylated monomer with strepavidin on an SDS-PAGE protein gel and routinely evaluated to 80-95%. Extravidin-PE (Sigma) was then mixed with biotinylated monomeric MHC to a 1:4 molar ratio.

FACS analysis. Cells cryo-preserved in liquid nitrogen were thawed into RPMI 1640 Glutamax-1 medium containing 10% inactivated fetal calf serum (R-10)(Gibco BRL, Life Technologies). Washes between stainings were performed with standard PBS-2% FCS. Cells were stained with PE-labeled tetramers for 15 min at 37°C prior to 30 min incubation at 4°C with other cell surface antibodies. Rat anti-human CCR7 antibody (3D12, rat IgG2a) staining was followed by goat anti-rat IgG (H+L)-FITC (Southern Biotechnologies Associates). The following mouse anti-human antibodies were used in different combinations for cell surface staining: anti-CD8 (IgG_1, Leu-2a) coupled to PerCP or CyChrome (Becton Dickinson). Data was acquired on a FACSCalibur™ system and analysed using CellQuest™ software (Becton Dickinson systems).

Results

The blood and lymph nodes of chronically infected patients contain HIV-specific CD8 T cells directed at multiple viral epitopes

Fifteen patients from the cohort previously characterized in Chapters 3 or 4 were selected on two criteria, namely 1) that, prior to any anti-retroviral therapy, they displayed at least one HIV-specific CD8 T cell reactivity, which could be studied in their blood and or lymph node cells using our panel of tetramers, and 2) that for each, corresponding blood and/or lymph node mononuclear cell samples were available for study 63-72 weeks following onset of anti-retroviral treatment, at a time when plasma viremia was below 50 HIV-1 RNA copies/ml. The blood and lymph node samples obtained at a "baseline" time point prior to any anti-retroviral therapy were first characterized using each of the available tetramers suited to their HLA.

In blood, the following reactivities were observed. Single CD8 T cell class I MHC-restricted, HIV-1 antigen-specific responses were observed and studied with tetramers in nine patients (Patients 1004, 1013, 1014, 1020, 2076, 2077, 2091, 2121, 2133). Meanwhile, a further five patients responded to two epitopes presented by the same (Patients 1001, 2084 and 2123), or different (Patients 2049 and 2066), HLA alleles that we could study using our panel of tetramers. Singularly, Patient 1011 displayed three significant HIV-directed responses, two of which were restricted by HLA-B8 and one by HLA-A2. The size of the tetramer positive (Tet^+) populations specific to a single antigen and evidenced in the blood of the various patients varied from 0.04% to 4.37% of the $CD8^+$ T cells (Mean = $1.19^{\pm}1.2\%$)(Table1). For Patient 2123, the sum of HIV-specific Tet^+ CD8 T cells responding to the two epitopes characterized in blood encompassed a remarkable 4.95% of all CD8 T cells present therein. Admittedly, these responses likely represent only a fraction of the HIV-specific CD8 T cell responses being orchestrated by the patients.

All patients with characterized responses in their blood had also undergone a "baseline" excisional lymph node biopsy upon study entry. The presence of HIV-1 directed CD8 T cell responses in the lymph node mononuclear cell (LNMC) samples of 15 patients in who peripheral HIV-specific $CD8^+$ Tet^+ T cell responses had been observed was also assessed. As in blood, a few patient LNMC samples contained CD8 T cells targeting several different HIV-1 epitopes (Table 1). In all, 19 of the 22 reactivities present in patients' baseline PBMC were also detected in the matching baseline LNMC. Significantly, Patient 2123 displayed an expanded HLA-B8-restricted, HIV-1 p17-specific CD8 T cell population representing 5.54% of the lymph node $CD8^+$ T cells that was not present in blood at that time. Detectable Tet^+ populations observed in the lymph node compartment represented between 0.36% and 6.01% of $CD8^+$ T cells, with a mean of $1.73^{\pm}1.88\%$ (Table 1). Overall, and as observed in Chapter 4, the proportion of HIV-specific Tet^+ cells within the pool of CD8 T cells present in either blood or lymph node did not differ significantly ($P>0.05$, t-Test). Furthermore, considering each HIV-specific CD8 T cell reactivity individually, greater proportions of Tet^+ cells were observed equally often in blood and lymph node compartments of patients characterized in this cohort prior to anti-retroviral treatment.

Effective resolution of HIV-1 viremia with Highly Active Anti-Retroviral Therapy (HAART) results in resorption of the HIV-1 specific $CD8^+$ T cell expansions in both blood and lymph node

We sought to further characterize the evolution of antigen-specific CD8 memory T cell subsets in the blood and lymph nodes of patients following HAART. As shown for representative Patient 1014 (Figure 1a and 1b), the long-term and sustained resolution of HIV-1 viremia following 63-72 weeks of HAART resulted in a clear tendency towards normalization of the relative expression of CCR7 in HIV-1 infected patients when compared to that of HIV-negative subjects presented in Figure 1 of Chapter 4. Throughout our cohort, this normalization was evident for CD8 T cells in blood and lymph node (10 and 8 patients, respectively). Following HAART, the proportion of $CCR7^+$ CD8 T cells increased to an average $42.10^{\pm}15.03\%$ in blood and $67.34^{\pm}11.20\%$ in

lymph node following therapy, compared to 25.33±12.50% and 39.66±13.22%, respectively at baseline and indiscernible from that in the blood and lymph nodes of uninfected individuals (Student's t-test, $P>0.05$).

Changes in the $CCR7^-$ and $CCR7^+$ HIV-specific $CD8^+$ T cell subsets were also analyzed following long-term suppression of virus replication with HAART. At baseline prior to therapy, Patient 1014 had a plasma viremia of 416 096 HIV-1 RNA copies per ml and displayed an HLA-B8-restricted response to a Nef epitope whereby Tet^+ cells accounted for 2.42% of $CD8^+$ T cells in blood and 3.19% in the lymph node (Figures 1a and 1b, respectively). In both anatomical compartments, the majority of these HIV-specific $CD8^+$ T cells did not express CCR7. Following 63 weeks of HAART, the plasma viremia of Patient 1014 had been suppressed and maintained below 400 HIV-1 RNA copies per ml (data not shown). The successful resolution of viremia in this patient resulted in a reduction of the proportion of this HIV-specific Tet^+ CD8 T cell population to 0.21% and 0.22% of $CD8^+$ T cells in blood and lymph node, respectively. Furthermore, the long-term suppression of HIV-1 replication in Patient 1014 was associated with a significant reduction of the frequency of both Tet^+CCR7^- and Tet^+CCR7^+ CD8 T cells, in both blood and lymph node (figures 1a and 1b).

With the exception of Patients 1004 and 2124, such a reduction of the frequency of HIV-specific Tet^+ CD8 T cells was confirmed in blood of an additional 7 patients (Figure 2). In lymph nodes, such a reduction in the number of HIV-specific CD8 T cells as detected by tetramer staining was evidenced in all 7 patients characterized (Figure 2).

Notably in the cohort, following therapy, the average proportion of the characterized Tet^+ cells in the $CCR7^+$ and $CCR7^-$ CD8 T cell subsets fell to 0.15±0.33% (median = 0.07%), and 0.28±0.33% (median = 0.14%) in blood. In lymph nodes, the corresponding populations fell to 0.31±0.62% (median = 0.02%) and 0.26±0.28% (median = 0.22%). The extent of the decrease in the proportion of HIV-specific $CD8^+$ T cells present in blood and lymph nodes of patients following effective anti-retroviral treatment was similar in both $CCR7^+$ and $CCR7^-$ cell subsets and the remaining proportions of Tet^+ populations of

either were similar in blood as in lymph nodes. Clearly, these observations warrant further investigation, as is argued in the discussion of this thesis.

Table 1. Proportion of HIV-specific tetramer positive cells in blood and lymph node CD8 T cells of HAART-naïve, chronically HIV-1 infected patients.

Patient	Tetramer (HLA-peptide)	Tetramer positive CD8 T cells	
		PBMC	LNMC
1001	**B8 Nef**	3.76%	4.85%
	B8 p24	0.04%	ND[1]
1004	**A2 Pol**	0.43%	ND
1011	**A2 Gag**	0.74%	0.80%
	B8 Nef	1.28%	3.72%
	B8 p24	0.20%	0.36%
1013	**A2 Gag**	0.45%	0.36%
1014	**B8 Nef**	2.42%	3.19%
1020	**A2 Gag**	1.55%	0.78%
2049	**A2 Gag**	1.00%	0.54%
	B8 Nef	2.36%	0.93%
2066	**A2 Gag**	0.59%	0.65%
	B7 Nef	0.30%	1.20%
2076	**B8 p24**	0.19%	ND
2077	**A2 Gag**	2.62%	0.53%
2084	**B8 Nef**	1.12%	0.47%
	B8 p24	0.83%	0.54%
2091	**B7 Nef**	0.24%	2.56%
2121	**A2 Gag**	0.91%	0.81%
2123	**B8 Nef**	4.37%	6.01%
	B8 p17	ND	5.54%
	B8 p24	0.58%	0.61%
2133	**B7 Nef**	0.12%	0.24%

[1]ND, not detected

Figure legends

Figure 1. Distribution of HIV-specific CD8 T cells in $CCR7^+$ and $CCR7^-$ CD8 T cell subsets within the blood (A) and lymph node (B) of representative patient 1014 prior to and following HAART. At baseline, prior to therapy, circulating and lymph node CD8 T cells are predominantly $CCR7^-$ and significant numbers of $CCR7^+$ and $CCR7^-$ B8-Nef (FLKEKGGL) tetramer-specific CD8 T cells are observed in both anatomic compartments. At study endpoint, 63 weeks following onset of HAART and after the stable reduction of HIV-1 viremia, the proportion of circulating and lymph node $CCR7^+$ CD8 T cells has significantly increased. However in both anatomic compartments, the overall proportion of tetramer-specific CD8 T cells, as well as their proportion within $CCR7^+$ and $CCR7^-$ pools is significantly reduced.

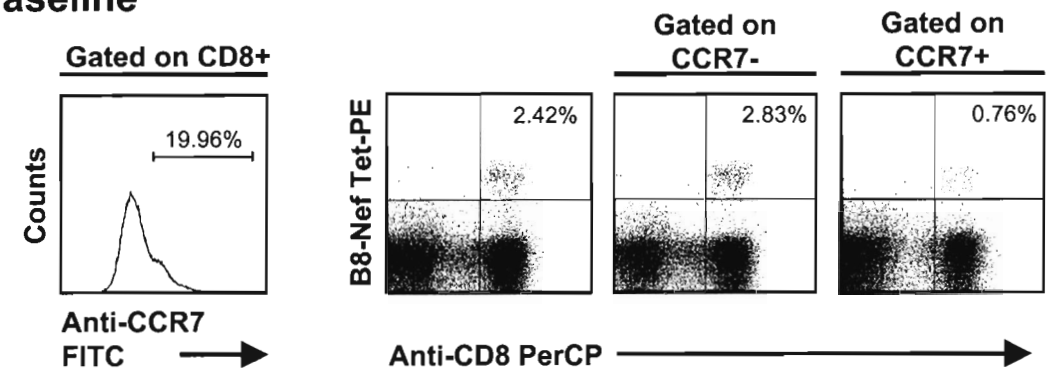
Figure 2. In blood and lymph node, the relative frequency of HIV-specific $CCR7^+$ and $CCR7^-$ CD8 T cells, is significantly reduced following the instigation of HAART during chronic HIV-1 infection. Data from 10 and 7 patients are compiled for blood and lymph nodes, respectively. Symbols identify individual tetramer-specificities of each patient.

A

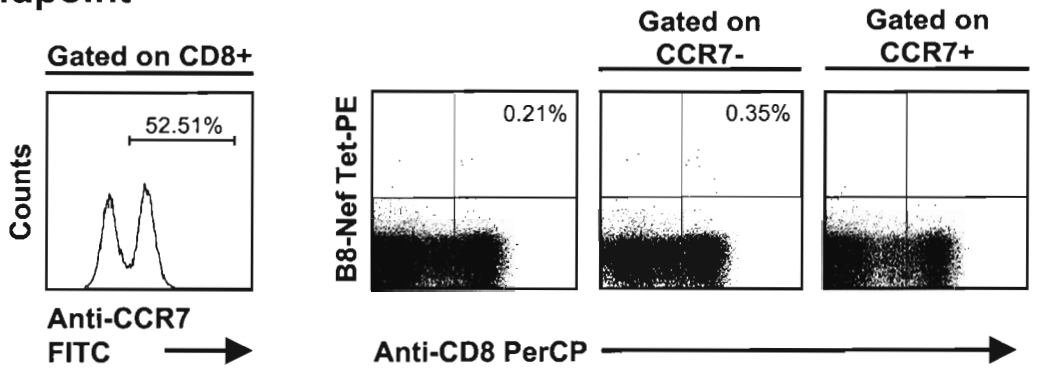
Patient 1014

PBMC

Baseline



Endpoint

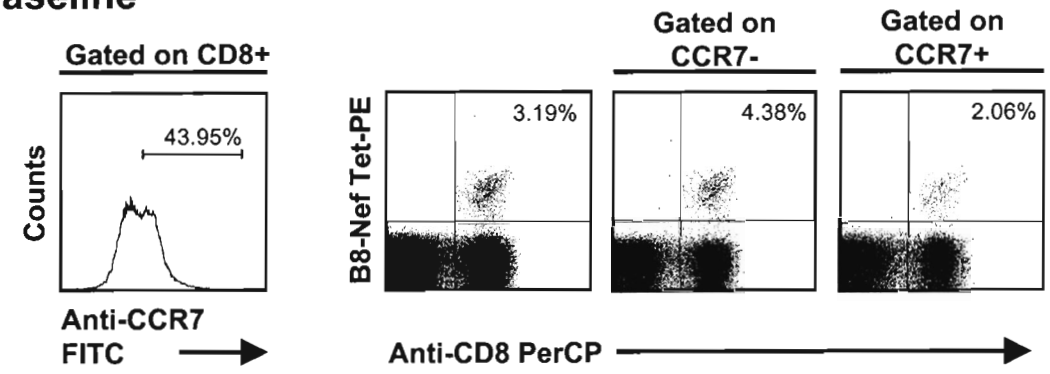


B

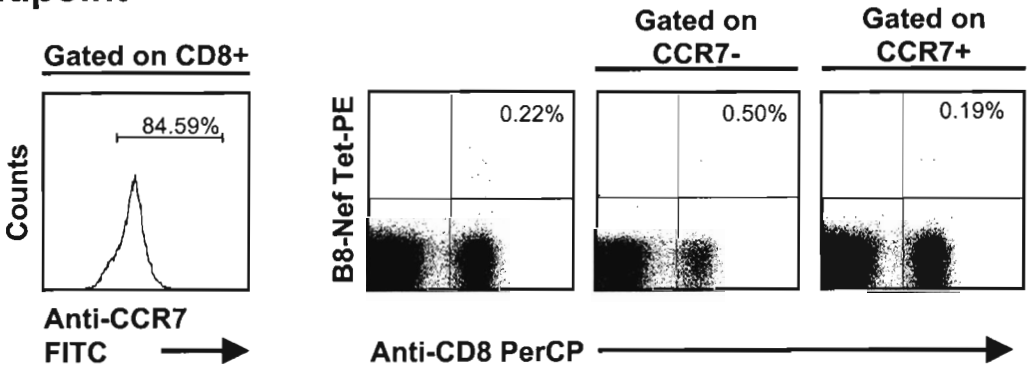
Patient 1014

LNMC

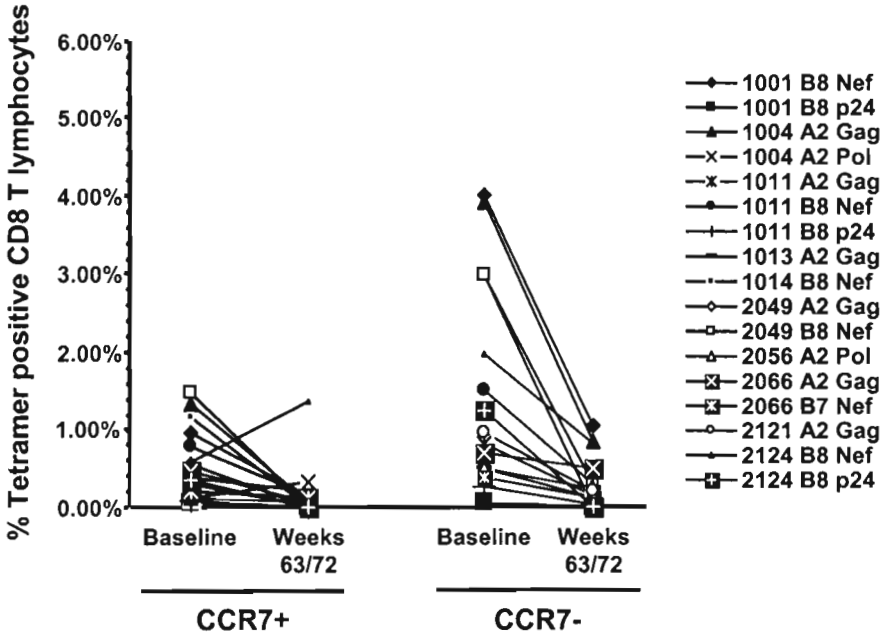
Baseline



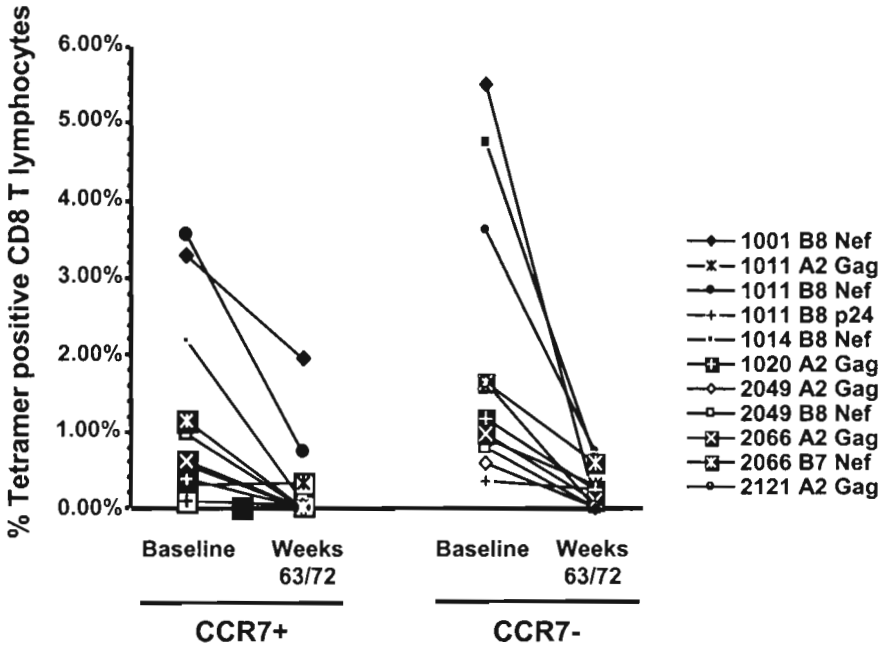
Endpoint



Blood



Lymph Node



6. Original contribution to scientific knowledge

The results presented in this thesis contribute to scientific knowledge in several aspects.

The work presented in Chapter 2 is an original report of the molecular characterization of TCR repertoires of CD4 and CD8 T lymphocytes in the context of pediatric HIV-1 infection. This report evidently displays the distinct characteristics of CD4 and CD8 T cell expansions; with successions of CD8 T cell clonotypic over-representations persisting for prolonged periods of time. Chapter 2 also showed the similarity of pediatric immune responses to that of adults when compared on the basis of the molecular TCR repertoire.

The results presented in Chapter 3 support an original lineage differentiation pathway of memory CD8 T lymphocytes on the basis of CD45RA/CCR7 phenotype. Critically, a population of antigen-specific $CD45RA^{+}CCR7^{+}$ CD8 T cells with strong proliferative potential was described for the first time. HIV-specific $CD45RA^{-}CCR7^{+}$ and $CCR7^{-}$ central and effector memory CD8 T cells were also observed in the blood of chronically HIV-1 infected patients. Finally, a minor population of terminally differentiated antigen-specific CD8 T cells with little proliferative capacity was also observed. While CMV-specific CD8 T cells of each subset were also observed, the relative distribution of these cells between the different memory subsets was significantly different from that of the HIV-specific cells. Specifically as compared to that of CMV-specific T cells, the work presented in Chapter 3 demonstrated a skewed distribution of HIV-specific memory CD8 T lymphocytes amongst different maturation subsets.

For the first time, the work presented in Chapter 4 described a significant enrichment of effector $CCR7^{-}$ CD8 T cells into lymph nodes during chronic HIV-1 infection. $CCR7^{-}$ HIV-specific CD8 T cells were shown to be present in significant proportions within lymph nodes and the distribution of HIV-specific CD8 T cells between these two compartments was characterized for the first time. The results presented herein argue strongly against a redistribution of terminally differentiated cells towards lymph nodes; on the contrary, in the context of chronic HIV-1 infection, the skewed distribution of HIV-specific CD8 T cells appears to be proper to both secondary lymphoid organs and

blood. Finally, in Chapter 5, successful treatment of patients from our characteristic cohort with HAART was shown to result in a reduced frequency of HIV-specific CD8 T cells from both $CCR7^+$ and $CCR7^-$ compartments.

Altogether, the studies presented herein provide novel insight into the characteristics of the HIV-specific repertoire of T lymphocytes and contribute to our understanding of the dynamic evolution of HIV-specific CD8 T cell immune responses. As will be discussed in greater detail, these studies further our understanding of CD8 T cell-mediated immunologic memory and form a sound basis for investigations seeking potential correlates of HIV-1 disease progression. Finally, the findings presented herein provide new avenues to be explored in search of novel correlates of immune protection that may be sought in therapeutic and vaccine strategies.

7. Discussion

7.1. Characteristics of the phenotypic and molecular TCR β-chain repertoire of pediatric subjects during HIV-1 infection

Pediatric HIV-1 infection is characterized by a faster course of natural progression to disease than is observed in adults infected with HIV-1[796]. The peak of viremia during primary HIV-1 infection also often persists for a longer period of time in infants as compared to adults: on average, 2 years in the absence of anti-retroviral therapy, compared to a few months in adults[796,797]. Despite the inherent difficulty of characterizing paediatric antiviral immune responses due to the very limited amounts of sample that can be obtained for study, detectable HIV-specific cellular immune responses have been measured and characterized[577,714,798-804].

An often proposed argument put forth to explain the faster course of natural disease in children is that the pediatric immune system might be less efficient than that of older individuals[805]. Such a theory may be supported by the fact that the protective advantage of immunologic memory is one that is created and enhanced by the experience of one's immune system having been subjected to various stimuli. The study presented in Chapter 2 nevertheless suggests that infants are capable of mounting immune responses having similar molecular signatures as that elaborated by adults, as far as is indicated by the analysis of their TCR repertoire.

As had been observed in adults mounting T cell-mediated immune responses to a variety of viruses including measles, EBV, CMV and HIV-1, infants vertically infected with HIV-1 display detectable TCR repertoire biases as they respond to infection[222,223,226,323,324,326,583,802,806-808]. These repertoire perturbations were observed in the CD4 and CD8 T cell compartments of all HIV-1 infected children, more frequently and to a greater extent than those seen in HIV-1 uninfected children born to HIV-1 positive mothers. Significantly, the observed Vβ perturbations were detected as early as the first month of life and exhibited amplification and resorption patterns similar to those seen in adults. The diversity of the Vβ segments differentially over-represented in

different subjects of the cohort, within same subjects, or between the CD4 and CD8 compartments of a same subject pre-empts suggestion of a superantigenic effect to explain the TCR amplifications observed in Chapter 2. Rather, antigen-driven responses are more likely to be at play.

7.2. The dynamics of antigen-specific T cell responses in the context of HIV-1 infection

7.2.1. The TCR repertoire of the CD8 T lymphocyte subset is characterized by a dynamic succession of over-expressed and persistent clonotypes during HIV-1 infection.

The TCRβ-chain repertoire of Child 9 was characterized by CD8-specific over-representation of Vβ14. As expected from antigen-driven CD8 T cell clonotypic expansions, the molecular cloning and sequencing of Vβ14 TCR chains revealed significant clonotypic over-representations at studied time-points. Furthermore, these over-representations were often stable through time as they were persistently detected over spans of several months. Although such an experimental approach based on the molecular cloning and sequencing of TCR chains constitutes the most precise characterization of the TCR repertoire, it is nevertheless limited by a lack of sensitivity due to the limited number of sequences that could be characterized (up to 31 per time point). The more sensitive diversity-specific PCR assay confirmed the amplifications that had been characterized more thoroughly by cloning and sequencing.

A strikingly dynamic pattern of response is evident from the succession of different CD8 T cell clonotypes observed through time. While no single Vβ14 clonotypic over-representation was observed in Child 9 at 17 days after birth, one of the clones then sequenced was detected again two weeks later, a time point at which a different clone was over-represented. At this time point, yet a third TCRβ-chain clonotypic sequence appeared that was later observed to be amplified and persist for a further 4 months. As of 2.5 months postpartum, multiple clonotypes were observed at each time point,

representing as much as 80% of the pool of TCRβ-chains sequenced. What is the fine specificity of the expanded CD8 T cell clonotypes? The contribution of the β chain of the TCR to the recognition of a peptide-MHC combination is principally through hypervariable regions of the Vβ and CDR3β. Within the pool of Vβ14-using T cell clones, the CDR3β thus remains the only source of variability for the βTCR chain. In this context, along with the αTCR chain pairing, it is the molecular features of the CDR3β that determine the T cell's specificity. Notwithstanding biases introduced by the over-representation of any of the amplified clonotypes, common molecular features of the sequenced TCRs at various time points sometimes included the focusing of CDR3 length (first to 8 residues, then to 9 or 13, then to 8 residues again), a characteristic of antigen-driven immune responses[217]. Nevertheless, no biased use of particular Jβ regions was observed. Furthermore, the physicochemical characteristics of the CDR3 regions did not appear to be skewed over time and the lack of amino acid conservation at any of the CDR3 residue positions of the different T cell clonotypes analysed strongly suggests that the expanded clonotypes herein observed might exhibit different specificities.

Are the observed expansions indeed HIV-specific? Although HIV-1 infected children exhibit more prominent biases in their TCR repertoire of $CD8^+$ T lymphocytes when compared to HIV-1 uninfected children born to HIV-1 positive mothers, direct evidence that the expanded clones are HIV-specific is not provided and the possibility remains that they might have evolved in response to other antigen. Nevertheless the data presented in Chapter 2 suggests that different T cell clonotypes appear to succeed each other in what can be perceived as an apparent relay of responding antigen-specific $CD8^+$ T cell immune specificities.

7.2.2. What are the mechanisms underlying the observed sequence of diverse expanded CD8 T cell clones?

This succession of responding clonotypes may underlie a switch in immunodominance of the CD8 T cell responses or a relay of responding clones. This dynamic evolution of CD8 T cell responses could result from several mechanisms, including:

i. The redistribution of HIV-specific T cells out of the circulating pool of lymphocytes and into tissues or lymph nodes is a possibility.

ii. A natural shift in the HIV-specific T cell response due to the recruitment of newly produced T lymphocytes. This mechanism is postulated to be relevant in adults, and might be even more so in infants that display elevated thymic output[629]. The production of T cells expressing new and diverse T cell receptors may result in the appearance of T cell clones possessing specificity for previously unrecognized antigen-MHC combinations. This ongoing diversification of the T cell pool could therefore explain the delay that characterizes the appearance of some CD8 T cell expansions.

iii. A natural shift in the primary viral targets of HIV-specific response throughout the course of disease progression. In SIV infection models and HIV-1 infected adults, the recent characterization of viral epitopes that are preferentially recognized in acute as opposed to chronic immunodeficiency virus infection suggests that the dynamics of T cell clonotypic expansion and resorption may be the result of such a switch of preferred specificity[763,777,809,810]. The quantity and quality of given epitopes being presented during different phases of infection may be hypothesized to contribute to the phenomenon[777]. The underlying mechanisms appear complex and remain unclear. They may result from inherent viral properties such as viral tropism and a propensity for active or latent life cycle strategies. The shift could also result from HIV-1 instigated immunopathology, including the altered architecture of secondary lymphoid organs during acute HIV-1 infection and disease progression. Alternatively, the switch may be re-directed by a natural modulation of the immune environment during an immune response. Here, hypothetical mechanisms include the alteration of the signals provided by the environment, such as the shifting dominance of various cytokines or the availability and identity of co-stimulatory signals, as well as changes in the available T cell help.

iv. A natural shift in the predominance of expanded clones due to viral escape from immune responses. The modification of protein-encoding viral sequences may result in the disappearance of a T cell epitope and the attrition of its specific T cell clonal expansion. Alternatively, variations in the sequence of the viral peptides presented could result in a shift of CD8 T cell responses towards other reactivities and the recruitment of distinct T cell clones. The clonal exhaustion of recruited HIV-specific CD8 T cells is one potential mechanism. Admittedly, the sensitivity of the assays used in Chapter 2 pre-empts us from affirming that the clonotypes are indeed physically deleted from the pool of T lymphocytes, however their significant reduction within the pool of cells suggests that the specific attrition of HIV-1 responding clones is highly probable. Mechanisms that could lead to specific clonal exhaustion include activation-induced cell death, senescence, lack of T cell help and an increased sensitivity to induced apoptosis. Beyond these, any of the mechanisms outlined in 1.3.7 are possible contributors.

v. A natural shift in the dominance of existing HIV-specific CD8 T cells on the basis of a maturation of the immune response. The functional maturation of T cells taking place throughout immune responses results in the selection of memory T cell pools. Various stages of differentiation of T cells are characterized by differential ability to proliferate and may thus result in transient clonotypic overrepresentations.

7.2.3. Molecular features of the molecular TCR repertoire of the CD8 and CD4 T lymphocyte compartments are differentially modulated during HIV-1 infection

Although there was no significant difference in the number or magnitude of TCRβ repertoire perturbations within the CD4 and CD8 compartments of HIV-1 infected pediatric subjects when analysed by cytofluorometry, molecular characterization of the clonotypes present therein revealed that the pattern of response within these two T cell compartments differed significantly. Child 6 exhibited TCRBV 6S7 and TCRBV 20 amplifications that were almost exclusively contained within the CD4 subset. Cloning

and sequencing of these βTCR-chains revealed no evidence of clonotypic over-representation or persistence of specific T cell clones. Furthermore, attempts using the more sensitive DS-PCR approach also failed to unveil CD4 T cell clonotypic expansions or identify clones that persisted through multiple time point samples. Altogether, this pattern of CD4 T cell response in pediatric HIV-1 patients is in agreement with the fact that CD4 T cell expansions have been extremely difficult to detect as compared to CD8 T cell expansions.

A first possible explanation for the difference we have observed pertains to the sensitivity of the assays used and the intrinsic diversity of the TCR repertoires of blood-circulating subsets of CD4 and CD8 T cells. Because our methods do not circumscribe total body diversity, should the overall TCR repertoire diversity of the CD8 compartment be significantly less than that of the CD4 compartment, it would then be possible for our experimental approach to be sensitive enough to detect expansions within the CD8 T cell subset, but not within the CD4 T cell subset. Thus hypothetically, CD4 T cell clonal expansions in HIV-1 infection would not be detected because of homeostatic characteristics that generate and maintain greater diversity in the repertoire of CD4 T cells, as compared to that of CD8 T cells. Alternatively, $CD4^+$ and $CD8^+$ T cells may clonally expand in discrete anatomical sites or disseminate differently into blood. Thus, analysis of the lymph node compartment or tertiary peripheral immune response sites may reveal a different repertoire of T cells than that seen in PBMC.

Beyond these potential technical and sampling caveats, different phenomena may result in the perturbation of the observed βTCR repertoires and could suggest why clonotypic expansions are detected in the CD8, but not in the CD4 T cell compartment. First, the antigen-driven expansion of clonally selected T cells is evidently expected to result in their over-representation within the total pool of T lymphocytes. This is most probably the case for the observed CD8 T cell expansions: CD8 T cells have been shown *in vivo* to undergo a programmed pattern of response implicating differentiation and several rounds of proliferation following brief antigen encounter[811-813]. Whether this is also a characteristic of CD4 T cells remains a matter of debate. Nevertheless, CD4 T cells are

postulated to behave differently, with a more restrained response possibly characterized by lack of differentiation and fewer cell divisions[814-816]. These potential intrinsic differences should be confirmed *in vivo*, avoiding TCR transgenic systems, using purified T cell populations in order to discriminate for their differentiation stage, and accounting for the variations in signal that may result from differences in TCR-MHC affinity or the diversity of other stimuli that are integrated in order to guide the response. Intrinsic differences between CD4 and CD8 T cell responses may thus underlie our observations.

Finally, perturbations may result from the selective depletion of T cell clonotypes. Such selective depletion requires a mechanism that leads to the preferential targeting of one T cell population over another. Particularly, retroviruses are known to infect replicating cells more efficiently than non-cycling cellular counterparts[487]. The clonal selection and activation of antigen-specific CD4 T cells thus renders them more susceptible to infection by HIV-1[489,635]. Thus, the preferential targeting of CD4 T cells undergoing expansion would be on the direct basis of the virus' preferred tropism for activated cells; this would indirectly result in the depletion of recruited CD4 T cells and the obliteration of CD4 T cell clonal expansions. Notably, HIV-1 has been shown to infect HIV-specific CD4 T cells more proficiently than their activated, non-HIV-specific counterpart as represented by a pool of CMV-specific CD4 T cells[817]. As previously overviewed in section 1.3.4, the selective elimination of HIV-specific CD4 T cells through viral pathogenesis or immunopathogenesis may underlie the absence of detectable expansions in the CD4 T cell subset.

The dynamic pattern of response observed in the study presented in Chapter 2 is strongly indicative of an ongoing antiviral immune response. As is proposed in section 6.7, the advent of approaches permitting for the direct evaluation of antigen-specific T cell pools combined with the high-throughput TCR analysis strategies that have been developed, now allow thorough and promising forays.

The detailed molecular characterization of the TCR repertoire of T lymphocytes undertaken in Chapter 2 displays attributes that shape immune response dynamics, yet it

presents several questions to attention. If the observed clonal expansions are evidence of the proliferation of antigen-specific T cells that is taking place during an ongoing anti-HIV-1 immune response, how effective is this response? Beyond proliferation, what are the functional properties of the responding HIV-specific T cells? Particularly, how do the properties of antiviral CD8 T lymphocytes evolve following antigen-specific recruitment and a protracted confrontation with virus? The studies presented in Chapters 3 and 4 begin to address these questions and evidence the evolution of the phenotypic and functional attributes of HIV-specific CD8 T cells in the context of memory CD8 T cell differentiation.

7.3. Creating a framework to conceptualize the lineage differentiation of antigen-specific CD8 T cells and the maturation of memory T lymphocytes

The mechanisms underlying memory T cell differentiation are still poorly understood. Two theoretical frameworks attempting to explain the generation of memory have been generated from experimental results. The first is a linear differentiation model whereby cells undergo necessary maturation through an effector stage before becoming memory; the second is a dichotomic differentiation model whereby memory cells are made such without having necessarily been effectors at any point. These are illustrated in figure 9.

The linear differentiation model

Independent groups have demonstrated that, upon encounter with antigen, T cells must undergo rounds of division before exerting effector function and having the potential of becoming memory[818-821]. These experiments typically involved the sorting of CFSE-labeled cells having undergone set numbers of *in vitro* peptide-driven cell divisions and their tracking following re-infusion into syngeneic hosts. Adoptive transfer experiments using cells from TCR- transgenic mice also support this model[822-824]. For all these studies, it is unclear whether all transferred cells truly were constituted of a homogenous pool of identical effector cells or whether the seeding of memory was the result of a subset of the transferred cells sharing phenotypic properties with transferred effectors.

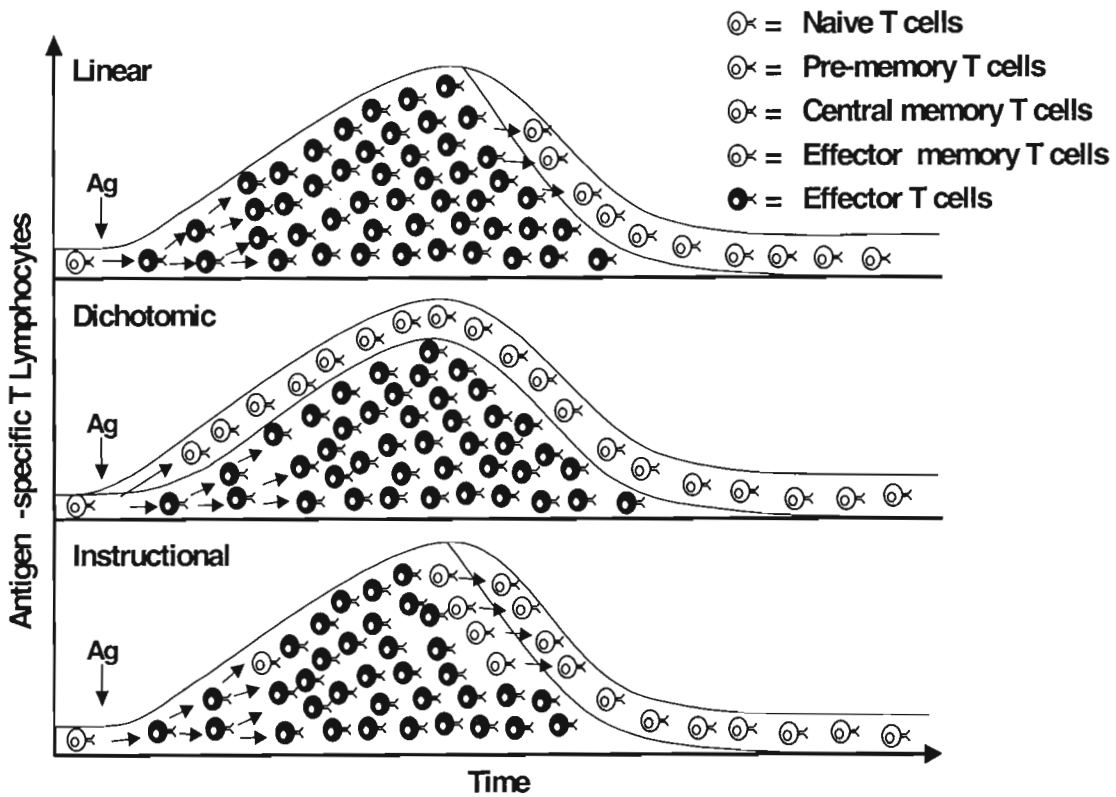


Figure 9. Models for memory T cell generation. **A)** Linear differentiation model. Following antigen encounter, naïve T cells proliferate and differentiate into effectors; after antigen clearance, only a small proportion survive and constitute the memory T cell pool. **B)** Dichotomic model. A subset of naïve T cells directly differentiates into memory without acquiring effector functions. At the end of the immune response, effector cells undergo apoptosis while the memory subset persists. **C)** Instructional model. A subset of naïve T cells receives a signal that drives them to differentiate into "pre-memory", further differentiation requires proliferation. These "pre-memory" cells form the pool of Tcm and Tem memory T cells.

The dichotomic differentiation model

Jacobs and Baltimore have developed an original transgenic mouse system that allow the permanent labelling of activated and memory T cells [825]. In these mice, the Cre-

recombinase gene was introduced under the control of a truncated human granzyme B promoter. A placental alkaline phosphatase (PLAP) reporter gene was also introduced such that its synthesis is induced only in T cells and only following a Cre-mediated recombination event. Activation of the T cell normally leads to induction of the granzyme B gene promoter; thus, in these double-transgenic mice, it ultimately results in the transduction of human PLAP that permanently labels the T cell having undergone activation. Surprisingly both during the peak of an LCMV infection and >30 days after the infection, only a small persisting fraction (5%) of activated T cells are $PLAP^+$ and thus show the potential to become long-term memory. Though these "pre-memory" T cells display functional effector properties similar to those associated with effector T cells, this subset of T lymphocytes having probably received a distinct signal early during the immune response will persist to memory. In a different experimental system, the expression of IL-2 by a subpopulation of naïve T cells has been linked to an enhanced memory/effector function [826]. Using an IL-2 promoter/GFP-reporter transgenic model, it was shown that $CD4^+$ T cells that reach the activation threshold associated with IL-2 gene transcription during the primary response later have an enhanced ability to produce effector cytokines, provide better B cell help and show increased sensitivity to antigenic stimulation as compared to those that did not reach the threshold.

A matter of perspective...

To date, the key difference between experiments supporting a linear differentiation model and those supporting a dichotomic model is in the original property used to isolate the cells to be characterized for their ability to seed memory. Experiments using cells sorted on the basis of BrdU or CFSE-labelling rely on their proliferative history but fail to demonstrate the functional homogeneity of the sorted population. While enrichment of measured effector function in the sorted populations correlates with enrichment of memory precursor content, direct demonstration that the cells exerting the function are the same which later seed memory remains to be performed. Conversely, experiments relying on the study of T cell populations selected on the basis of their demonstrated function are limited by the transgenic nature of the systems used and the unknown history

of individual cells. Finally, our limited knowledge of the complexity of factors that modulate the promoters and enhancers that direct the expression of tracer-proteins in transgenic reporter systems complicates interpretation of studies making their use. Altogether, the complexity of the memory phenotype with regards to function as defined either by proliferation or by which cytokine/effector molecule is up-regulated, is such that even the most elegant systems available force the bias of which property to primarily target. The combined use of newly developed reagents allowing for the tracking and multi-parametric functional analysis of viable T cells will begin to resolve this problem.

The instructional differentiation model

Recent studies have shown that a brief initial antigen encounter is sufficient to induce naïve CD8 T cells to expand and mature to differentiated effectors that display memory properties in vivo[827,828]. Though a few hours of antigen stimulation in the presence of APCs expressing B7 appear to be sufficient, other factors contributing to the appropriate costimulation remain to be identified. The results obtained by various groups investigating the CD4 T cell response suggest that CD4 T cell proliferation may also be induced by a brief encounter with antigen, but that differentiation requires protracted exposure[815,816]. Both for CD4 and CD8 T lymphocytes, signals of a certain nature or quality may therefore confer some cells with the potential to become memory, a potential that is then exploited only if these "pre-memory" cells receive further signal. The maturation of memory cells would thus be a multi-step process requiring a timely succession of key stimuli.

Critically, studies by Manjunath et al. have demonstrated that the generation of memory T cells does not require prior linear differentiation through an effector stage: alone, the cytokine environment at the time of recruitment may be sufficient to bias maturation of the T cells as central or effector memory[829]. These experiments made use of an original transgenic system of T-GFP mice, in which a green-fluorescent protein (GFP) marker was introduced under the control of the CD4 promoter and proximal enhancer[830]. Original observations using these mice were that GFP was expressed in naïve CD4 and CD8 T

cells but lost from a significant proportion of CD8 T cells having been stimulated, and that the effector properties of cytotoxicity and IFN-γ secretion were restricted to GFP$^-$ cells. While *in vitro* restimulation of the GFP$^-$ cells in the absence of exogeneous cytokines resulted in their apoptosis, exogenous provision of IL-2 rescued these cells and supported their proliferation[830]. In a follow-up study, T-GFP mice were crossed to TCR transgenic mice in order to study T cell differentiation in an antigen-dependant manner. *In vitro* peptide-specific stimulation of the naïve double transgenic CD8 T cells in the presence of high concentrations of IL-2 resulted in the formation of GFP$^-$ effectors that did not express CCR7. The subsequent resting of the cells (7 days) and switch to IL-15-containing medium resulted in their apparent reversion to a memory phenotype that nevertheless did not express GFP or CCR7. Meanwhile, *in vitro* stimulation of naïve CD8 T cells in the presence of low doses of IL-2, or in the presence of IL-15 directly resulted in the formation of memory cells that secreted IFN-γ, lacked immediate cytotoxic function and phenotypically remained positive for GFP and CCR7 expression. Thus, distinct pools of memory CD8 T cells can be generated by linear differentiation through an effector stage, or directly as per the dichotomic differentiation model.

Conceptually, it may therefore be possible to reconcile data supporting the linear and dichotomic models discussed above with an instructional differentiation model for the generation of memory T cells (figure 9c). This model is based on the premise that the stimuli received by different naïve T cells are variable both in nature and intensity. This variability would result from differences in signals provided by different affinity/avidity of the interaction between peptide/MHC and TCR, by costimulation and the cytokine environment in which the cells are engaged. While summation of the memory-inducing signals through multiple descendent generations of a precursor cell would result in a "linear" pattern of memory generation, provision of the necessary signals to a non-proliferating T cell would result in a pattern compatible with an intrinsic dichotomy of memoryT cells (figure 9). The lineage differentiation pathway established in Chapter 3 is most compatible with an instructional differentiation model of memory CD8 T lymphocytes. An instructional differentiation model supports a notion that serves to interpret the results of the studies herein presented: that the maturation of CD8 T cells

into memory subsets is undertaken in the context of ongoing immune responses (ie. in the presence of antigen), and not solely following their resolution.

7.4. T cell memory in the presence of antigen: is there a role for antigen in the maintenance of T cell memory?

Memory T cells survive for a long period of time. Whether persistence of the specific antigen is required for the survival of the long-lived memory T cells has long been the subject of debate [823,831-835]. While some groups, using adoptive transfer experiments with or without priming antigen, observed that both CD4 and CD8 memory T cells are short-lived without the antigen [836,837], recent work exploiting stringent experimental protocols to dissect this question are increasingly arguing for an antigen-independent survival of memory T cells. Through use of adoptive transfer experiments with *in vivo*-generated effector cells, some have shown that a resting memory population could persist for prolonged periods of time in the absence of further antigenic re-stimulation [823,832,834]. Tanchot *et al.* used HY-specific TCR transgenic RAG2$^{-/-}$CD8^{+} T lymphocytes in transfer experiments to irradiated hosts and similarly showed that the specific antigen is not necessary for the survival of memory cells [831]. However, their data suggest that the minimal interaction of the TCR with non-specific class I MHC is essential for long-term maintenance of memory T cells [831]. Similar results supporting a role for class I MHC in the maintenance of memory are put forth by Markiewicz *et al.* who showed that HY-specific TCRB6.2.16 memory cells do not persist past 70 days following transfer into TAP1$^{-/-}$RAG1$^{-/-}$ mice: cells from these mice have fewer than 10% of the normal numbers of MHC class I on their surface [837].

The requirement for TCR-MHC interactions to maintain memory T cells is nevertheless questioned by recent results published for both CD4 and CD8 memory T cells [838 839]. Swain *et al.* generated polarized CD4 T cell effectors *in vitro* from naïve cells and transferred them to MHC class II-deficient hosts. They found that the transferred effector cells gave rise to long-lived, small resting memory cells (CD44^{hi}). Meanwhile, Murali-Krishna and colleagues similarly demonstrated that LCMV-specific memory CD8^{+} T

cells transferred into $\beta_2m^{-/-}$ and/or $D^{b-/-}$ x $K^{b-/-}$ x β_2-$m^{-/-}$ mice for more than 300 days can persist and retain the ability to rapidly respond even if maintained in absence of both antigen and signals provided by classical MHC class I contact. Furthermore, these experiments do not pre-empt that a signal may be transduced through the TCR either by non-classical MHC or undetermined ligands.

Significantly, although MHC is most probably not required for the survival of memory T lymphocytes, MHC has been shown to be necessary in order to sustain the qualitative features of memory CD4 T lympohocytes[840]. Furthermore in a setting where MHC is present, memory T cells may possibly be maintained in a ready effector-mode by sub-optimal, non-specific TCR stimulation, either by interaction with cross-specific peptide or self-peptide MHC. Using limited dilution analysis in order to assess T cell reactivity for Pichinde (PV), vaccinia (VV), and murine cytomegalo (MCMV) viruses in the spleens of mice having previously been immunized with LCMV, Selin *et al.* showed the significant cross-reactivity raised against these related viruses in immune, as opposed to non-immune mice[841,842]. A consequence of this cross-reactive stimulation of memory T cells is that, in certain cases, an infection with a second virus can reactivate memory cells specific for a first[841]. In further studies, they showed that prior immunity to LCMV could significantly enhance clearance of an unrelated virus (VV). In these studies, memory cells specific for LCMV were engaged against VV even before recruitment of the high affinity VV-specific T cell clones normally stimulated in a naïve host[842].

The complexity of the potential outcomes resulting from cross-specific priming of memory T cells is illustrated by work performed by Klenerman and colleagues. In a murine system, Klenerman *et al.* showed that activated $CD8^+$ T lymphocytes specific for an H-$2D^b$-restricted w.t. gp33 epitope of LCMV could also respond to a mutated gp33 epitope[843] . As expected, following two subsequent infections with the w.t. virus, the memory T cell response was observed to be mainly directed against the w.t. epitope. Surprisingly, an infection with the mutant virus after a first infection with the wild type was shown to lead to the efficient recruitment of a functional response mainly directed against the wild type viral epitope rather than the mutated epitope itself. This

immunologic phenomenon of "original antigenic sin" is therefore a potential viral mechanism for immune evasion. This is particularly relevant in the case of HIV-1 infection, due to the virus' high propensity to mutate.

Following its establishment, T cell memory is thus constantly subject to modulation by an individual's immunological experience. These modulations may lead to changes in peptide immunodominance, reactivation of cross-specific memory T cells, as well as changes in the absolute number of precursors specific for a given epitope within the total pool of persisting memory T cells[844]. Furthermore, the presence and persistence of a specific antigen as is the case during chronic viral infections and HIV-1 infection in particular, may modulate the evolution of immunologic responses and memory, and alter the maturation of virus-specific memory CD8 T lymphocytes.

7.5. Heterogeneity of the repertoire of antigen-specific T cells and differentiation of antiviral memory CD8 T lymphocytes

In Chapter 3, a pathway of memory T cell differentiation was established whereby, following antigen-encounter, T cells proliferate and gain effector properties while undergoing phenotypic changes that modify their tissue homing properties as originally suggested by the work of Sallusto and colleagues[792,795]. According to this differentiation pathway, antigen-specific cells are first recruited into a pre-memory subset possessing a naïve-like phenotype ($CD45RA^+CCR7^+$) before reaching the Tcm and Tem maturation stages ($CD45RA^-CCR7^+$ and $CD45RA^-CCR7^-$, respectively). Ultimately, they may reach a terminally differentiated effector stage ($CD45RA^+CCR7^-$). Progression through these subsets is associated with a loss in proliferative potential, in combination with a gain in the potency of effector functions. The prevalence of antigen-specific T cells from each memory subset in an individual may therefore be hypothesized to evolve throughout the progression of immune responses (figure 10).

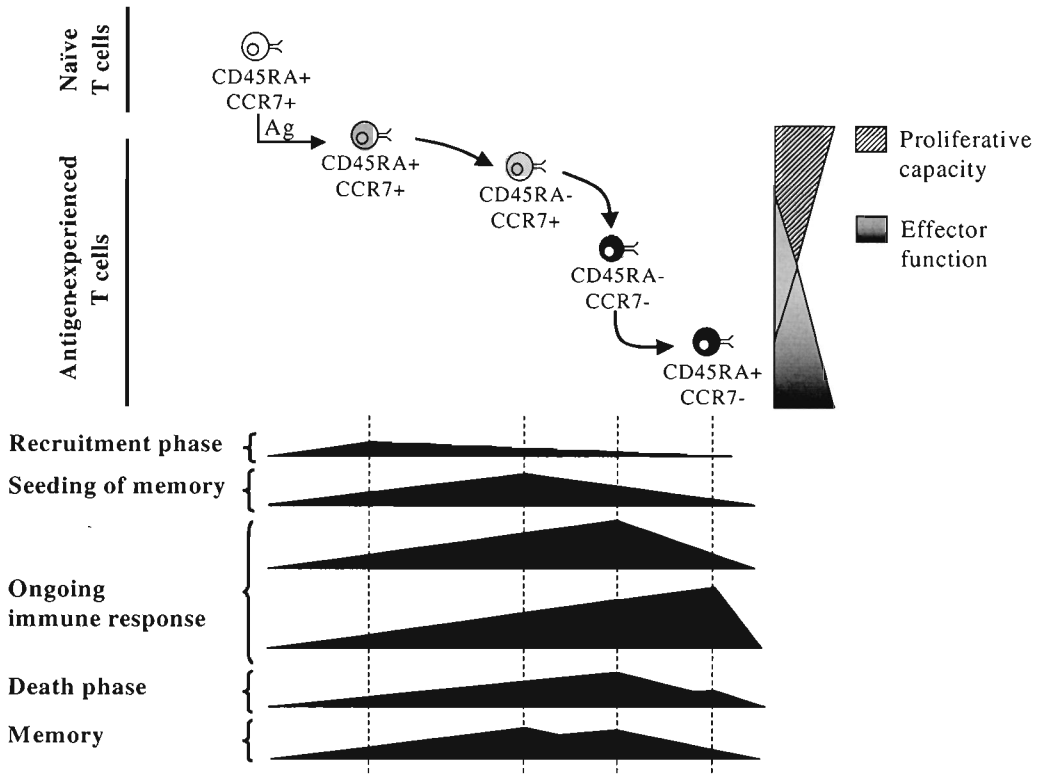


Figure 10. Heterogeneity of the T cell pool. *The differentiation of antigen specific T cells results in a heterogenous population of T cells characterized by different proliferative capacity and effector function. Antigen-specific T lymphocytes that are endowed with high proliferative capacity and share phenotypic markers ($CD45RA^{+}CCR7^{+}$) with naïve cells are first recruited into the response. Central memory (Tcm) and effector memory (Tem) T cell pools displaying differential phenotypic ($CD45RA^{-}CCR7^{+}$ and $CD45RA^{-}CCR7^{-}$, respectively), functional and homing properties are formed. Differentiated functional effector T lymphocytes are also generated ($CD45RA^{+}CCR7^{-}$). The prevalence of antigen-specific T cells from distinct antigen-experienced subset is hypothesized to evolve throughout the progression of immune responses and may vary due to the nature of the pathogen, the level of antigenemia and the immune (co-stimulation, cytokines) environment. Memory compartments are seeded during the immune response and persist following resolution of detectable antigenemia.*

The subset of CD45RA$^+$CCR7$^+$ CD8 T cells encompasses a population of antigen-experienced, antigen-expanded T cells endowed with strong proliferation potential

The antigen-specific CD45RA$^+$CCR7$^+$ CD8 T cell subset originally identified in Chapter 3 is of particular interest because these cells express a combination of markers normally associated with naïve T cells[430,792]. Notably, Zippelius and colleagues have recently published a thorough characterization of the phenotype, TREC measurement and telomere length parameters of the CD45RA$^+$CCR7$^+$ CD8 T cells specific of an HLA-A2-restricted self-antigen and demonstrated the close relationship of this subset with naïve T cells[845]. Nevertheless, one must be careful before projecting the conclusions of these findings to CD8 T cell populations that are specific of foreign antigen, such as is the case here, and assuming that HIV-specific CD45RA$^+$CCR7$^+$ T cells represent a pure naïve subset. In fact, the significantly large proportion of HIV-specific CD8 T cells bearing this phenotype (nearly 20% of the antigen-specific CD8 T cells) and observed in Chapters 3 and 4 strongly suggests that these cells are antigen-experienced and that they have been driven to clonal expansion[795].

The probability that HIV-specific CCR7$^+$CD45RA$^+$ T cells may not be naïve could be tested using a four-part strategy similar, but expanded from, the approach used by Zippelius and colleagues. This strategy would also entail the characterization of sorted antigen-specific CD8 T cells of this phenotype and a comparison with certified naïve cells such as those isolated from cord blood. First, the analysis of phenotypic markers should be extended beyond CD45RA, CCR7 and CD62L to include other naïve cell-associated markers such as CD27, CD28, and CD31 as indicators that are neither sufficient nor necessary criteria for exclusion from the "truly" naïve subset of T cells[248,846]. Second, quantification of TRECs within antigen-experienced CD45RA$^+$CCR7$^+$ CD8 T cells should not reveal a significant enrichment of these TCR recombination by-products, thereby suggesting that TRECs have been diluted by T cell proliferation[167,168]. Importantly, alone, detection of an enrichment of TRECs within this T cell subset would only suggest a close lineage relationship to naïve cells. Third, measurement of the telomere length of these HIV-specific CD8 T cells should reveal that they are shorter than

that of truly naïve cells[461,847-850]. Here, interpretation of the results would require that the potentially confounding effect of the activation of telomerase activity, which takes place following the specific TCR engagement, be accounted for[851-853]. Furthermore, telomere length-measurement assays may not be sufficiently sensitive in order to distinguish genuine naïve cells from their recently recruited counterparts. Finally, the molecular characterization of the TCR repertoire of these antigen-specific cells would be expected to reveal a strong bias and perhaps contain a single, or few highly represented clonotypes (monoclonal or oligoclonal T cell expansion), in a proportion significantly discordant from what may have been expected when considering the number of TRECs identified therein.

Our studies provide a distinct line of evidence that pre-empts the assumption that the numerous HIV-specific CD45RA$^+$CCR7$^+$ CD8 T cells detected in peripheral blood and lymph nodes might represent truly naïve cells. In fact, results reported in Chapters 3 and 5 strongly suggest that they are indeed expanded in an antigen-dependant manner. Interestingly, stimulations conducted *in vitro* and presented in Chapter 3 suggest that cells of this phenotype may be regenerated; whether they will exhibit the same properties as those identified *in vivo* is uncertain[795]. Critically, in Chapter 5, the successfully maintained reduction of HIV-1 viremia to levels below 50 RNA copies per millilitre of blood after prolonged HAART chemotherapy (72 months) results in the resorption of the HIV-specific CCR7$^+$ T cell population to below the threshold of detection attained by tetrameric MHC reagents. Because the frequency of cells bearing this phenotype falls below the threshold of detection of our experimental approach, it remains to be determined whether the cell subset of recently recruited CD45RA$^+$CCR7$^+$ CD8 T lymphocytes exists solely as a result of an ongoing antigen-specific recruitment of T cells or whether a minor population of these cells persists in the absence of antigen. Finally, no matter their phenotype, HIV-specific CD8 T cells are seldom observed in HIV-1 uninfected subjects, pre-empting speculation as to our ability to document a detectable frequency of HIV-specific naïve T cells.

Altogether, the attributes of the antigen-specific CD45RA$^+$CCR7$^+$ CD8 T cell subset during HIV-1 infection is that it contains a significant proportion of cycling cells *in vivo*,

that it can be stimulated to rapidly proliferate following stimulation *in vitro*, that cells from it have the capacity to secrete IFN-γ and perforin, and that cells from this subset can differentiate into the various memory CD8 T cell subsets: it therefore constitutes a memory cell precursor with limited effector function and a high proliferative capacity. Several questions remain with regards to this antiviral antigen-specific $CD45RA^{+}CCR7^{+}$ CD8 T cells, which represent some 20% of HIV-specific CD8 T lymphocytes during chronic HIV-1 infection. Are these "pre-memory" cells already committed to become memory? Is a dichotomy of memory T cells already established by this maturation stage? Is their further differentiation antigen-dependent? In Chapter 3, the *in vitro* peptide-specific stimulation of $CD45RA^{-}CCR7^{-}$ CD8 T cells did result in the appearance of a $CD45RA^{+}CCR7^{+}$ revertant population. Some of the HIV-specific $CD45RA^{+}CCR7^{+}$ CD8 T cells present in PBMC during HIV-1 infection can readily secrete cytokines upon stimulation and have granules of perforin: are they the result of a similar phenotypic reversion having occurred *in vivo*? Could cells of this phenotype be treated differently in order for their differentiation to be preferentially directed for arrest at a specific maturation stage? The further dissection of this $CD45RA^{+}CCR7^{+}$ CD8 T cell subset using other phenotypic markers known to enrich for naïve cells in strategies that keep track of functions associated with memory or effector status will shed light on the nature of this subset.

Central memory, Effector memory and terminally differentiated effector CD8 T cells in the context of HIV-1 infection

Antigen-specific CD8 T cells bearing the phenotypic properties of Tcm and Tem cells were observed during the ongoing immune response to HIV-1[792,795]. While the Tcm subset represented some 5% of the HIV-specific CD8 T cells, the Tem subset represented around 70% of the HIV-1 epitope-specific CD8 T lymphocytes in blood. Proof of the presence of these memory T cell subsets during an ongoing immune response provides insight into the mechanisms that underlie the generation of T cell memory and suggests a possible role for persisting antigen in the modulation of T cell-mediated immunologic memory, as will be discussed.

A subset of antigen-specific CD8 T cells expressing CD45RA, but lacking CCR7 expression, was also identified. This phenotypic subset significantly, but incompletely overlaps the previously described $CD45RA^{+}CD27^{-}$ T cell subset (K. Ellefsen, P. Champagne, and G. Pantaleo, unpublished observations)[854,855]. This effector subset is functionally potent, as it effectively produces cytokine mediators and possesses high exocytic granule stores of perforin[856]. Our *in vitro* attempts failed to induce the proliferation or differentiation of $CD45RA^{+}CCR7^{-}$ CD8 T cells when stimulated in an antigen-specific manner in the presence of feeder PBMC and exogenous IL-2. Furthermore, these cells did not respond under polyclonal stimulation conditions when plate-bound anti-CD3 purified antibodies were used in the presence or absence of soluble anti-CD28, and whether or not exogenous IL-2 was added. Altogether, this subset of antigen-specific CD8 T cells behaves as terminally differentiated[795]. It will nevertheless be of interest to further attempt to manipulate this terminally differentiated subset by subjecting it to various cytokines, candidates including IL-2, IL-4, IL-7, and IL-15, or to a pool of these cytokines. Furthermore, the use of mature dendritic cells as feeders may rescue these lymphocytes' potential to differentiate by providing co-stimulatory signals other than CD28 engagement.

7.6. Skewed maturation of HIV-specific memory CD8 T lymphocytes

7.6.1. HIV-specific memory CD8 T lymphocyte distributions in blood

The characterization of antigen-specific CD8 T cells within the peripheral blood compartment of chronically HIV-1 and CMV co-infected patients allowed for two significant observations. First, CMV-specific CD8 T cells belonging to each of the four memory CD45RA/CCR7 CD8 T cell subsets are also present during chronic CMV infection, and second, a skewed distribution of HIV-specific CD8 T cells as compared to that of CMV specific cells is readily observable. Whereas CD8 T cells specific for epitopes of either viral agent were often present to similar frequency within the PBMC of a same patient, phenotypic characterization of the cells revealed significant qualitative

differences within the heterogeneity of each specific cell pool. Whereas only 5% of the CMV specific cells were of the $CD45RA^{+}CCR7^{+}$ subset, some 20% of HIV-specific cells bore this phenotypic combination. Respectively, 90% and 75% of the CMV and HIV-specific CD8 T cells did not express CCR7: the majority of these antigen-specific cells therefore belonging either to the Tem ($CD45RA^{-}CCR7^{-}$) or terminally differentiated ($CD45RA^{+}CCR7^{-}$) CD8 effector T cell subsets. Significantly, whereas nearly 50% of CMV-specific cells belonged to the terminally differentiated effector subset, only ~5% of HIV-specific cells were $CD45RA^{+}CCR7^{-}$[795]. The HIV-specific pool of CD8 T cells was observed to consist overwhelmingly (~70%) of Tem cells, while some 40% of CMV-specific cells were Tem.

Few studies published until now have made use of the CCR7 marker, mostly due to its recent availability: because CCR7 expression correlates with that of CD62L, studies making use of either marker form a sound reference base to better understand the skewed distribution of HIV-specific memory CD8 T cells[792,854]. However, it is important to note that, just as with the CD27 marker, neither of these molecules is a perfect phenotypic surrogate of the others: CCR7, CD27 and CD62L are not always co-expressed[854,857]. Nevertheless, expanding our consideration to studies having used CD62L or CD27 where we have used CCR7, several published reports corroborate our observation that the distribution of HIV-specific CD8 T cells in blood differs significantly from that of CD8 responses directed against CMV[756,858,859]. Furthermore in accord with work published by other groups, our unpublished observations also suggest that the distribution of memory CD8 T lymphocytes specific to other viruses, including Epstein-Barr virus (EBV) during chronic infection and influenza in healthy subjects, resembles that directed against CMV; but differs from the distribution of HIV-specific cells, when memory T cells are tracked by their expression of CD45RA and CCR7. The phenotypic dissection of the virus-specific T cell responses directed against HIV-1, CMV, EBV and influenza, as well as tumour-specific $CD8^{+}$ T cells, suggests that immune responses may yield very different distributions of responding and memory cells[756,795,857,859-863]. This diversity has further been dissected recently with respects to CD27 and CD28 phenotypes when HIV-1, CMV, EBV and HCV are compared[855].

7.6.2. HIV-specific memory CD8 T lymphocyte distributions in lymph nodes

As presented in Chapter 4, $CCR7^-$ effector CD8 T cells were significantly enriched in the lymph nodes of HIV-1 infected patients as compared to the lymph nodes of normal subjects. Particularly, Tet^+ HIV-specific CD8 T cells were present in the lymph node biopsies as in blood. Furthermore, on the basis of the functional assays performed using HIV-specific peptide stimulation of cells from either the lymph node or blood of HIV-1 infected individuals, HIV-specific IFN-γ producing cells can be induced from both anatomic compartments (Chapter 4)[778,864].

Interestingly, using our experimental approach to analyse the distribution of HIV-specific CD8 T cells didn't reveal a significant preferential accumulation of HIV-specific cells within or away from lymph nodes. These findings nevertheless contrast with the recent observation that, using a significantly more diverse panel of epitopes and larger number of patient reactivities in an ELISpot-based approach, the overall proportion of HIV-specific IFN-γ secreting cells in lymph nodes is larger than that in the blood of chronically-infected patients[724]. In perspective, it is difficult to assess the significance of the differences observed between the present cohort of patients and the results published by Altfeld *et al.*. Admittedly, the restrictive bias introduced in the present characterization, as compared to the extensive testing performed by Altfeld *et al.*, calls for consideration when interpreting the data presented here in Chapter 4. Beyond a potential sampling error, possible explanations for a discrepancy in the amount of effector activity present in mononuclear cells from blood as compared to from lymph nodes could eventually be a result of biases in the maturation of the HIV-specific CD8 T lymphocytes or the preferential homing of HIV-specific CD8 T cells at different stages of maturation towards or away from lymph nodes.

7.6.3. Memory CD8 T cell maturation in the context of pediatric HIV-1 infection

Practical elements greatly restrain investigators' ability to perform the original comprehensive evaluation of pediatric immune responses as we chose to undertake in

adults with chronic HIV-1 infection in Chapter 3 and extended in Chapter 4. These practical limitations pertain mainly to the availability of pediatric blood samples and the limited quantity that can conceivably be obtained. Nevertheless, our knowledge that the characteristics of pediatric immune responses to HIV-1 parallel those of adult subjects suggests that our findings will also shed light on the attributes of antiviral pediatric immune responses.

The FACS-based characterization of the antigen-specific CD8 T cells from pediatric subjects on the basis of CCR7 expression and CD45 isotype will be of interest. Significantly, recent results by Scott *et al.* suggest that the phenomena uncovered in adult HIV-1 infection and presented in Chapters 3 and 4 may also be characteristic of the pediatric condition. Using ELISpot assays in order to screen for CMV and HIV-specific T cell responses in HIV-1 infected children 1-23 months of age, CMV specific responses were consistently observed in all CMV-infected children while HIV-specific responses were infrequently detected[577]. Furthermore, whereas the stable reduction of HIV-1 antigenemia with HAART resulted in the disappearance of previously observed HIV-specific responses in pediatric subjects, CMV specific responses persisted following successful resolution of HIV-1 viremia[577]. We have already made similar observations in HAART-treated chronically HIV-1 infected adults[589]. In conjunction with the functional data already available, these observations suggest the possibility that a similarly skewed distribution of memory HIV specific CD8 T cells will also be characteristic of pediatric responses. How do antigen memory CD8 T cell responses evolve in children and infants? Cross-sectional and longitudinal studies of virally infected pediatric subjects, including subjects vertically infected by HIV-1 at various stages of disease and those exposed to the virus yet uninfected, should be undertaken.

7.7. Potential mechanisms underlying the skewed maturation of HIV-specific memory CD8 T lymphocytes

During a protracted virus *vs.* host interaction, as is the case here for both HIV-1 and CMV infections, multiple parameters may influence the resulting distribution of cells between

different memory subtypes. These include the antigen load, the nature of the antigen, the nature of the infection, as well as the site of antigen production and the site of immune response [865-867]. In the context of chronic HIV-1 infection, the destruction of the CD4 T cell compartment and the generally reduced HIV-specific CD4 T cell help can be hypothesized to be a significant driving force behind the observed skewed distribution of HIV-specific CD8 T cells, as compared to CMV-specific CD8 T cells.

The observed skewed maturation of HIV-specific CD8 T lymphocytes may be the result of the preferential deletion of terminally differentiated effectors and/or of a relative or absolute enrichment of the Tem subset. Interestingly, the observation that there is increased apoptosis of T lymphocytes in the context of HIV-1 infection has recently been refined with the demonstration that HIV-specific CD8 T lymphocytes have an increased propensity for Fas-induced apoptosis as compared to CMV- or EBV-specific CD8 T cells within a same donor; a phenomenon that is not simply related to the level of Fas surface expression[756]. This critical finding concretely demonstrates distinct properties of the different antigen-specific CD8 T cell pools that may pertain to their differentiation status. Finally, the observed relative enrichment of the HIV-specific pre-memory $CD45RA^{+}CCR7^{+}$ subset further suggests an increased turnover associated with the ongoing immune response to HIV-1 as compared to CMV.

What mechanisms may underlie these phenomena? Altogether any parameter normally known to influence the formation and maintenance of T cell immunologic memory, including but not restricted to CD4 T cell help, may be hypothesized to play a modulatory role on the antigen-specific memory T cell pools and could result in a skewed T cell maturation. The contributing parameters may be divided into those that result directly from the peptide-MHC/TCR interaction and those that result from the environmental modulation of this interaction. This latter subset includes co-stimulatory molecules and the cytokine environment.

7.7.1. Focusing on the peptide/MHC-TCR interaction: affinity-based selection of memory T cells

The central role of the peptide-MHC/TCR interaction in the generation of T cell memory is highlighted by the antigen-specific nature of T cell memory. Here, as during the recruitment of antigen-specific effector responses, the clonal expansion of antigen-specific T lymphocytes results in the amplification of a pool of memory T cells primarily selected on the physicochemical properties of their TCRs. Using the murine P815 tumour transplant system, Maryanski and colleagues observed little difference between the primary and secondary immune response T cell repertoires raised against the tumours[868]. Sourdive *et al.* studied the LCMV-specific TCR repertoires involved in both primary and secondary responses[869]: through molecular analysis, they established that the TCR repertoire was conserved for the memory response as measured by TCR Vβ family usage and βchain CDR3 length analysis. These results suggest that the selection of CD8 T cells into the memory pool is a stochastic process that is not evidently biased by the affinity of the TCR for the specific antigen-MHC.

Nevertheless, a favoured observation is that the recruitment of memory T cells sometimes results in the selection of a narrower subset of specific T cells than that which is first recruited into the primary response[870]. Recent *in vivo* evidence suggests that persisting memory T cell populations have a greater affinity for the specific peptide/MHC ligand compared to the naïve population. Using a murine model, Busch *et al.* observed a narrowing of the TCR repertoire during the secondary response to *Listeria monocytogenes*, an intracellular microbial pathogen[871]. The cells mediating this secondary response were more sensitive to antigen in functional assays and were characterized by an overall higher affinity for peptide/MHC complexes as measured by class I MHC tetramer- dissociation assays. Similarly, using a murine model studying the response to an I-E^K-restricted moth cytochrome C (MCC) epitope, Savage *et al.* observed a focusing of the secondary repertoire resulting from the loss of cells expressing TCRs which have the fastest dissociation rates from their specific-peptide/MHC[872]. Studying the response to pigeon cytochrome C (PCC), McHeyzer-Williams *et al.* also described the

selection of T cells bearing TCRs with CDR3 sequences having the greatest specificity or the "best fit" for the antigen during the secondary response[217,219]. The result of a primary immune response therefore appears to be a preferential selection of high affinity T cells into the memory pool, a process defining the "T cell affinity maturation" hypothesis. The key outcome of this process is the recruitment of a greater frequency of high affinity T cells during secondary responses, in part explaining the speed and potency of the memory response.

Immune responses raised against different pathogens differ in the kinetics with which they take place. The apparently conflicting results thus far published with regards to the affinity-based selection of responding and memory T cell populations may in fact reflect the dynamic nature of memory T cell selection. Time-course analyses of the responding repertoires may yield a better understanding of the dynamic processes at hand.

How and why could high affinity T cells be preferentially selected to become memory over the pool of responding cells?

A possible explanation may be that high affinity T cell clones are activated at much lower concentrations of antigen, allowing them to proliferate earlier during the primary response and to rapidly become prevalent over low affinity T cells (A.D., Lavoie, PM, and R.P.S., unpublished results). Such a kinetic advantage could favour, in a purely statistical manner, their selection to become the predominant memory cells in a model of stochastic selection from a pool of specific T cells. Indeed, it has been shown that the contribution of a T cell clone to the immune response is mostly determined by the time of first cell division following antigen encounter[873].

High affinity T cell clones may alternatively receive a qualitatively different signal compared to low affinity clones. As presented in section 1.1.10, in the context of a kinetic model of T cell activation, the duration of the interaction between TCR and peptide-MHC determines the outcome for the T cell. The half-life of this interaction must be long enough to allow TCR cluster formation in order to transduce a complete activation

signal[277]. However, it has been demonstrated that too long a half-life can impair T cell activation, probably by interfering with the serial triggering process[270]. The kinetics of peptide/MHC-TCR interactions determines whether the threshold for activation of different intracellular signalling pathways is reached and thereby direct the scope of the effector response mobilized[329]. Differences in the quality of the signal received by the low versus high affinity T cells may therefore be hypothesized to drive the development of memory cells or to influence their maturation.

Little is known about the properties of the TCR repertoire of virus-specific lymphocytes in humans. Additionally, the TCR repertoire of the antigen-specific memory CD8 T lymphocytes recruited within each of the CD45RA/CCR7 subsets has yet to be characterized. Two extreme scenarios may be envisaged, the probability being that a composite image will be observed. The spectrum would then lie between 1) TCR clonotypes being restricted to each maturation subset and 2) observed clonotypes being present within all subsets. While the first scenario would suggest the parallel maturation of the specific T cells and a dominant role for the affinity of the specific-TCR engagement, the second, favoured scenario would implicate a role for the environmental modulation of this selection.

Could the skewed maturation of memory T cells be the result of differential peptide-MHC/TCR affinity?

A first corollary of the hypothesis that peptide-MHC/TCR affinity influences the maturation of memory T lymphocytes is that the diversity of the selected memory population is peptide-dependant. In the context of the studies presented in Chapters 3 and 4, we have had the opportunity to study subjects presenting strong CD8 T cell immune responses to more than one HIV-1 derived epitopes; for each of these responses, the involved CD8 T cells can reasonably be expected to differ in their affinity for their respective ligands, although these measurements need to be performed in order to experimentally confirm this affirmation. No biased trends were observed in the distribution of the various antigen-specific memory CD8 T cells between memory subsets

when Tet^+ cells were separately analyzed with respect to the epitopes they recognized or the MHC context of these epitopes. Nevertheless, a more significant number of these multi-epitope responders need to be characterized. Furthermore, it will be interesting to compare the TCR repertoire of lymphocytes responding to the various defined peptide epitopes in order to analyse the breadth of each response and, ideally, to assess a potential role for affinity.

A second corollary of the hypothesis that peptide-MHC/TCR affinity influences the maturation of memory T lymphocytes is that the diversity of the selected memory population will differ between individuals based on their pre-infection TCR repertoire and immunological experience. In various individuals, qualitatively different breadths of TCR repertoires may characterize the specific CD8 T cell response to HIV-1 derived epitopes; parameters of potential impact being the age of the patient at the time of infection, and the length of time that a subject has been infected by HIV-1. The recent observation that preferred target epitopes of immunodeficiency virus-specific CD8 T cells evolve as of acute infection and that antiviral-specificities are lost early in infection may suggest affinity-based deletion of CD8 T cell clonotypes[777,810]. A longitudinal study making use of an original approach based on peptide-MHC tetramer competition assays could allow for assessment of the affinity characteristics of the pool of HIV-specific CD8 T cells in humans throughout the course of disease progression and evidence a role for TCR affinity in clonal exhaustion. In a subsequent step, such an assay could also be used in order to unveil a role for TCR affinity in the maturation of antigen-specific CD8 T cells.

Finally, it has been demonstrated that T cells compete for access to antigen presenting cells; this competition for a limited number of antigen/MHC complexes can influence the repertoire selection by favouring high affinity T cells[874,875]. This selection process would be particularly relevant when the number of specific antigen/MHC at the surface of APCs is low, as suggested by *in vivo* results showing that the average TCR affinity of the memory population is an inverse function of antigen dose during the primary response[876]. Competition for a limited number of surface interactions may also underlie the

establishment of immunodominance during the immune response[877]. A role for such competition has therefore also been hypothesized to be that it results in the functional diversification of the responding T cells[878]. For now, the impact of HIV-1 infection on the competition of T cells for APCs can only be presumed. During primary HIV-1 infection, could the exceptionally elevated viral antigenemia result in the elimination of HIV-specific CD8 T cell responses by an overwhelming effective avidity? Throughout the natural course of HIV-1 disease progression, during the chronic phase, HIV-1 antigenemia is not expected to be a limiting factor; nevertheless, the systematic destruction of secondary lymphoid organ structures as well as the the virus' tropism for the APCs, macrophages and dendritic cells, can be foreseen to have a potential pathologic impact on the differentiation of memory HIV-specific T cells.

7.7.2. Beyond peptide/MHC and TCR: environmental modulators of the maturation of memory T cell

Although primary selection of T cell clones that will form T cell memory is based on its peptide/MHC specificity, the immediate environment where the recruitment takes place may also modulate the selection. Perhaps the simplest illustration that peptide/MHC-TCR interactions do not solely govern the generation of memory lies in that not all T cells of TCR-transgenic mice develop into memory following *in vivo* challenge with their specific antigen[823,879]. Despite having originated from a unique precursor, not all progeny of a stimulated antigen-specific clone are exposed to identical microenvironments: subjected to different stimuli, some perhaps more adequate to the evolution of memory, T cells mature differentially. Critically, "helper" CD4 T cells are key players in modulating the immune environment, both directly and indirectly, and HIV-specific CD4 T cells are specifically impaired during HIV-1 infection. This may perhaps result in a vulnerability of HIV-specific CD8 T cells to a diverted or incomplete maturation: the virus-specific restriction of the altered memory CD8 T cell differentiation pattern could then be explained if antigen-specific CD4 T cell help is naturally more efficient than help obtained randomly from bystander CD4 T cells.

Contributing cell-cell interactions

From a molecular perspective, a first set of interactions to be considered are those taking place within the immunological synapse, the area of physical contact between the T cell and its APC where the specific signal first originates[300,301]. Beyond interaction between peptide/MHC and TCR, selection of memory T lymphocytes may result from specific signals transduced through accessory molecules at the synapse. Alternatively, selection may result from an increase of the overall avidity of the T cell for the APC, allowing for an enhancement of the TCR-mediated signal and/or compensating for a lower peptide/MHC-TCR affinity/avidity. T cell accessory molecules involved may include CD2 and LFA-1, as well as members of the expanding family of co-stimulatory molecules including CD28, CTLA-4, ICOS, and PD-1[304,880-882]. Some of these interactions are known to play roles in the recruitment of naïve T cells and the regulation of activated T cells: they may also play a role in directing the maturation and maintenance of memory T cells. In the context of HIV-1 infection, an enrichment of CD28-negative cells has been repeatedly observed and defects in the expression of the ζ-chain of the TCR has also been detailed: these maybe hypothesized to play a role in the phenomena at hand[756,785,786,883,884].

Contributing soluble mediators

Beyond direct cell-cell interactions, several groups have highlighted the important regulatory role of soluble mediators in the generation and maintenance of memory T cells. Cytokines have pleiotropic effects on T cells: IL-2, IL-7 and IL-15 are now under much scrutiny. The receptors for these cytokines have the particularity of all using the common γ chain[885-888]. The functional receptors for IL-2 and IL-15 also share the β chain, fine specificity being defined by the further expression of private α chains. Signaling from the IL-2R and IL-15R has so far only been associated with the β and γ chain components they share. In spite of this, engagement of these receptors leads to different outcomes. In fact, while IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ mice suffer from lymphadenopathy and autoimmunity, IL-$15R\alpha^{-/-}$ mice display lymphopenia and a selective reduction of memory

phenotype $CD8^+$ T cells. IL-2 has been linked to T cell survival and proliferative expansion early in an immune response, however IL-2 is also required later on for triggering the death pathway[889]. IL-$2^{-/-}$ mice and IL-$2R\alpha^{-/-}$ mice show accumulations of activated T cells associated with reduced AICD[890-893]. In contrast, both IL-$15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice show a 2-3 fold decrease in the number of CD8 T cells with a $CD44^{hi}$ memory phenotype[894,895]. Moreover, IL-15 transgenic mice have 3 times more memory cells compared to syngeneic wild-type mice[896,897]. In fact, using combinations of blocking antibodies specific for IL-2, IL-2Rα and IL-2Rβ, Ku *et al.* demonstrated that, whereas IL-15 drives the slow proliferation of memory $CD8^+$ T cells *in vivo*, this proliferation is increased by the inhibition of IL-2[898]. This increase is attributed both to an enhanced proliferation of the memory cells and to reduced AICD. Finally, IL-2 and IL-15 have been shown to differentially modulate both of the recruitment and of the maturation of memory CD8 T lymphocytes, as previously discussed[829].

Another cytokine, IL-7, appears to be necessary for the establishment of T cell memory and may play a role in the maintenance of memory T cell homeostasis[899]. Blocking of IL-7 and IL-7R appeared to have only a modest effect on memory $CD8^+$ T cell proliferation[898]. Nevertheless, IL-7 is also linked to memory T cell homeostasis. IL-$7R^{-/-}$ CD8 T cells transferred into non-irradiated wild type recipient mice were shown to proliferate at levels comparable to that of w.t. cells following subsequent in vivo stimulation. However, the survival of the IL-$7R^{-/-}$ CD8 T cells was greatly impaired as compared to their wild-type counterpart as the former failed to persist in large numbers[899]. Interestingly, this impairment in survival of the IL-$7^{-/-}$ CD8 T cells was correlated with a failure to re-express Bcl-2 to normal levels following the peak of proliferation associated with the antigen-driven response[899].

Most importantly, a combination of IL-7 and IL-15 has been shown to efficiently homeostatically expand $CD45RO^+CCR7^-$ Tem cells, but to have a lesser effect on $CD45RO^-CCR7^+$Tcm cells[888]. Moreover, *in vitro* treatment of sorted $CD45RA^+CCR7^+$ Tcm cells for 7 days with a pool of cytokines consisting of IL-7, IL-15, TNF-α, IL-6, and IL-10 was shown to induce their proliferation and differentiation to $CD45RA^-CCR7^-$

Tem-like cells. These cytokine-induced effects on T lymphocytes were observed without TCR-engagement[888]. Interestingly, increased IL-7 expression has been documented in HIV-1 infection and is thought to influence T cell turnover in CD4 lymphopenic HIV-1 infected patients[900]. It is therefore tempting to hypothesize that the cytokine imbalance that characterizes HIV-1 infection could result in a differential modulation of the homeostasis of memory CD8 T cells. The state of activation of HIV-specific CD8 T lymphocytes could possibly further subject them to a bias in their maturation pathway.

7.8. What is the significance of the skewed maturation of HIV-specific CD8 T cells during chronic HIV-infection?

Its significance pertains to a number of immunologic phenomena. As discussed in 1.1.13, immune responses and immunologic memory are orchestrated to a significant extent within the specialized environment of the lymph node, where naïve and memory T cells are first brought into contact with antigen. Infection by pathogens that localize to peripheral anatomic sites are characterized by the recruitment of a T cell response within secondary lymphoid organs that is followed by the selective migration of effectors to the tertiary peripheral immune sites. While HIV-1 does replicate in the immunologic "periphery", secondary lymphoid organs are a primary site for HIV-1 replication: during HIV-1 infection, the lymph node therefore constitutes a site of virus-specific CD8 T cell recruitment and a site of intense anti-HIV-1 immune response.

The characterization of HIV-specific CD8 T cell immune responses within the lymph nodes of chronically infected patients is therefore critical to further understanding the skewed maturation of HIV-specific memory CD8 T lymphocytes. In Chapter 4, the primary characterization of the distribution of HIV-specific CD8 T cells using CCR7 and CD45RA revealed that these distributions were very similar in the blood and lymph nodes of same patients. These results exclude the possibility that the skewed maturation of HIV-specific memory CD8 T lymphocytes in blood is the result of biased homing of the $CD45RA^{+}CCR7^{-}$ terminally differentiated HIV-specific CD8 effector cells away from blood and towards lymph nodes[901]. Furthermore, ongoing studies undertaken to similarly

characterize CMV specific CD8 memory T cell subsets and responses in blood and lymph nodes will further dissect the mechanisms of memory T cell maturation. Critically, the comparison of HIV-1 and CMV specific memory CD8 T cell distribution in both blood and lymph node will further substantiate whether the skewed maturation of peripheral memory CD8 T cells which has been described is a phenomenon that is centrally shaped or whether it is peripherally induced. Finally, it will contribute to clarify whether the observed distribution of HIV-specific memory CD8 T cells represents a normal or pathologic aspect of the antiviral response.

7.9. Towards the comprehensive delineation of an effective repertoire of antigen-specific CD8 T lymphocytes

The studies presented herein sought to characterize immune responses to HIV-1 in order to better understand HIV-1 pathology and identify parameters with the potential to serve as immunologic reference points in the monitoring of disease progression, in the elaboration of therapeutic approaches and in the evaluation of potential vaccination strategies.

The following reflections and proposed experimental approaches may also be of interest in order to guide future forays in the characterization of memory cells and their maturation within healthy individuals and HIV-1 infected subjects:

i. With regards to the molecular characterization of TCR repertoires. The methods described in Chapter 2 could be improved by using high-throughput sequencing to increase the number of clones to be analyzed. Subsequently, a diversity-specific real-time PCR assay based on the quantification of selected genomic DNA βTCR-chain amplicons could be performed. The β-chain TCR repertoire of antigen-specific cells should be assessed so as to compare the diversity of clonotypes present within each of the maturation subsets we have identified in peripheral blood. Furthermore, the direct *in vivo* demonstration that distinct memory subsets home peripherally as compared to centrally in mice[793,794], and our *ex vivo* results

in Chapter 4 pose the question of whether the antigen-specific TCR repertoire of lymph nodes overlaps that of blood. This may now be investigated, both for bulk HIV-specific CD8 T cells as identified by tetrameric MHC complexes in lymph node and blood, and for the various memory T cell subsets within these distinct anatomic pools.

ii. Considering the number of hypothesized αβ chain pairings[173], a pending question with regards to the study of β-chain TCR repertoires is whether an amplified βTCR-chain represents a single T cell clonotype, or a family of T cell clones with distinct α chains. For investigations that require the identification of the α and β chains of the TCR expressed by a single T cell, individual antigen-specific CD8 T cells could be separately FACS-sorted: an antigen-specific αβTCR repertoire could thus be characterized for the first time.

iii. Are antigen-specific CD8 T cells selected to mature differentially into the various memory T cell subsets on the basis of the affinity of their TCR for their cognate peptide-MHC ligands? The parameter of TCR affinity may underlie the establishment of efficient T cell memory. Experimentally, this could be assessed in cross-sectional samples using a novel approach modified from the tetramer competition assay that has already been used by several groups[871,902,903]. This assay determines the affinity of a heterogeneous population of T cell receptors by monitoring the kinetics with which a fluorochrome-labeled MHC tetramer can be competed-off by an unlabelled ligand or blocking antibody. The net result is that antigen-specific tetramer labeling is gradually lost from the various T cells of the pool in a sequence that should mostly parallel the increasing affinity of the TCR-MHC interaction. By arresting competition at set increments of time and using a staining strategy for phenotypic markers of maturation, the putative relation between TCR affinity and the maturation stage of memory CD8 T lymphocytes could be explored. Furthermore, a similar characterization of longitudinal samples within a same individual would offer a dynamic view of memory CD8 T cell maturation through time.

iv. Pathologic mechanisms such as the clonal exhaustion of HIV-specific CD8 T cells may also result from an affinity-based deletion process. By performing similar arrested-competition assays at set increments of time and FACS-sorting the cells still labeled by MHC tetramers, sorted populations of CD8 T lymphocytes will have been selected on the basis of the affinity of their TCR affinity for the peptide-MHC of the tetrameric complexes. Molecularly cloning and sequencing the βTCR chains of tetramer-positive cell populations sorted in this fashion will then allow for the characterization of the TCR repertoire of CD8 T cell clones typified by similar TCR affinity; albeit, a range of affinities because the number of TCRs on the surface may vary and this ought to be taken into account. First, the extensive characterization of the TCR repertoire of a pool of CD8 T cells specific for a peptide-MHC tetramer at a given point in time during the natural course of disease progression would establish a comprehensive relative *ex vivo* frequency of specific clones. Thereafter, a similar characterization undertaken for samples of the same time point subjected to various extents of competition would reveal a gradient where clonotypes of lesser affinity are lost from detection to a greater extent after less competition than clonotypes of higher affinity. A controlled proof of concept would certainly be required and an assessment of the realistic applicability of such an assay to clinical samples would be essential. Nevertheless, should this approach be feasible, its subsequent application to multiple time point samples in intra-patient longitudinal studies would provide insight into the mechanisms of clonal exhaustion.

v. With regards to the design of an experimental approach that seeks the comprehensive characterization of the repertoire of antigen-specific CD8 T cells. First, the fine antigen-specificity of the sub-population of CD8 T cells of interest could be assessed using multi-parametric assays. Ideally, this could be done using recently developed experimental approaches that would require limited *in vitro* manipulation and a reduced quantity of cells. This functional characterization could be undertaken using cytokine production measurement assays following *in*

vitro stimulation with pools of overlapping peptides derived from HIV-1 proteins[584,748,904-906]. ELISA, ELISpot, FACS-based cytokine bead arrays or intracellular cytokine assays could be used for these functional characterizations, although approaches that simultaneously assess a broad variety of effector functions should be preferred, at this stage. Observed responses elicited by a given pool of peptides could then be further dissected by performing a second round of functional assays using subgroups of the peptides from the original response-inducing pools, and distributed in partially-overlapping combinations that would allow for the fine epitope mapping of reactivities. While the same functional assay as first used could be applied again, the previous round of functional assays should have identified effector functions of particular interest: these should be targeted using more focused functional assays. Knowledge of the specific epitope responses present and of the subject's HLA type would then allow for the production and use of tetrameric MHC molecules to be used for single-cell surface molecule phenotyping, for the assessment of other cellular functions, or in order to sort out HIV-specific $CD8^+$ T cells whose TCR repertoire could then be characterized phenotypically and molecularly as mentioned above. Such a characterization could be performed in cross-sectional and longitudinal studies of natural infection, as well as in the assessment of immune responses elicited by a vaccine administered with either prophylactic or preventive intent.

7.10. Implications for disease prognosis, immune modulation and vaccine development

The phenotypic characterization of the TCR repertoire as was undertaken in Chapter 2 has already been shown to be of good prognostic value for progression to disease in adults infected with HIV-1. Furthermore, the recruitment of a broad TCR repertoire of CD8 lymphocytes has been associated with successful immune responses to other viral infections, including hepatitis B and C[907-913]. As such the induction of a broader diversity of a recruited antigen-specific TCR repertoire of CD8 T lymphocytes is a quality that should be sought in therapeutic and preventive anti-viral vaccines, including those

elaborated against HIV-1. The characterization of pediatric samples undertaken in Chapter 2 suggests that the pediatric immune system may respond much in the same way as that of adults; although kinetic parameters may differ, the qualitative features of the pediatric response may also serve in prognosis and vaccine development. Significantly, the characterization of the molecular TCR repertoire in Chapter 2 has revealed crucial differences in the response of the CD4 as compared to CD8 subset of T lymphocytes, dictating that immunologic parameters that will be useful for the evaluation of CD8 T cell responses may not be so for CD4 T cell responses.

To what extent does the differential modulation of antiviral CD8 T lymphocytes impact on their effectiveness in resolving infection and their ability to establish enduring control of virus? The characteristics shown to contribute to the successful resolution of a natural infection by a specific pathogen then should be sought in vaccine-induced responses. This ought to be taken in consideration in the design and evaluation of novel pathogen-customized vaccine strategies.

The observed skewed maturation of HIV-specific memory CD8 T lymphocytes should be further investigated and its ramifications remain to be determined. A pending question is: what extent is the immunologic phenomenon of the skewed maturation of HIV-specific memory CD8 T cells also a pathologic phenomenon of HIV-1 infection? Several potential lines of investigations have already been mentioned throughout this discussion. Furthermore:

i. With regards to the validity of comparing HIV-specific responses to that directed against other viruses including herpesviridae, hepadnaviridae and RNA viruses. Admittedly, the unique nature of each virus and the pathologies they induce are caveats that restrain the interpretation of the observation that these vary greatly. Nevertheless, the compound picture resulting from the eventual characterization of multiple different immune responses will allow for the best understanding of the phenomena at hand and, along with the data of others, the work presented in Chapters 3 and 4 pioneer this notion.

ii. There is a critical need to establish immune correlates of protection that may be used for prognosis of disease progression and to eventually assess the effectiveness of vaccines. As the maturation of antigen-specific CD8 T cells dictates their proliferative and functional properties, skewed maturation of CD8 T cells is expected to impact on the efficiency of the CD8 T cell-mediated immune responses. The significance of the skewed distribution of HIV-specific CD8 T cells remains to be assessed but it is now a promising avenue to explore in the quest to establish such correlates of protection. Here, the skewed maturation of HIV-specific memory CD8 T lymphocytes was established in a cross-sectional study of adults experiencing chronic HIV-1 infection. The characterization of well defined cohorts with distinct particularities will be of great interest; these include:

   a. HIV-1 infected individuals who are long-term non-progressors
   b. Individuals infected with attenuated strains of HIV-1
   c. HIV-1 exposed, yet persistently uninfected individuals

iii. Is the skewed maturation of HIV-specific CD8 T lymphocytes the result of persistent infection or a characteristic of HIV-specific responses that is established early in infection? The subjects studied in Chapters 3, 4 and 5 had been infected by HIV-1 for years prior to their characterization, the distribution of HIV-specific memory CD8 T cells was therefore observed following a protracted interaction between the virus and the host: this distribution may differ significantly from the response first recruited in acute infection. The cross-sectional characterization of the following types of cohorts will be informative to answer this question:

   a. Individuals experiencing acute primary HIV-1 infection
   b. Individuals experiencing primary HIV-1 infection
   c. Individuals experiencing early chronic HIV-1 infection

iv. How do antigen-specific memory CD8 T cell subsets evolve throughout the natural course of viral infection? Critically, the longitudinal follow up and characterization of subjects within the different cohorts listed in paragraphs ii and

iii will yield invaluable information as to the evolutionary dynamics of antigen-specific memory CD8 T cell responses throughout the course of the natural progression of HIV-1 disease.

v. Is the skewed maturation of HIV-specific CD8 T lymphocytes a reversible process? Should the distribution of HIV-specific CD8 T cells be demonstrated to influence the efficiency of the antiviral immune response and to have protective value against disease progression, then the answer to this question will be of utmost importance to indicate whether therapeutic vaccine strategies may be of eventual assistance in curbing disease progression. Informative clues to answer this question will be povided by the study of:

   a. HIV-1 infected individuals compliant with HAART regimens instated at different times following infection
   b. HIV-1 infected individuals who are non-compliant to HAART
   c. HIV-1 infected individuals who discontinue HAART following successful reduction of viremia. Significantly, scheduled treatment interruption (STI) studies offer unique opportunities to monitor the dynamics of memory T cell subsets through cycles of repeated antigenic exposures and withdrawal[617,618]. Is the skewed distribution observed the result of incessant challenge by elevated antigenemia? Is the reported control of viremia by patients on STI regimens associated with evidence of the modulation of their memory T cell subset distribution?

vii. Distinct memories? Memory T cells were once thought a homogenous population of quiescent cells that persisted after an infection and had the capacity to generate a rapid and enhanced effector response following antigen re-encounter. In fact, the generation and maintenance of immunologic memory is a dynamic process subject to modulation as of the moment of antigen-encounter: this nuance is critical as the rational manipulation of the immune system is attempted through therapeutic and prophylactic vaccination. Distinct stages of memory T cell maturation characterized by different trafficking and

functional properties have been identifed, and we have shown that the maturation of cells into these subsets may be differentially modulated. Subsets of memory T cells at distinct maturation stages may not only differ in functional and homing properties but may also differ in the conditions they require for generation and survival. As such, it is interesting to hypothesize that different memory T cell populations may result from the nature and timing of the signals provided by their environment, indicating that:

a. The immunological environment following exposure to antigen is critical as it dictates the pool of memory cells seeded and introduces the biases that confer set characteristics to the specific memory generated.
b. Distinct memory subsets may be maintained by separate stimuli and would therefore occupy distinct homeostatic niches.

What is their reason for being? Distinct memory T cell subsets as herein characterized, perhaps occupying distinct memory T cell niches, could support a bi-phasic model for memory response. In an analogy to neurologic memory, this model puts forth that immunologic memory responses could then be the result of the rapid recruitment of a "short-term" $CCR7^-$ effector memory component, accompanied by the engagement of a "long-term" $CCR7^-$ memory component, whose enrollment is more intricate. This remains to be determined.

Conceptually, the maintenance of such distinct memory pools may be postulated to result from different stimuli requirements. Over a prolonged period of time, presence or absence of antigen is one potential parameter. The results presented in Chapter 5 demonstrate that the sustained resolution of HIV-1 viremia following 15-18 months of HAART instigated during the patients' chronic phase of infection results in a significant reduction of the size of all HIV-1 antigen-specific memory CD8 T cell populations. Nevertheless, whether all memory subsets disappear at the same rate upon gradual antigen withdrawal is not known. Furthermore, HIV-specific CD8 T cell responses may be preserved following effective treatment with HAART, depending on the rapidity with which treatment

is undertaken following infection: are HIV-specific memory CD8 T cell subsets differentially preserved? Is the early instigation of HAART of benefit for the preservation of memory T cells? The longitudinal tracking of HIV-specific memory CD8 T lymphocytes through time, prior to and throughout HAART, in conjunction with the monitoring of phenotypic markers of maturation will evidence the kinetics of attrition of HIV-specific memory CD8 T lymphocytes during HAART and might provide insight into the extent of antigen-dependency of each memory subset. In turn, distinct cellular half-lives may be observed for the various memory CD8 T cell subsets following antigen-removal.

7.11. Conclusion

The studies presented in this thesis provide novel insight into the dynamics of antigen-specific T cell immune responses and the maturation of antigen-specific T cell memory. As such, our characterization of memory CD8 T cell maturation subsets is inscribed in a broader effort to begin the comprehensive delineation of the properties of antigen-specific CD8 T cells involved in effective antiviral responses and immune protection. Beyond the original knowledge gained through the studies herein presented, this work's greatest contribution to research efforts undoubtedly lies in the questions that it now raises.

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