To my mother

Acknowledgments

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Abstract

HIV-1 infection is characterized by immunological and virological events that lead to the demise of the immune system and the establishment of AIDS. Infection with HIV has been associated with perturbations in the CD8 and CD4 T cell receptor BV (TCRBV) repertoire. However, the nature of these perturbations, the viral factors that initiate those disruptions whether antigen or superantigen-driven, and their consequences on the outcome of HIV infection are only starting to unravel. To verify whether HIV encodes a superantigen, we analyzed the TCRBV repertoire of SCID-hu Thy/liv mice infected with either HIV or staphylococcal enterotoxin B (SEB) by flow cytometry. Infection of thy/liv implants with HIV has caused a depletion of T cell subsets; however, this depletion was not TCRBV-specific, as compared to the one induced by SEB. These results suggest that perturbations in the TCRBV repertoire of HIV-infected patients are most likely not mediated by an HIV-encoded superantigen. In order to evaluate whether perturbations in the CD4 repertoire and disease progression are associated with viral replication, three SIV strains (SIVmacJ5, SIVmacC8 and simian-human immunodeficiency virus 89.6P (SHIV89.6P)) with variable pathogenic potential were used to infect cynomolgus macaques. The patterns of CD4+ T cell expansions detected by flow cytometry were variable among animals of the same group, indicating the lack of correlation with the relative pathogenicity of the viral isolates. Instead, a strong prognostic factor for disease progression was evident. Indeed, the magnitude of the expansions in the CD4+ TCRBV repertoire in fast progressor macaques is significantly more important ($p=0.025$) than in slow progressors during primary infection (PI). Characterization of the clonality of the CD4 expansions by sequencing and the longitudinal follow up of the persistence of CD4 clonotypes by heteroduplex tracking assay (HTA) has revealed that slow progressors with oligoclonal CD4 expansions are able to maintain their clonotypes and generate a secondary immune response upon challenge. However, fast progressors lose their primary CD4 clonotypes during the transition into chronic infection and, slow progressors with no detectable CD4 responses during PI are susceptible to superinfection by SHIV89.6P or PBj14 Sooty Mangabey (SIVsmmPBj14) upon challenge. These findings provide a better understanding of the dynamics of CD4 T cell responses and their involvement and importance in the pathogenesis of HIV infection.

Résumé

Le VIH est caracterisé par des évenements immunologiques et virologiques qui aboutissent à la détérioration du système immunitaire et l'établissement du SIDA. L'infection avec le VIH a été associée à des perturbations dans le repertoire des recepteur de cellules T (RCT) du compartiment CD4 et CD8. Pourtant, la nature de ces perturbations, les facteurs viraux qui les causent tant de sources antigéniques ou superantigéniques, de même que leurs conséquences sur le devenir de l'infection avec le VIH, commencent seulement à s'éclaircir. Afin de vérifier si le VIH encode un superantigène, nous avons analysé le repertoire des RCT de souris SCIDhu thy/liv qui ont été infectées avec le VIH ou avec staphylococcal enterotoxin B (SEB) et ceci par cytofluorometrie en flux. L'infection avec le VIH des organes implantés a causé une depletion de cellules T, par-contre cette depletion n'était pas spécifique a certaines familles BV comme l'est celle induite par SEB. Ces résultats suggèrent que les perturbations dans le repertoire des RCT chez les patients infectés avec le VIH ne sont probablement pas declenchées par un superantigène provenant du VIH. Dans le but d'évaluer si les perturbations des RCT du compartiment CD4 et la progression à la maladie sont associées au niveau de la replication viral, trois souches du virus SIV (SIVmacJ5, SIVmacC8 et simian-human immunodeficiency virus 89.6P (SHIV89.6P)) ayant different potentiel pathogénique ont été utilisées pour infecter des macaques cynomolgus. La nature des expansions detectées par cytofluorometrie dans le compartiment CD4 étaient variable suivant les animaux et le groupe, ce qui indique l'absence de correlation avec le potential de pathogenicité de la souche viral. Plutôt, ce dernier constitue un indice indiquant la vitesse de progression de la maladie. En effet, la magnitude des expansions CD4 dans le repertoire des RCT chez les progresseurs rapides est significativement plus importante ($p=0.025$) que celles detectées chez les progresseurs lents durant la primo-infection. La caractérisation de la clonalité des expansions CD4 par sequencage, de même que l'étude longitudinale de la persistance des clonotypes CD4 par HTA ont révélés que les progresseurs lents sont capables de maintenir leur clonotypes CD4. Suite a une re-exposition avec un virus virulent, ses derniers regénèrent une réponse secondaire. Par contre, les progresseurs rapides perdent leurs réponses primaires CD4 durant la transition dans la phase chronique de la maladie. Les progresseurs lents n'ayant pas de réponse CD4 detectable sont susceptibles à une superinfection par PBj14 Sooty Mangabey (SIVsmm PBJ14(6.6)sm) ou SHIV89.6P suite à une infection avec ces virus. Ces données offrent une meilleure comprehension de la dynamique de la réponse CD4, de son rôle et son importance dans la pathogénèse de l'infection avec le VIH.

Chapter I

General introduction

1. T lymphocyte responses during viral infections

1.1. The T cell receptor

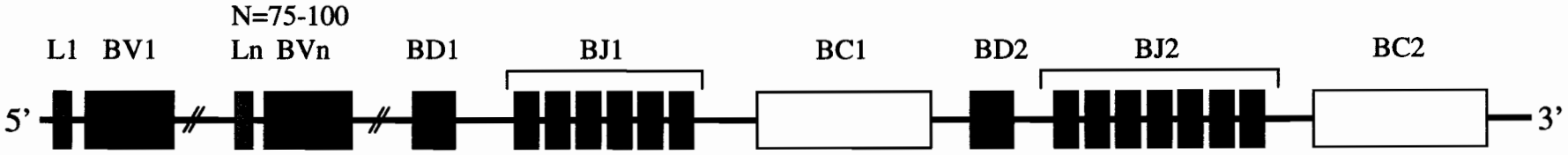
1.1.1. Genetics

The T cell receptor (TCR) is a heterodimer composed of two polypeptide chains, either α and β or γ and δ chains. TCR heterodimers are expressed on the surface of T cells in a mutually exclusive way. These two chains resemble those of Fab fragments of immunoglobulins (Ig), in that they have variable (i.e. BV and AV) and constant (i.e. BC and AC) domains. Their specificity and diversity are both dictated by the rearrangement of their gene segments V, D, and J, in a process that is similar to the generation of antibody diversity. Comparison of the TCR sequence with that of Ig has shown that the corresponding domains of the two sets of molecules have similar folding (1-6). Studies involving the isolation and characterization of TCR cDNA libraries (7-20) or genomic cloning (15;19-24) have revealed that the TCR β chain locus is comprised of at least 53 segments that are all functionally expressed. All those members were grouped according to their sequence homologies into 25 families that share 75% identity and contain subfamilies. In human, the β chain locus occupies about 685 kb of DNA (25) that is located on chromosome 7 (26). Among the 64 BV segments that are encoded by this locus only 53 are functional, whereas 11 are pseudogenes containing stop codons, frame shifts or splice site defects (25) (table 1). There are two very similar BC segments, each containing four exons. Each BC is associated with a 5' cluster of six BJ1 and seven BJ2 segments and one-D segment. The BV genes are located 5' from the clusters BD1-BJ1-BC1 and BD2-BJ2-BC2 except for BV20S1, which is located at the 3' end of the β locus. As for the TCRAV located on chromosome 14, it is comprised of 75 AV segments grouped into at least 12 families, all located 5' of the J and C regions. There is a single AC gene composed of four exons which is associated with a large 5' cluster of up to 60 different J segments. No D segments have been identified in the α locus (figure 1).

1.1.2.1. Rearrangement

The generation of TCRs takes place during T lymphocytes differentiation by a series of regulated site-specific DNA recombination reactions termed V(D)J recombination. In order for a TCR to be expressed on the cell surface, three recombination events have to be completed. These events

Human TCR b chain locus (chromosome 7)



Human TCR a,d chain locus (chromosome 14)

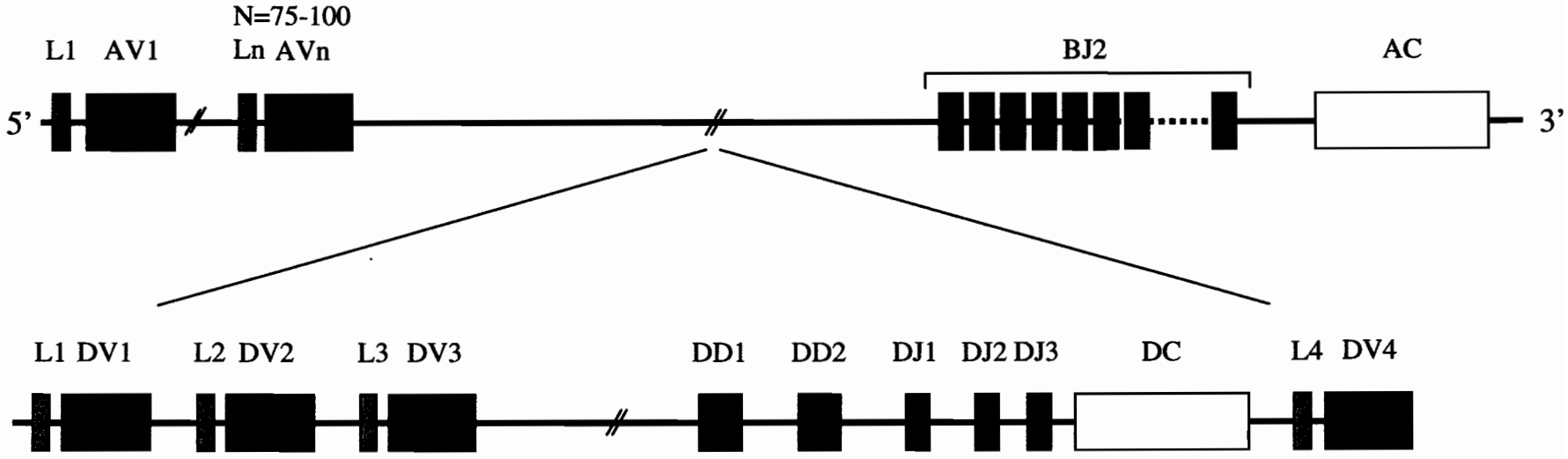


Figure1: Organization of human T cell receptor genes in the germline.

TCRBV Family	Bands on Southern Blots	Gene number	Number of functional genes
BV1	1	1	1
BV2	2	2	1
BV3	1	1	1
BV4	2	2	1
BV5	5-8	7	7
BV6	8-10	9	7[a]
BV7	3	3	3
BV8	5	5	3
BV9	2	2	1
BV10	2	2	1
BV11	2	2	1
BV12	3	3	3
BV13	6-9	9	9
BV14	1	1	1
BV15	2	2	1
BV16	1	1	1
BV17	1	1	1
BV18	1	1	1
BV19	2	2	1
BV20	1	1	1[a]
BV21	3	3	3
BV22	1	1	1
BV23	1	1	1
BV24	1	1	1
BV25	1	1	1
Total	58-66	64	53

[a]Common null exist for a TCRBV gene in these families

S. Wei, P. Charmley, M.A. Robinson, P. Concannon. *Immunogenetics. 1994;40(1):27-36.*

S. Wei and P. Concannon. *Hum Immunol. 1994 Nov;41(3):201-6*

Table 1: Size estimate of the human germline TCRBV repertoire

consist of the joining of D-J, then V-DJ segments for the β chain and V-J segments for the α chain.

TCR rearrangements are mediated by a recombination signal sequence (RSS) that flanks the gene segment to be rearranged. The consensus RSS consists of a heptamer and a nonamer sequence and a spacer of 12 or 23 base pairs that separates those two elements. During a recombination process, an element with a 12bp spacer is joined with one that has a 23bp spacer (27;28). This 12/23 rule restricts therefore segment joining to events that are biologically productive. The recombination-activating genes (RAG)-1 and (RAG)-2, are two lymphoid-specific genes that makeup the V(D)J recombinase complex and are responsible for recognizing the RSS sequence and introducing a cleavage between it and the coding segment (29). First they recognize and bind to the properly spaced heptamer and nonamer sequences of the RSS, and a nick is introduced at the 5' end of the heptamer at the signal/coding boundary. In a second step, the free hydroxyl on the coding DNA binds to the phosphodiester bond of the opposing strand to form a hairpinned coding end and a blunt signal end (30). The RAG proteins have been shown to perform the cleavage reactions on their own *in vitro* (30), but not *in vivo* (31). High mobility group (HMG) proteins have been identified as cofactors for the V(D)J cleavage (32). Coding and DNA hairpins are resolved through an endonuclease action that results in the generation of palindromic repeats (P region) that are later trimmed by exonucleases. A terminal deoxynucleotidyl transferase (TdT) activity is then responsible for adding non-templated bases (N-region) (33;34). The rearrangement is then completed by ligating the coding and signal joints by reactions that involve ubiquitously expressed factors such as (XRCC4, Ku-80 antigen) (35) and the large catalytic subunit of DNA-dependent protein kinase (DNA-PKcs, XRCC7) (36). Although some earlier findings have suggested that RAG-1 and RAG-2 play a predominant role in the rearrangements initiation, recent evidence suggest that the role of RAG proteins is not limited to initial cleavage stage. Post-cleavage complexes containing the RAG proteins bound to either both signal and coding ends or just signal ends have been found (37), suggesting that the RAG proteins remain available for future activity. Moreover, Besmer et al. (38) have reported that the RAG proteins can open synthetic hairpins *in vitro*.

The recombination takes place in a cell-specific manner through the restricted and temporal expression of RAG proteins as well as the differential chromatin locus accessibility potential (28). TCR sequences recombine only in pre-T lymphocytes while Ig sequences are restricted to

pre-B lymphocytes. Within each lymphocyte lineage there is a clear-cut order of rearrangement: Ig heavy chain (IgH) precedes Ig light (Igκ and Igλ) chain and TCR β chain rearrangement comes before the TCR α chain. Also within the IgH and TCR β chains, D-J joining precedes V-DJ joining (39) (figure 2).

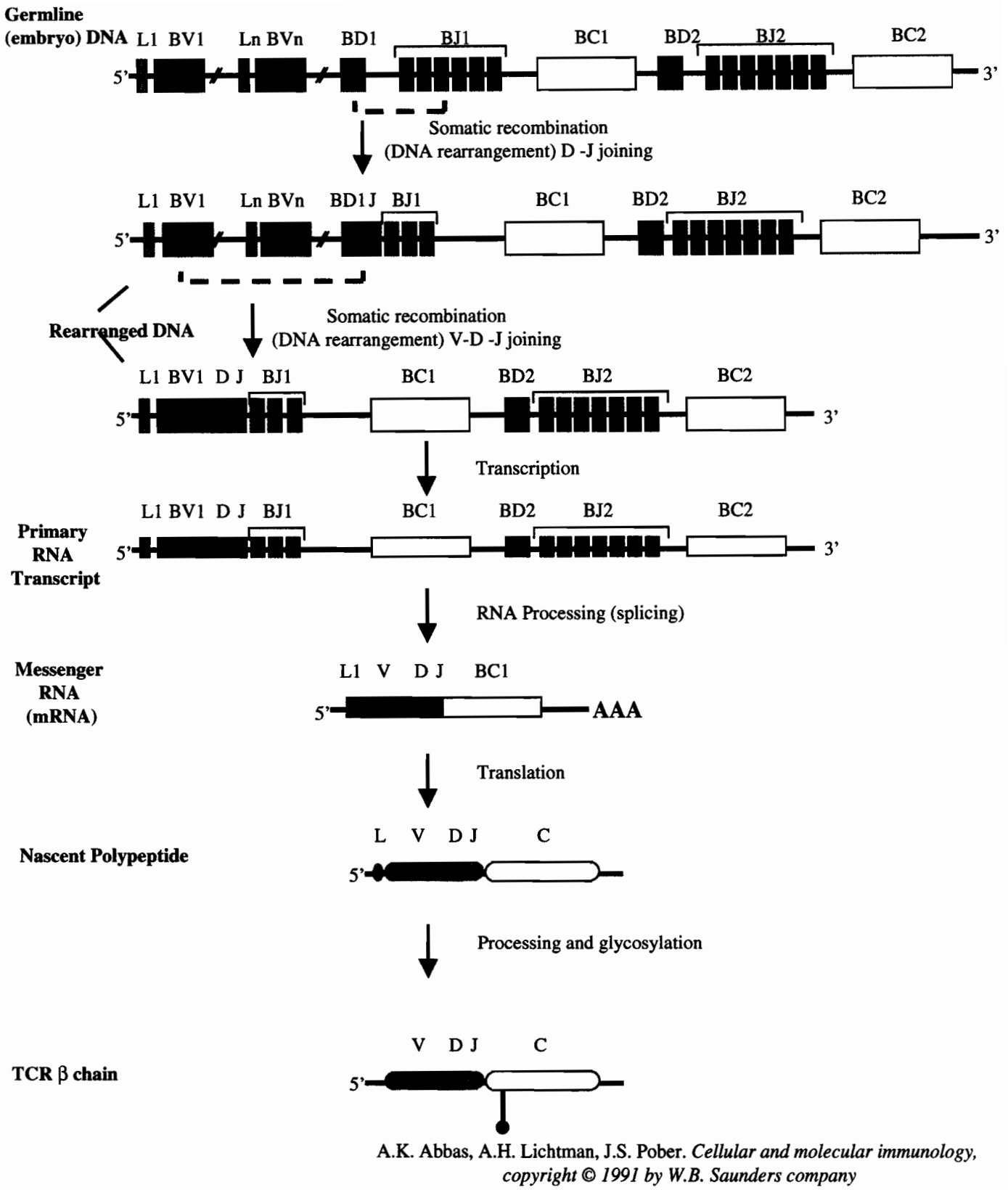
1.1.3. Diversity

Several mechanisms involved in the V(D)J rearrangement and cell surface expression of a functional αβ TCR are responsible for the generation of a repertoire endowed with an extended diversity. This diversity allows the recognition of a large panel of foreign antigens that are encountered during a lifetime in order to mount an immune response against them. These mechanisms are as follow:

A large degree of polymorphism is observed in the β locus, this polymorphism is mainly restricted to the V region exons, with some V regions showing more allelic polymorphism than others are (19). Such as BV6S7 has two alleles a and b that differ in such a way that two nucleotide substitution are able to abrogate/enhance their responsiveness to superantigens (Sags) (40), indicating that allelic polymorphism has a direct influence of TCR function. As a result, the extensive number of BV alleles is segregated within the general population, creating therefore distinct TCRBV repertoire for each individual.

During TCR rearrangement, several recombination assortments are possible between gene segments that result in the generation of diversity within the TCR repertoire. For the β chain, the two BD1 and BD2 segments can rearrange with either one of the 6 or 7 different BJ1 or BJ2 segments respectively. However, some rearrangement that brings together BD1 and BJ2 has been documented, as well as the joining of BD1 with BD2-BJ2 (14). Subsequently, the 53 different BV genes can rearrange with any of the DJ combinations adding another level of diversity. As for the α chain, to surpass the lack of D segments, diversity is achieved through the presence of 60 different AJ segments that can rearrange with any of the 75 AV segments.

The activity of the enzymes involved in the V(D)J recombination are site specific with a certain degree of variability. This variability can change the original gene segment and generate a panel of different variants from the original germ-line sequence. This is performed by exonucleases that trims coding junctions at several possible nucleic acid positions within the vicinity of the junction sometimes loosing entirely or partially the BD segments. TdT activity that is involved



A.K. Abbas, A.H. Lichtman, J.S. Pober. *Cellular and molecular immunology, copyright © 1991 by W.B. Saunders company*

Figure 2: Schematic representation of the events leading to the rearrangement of the TCR β chain. In this case the β chain is composed of the BV1, BD1 regions and BJ1.3 the third exon of the J1 cluster, as well as BC1. The unused segments located between the rearranged genes are deleted.

in the addition of random bases to the coding ends (N-region). Similarly, endonucleases attack hairpins at several possible regions distal from the apex of the hairpin, creating palindromic repeats of variable sizes (P-region). Apart from changing the germ-line sequence, all these processes have other implications, such as creating frameshifts or stop codons following the CDR3 region, which results in a nonfunctional β chain not expressed at the surface.

The D region in the β chain can be translated in three reading frames, a property that is reserved for T cells which can provide them with an additional level of diversity. Rowen et al. reported however that not all reading frames are used in a similar fashion; apparently the third reading frame is used 2.5 times less than the first or second reading frames (25).

Following the productive rearrangement of one of the two α and β alleles in each T cell due to allelic exclusion, pairing of the two productively rearranged chains is random, creating therefore four different possible combinations. In most cases one β chain is paired with one α chain. However a fraction of T cells can display two productive AV gene rearrangements or even express two surface TCR β chains with distinct superantigenic reactivities. This suggests that the level at which allelic exclusion operates is not fully efficient and that some T cells can escape this allelic control (41-43).

Finally mutations in BV genes that resemble somatic hypermutations in Ig following several rounds of exposure to antigen have been documented in TCR located in germinal centers and it has been observed in the α and β chains. Nevertheless, it remains to be proven whether T cells experience affinity maturation (44;45).

Considering that all these factors contribute to the generation of the TCR diversity, it is theoretically possible that as many as 10^{16} different TCRs may be present in the periphery (table 2). However, studies in mouse (46) and human (47) in which the size of the T cell pool in the periphery has been investigated accounted for merely 10^6 different naïve T cells. This suggests that certain structural factors can potentially limit the TCR diversity. One factor can be that the BD region is small in length and GC-rich in the center (9). Considering that rearrangements usually occur at the two extremities of these regions, a motif containing glycine residues will be mostly represented within the CDR3 region of TCRs. The use of certain BJ segments more frequently than others has been documented. For instance, BJ2.1 and BJ2.7 are used 40% of the time (48;49). Moreover, some T cells can lack TCRs due to the inefficient pairing of α and β chains (50). Altogether, these factors can alter the overall theoretically estimated TCR diversity.

Mechanism	Immunoglobulin		TCR αβ		TCR γδ	
	Heavy chain	κ	α	β	γ	δ
Variable segments	250-1000	250	75	25	7	10
Diversity (D) segments	12	0	0	2	0	2
D segments read in all three reading frames	Rare	-	-	Often	-	Often
N region diversity	V-D,D-J	None	V-J	V-D,D-J	V-J	$V\text{-}D_1$, $D_1\text{-}D_2$, $D_1\text{-}J$
Joining segments	4	4	50	12	2	2
Variable segment combinations	62,500-250,000		1875		70	
Total potential repertoire with junctional diversity	$\sim 10^{11}$		$\sim 10^{16}$		$\sim 10^{18}$	

M.M. Davis and P.J. Bjorkman. *Nature 334-402,1988*

Table 2: Mechanisms that contribute in the generation of diversity in the TCR and the immunoglobulin repertoires.

1.1.4. Structure and Function

As described earlier, the TCR is composed of either αβ, or γδ heterodimers which represent a smaller percentage of T cells. The function of the TCR involves its interaction with other proteins, such as processed antigens as well as the polymorphic MHC to which the peptide binds. Moreover, they bind within their own cells to CD4 molecules to mediate helper responses or to CD8 proteins in case of cytotoxic T cell responses (CTL). While T cells expressing αβ TCRs recognize peptides that are found in complex with either MHC class I or class II molecules, γδ TCRs bind to soluble or tissue-associated peptidic and non-peptidic antigens, such as breakdown products from bacterial cell walls (51).

The TCR α and β or γ and δ chains have variable (V) and constant (C) domains analogous to antibodies. Indeed, when TCRs are aligned with one another, regions of homology and diversity are found (52). The hypervariable segments of polypeptides in the TCR V domains are called complementary-determining regions (CDRs) and they are functionally equivalent to CDRs found in antibodies. There are three hypervariable regions CDR1, CDR2 and CDR3 and a less hypervariable region termed the CDR4 that is only found in the β chain. In the α chain, the CDR1 region is located between residues 25 to 31, the CDR2 region between 48-56 and the CDR3 between 93-104. In the β chain, CDR1 segment covers residues 26-31, CDR2 covers residues 47-60 while CDR3 region spans positions 95-106 (52-54). The constant regions within the V domains of both AV and BV are called framework.

The overall structure of the TCR has been mainly predicted from primary sequences analysis that were similar to Fab fragment of the Ig. However, recently the crystal structure of isolated α (55) and β (56) chains have been determined followed by the structure of the αβ heterodimer (57;58). The three dimensional structure of the β chain has revealed that the β-sheet of the BV and BC domains are very similar to those found in Ig. In contrast, the CDR loops have different conformations that are not found in Ig. The CDR1 and CDR2 are less structurally diverse than the corresponding regions in Ig and they are thought to contact the α-helical regions of the MHC molecule (59). The BC domain of the β chain has a large insertion between residues, 219 and 232, which forms a loop that is though to be involved in contacting CD3 components. The BV and BC domains are in close contacts with each other in the crystal structure however the biological functions behind this association is not known.

The crystal structure of the α chain has revealed that the AV domains exist as a homodimer just like IgL chain dimers. The difference between V domain of the α chain compared to that of the BV domain is a switch of a polypeptide strand from one β sheet to the other, which removes a surface protrusion from the molecule and enables two AV homodimers to pack as parallel dimer-of dimer in the crystal structure (55).

In the two studies that illustrated how the TCR binds to the peptide plus MHC complex (57;58), the TCRs are oriented diagonally over the MHC molecule, with the α chain over the amino-terminus of the peptide and the β chain over the carboxy-terminus. Through this orientation, the CDR2 loop of both chains were found in contact with the MHC molecule alone, while the CDR1 and CDR3 loops were contacting both the MHC and the peptide. The CDR3 domain was found in contact with the centrally located portion of the peptide. This representation is in agreement with the large body of evidence that identified the CDR3 region as the main site directly contacting the peptide. This was demonstrated by several techniques such as site directed mutagenesis (59;60), PCR analysis of T cell clones (61) and peptide immunization of TCR transgenic mice (62).

1.1.4. The T cell receptor repertoire

Within an individual, each T cell clone has a unique TCR αβ combination possible on their cell surface, and the sum of all the T cell clones make up the TCR repertoire. The naïve TCR repertoire is shaped in the thymus during positive and negative selection, its identity and diversity at this stage is based on the MHC of the individual. In cases where several MHC are present, the diversity observed within the repertoire is higher (63). An evidence for this phenomenon is the finding that monozygotic twins have very similar repertoires than two unrelated individuals (64). Perturbations in the TCR repertoire during infections and their impact on disease progression namely HIV, will be discussed in the following sections.

1.2. Dynamics of antigen-specific T cell responses

The ability of an organism to mount an orchestrated defense mechanism against an invading foreign pathogen is called specific immunity. The establishment of an immune response is regulated by the specific recognition of TCRs with their ligands; peptides bound to MHC molecules. This initial encounter is sufficient to trigger the activation and expansion of CD4

and CD8 antigen-specific T lymphocytes, known as the primary T cell responses. This initial proliferation is evidenced by an increase in the number of antigen-specific T cells within the relevant secondary lymphoid organs. The time, magnitude, and composition of this primary response can be substantially diverse depending on the stimuli. Nevertheless, as the pathogen is controlled at least partially and antigen output is reduced, these T cell subset decline in activity and number, leaving the host with a sufficient number of pathogen-specific memory T cells. Memory T cells have the advantage of being able to respond more efficiently than naïve T cells do. This is probably due to T cell frequency as well as qualitative changes that they acquire through their differentiation process. Therefore, upon re-exposure to the same antigen, memory T cells go through a second round of proliferation, only this time their progeny is more robust and rapidly generated.

Some important parameters that determine the composition, requirements and fate of primary responses during the course of viral infections will be discussed. How effector T cells evolve into the memory pool and whether they are maintained and in which conditions are also issues that will be addressed.

1.2.1. Initiation of primary T cell responses

Initiation of primary immune responses is restricted to secondary lymphoid organs such as lymph nodes, spleen, and Peyer's patches (65). Within these compartments, naïve T cells are located in T cell-rich areas known as the paracortex in the lymph nodes (66). The blood vessels contacting lymph nodes are known as high endothelial venules (HEV). These HEV express a number of specific ligands (Glycam-1, CD34, SLC and ELC) which play an important role in attracting naïve T cells that express the appropriate receptors. Among the known receptors, CD62-L (67) is required for rolling on vessel walls, whereas CC chemokine receptor (CCR) 7 (68) is used for integrin activation and extravasation. During their migration into secondary lymphoid organs, naïve T cells enter through HEV in case of lymph nodes then into the paracortex where they will reside for one day due to the continuous presence of SLC and ELC chemokines that are produced (69;70). In case they do not encounter their specific antigen on antigen presenting cells (APC), naïve T cells migrate back into the blood to reach other secondary lymphoid tissues.

Within secondary lymphoid organs, dendritic cells express the most abundantly MHC class I and class II molecule-peptide complexes in T cell areas (71), other MHC class II rich APCs are B

cells and macrophages, which are both located outside of the T cell area (72). Therefore due to their anatomical distribution and functional properties, dendritic cells (DCs) are the first to initiate antigen presentation to naïve CD4 and CD8 T cells (73). There are at least 2 sets of DCs in T-cell areas, one that takes a migratory myeloid pathway which brings in antigens from the periphery and induces immunity, and another that takes a more residual lymphoid pathway that presents self-antigens and maintains tolerance (71;74). Due to the expression of surface molecules (DEC205 and CD8α or CD11b) (75;76), lymphoid DCs are thought to derive from migrating Langerhans whereas myeloid DCs come from differentiated monocytes respectively (77).

In nonlymphoid tissues such as the skin, DCs are immature and capable to endocytose and process antigens as well as apoptotic cells through a variety of receptors (78-80). It is through this process that DCs mature and migrate via the afferent lymph to T cell areas of secondary lymphoid organs and act as APCs to naïve CD4+ T cells (71). To activate cytotoxic killer cells, DCs at the site of deposition have to present peptides in complex with MHC class I molecules to naïve CD8 T cells in T cell areas of secondary lymphoid tissues. They do so by being infected themselves, such is the case with influenza virus (81), which uses the cellular machinery to express its viral proteins that are in turn degraded in proteasomes. Transporters guide these peptides from the cytosol to the cell surface in complex with MHC class I by passing through the endoplasmic reticulum. In cases like tumor antigens, antigens from transplants, or viruses that can not infect DCs and have no access to their cytosol, they can still manage to express their specific peptides in complex with MHC class I molecules by a mechanism not well understood (82;83).

The initiation of full activation potential instead of tolerance, requires a danger signal to the T cells. This is provided through inflammatory cytokines by the innate immune system comprised of natural killer cells (NKs) and others (84;85). At the site of inflammation, the innate immune system causes the release of TNF-α and IL-1 cytokines, which signal the local tissue DCs to present peptides either in the context of MHC class I or class II (86). Levels of co-stimulatory molecules such as CD40, B7-1 and B7-2 are thus increased and mature DCs, that have migrated to T cell areas of secondary tissues, loose their potential to capture particles and activate naïve T cells (87). If a naïve T cell recognizes its given antigen on a DC, it will produce high levels of IL-2 and an unidentified T cell growth factor, and start to proliferate. DCs have the ability to

instruct T cells to differentiate along the Th1 or Th2 pathway, through the secretion of cytokines such as IL-12 (88) or IL-4 (89).

Of the surviving effector T cells that are able to proliferate extensively in the presence of IL-12, they become CCR7- and acquire the ability to secrete IFN-γ. At this stage, they exit from the secondary lymphoid organ into the blood or nonlymphoid tissues (90) guided by the expression of specific adhesion molecules such as CLA, VLA-4 and LFA-1 (91), where they exert their specific activity as effector T cells at the site of antigen deposition.

To stimulate the humoral immune response, activated CD4 T cells from T cell areas and B cells from primary follicles of secondary lymphoid organs both have to migrate and meet at the intersection of both compartment where CD4 T cells can then provide help for B cells that express epitopes from the same antigen (92;93). The transit into B cell-rich areas are adjuvant dependant and requires CXCR5 expression by activated CD4 T cells, which is a chemokine specific for BLC produced by follicular stromal cells. Activated T cells gain their CXCR5 expression several days after antigen exposure through CD28 and OX40 interaction with their ligands on DCs after inflammation (94). Through this pathway, activated CD4 T cells loose their CCR7 receptor, preventing them from staying in T cell areas that are rich in SLC and ELC chemokines.

1.2.2. The Clonal composition of primary T cell responses

Studies monitoring the complexity and distribution of antigen-selected TCR repertoires in response to primary infection have revealed that some are extremely diverse while others are limited (95-99) and (100-104).

Upon infection, several factors may play a role in the generation of a primary immune response. It is the combined influence of all those factors that ultimately creates variations between antigen-selected repertoires, whether a repertoire is broad (polyclonal) or restricted (oligoclonal), persistent or short-lived, early or late in infection. Among these factors we can consider: the frequency of each individual T cell clone that form the precursor repertoire for a given antigen, the rate of expansion and the time of recruitment of those T cells after the encounter with the foreign antigen (figure 3).

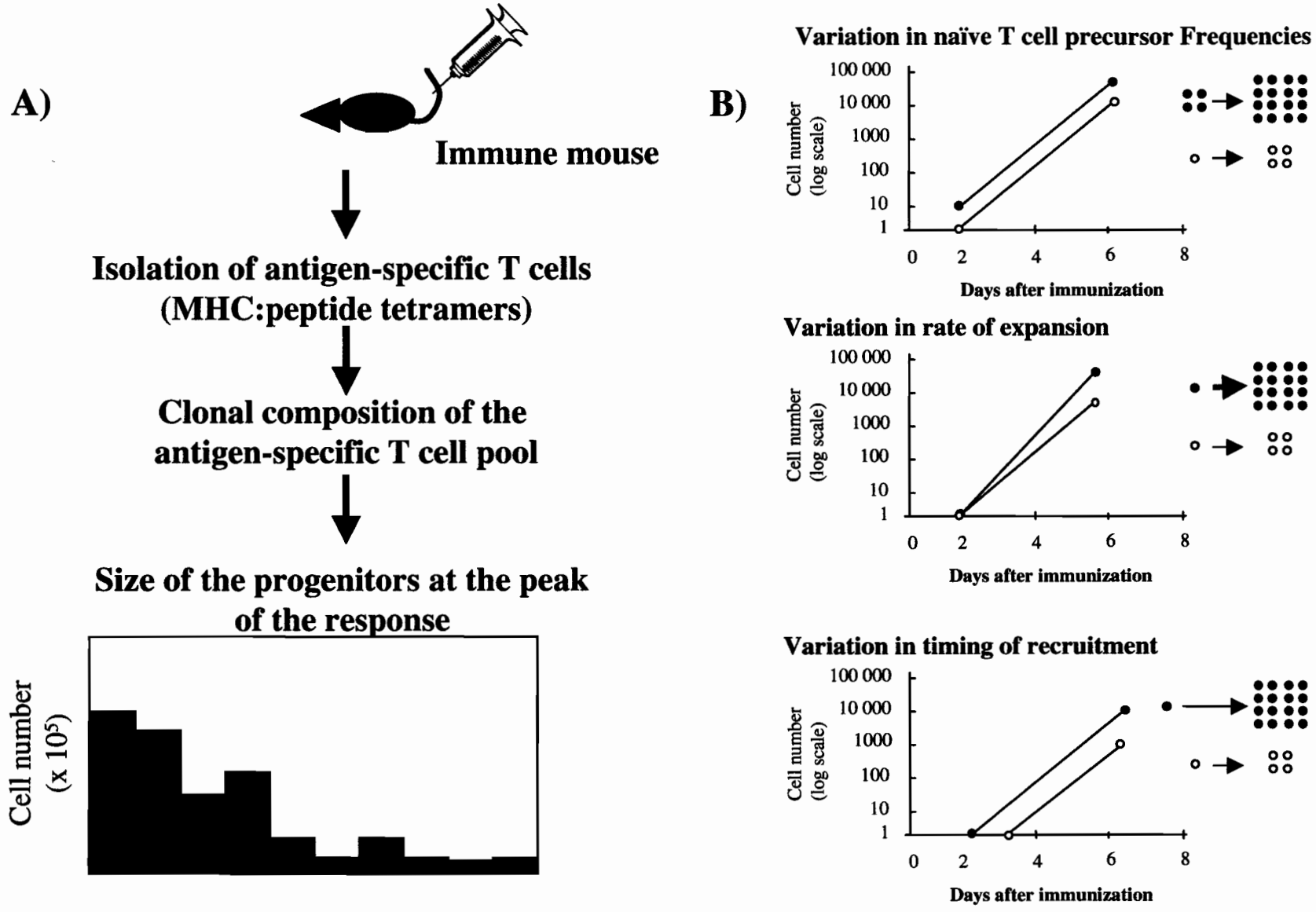


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Figure 3: Composition of the primary immune response. A) The primary Ag-specific immune response is composed of dominant and minor clones at the peak of activation. B) The abundance of each clone depends on three factors: the precursor frequency, the rate of expansion and the timing of recruitment.

1.2.2.1 Variations in the frequency of the precursor repertoire

Considering that the recombination of α and β chains of the TCR is a random process, the theoretical diversity estimated would be in the order of 10^{15}. The total number of T lymphocytes in the periphery however, is estimated around 10^6 in a mouse (46). This suggests that only a small fraction of the potential T cell repertoire can be used at a given time in a mouse (105). Moreover, since the size of the T cell compartment is restricted in the periphery, one would expect a competition for space between precursor T cell clones. Consequently, T cell clones derived from a large precursor pool would be more susceptible to be recruited and give rise to a more abundant progeny compared to the ones derived from a single precursor. Several factors can account for differences in the size of the precursor pool: Accumulation in the periphery of certain clonotypes due to the cross-reactivation with antigens derived from food or other environmental components. Recombination and positive or negative selection in the thymus may be biased in the manner they recruit naïve T cell clones in the periphery, favoring more abundantly some clonotypes than others. Among others, Sant'Angelo and colleagues (106) showed that the intrathymic self-peptides have a strong impact on the mature naïve TCR repertoire. By eliminating a peptide exchange protein (H2-Ma), an element of the intrathymic self peptide repertoire, the structure and specificity of the mature TCR repertoire was dramatically altered.

Several studies have attempted to quantify the precursor frequency of CTLs. First by limiting dilution assays (LDA), which are based on the *in vitro* stimulation of precursor CTLs with specific antigens. Although the use of tetramer has shown that this strategy can underestimate the level of antigen-specific T cells because its level of sensitivity does not take into account clones with a low rate of cell division (107). Second, adoptive transfer experiments of various amount of monoclonal naïve CD8 T cell populations derived from TCR-transgenic mice (P14) that are LCMV gp33-specific, into host mice later infected with LCMV, was performed (108). The total host-plus-donor T cell response to gp-33 remained constant, but the composition of this response changed. The more TCR-transgenic cells were transferred the more they dominated the anti-gp33 response. Bousso et al. (109), have used a strategy based on the extensive sequencing of BV10-JB1.2 rearrangements size selected to encode 6 aa long CDR3 segments typical of TCRs expressed by CD8 T cells specific for CW3. The TCRs from those libraries were very different from one mouse to another, and very few shared the same CDR3 sequences, suggesting

that their preimmune repertoire was not the same. In a following study in which they attempted to follow the selection of these repertoires in hemisplenectomized mice immunized with P815-CW3 tumor cells, they concluded that most expanded T cell clones did not derive from high-frequency precursors (110). However, the antigen specificity of the cells expressing the different TCRs identified in the preimmune repertoire is unknown, since the criteria for comparison was only CDR3β sequences that are BV10, BJ1S2 and are 6 aa long. It is possible that the most frequent T cell clones in the naïve half spleen that display this criteria are specific for another unrelated peptide.

1.2.2.2 Variation in the proliferation capacity

Upon activation, the progeny of a T cell clone depends on the rate of expansion. The more rapid a T cell clone divides upon triggering the more advantage it would have to dominate the repertoire. The extent of proliferation of a given T cell clone is likely to be a function of its affinity to the peptide-MHC complex, but also the density of peptide-MHC complex on the surface of APCs.

During antigen processing, peptides are degraded from their original protein and subsequently transported to the cell surface in association with MHC class II or I molecules. Therefore, the efficiency with which different peptide epitopes complete each rate-limiting step of the antigen processing and recognition pathway can account for the relative complexity of antigen-selected repertoires during primary infection. Indeed, Restifo et al. (111) have demonstrated that the efficiency of CTL induction *in vivo* is influenced by proteolysis as well as peptide transport into the ER. By bypassing the need for proteolysis or peptide transport, they have shown that more complexes are found on the cell surface of APCs and, more CTLs are recruited in this process. Similarly, this correlation was also observed in a study in which the effect of abundance of HLA-A2 restricted HIV-1 derived epitopes on infected cells was associated with a better CTL activity (112). Variations in the density of the epitope on infected cells could be due to several factors, in the case of HIV for-example, a single mutation in the epitope could abrogate or limit the recognition by the MHC class II molecules, and accordingly affect the HIV-specific CD4 T cell recognition (113).

However, the density of peptide-MHC complexes is not the only factor that determines the extent of T cell proliferation. Increasing their amount will eventually cease to improve the size of

the T cell response. This suggests that the proliferation capacity is constrained by the availability of the precursor pool, which has a threshold for the engagement with peptide-MHC complexes. Butz et al. (108) have demonstrated that after a certain amount of adoptively transferred naïve Tg T cells, the overall size of the specific response is no longer amplified. Apart from the epitope density, the avidity of the interaction between T cells and their target cells can also control T cell proliferation. Indeed, when equal amounts of CTLs specific for three LCMV peptides were adoptively transferred into mice that were infected few hours before with LCMV, the best degree of protection was not in concordance with the relative density of the peptides found on infected cells but with the sensitivity of the CTLs for their peptides (114). The effect of high versus low affinity interactions on the diversity of the antigen-selected repertoire has been investigated. Campos-lima et al. (95) have reported that two, immunodominant and subdominant epitopes both restricted by the same HLA molecule elicit either a broad or a restricted response respectively. CTLs specific for the subdominant epitope were shown to exhibit high affinity for their epitope, while CTLs specific for immunodominant epitope were shown to exhibit both high and low affinities for their antigens. It is therefore possible to conclude that in situations were the density of the epitopes are low, the proliferation capacity of CTLs will be determined by their precursor frequencies as well as the affinity of TCRs for their ligands because a competition for ligand engagement are imposed. In this case, CTLs with high affinity and high frequency will dominate the repertoire. However, in situations where the density of the epitope is high, no competition for ligand interaction are imposed and CTLs of high and low affinity can be generated, creating a diverse repertoire.

1.2.2.3 Timing of T cell recruitment

Until they encounter their target, naïve T cells will circulate through secondary lymphoid organs. Considering that their turnover is very slow at this stage, the ones that will meet their target earlier will probably have a selective advantage to dominate the antigen-selected repertoire over the ones that are recruited at a later time. The reason being that an immune response to a given antigen has a defined window of time after which no additional T cell clones can be engaged. Indeed, this hypothesis was first addressed through the use of a fluorescent dye CFSE. The life-span of T cell proliferation during an antigen-specific response has shown that there is marked asynchrony in the kinetics of cell division exhibited, with some T cells having divided as many

as six times two days later, while others remained undivided throughout the same time period (115;116). Therefore, the period of T cell expansion is delineated by the first naïve T cell that gets activated and starts dividing and, by the end of that response. This was elegantly demonstrated by Bousso et al. (110), who followed the antigen-specific response to P815-CW3 injection in DBA/2 mice at various time points. The peak of the response is at day 11 of immunization, where 50% of CD8+ T cells are CW3 specific. Taking this into consideration, they monitored the CW3-specific repertoire at earlier time points by following the CDR3β size distribution bearing different BJ segments in order to have a global view of the CW3 response. The first clonal expansion was detected 5 days before the peak of the response and a conservation of this response was evident since the same pattern of TCR usage was conserved between the time of first detection and the peak of response on day 11. Finally, only those T cells that got activated at early time points managed to proliferate the most and were able to dominate the immune response.

1.2.3. Evolution of antigen-specific immune responses: entry into the memory compartment

Following their expansion in secondary lymphoid organs, effector T cells reach a peak of proliferation after which they undergo apoptosis. Indeed, using flow cytometry, gene amplification and a colony screening assay, McHeyzer-Williams et al. (117) were able to monitor in mice the expansion and contraction of the specific T-cell response against pigeon cytochrome C. The extent of antigen-driven clonal expansion was estimated to be greater than 1000-fold over the first six days of a primary response, which then falls to 40-fold above naive levels eight weeks later. The remaining T cells that have reached a hierarchical level of differentiation characterized by distinct functions, homing and survival capacities are now memory cells.

1.2.3.1 Memory versus naïve T cell responses

Naïve T cells can be readily distinguished from effector and memory T cells through the surface expression of adhesion molecules that are downregulated or upregulated throughout the differentiation process. Among them, CD45 with a high molecular weight (RA) defines naïve cells whereas the low molecular weight (RO) is expressed on memory cells in humans. In mice,

CD45 isoform is known as RB high in naïve T cells and RB low in memory cells (118). Other molecules are also used to identify the two T cell subsets, such as CD44 and CD62L. Naïve T cells express low levels of CD44 but high levels of CD62-L whereas memory T cells are CD44 high and CD62L low (119). On the other hand effector T cells have also lost CD62L and gained CD44, however in contrary to resting memory T cells, effector cells are activated and express CD69, CD25, HLA-DR.

Memory T cells being antigen experienced, require less stimulation than naïve T cells. Indeed, naïve T cells need to be stimulated for ~20 hours *in vitro* in order to be committed to proliferate, whereas memory T cells require a short TCR stimulation ~ 30 minutes (120). This is due to the fact that memory T cells do not need costimulation to get activated, and they can respond to very low doses of antigen (121-123). However, optimum responses of T cells still depend on costimulation that involves CD28 interaction with B7-1 and B7-2 as well as integrins such as CD54/CD11a (124). These requirements are important probably because they allow only those professional APCs that have already been activated by infectious agents and that express the proper costimulatory ligands to trigger safely and rapidly the reactivation of primed T cells.

Memory T cells are capable of secreting a large variety of cytokines, whereas naïve T cells can only produce IL-2, TNF-α, but not IFN-γ, IL-4 or IL-5 (125). However, effector T cells secrete these cytokines at high titers and faster than resting memory cells do. Adoptively transferred effector cells can generate resting T cells that retain the polarized patterns of responses, suggesting that memory T cells perform the functions that are dictated by the patterns of cytokines they produced earlier. On the other hand, *in vitro* studies have shown that naïve T cells can differentiate into cells capable of producing Th1 or Th2 pattern of responses depending on the cytokine milieu during priming. For-example, naïve T cells with antigen in the presence of IL-12 or IFN-α differentiate into cells producing inflammatory cytokines IFN-γ and lymphotoxin upon recall. Whereas stimulation in the presence of IL-4 will lead to Th2 producing cells i.e. IL-4, IL-5, IL-6 and IL-10 (126).

1.2.3.2 Migration of memory T cells

One of the major difference between naïve and memory T cells is the ability of memory T cells to extravasate through both normal and activated endothelium of secondary lymphoid organs, whereas naïve T cells do not (127). This property allows only effector memory T cells to

migrate into nonlymphoid tissues through endothelium. In support of this concept is the fact that through their differentiation process, antigen-experienced T cells found in the blood are comprised of two population (128). The first population is CD45RA-, CCR7+, CD62L high and CLA and $\alpha 4\beta 1$ integrin low suggesting that it is capable to circulate through lymph nodes through its homing receptors. The second population is CD45RA-, CCR7- and CD62-L low but has high levels of the cutaneous lymphocyte antigen CLA and $\alpha 4\beta 1$ integrin which indicates that these cells are probably excluded from lymph nodes but they are able to circulate through nonlymphoid tissues. Moreover, the localization of activated CD8 T cells in intestinal mucosa was first identified by Kim et al. (129) by using an adoptive transfer system that enables the tracking of OVA-specific T cells in mice following primary infection. More recently, Lefrancois and coworkers (130) showed that mice infected with either VSV or listeria monocytogenes expressed higher level of effector T cells in nonlymphoid tissues than in lymphoid tissues, which were able to reside as long-lived memory T cells. These effector cells were able to exhibit lytic activity ex vivo. Upon *in vitro* stimulation with anti-CD3, CCR7- T cells are able to produce INF-γ, as well as IL-4 and IL-5, a function that is not observed in CCR7+ T cells. However, the later can loose their chemokine receptor after 10 days of stimulation, which enables them to produce lymphokines. These results suggest a mechanism of T cell differentiation whereby upon encounter with antigen, naïve T cells give rise to CD45RA-, CCR7+ cells that retain the ability to home into lymph nodes until they reach a threshold of stimulation enough to downregulate CCR7 expression and start to circulate into the blood and nonlymphoid tissues (131;132).

1.2.3.3 Maturation of T cell responses

The peak of response generated after reactivation of resting memory cells will occur faster and will give rise to a much more abundant progeny than they were able to during their primary encounter with antigen (133). This is explained by the fact that the frequency of memory T cells is larger than precursors. A large body of evidence supports the notion that repertoire selection characterized by the recruitment of the highest-affinity TCRs through an affinity maturation process, is observed during persistent infection (117;134-137) (figure 4). Busch et al. (138) have used the murine infection with *Listeria monocytogene* as a model to study the antigen-specific evolution of CD8 T cell responses. During primary infection the immunodominant

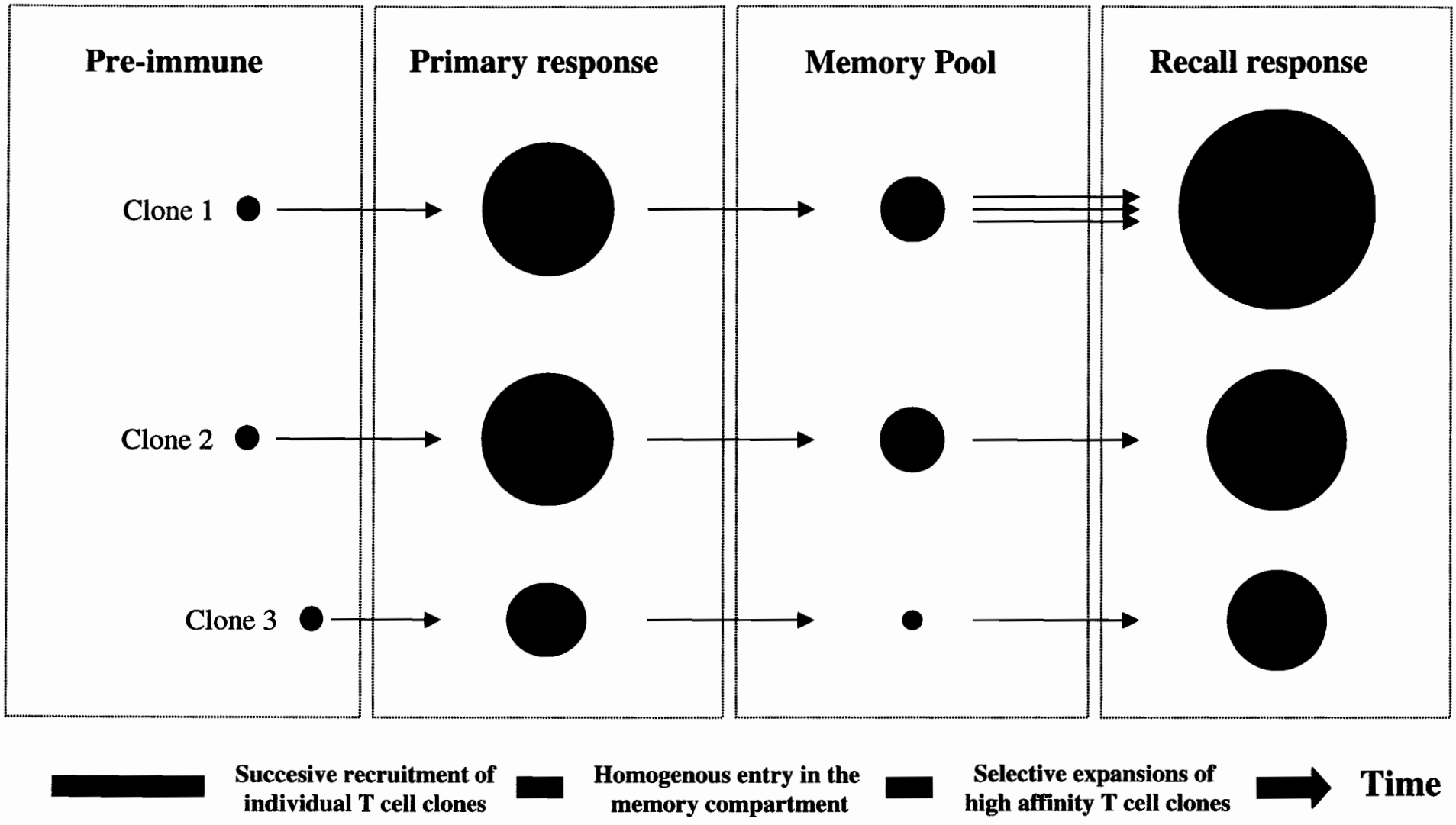

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Figure 4: Maturation of the T cell response: Selective recruitment of T cell clones with the highest TCR affinity for the MHC-peptide complex. In this case clone 1 is the dominant clone because it has the highest affinity, and is the most abundant compared to the other two clones.

epitope of *Listeria* listeriolysin O $(LLO)_{91-99}$ elicits a very diverse repertoire that is maintained in the memory pool. This response is accompanied by a clearance of viral burden and a long lasting immunity. Upon challenge, it appears that the diverse repertoire selected during primary infection evolves to include clonotypes with greater affinity to the antigen in question. This finding is demonstrated by the increase in the level of binding and the rate of dissociation of H2-Kd/LLO_{91-99} that are higher in secondary responses, indicating that their T cell lines are of a higher affinity. Later in the same year, Mark M. Davis and colleagues (137) also confirmed these results by comparing the kinetics of peptide/MHC tetramer dissociation rates between primary and secondary effector population in transgenic mice immunized with cytochrome C. These observations imply that TCR repertoire maturation during a secondary response is based on the selection of TCRs that bind peptide/MHC complex for the longest duration. However, this is not a general concept since in some circumstances the dominant clonotypes selected during primary infection appear to be replaced by new clonotypes later in disease. The immunodominant HLA-A2.01 restricted epitope BMLF1 was monitored in patients with acute infectious mononucleosis IM were during the primary immune response as well as the preservation of this response long-term (139). Clonotypes dominating the primary response were not detected after *in vitro* re-stimulation of memory T cells collected at later time points in the same individuals; instead subdominant clones managed to surmount the initial response and prevailed. When testing the specificity of these clonotypes by substituting alanine residues of the peptide, memory clones appeared to be more tolerant to sequence variations than primary clones.

On the other hand, apart from a narrowing or repertoire diversification upon antigen rechallenge, some studies have also reported no changes between primary and secondary immune responses (97;140-142). Sourdive et al. (143) demonstrated in the LCMV model that an oligoclonal response selected during acute infection is recruited again upon re-encounter with the same antigen which has the same structure and function. The same observation was reported in a study monitoring the evolution of the immunodominant response to CW3 peptide in mice injected with P815-CW3 tumor cells (140). In fact the repertoire of the immunized animal remained stable with or without antigenic challenge, suggesting that re-exposure to the antigen does not skew the primary antigen-selected repertoire.

Factors that determine selection into the memory pool and shape the repertoire to include those clonotypes that are recruited earlier or engage new ones, are probably identical to those that are implicated during primary infection. It is highly likely that memory T cells would preferentially re-expand upon encounter with the antigen in an anamnestic response compare to minor clones since they are more abundant than the later. However, in situations were the antigen-selected repertoire is diverse and includes several clonotypes with high and low affinities to peptides/MHC complexes, then T cells with the highest affinity survive preferentially over T cells with lower affinities. Also, the density of the antigen in question can influence the evolution and outcome of the primary response to favor new determinants. This is because the original clonotypes selected during acute infection were exhausted or anergized due to high antigen concentration or simply because of the absence of antigen that prevents primary clonotypes from persisting.

1.2.3.4 Maintenance of memory T cells

The definition of memory pool has been controversial for many years. Two opposite views are the source of this debate. The initial view postulates that memory is due to long-lived non-dividing memory cells, and the second envisions long-term survival due to the continuous replication of memory cells that provide new members as fast as old ones are lost by apoptosis.

In support of the first concept, are findings by Lau et al. (144) who demonstrated that purified memory CD8 T cells adoptively transferred into uninfected mice persisted and provided protection for a period of over two years. Also transfer experiments with H-Y-specific TCR-transgenic cells have also documented persistence in the absence of antigen (145). Vaccinia virus-specific memory CTLs can be detected in individuals that were vaccinated more than 30 years earlier (146). Considering that vaccinia virus does not cause a chronic or latent infection and there are no possibilities of re-exposure since vaccination was stopped in 1977, it was suggested that long-term memory could not be maintained by antigen deposition for all these years.

Whereas the first concept does not require any antigen for survival, the second concept depends on defined mechanisms that make sure T cell renewal are well regulated. Among the mechanisms that regulate long-lived survival of dividing T cell memory are, the persistence of antigen and the interactions between cell lineages.

Among the evidence supporting a role for antigen in maintaining T cell memory, Zinkernagel and colleagues (147) have demonstrated that in the absence of specific neutralizing antibodies and CD4 helper function, mice vaccinated with VSV develop a specific CD8 memory response that confers protection against reinfection as long as additional antigen was provided. Upon removal of antigen, specific CTL responses were abrogated. In a more recent study (148), adoptively transferred 318 TCR-transgenic T cells expressing a GP33 specific TCR, into naïve C57BL/6 recipient mice that were later either immunized with LCMV or with H8-DC, which are dendritic cells that present solely GP33 CTL epitope. The long-term CTL activity was compared in the two systems. While infection with LCMV-WE induced a long lasting immunity until 120 days after infection, the DC-activated CTL population quickly diminished and was eliminated after 30 days. The authors suggested that this is observed due to the short half-life of the peptide presentation by dendritic cells, which disappeared after 2-3 days. On the other hand, infection with a continually replicating LCMV-WE strain, even at low levels, ensures antigen output for a prolonged period of time.

Maintenance of memory T cells also requires the interaction with lymphocytes from other lineages. However, the rules for retaining CD4 and CD8 memory T cells are not the same. For-example CD8 T cells do not require B cells for their maintenance, however, CD4 helper function is essential. CD4 memory T cells are thought to require DC and/or B cells for their survival which function as professional APCs. Alternatively, CD4 as well as CD8 and B cells need to be in some sort of balance to maintain the equilibrium of all parties at a constant status quo.

Evidence for CD4 help is provided in experimental models in which CD4 T cells were depleted and the level of CTL activity and protection was examined overtime in mice that were immunized and challenged with a persistent virus. Von Herrath et al. (149) first showed that protection is affected following challenge and the CTL activity is reduced in this model. More recently, Zajac et al. (150) showed that CD4 deficiency impair CTL responses by making them unresponsive, even in the presence of continuous antigen deposition. This is demonstrated by the fact that after PMA stimulation of these cells, very few are able to secrete IFN-γ and were recycling *in vivo*. Transfer of IFN-γ to CD4-deficient mice can restore CTL function, suggesting that the element of help provided by CD4 T cells is the secretion of IFN-γ (151). Although some *in vitro* studies suggest that help for CTLs is provided by CD40-CD40-L

interactions (152-154). The progressive loss of CTL late in disease has also been documented in HIV infection (155), Friend virus infection (156) and murine γ-herpes virus infection (157) in which CD4 helper function has been compromised or lost.

Fewer studies have investigated the factors required for the maintenance of memory CD4 T cells. Among them, the role of B cells has been addressed in B cell-deficient mice that were infected with keyhole limpet hemocyanin and CD4 specific responses were monitored over a period of 6 month. Results have shown that IL-2 production was affected due to a lower frequency of responsive CD4 T cells that managed to survive in the absence of B cells (158). Antigen immunization did not restore CD4 proliferation but transfer of B cells restored memory development again. These results suggest together with a recent study that antigen presentation by B cells is not required for long-lasting CD4 memory T cells, B cell interaction are (159;160). DCs have also been shown to be implicated in the maintenance of CD4 memory cells through MHC class II/peptide stimulation (161). The role of B cells is though to be the trapping of antigens for long periods of time, in the form of immune complexes on the surface of follicular dendritic cells (FDC) (162).

The reason behind the differences between the two concepts of memory and their maintenance is probably because of the use of different protocols. Such as the use of models in which viral replication is persistent versus others that are not; the route of immunization with or without adjuvant could also effect antigen deposition and finally the type (peptide versus replicating virus), half-life and dose of antigen uptake. Moreover, the confusion between effector and resting memory T cells that have different requirements, could lead to different interpretations and conclusions. However, from recent data on cell cycling (163) as well as state of the art molecular tools, researchers are more inclined to believe the continuous cycling of memory T cells that are regulated by a constant antigen stimulation and cellular interactions.

2. Immunopathogenesis of HIV-1 and SIV

2.1. Clinical features of HIV and SIV infections

The clinical course of HIV infection is comprised of three different stages: 1) Primary infection which is characterized by clinical symptoms that occurs up to 70% of the cases; 2) a period of clinical latency that can vary in time; and finally 3) an AIDS-defining illness. A newly infected individual can start to present symptoms within 1 to 4 weeks after infection; these symptoms

consist of headache, retro-orbital pain, muscle aches, sore throat, fever, swollen lymph nodes, and rash. In some cases oral candidiasis and ulcers in the esophagus or anal canal may occur, as well as disorders in the central nervous system (CNS) such as encephalitis, or even gastrointestinal disorders such as pneumonitis or diarrhea. All these acute mononucleosis-like illnesses are reported in some of the infected patients. Not all patients represent the same symptoms, for the same period or in the same severity, which makes this disease very difficult to diagnose (164-166).

There are striking similarities between HIV infection in human and SIV-induced diseases in macaques, which makes the SIV/macaque model very useful for understanding the pathogenesis of HIV. Among the reported clinical symptoms, SIVmac-infected macaques can develop skin rash, and histopathologic changes that can occur in lymphoid organs, brain and lung. Sequential lymph node biopsies performed on infected rhesus macaques followed by *in situ* hybridization has shown that the virus is detected in these sites as early as 5 days postinoculation, in the form of cells expressing viral RNA (167). This is followed in the next months by a generalized lymphodenopathy of peripheral and visceral lymph nodes. These enlargements could persist for several weeks, months or they can even remain throughout the course of the disease. In some macaques, lymph nodes and spleens may return to normal or smaller size following primary infection.

These clinical symptoms whether in infected humans or macaques coincide with a transient peak in HIV/SIV viremia as well as a transitory drop in circulating CD4 T cell levels and a rise in the number of CD8 cells, which results in the inversion of the CD4:CD8 ratio (166;168;169). Within months following primary infection, the CD8 T cells return to baseline or slightly higher levels. On the other hand, the CD4 count returns to normal levels for a very short period before decreasing progressively and permanently, causing the infected hosts eventually to become immunodeficient and susceptible to infection by opportunistic diseases.

Rates of disease progression can be substantially different among infected subjects. In both human and macaques they can vary from not having clinical signs of disease, to having some symptoms, to having very severe symptoms during acute infection and throughout the course of the disease. The duration of the disease can also vary. In SIVmac infected rhesus macaques, not all macaques evolve with the infection in the same way (170). Overall, HIV/SIV infection

can progress in four distinct patterns (171):1) typical progression, 2) slow progression, 3) Long-term-non-progression, and 4) fast progression (figure 5).

2.1.1. Typical progression

Between 70-80% of HIV-infected individuals are considered typical progressors. This group is characterized by having during acute infection clinical syndromes of variable severity that can last for several weeks (166). Usually, individuals that have CD4 counts below 500 per µl suffer from clinical symptoms, whereas individuals with CD4 levels higher that 500 cells per µl do not encounter any signs of disease. Chronic infection is characterized by a period of clinical latency, which usually lasts between 6 to 8 years. It is accompanied by a progressive loss of CD4 count and a persistent but low replication of the virus, which can be detected in plasma as low viral RNA copies. Progression to clinically apparent disease is usually observed when CD4 count reaches 200 cells per µl of blood and the viral load increases due to low immunological control, reaching very high levels in plasma. This occurs within 8 to 10 years after infection in typical progressors. At this stage, the immune system is compromised and increasingly sensitive to opportunistic infections such as pneumonia. Kaposi's sarcoma and other malignancies, as well as neurological abnormalities are observed (172).

2.1.2. Slow progression

A small percentage of individuals undergo progression to AIDS at a slower rate than typical and have less severe clinical symptoms. CD4 count remains stable for an extended period of time although viral replication is still sometimes detected in these individuals during the asymptomatic phase (173). Immunological responses detected in infected individuals and macaques have been shown to be stronger and responsible for the control of viral replication (174-177). Establishment of AIDS related-diseases are observed after 10 to 12 years of infection. Virological and immunological mechanisms responsible for prolonged survival period in this group will be discussed in the upcoming section.

2.1.3. Long-term non-progression

Long-term non-progressors are individuals that do not show any progression to AIDS for an extended period of time. They represent about 5% of HIV infected patients. No clinical signs

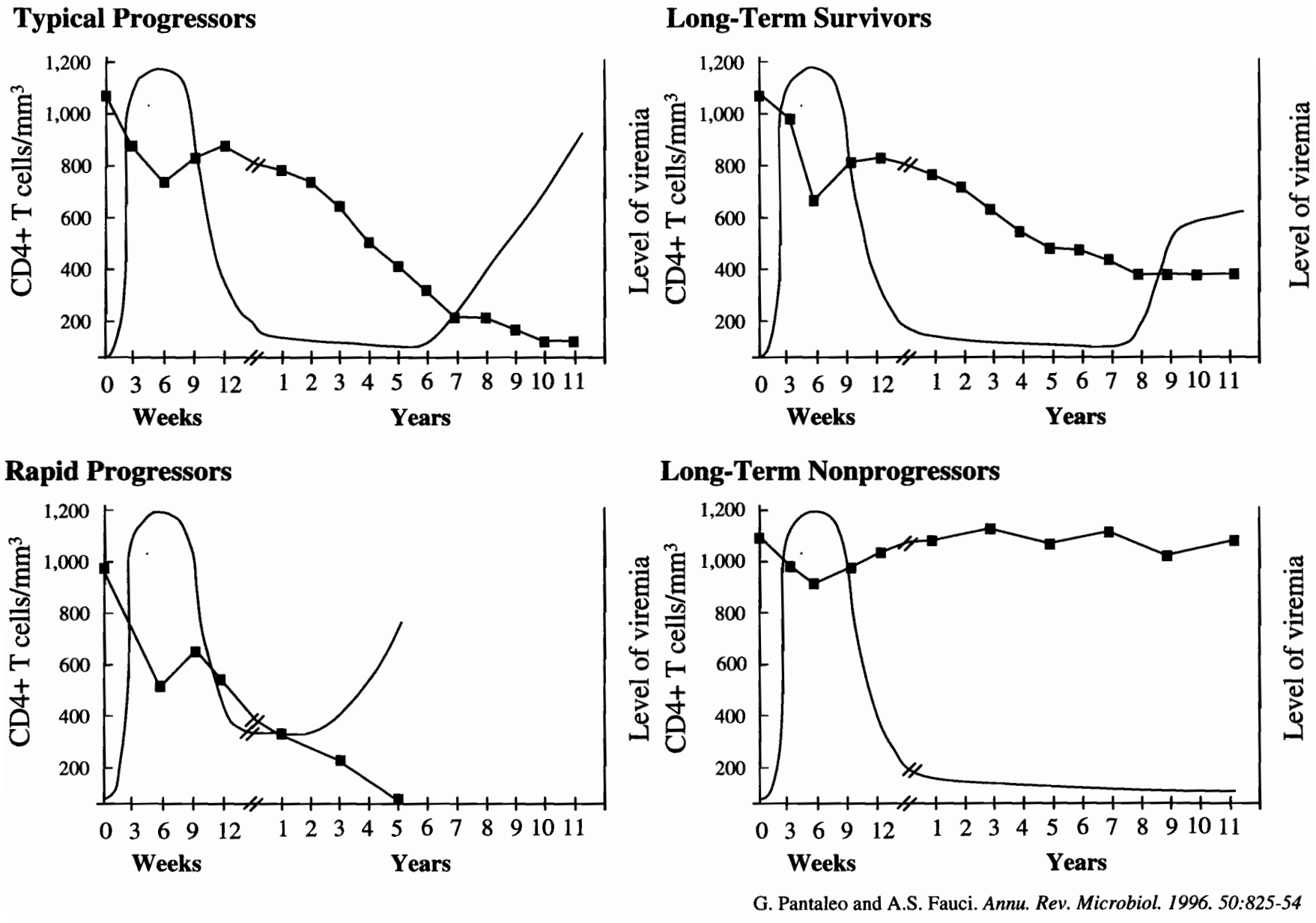


Figure 5: The course of HIV infection includes four types of disease progressions with regard to their CD4 count and viral load overtime.

of immunodificiency are observed in these patients even 10 years after primary infection. They display conserved lymph nodes architecture, a stable CD4 count and very low to undetectable levels of HIV viral RNA copies (178;179). Indeed, in some Gambian prostitutes highly exposed to HIV-1 and HIV-2 no evidence for HIV was apparent by PCR and viral culture (seronegative), although HIV-specific CTL activity was detected, indicating exposure to the virus (180). This unusual disease-free status has been related to several factors such as exposure to defective viruses (181-183). However, other factors could also contribute to the arrest of disease progression. These include a strong CTL activity, CD4 helper responses, neutralizing antibodies, and other host factors that will be discussed later.

2.1.4. Fast progression

Fast progressors include between 10 to 15 % of HIV-1 infected individuals who experience a fast and difficult progression to disease that could take about two to three years after the establishment of primary infection (184-186). Their CD4 count drops very drastically and the viral set point in these individuals is very high. The downregulation of the initial viral peak is not effective in fast progressors, indicating an inefficient immunological control. Indeed, in these individuals homogeneity in HIV isolates is found, whereas in typical progressors a change from viral homogeneity to heterogeneity and back to homogeneity is observed, indicating that viral escape mutants are absent most likely due to the lack of the immunological pressure. Rapid progressors usually suffer from severe and persistent symptoms such as fever during primary infection that may last throughout the disease course. These illnesses observed during acute infection may reflect a hyperresponding immune system with production of cytokines that give rise to the clinical signs. The more rapid progression to disease in symptomatic people may reflect the immune activation that could in turn enhance HIV replication and spreading.

2.2. Immunological and virological events during HIV/SIV infection

2.2.1. Virological events

2.2.1.1 Transmission

The early events of HIV transmission and the establishment of infection are achieved by mucosal or transdermal contact with contaminated bodily fluids. It is well accepted that the first cells to be infected and transmit the virus are dendritic or Langerhans cells located in the mucosal

epithelium. Within two days after intravaginal SIV inoculation of rhesus macaques, infected langerhans were found in the paracortex and subcapsular sinuses of the draining internal iliac lymph nodes. This finding indicates that langerhans present at mucosal sites are reservoirs for SIV that transport the virus to the most neighboring lymph nodes, that can spread the virus through the infection of activated CD4 T cells residing in those sites (187;188). In support of the migration into lymph nodes is the fact that in the absence of T cells, dendritic cells are unable to support HIV replication (189). Dendritic cells have two separate mechanisms to transport HIV (190): 1) by capturing the virus, a mechanism which has been shown to be CD4- and co-receptor-independent, or 2) through their infection by HIV since they do express CCR5 co-receptor (191) as well as CD4 molecules, which are the receptor for HIV entry (192). Interestingly, during the acute period, before strong immune responses can occur, replication of a predominant HIV strain can be detected as a relative homogeneous virus population that is macrophage-tropic (NSI) and hence uses the CCR5 co-receptor.

Following transmission of the virus to lymphoid organs, high-level virus replication takes place, which is reflected in a few days by p24 antigenimia and high viremia titers. Up to 5,000 infectious particles/ml, or 10^7 viral RNA copies per ml of plasma can be detected at the peak of viremia (193;194). These findings were shown by cross-sectional and longitudinal biopsies performed on lymph nodes collected from acutely HIV/SIV-infected individuals or macaques respectively. In both cases, the time at which cells in the lymphoid organs are infected coincide with or precedes the establishment of viremia observed in the blood, indicating that the spread of the virus to the periphery is due to its initial replication in lymphoid organs (167;171;195).

2.2.1.2 Mechanisms of HIV escape from immune responses

One of the main objectives of the HIV virus at this early stage is to replicate in and invade as much as possible target cells before the appearance of immunological responses that can restrain the virus from further dissemination into other sites. However, even though the generation of HIV-specific cellular responses has been shown to be responsible for the downregulation of the virus in the circulation (176), the virus has developed mechanisms to escape this immunological control. Mechanisms include 1) the formation of a stable pool of latently HIV-infected cells that harbors replication-competent viruses upon activation, 2) the potential to mutate and generate variants, and 3) trapping of viruses on the surface of follicular dendritic cells (FDC). All these

processes could be resistant to anti-retroviral therapy or to cytotoxic killing, providing means for the virus to persist.

The establishment of a reservoir for HIV in resting CD4 T cells occurs very early in infection, even though the frequency of this process is very low, its presence is stable in HAART-treated or untreated patients (196). This phenomenon occurs when activated CD4 T cells have undergone integration of proviral DNA but has returned to a resting G0 state in which no transcription of viral genes can occur. In contrast to the labile nature of the pre-integrated forms of the virus, post-integrated forms in resting cells may permit the survival of the DNA for the entire life span of the memory T cells in which they reside (i.e. months to years) (197). Frequency of these reservoirs does not correlate with either CD4 count or the viral load or time in cross-sectional studies and it has a very slow decay rate (198;199), indicating that this phenomenon is random and is not associated with disease status. Moreover, the fact that it is resistant to antiretroviral therapy comes from the finding that recovered viruses from those reservoirs did not show any mutations associated with resistance to the relevant aniretroviral drug used by the patient (196). However, attempts to reduce this pool have unraveled the possibility of activating and therefore releasing all the replication competent viruses from the resting CD4 T cells in combination with HAART. Although results have been positive at first, in that a reduction in the pool of resting CD4 T cells that contains replication-competent HIV was observed in HAART plus IL-2-treated patients compared to HAART alone (200), the complete eradication of HIV in infected individuals remains extremely problematic (201).

One of the most convincing evidence for the existence of HIV escape from CTL killing is when more than 10^{10} autologous cloned nef specific /HLA-A3-restricted CTLs were given back to an HIV infected donor with large doses of IL-2. In contrary to what was expected, a transient increase in viral load and deletions in the nef gene were apparent in a large proportion of the virus load collected from that patient. These deletions had removed the epitope for the CTL clone. These results suggest that the infused CTL clone had exerted a selective pressure on the virus to mutate by becoming immunodominant (202). Moreover, Couillin et al. (203) have observed that mutations in the epitopes recognized by HLA-A11+ CTL occurred more often in patients that were HLA-A11-positive compared to those that were HLA-A11-negative. The same observations were made for HLA-B7 and B8-restricted epitopes (204), (205). Similarly HIV can also escape pressure exerted by other mechanisms that are intended to control HIV replication

such as antiretroviral therapy (206), HIV-specific CD4 helper responses (113), or humoral immune responses (207).

During acute HIV infection before seroconversion plasma virus is relatively homogenous in sequence indicating that a dominant strain is replicating and CTL responses has only started to downregulate HIV replication. Soon after seroconversion, a fairly rapid production of viral variants takes place reflecting the emergence of the host immunological responses. However, few cases have reported that even in acute infection escape can occur, usually this is observed when a monospecific immunodominant CTL response is present (208;209).

HIV can escape from CTL killing or from CD4 TH1 helper responses by mutating at residues that are implicated in antigen processing or that are in direct contact with HLA binding. Indeed, these mutations can either abrogate binding altogether, bind weakly with a fast off-rate, or bind but changes the peptide orientation or alter residues that are exposed to the TCR (210). Usually CTL responses that are polyclonal are less effected by single epitope changes compared to monoclonal CTL responses. Mutations in the residues that contact the TCR can affect recognition and interaction in several ways. Either by abrogating the T cell response, induce a poor response which can shift the immunodominant epitope to other peptides, and in some cases changes in the epitope can antagonize CTL clones so that they fail to respond to the wild-type peptide (211).

In the early stages of HIV or SIV infection, lymphoid organs contain HIV-infected cells as the primary source of the virus. Following seroconversion, the appearance of complement (C)-binding antibodies specific for HIV proteins are programmed to bind with HIV protein, creating immune complexes that trap the virus on follicular dendritic cells (FDC) within germinal centers. These trapped virions are the dominant form of the virus present in lymphoid organs at this stage, they can provide a continuous source of virus for infection of susceptible target cells that migrate through lymphoid organs (212;213).

Another mechanism that HIV has adapted to escape from CTL lysis is through the dowregulation of the class I molecule by Nef protein (214). This is accomplished by modifying the transport of newly synthesized MHC class I molecule from trans-golgi into clatherin coated pits for endosomal degradation instead of allowing their regular path to the cell surface. The downregulation of the MHC class I molecules is mediated by a signal sequence located in the cytoplasmic tail of HLA-A and HLA-B heavy chains only (215), lack of this tyrosine-based

sorting signal will abrogate the effect of Nef, this is observed in HLA-C and HLA-E haplotypes that do not have this motif (216). Cynomolgus macaques infected with either SIVmacC8 a Nef negative strain, or SIVmacJ5 a wild type strain show SIV specific CTL responses only in the C8 group, the J5 group showed a increase in Fas-L expression. Interruption of the Fas-Fas-L interaction in J5 infected macaques was accompanied by a rise in CTL activity, indicating that Nef rescues infected cells from CTL killing by up-regulating the surface expression of Fas-L (217). This induction of Fas-L expression is mediated by the interaction of Nef with an ITAM motif located in the intracellular tail of the TCR zeta chain (218).

Furthermore the switch from macrophage tropic strains to T cell tropic strains that require the CXCR4 instead of the CCR5 co-receptor during disease progression, allows the virus to escape from CTL associated chemokine secretion which are MIP-1α, MIP-1β and RANTES that have been shown to compete with HIV for receptor engagement (219).

2.2.1.3 Putative superantigen effects of HIV

Superantigens are molecules that include microbial toxins and endogenous retroviral gene products that can stimulate proliferation or cause clonal anergy or deletion of a large number of T cells bearing a particular TCRBV sequence, depending on their environmental stage or the experimental condition used. All superantigens have the ability to stimulate the proliferation of T cells irrespective of their antigenic specificity, since they interact with monomorphic regions of MHC class II molecules on APCs, and with TCRBV regions of the TCR on T cells.

The issue of whether or not HIV encodes superantigens has engendered much interest. Several groups have observed deletions of CD4+ TCRBV T cells expression-specific BV segments. The selective elimination of CD4 T cells in AIDS patients was suspected to be driven by a superantigen of an HIV or an HIV-associated origin. Among these findings, Imberti et al. (220) have examined in PBMC the TCRBV repertoire of HIV infected and seronegative individuals by RT-PCR. Their results have indicated that patients with advanced HIV infection demonstrated a selective loss of T cells expressing TCRBV14, 15,16,17,18,19 and 20 compared with seronegative controls. Another group analyzing the TCRBV repertoire by three-color flow cytometry has come to the same conclusion, however they only observed a depletion in TCRBV19 (221). The ability to analyze TCRBV families in CD4 and CD8 subsets separately has demonstrated that these selective expansions or deletions are present in the CD4 but not in

the CD8 compartment, in some research groups (222). The authors concluded that HIV-1 or non-HIV-encoded superantigens cause a selective response by CD4 T cells in HIV infections. In search for evidence suggesting superantigen potential, Soudeyns et al. (223) have analyzed the TCRBV repertoire of HIV infected HLA-matched individuals in lymph nodes and peripheral blood. They have shown that overlapping sets of TCRBV segments were perturbed in both patients that share the same HLA-haplotypes, consistent with an indication of a superantigen function. Also, gp160 was suggested to display properties indicating a superantigen-effect because of its ability to stimulate in five different HIV infected individuals, overlapping sets of TCRBV segments (224).

Among the groups that negated the presence of such superantigen, Nisini et al. (225) have shown in a mixed lymphocyte reaction with peripheral blood mononuclear cells (PBMCs) from monozygotic-twins discordant for HIV, a lack of stimulation of the PBMCs from the healthy monozygotic-twin which shares the same HLA-haplotype. Whereas the superantigens encoded by the retroviral MMTV do in minor mouse stimulation (Mls)-positive mice. Moreover, depleted CD4+ TCRBV families in AIDS-patients known to engage SEB, were able to expand upon *in vitro* stimulation with SEB of PBMCs collected from these patients. This indicates that in the case of HIV-infected patients such cells could not have been deleted from the repertoire since they were able to re-expand upon stimulation *in vitro* with HIV-1 (226). In the intent to investigate a possible role of a TCRBV12-associated superantigen because of a preferential replication capacity of HIV in those cells (227), further experiments illustrated that a CMV and not an HIV gene product was responsible for this superantigen-driven TCRBV12 selective HIV-1 reservoir *in vivo* (228).

2.2.2. Immunological events

Immunological responses against HIV or SIV are detected within days after infection during primary infection. At this stage HIV has already invaded lymphoid organs and series of immunological events proceed in the attempt to control viral spreading to other sites. Among these events cellular responses are the first to be detected, which are characterized by a sharp increase in CD8 T cell numbers (164;178). Within two month after primary infection neutralizing antibodies against several epitopes of HIV namely gp120 are produced (229). As for CD4 helper responses, most of the reports in human or in SIV-infected macaques, suggest

that they are implicated during chronic infection (174;230). However, despite the generation of vigorous virus-specific immune responses, HIV replication is persistent and this would ultimately lead to the breakdown of the immunological responses and the establishment of AIDS-like diseases. Some of the strength and weaknesses of the immunological events that occur during HIV and SIV infection will be discussed in the following paragraph.

2.2.2.1 CD8 immune responses

During acute infection, CTL responses rise in association with increases of HIV in blood. When these cellular responses reach a peak, the level of viremia in blood falls accordingly. The most convincing evidence for the role of CTL responses in the dowregulation of HIV has been demonstrated in the SIV model. Schmitz et al. (176) have shown that in the absence of CD8 T cell responses during acute infection, through the administration of monoclonal antibodies against CD8 that results in their depletion, SIV infection of rhesus macaques results in a rapid and marked increase in viral load and a rapid progression to disease. However, when the antibody is infused during chronic infection, viral load increases again and remains high until the antibody has no effect on CD8 depletion. These results show that CD8 responses play a role in controlling HIV replication during acute infection. Several reports have identified CTL responses during this phase of the disease in humans as well as in SIV models. Among them, Borrow et al. (231) and Koup et al. (232) have quantified CTL activity against several HIV proteins, namely gag, pol, env, nef and tat, in patients experiencing acute infection. A correlation was made between the ability to mount a strong CTL response and the efficiency to control primary viremia, patients that had undetectable CTL activity had high levels of viral load whereas, patients with a strong CTL activity were able to dowregulate plasma viremia. The TCRBV repertoire has been extensively analyzed in acute (102) and chronic (101) HIV infection of adults, whereby major expansions in TCRBV families were observed in the CD8 compartment, these expanded T cells were shown to mediate CTL activity against HIV. Studies in HIV-infected children (233;234) have shown that during acute infection, HIV-infected children show more oligoclonal expansions in their CD8 TCRBV repertoire than children exposed to HIV through their mother but remained uninfected, suggesting that the generation of CTL responses against HIV depends on antigenimia. Macaques acutely infected with SIV

(235-237) have also shown oligoclonal expansions in their CD8 repertoire that mediate CTL activity against gag epitopes.

The early detection of CTL responses in HIV or SIV infected subjects were obtained through limiting dilution assays. Although this strategy enabled the identification of CTL responses during primary infection, it underestimated the magnitude of CTL responses during that period. A better quantification became possible with the use of HLA tetramers bound to HIV immunodomnant epitopes (238). This strategy confirmed the presence of CTL responses following peak of viremia, in SIV infected macaques (239) as well as in human (240).

A correlation was made between the qualitative nature of the CTL response generated during primary infection and progression to disease. As described by Pantaleo et al. (177), slow progression and low viral burden were associated with a diverse CD8 TCRBV repertoire, whereas individuals with restricted CD8 responses showed high viral load and rapidly progressed to AIDS. The same observations were made in SIV infected macaques in which both restricted and diverse repertoire against a single gag peptide were identified (235;236). Moreover, macaques with a detectable CTL response during acute infection have a better prognosis than those with no or late CTL responses (239). Differences in disease progression where also shown to correlate with the number of CTL clones present during primary pediatric HIV (241) or later during chronic infection (242). Although several host factors can play a role in determining the qualitative nature of HIV-specific CTL responses, their implication in disease progression could be explained by the fact that the more CTL clonotypes are mobilized against HIV, the less HIV can escape from the immunological checkpoint (see above). Furthermore, when the restricted CTL response is monoclonal, continuous stimulation and proliferation could result in the clonal exhaustion of this response (243).

CTLs control viral replication through several mechanisms. These mechanisms are either cytolytic or non-cytolytic, and include the secretion of cytokines and chemokines, as well as killing by perforin and granzyme proteases. CTLs do not use the same antiviral activities in all infections. Depending on the infecting pathogen, some antiviral factors are secreted more than others. For-example, perforin-knock-out mice are sensitive to killing by LCMV but not by hepatitis B or vaccinia (244). As for HIV infection, among the noncytolytic factors, CD8 T cells produce IFN-γ, TNF-α and TNF-β upon contact with infected cells. Whereas IFN-γ was shown to have inhibitory effects on the early steps of HIV replication, TNF-α up-regulates replication

by activating the promoter located in the 5' LTR (245;246). Other noncytolytic factors include the chemokines specific for CCR5, the co-receptor of HIV, which are MIP-1α, MIP-1β and RANTES (247). These chemokines inhibit viral entry by blocking the critical interaction between CCR5 and the V3 domain of the viral envelope glycoprotein gp120. These chemokines mediate CTL activity by different mechanisms, such as RANTES induces direct cytolytic activity via the interaction with CCR3 (248), which induces increases in Fas-L expression on HIV-specific CTLs. These cytolytic effects as well as increases in Fas-L could be inhibited by administration of monoclonal antibodies against Fas-L (249). Among other cytolytic pathways, perforin and CD8 antiviral factor (CAF) were identified. CAF was shown to be different from any known antiviral cytokine (250) in that it has inhibitory effect on virus production by interfering with the LTR-mediated transcription in infected cells, allowing the establishment of viral latency in infected cells (251). Nevertheless, the classical pathway for CTLs for lysing infected cells is mediated by perforin (252).

The immunological destruction and lack of appropriate control of viral replication is due to the inefficient function of CTLs. Some of the mechanisms that result in HIV escape from cellular immune responses are 1) exhaustion of HIV-specific CTL clones early in infection; 2) the establishment of a restricted HIV-specific CTL response; 3) lack of CD4 help during chronic infection; 4) not all HIV-specific CTL clones mobilized during primary infection have a strong cytolytic activity; 5) not all CTL clones generated during primary infection persist throughout the course of the disease; 6) and the persistence of CTL responses depends on antigen exposure, i.e., HIV replication.

The idea of the exhaustion of CD8 responses is not new. It was first demonstrated in LCMV infection in mice, whereby administration of the highly virulent strain CL13-Armstrong which tends to persist after acute infection and spread by activating all the CL-13-specific antiviral CD8+ T cells to the point that they disappear due to their continuous stimulation (253). Pantaleo et al. (243) have observed this consequence in HIV-infected patients during acute infection, by following in a longitudinal analysis the outcome of an HIV-specific CTL clonotype with the use of diversity sequence PCR (DS-PCR). Disappearance of the CTL clonotype was not caused by a mutation in the specific CTL epitope. Furthermore, the patients experienced increases in the levels of viremia. Taken together, these results suggest that in the presence of persistent viral replication, elimination of the specific CTL response could be advantageous for

the virus. In cases where the immunological response is directed towards a single epitope, or whether the response is monoclonal, the disappearance of that clonotype or the generation of mutations in the CTL epitope would result in the collapse of the entire HIV-specific cellular response. This indicates that a CTL response is empowered by its composition and less by its amplitude, the more CTL clones are mobilized the less HIV can escape and the better prognosis is.

The inefficient maintenance of HIV-specific CD8 responses during chronic infection has also been suggested to be caused by the lack of CD4 helper responses. The need for CD4 help to sustain a functional CD8 response was shown in other infections such as LCMV (150), whereby a depletion of the CD4+ T cells in LCMV infected-mice made the LCMVgp33-specific CTL clonotype unresponsive during chronic infection and, viral replication persistent (254). Mice infected with LCMV that have a depletion in class II molecules, lack CD4 help but still are able to mount a primary CD8 response. However, two month later no memory CD8 T cells are detected in these mice suggesting that the initial CD8 response was eliminated (149;255). These results indicate that in cases of non persistent infections CD4 T cells do not play an important role in acute infection since CTL activity sustained for 2 weeks is able to clear permanently certain strains of LCMV viruses. On the other hand, under conditions of persistent infection during chronic phase, whereby CTLs take several months to clear the infection, the interference of CD4 help is crucial.

2.2.2.2 CD4 immune responses

The implication and importance of the HIV-specific CD4 immune response are only starting to be recognized. The first report by Rosenberg et al. (174) demonstrated that an inverse correlation exists between viral load and gag-specific CD4 responses. Patients with advanced disease and high viral load did not have detectable p24-Gag-specific CD4 responses, whereas long-term-survivors did. Furthermore studies in SIV-infected macaques have shown that fast progressors have no detectable CD4 responses compared with slow progressors or macaques infected with attenuated strains of SIV, which induce low viral load and a non-progressor phenotype (230). These results indicate that SIV-specific CD4 responses are inversely correlated with both viral load and the pathogenicity of the virus. Through the use of intracellular detection of IFN-γ producing CD4 T cells after stimulation with HIV peptides, Pitcher et al. (256) have also

demonstrated that HIV-specific CD4 responses are more important in non-progressors than in rapid progressors.

Studies examining the CD4+ TCRBV repertoire of HIV infected subjects undergoing chronic or late stages of HIV infection, have shown perturbations in several TCRBV families (257). The number and magnitude of abnormalities within the CD4 repertoire were inversely proportional to their CD4 counts. Furthermore, perturbations were characterized by either clonal expansions or depletions among CD4 subsets, and these processes are exaggerated with disease progression (258-260). These observations indicate that disruptions in the CD4 compartment occur mostly at the end phase of the disease as a consequence of persistent replication and inefficient control by HIV-specific CTL responses. This is supported by the fact that no apparent perturbations in the CD4 compartment were observed during the early stages of the infection in untreated patients (259), a time when the immune system is at its best. Chen et al. (261) have demonstrated that in fast progressors, the CD4 clonotypes detected during chronic infection are dominant and persistent, however, considering the disease state in these animals, the presence of CD4 clonotypes were not accompanied by an improvement in the immunological control. Taken together these results suggest that in HIV infection the role of CD4 T cells are probably compromised due to the fact that HIV takes to its advantage the activation of these cells in order to replicate and any attempt from these subsets to accomplish their role as helper T cells would cause more damage than benefits. In this case, earlier finding that non-progressors are able to develop a strong HIV-specific CD4 response is probably due to the low replication capacity of the virus in these individuals. This observation is consistent with the finding that acutely HIV-infected patients undergoing HAART therapy manage to generate a strong HIV-specific CD4 response compared with untreated patients experiencing acute infection (262). The lack of CD4 response during primary infection at a time when viral dissemination is optimal (263), suggests that these patients most probably developed helper responses that were immediately eliminated by HIV, making it difficult to detect. Furthermore, early treatment of HIV/SIV infected subject was shown to be more beneficial in the preservation/generation of immunological responses than antiretroviral treatment conducted during chronic infection. (262;264;265). Indeed, initiation of therapy during chronic infection has shown to cause increases in CD4 counts as well as the recovery of proliferation capacities to recall antigens such as candida but not HIV (266). This phenomenon could be due to prolonged suppression of HIV replication that prevents antigenic

stimulation of the preexisting HIV-specific helper response (267). However, what we do not know is whether the preservation of the CD4 population during acute infection due to drug therapy reflects the rescue of an HIV-specific CD4 immune response that plays an important role in prognosis. Our own analysis of the CD4 repertoire in SIV infected cynolomgus macaques has revealed that the early expansions in the TCRBV families contain CD4 clonotypes that persist only when SIV replication is under control early in infection (chapter 3 and discussion). Results from our group and from others suggest that a possible elimination of CD4 responses early in infection tends to restrict the pool of SIV specific CD4 T cells when they are mostly needed, giving rise to either no response or a poor response that is not always strong enough to keep viral burden in check. When the virus has reached a distinct threshold of viral RNA copies in plasma, any activation of CD4 T cells specific for HIV or other antigenic stimuli will only provide a reservoir for HIV replication and persistence dissemination. On the other hand, patients treated during primary infection have a broad and strong SIV specific CD4 repertoire that are preserved due to the absence or the presence of low viral replication. In these cases they are able to accomplish their role in maintaining CD8 T cell and B cells responses.

2.2.2.3 B cell responses

With regard to the HIV-specific humoral immune response, the first antibodies generated against a variety of HIV proteins are mostly detected during seroconversion at the time of peak viremia or also during the downregulation of viremia. These antibodies lack any neutralizing activity and their appearance is followed by the sequestration of newly produced virions into lymphoid organs. Indeed, these antibodies have a complement (C)-binding ability that could mediate the formation of immune complexes. FDCs and the reticuloendothelial system express receptors for C that could retain these immune complexes in lymphoid organs and provoke the removal of the viral particles from the circulation. Therefore even though the initial primary humoral immune response may not be protective, it allows their deposition at sites were immunological responses are generated (268). The potential implication for trapping viral particles in lymphoid organs is the continuous source of virus for de novo infection of cells that reside or migrate through these lymphoid organs, or even inducing a chronic stimulation of resting cells that are harboring HIV particles including CD4 T cells. This process would result in the continuous activation of the immune system that constitutes the best environment for HIV replication (269).

Neutralizing antibodies are generated during or even after the transition from acute to chronic infection. In contrary to C-binding antibodies, neutralizing antibodies are thought to confer protection. These antibodies are directed mostly against cryptic epitopes that are not usually exposed early in infection, which explains why they are not detected during that period (270-273).

Studies monitoring the production of neutralizing antibodies have shown that long-term non-progressors produce a broad repertoire of antibodies against several HIV epitopes. On the other hand, rapid progressors have instead a very restricted repertoire or no neutralizing antibodies. Furthermore, neutralizing activity has been shown to protect against SIV Sooty Mangabey (SIVsm) infection of naïve animals through passive immunization of antibodies from one long-term non-progressor macaque (274). The escape of virus variants from neutralizing antibodies usually occurs through mutations in the loop and flanking sequences in variable region 3 of the HIV-1 envelope. HIV-infected individual may harbor 10^{10}-10^{12} different proviruses that can provide a large reservoir for the outgrowth of the immune response escape mutants.

3. Host and viral factors modulating HIV-1/SIV disease progression

3.1. Host factors

As discussed earlier, the course of HIV infection can be substantially diverse among infected individuals. Factors responsible for these differences can be due to individual variations that may profoundly influence TCR mediated immune responses against HIV and disease progression. These individual variations can be observed at several levels: 1) the TCR repertoire that initiates the responses, 2) the MHC class I and II molecules that present the appropriate peptides, 3) the chemokine receptor expressions that may confer resistance to infection, and 4) the state of activation of the immune system due to ongoing infections that can influence HIV replication.

3.1.1. The T cell receptor repertoire

The TCR repertoire is shaped by genetic factors that are ultimately responsible for individual variations. Studies that assessed variations in the TCR repertoire between two unrelated individuals compared with two twins have shown that a substantially lower variation is observed between identical twins compared with two unrelated subjects. These comparisons have been

addressed by calculating the Δ score obtained by summing the absolute differences in frequencies for all 22 TCRBV families between any two individuals being compared. Within the same individual, these variations can be introduced between subpopulations of cells such that TCRBV families are differentially expressed in CD4 and CD8 T cells, or between T cell populations collected from different anatomical sites (275). Variations in the frequency of each TCRBV family can be found among different individuals, indicating that the composition of the TCR repertoire is different (276).

The principle genetic factors that shape the TCR repertoire include MHC class I and II molecules which are the most numerous, divergent, and evenly distributed within the population. They play an important role during thymic selection by presenting at the surface of epithelial or dendritic cells in the cortex or macrophages in the medulla a defined set of self peptides that will ultimately govern which T cells are positively and/or negatively selected. Akolkar et al. (277) have assessed the influence of such molecules on HLA-identical sibs. Results have indicated that more similar patterns of V segment frequencies have been found compared with totally mismatched or haplo-identical sibs, as measured by mAbs specific for a panel of nine TCRBV families, and by semi-quantitative PCR using primers specific for these TCRBV families. However, when comparing monozygotic twins with HLA-matched siblings, the TCR repertoires of identical twins were shown to be more similar to each other than to their HLA-identical sib, indicating that non-HLA genes also contribute to influence the expression levels of TCRBV families. The authors suggested that polymorphism in the TCR genes may be implicated (278).

Apart from genetic factors that can alter the TCR repertoire and influence immune responses, other environmental factors can play a role in shaping the mature TCR repertoire in favor of the host or the invading pathogen. Environmental factors include previous infections that induce permanent changes in the TCR repertoire such as deletions of T cell clones or even a whole TCRBV family in cases of superantigens such as SEB (279). In both cases holes in the repertoire are introduced which alters the composition of the native TCR repertoire (243;280-282). Because HLA and TCR genes are known to have a profound effect on the repertoire, assessing whether environmental factors influence the composition of the TCR repertoire requires experimental design that precludes or minimizes such genetic factors. Therefore, disease-discordant identical twins were analyzed for their TCR repertoire (283;284). Results have shown that when comparing a diseased TCR repertoire to the one from its normal twin,

significant differences in both CD4+ and CD8+ T cell populations were observed in two independent studies that were analyzing either HIV- or rheumatoid arthritis-discordant twins. In both studies when the TCR repertoire of pairs of normal monozygotic twins were compared, the two sibs in each pair were found to have similar TCR repertoires.

Taken together, we can conclude that the TCR repertoire is constantly exposed to changes from a genetic aspect during T cell selection and from the environment. Upon infection with HIV, the diversity and composition of the repertoire may influence disease progression by modulating the efficiency and the breath of the CD8 and the CD4 immune response. Among these effects, the nature, size and diversity of the preimmune HIV-specific repertoire give rise to an immune response that is more or less sufficient to control HIV replication. Indeed, it has been shown by Pantaleo et al. (177) that an inverse correlation exists between the diversity of the primary HIV-specific CD8 immune response and disease progression. As for the CD4 immune response Rosenberg et al. (174) have also shown that a strong CD4 immune response is associated with low viral load in slow progressors during chronic infection. Consequently, viral evasion and persistence are less observed in slow progressors that are able to maintain a strong and diverse TCR repertoire against HIV compared with fast progressors.

3.1.2. Genetic factors

The principal components of an effective immune response are APCs that process the infectious pathogen's proteins, the MHC class I and II that bind to those processed peptides and present them to the immune system, and finally the TCR repertoire that initiates an immune response against this pathogen. Variability among individuals in containing and handling HIV persistence is directly associated with polymorphism in the components of the immune response. Apart from variability in the TCR repertoire within the population already discussed, an extensive polymorphism in MHC molecules is also important. Since MHC molecules act as filters driving the recognition of epitopes by the host immune system, polymorphism in the MHC molecules will thus result in variations in HIV-specific immune responses among infected individuals.

Many studies have looked for associations between HLA alleles and HIV progression. HLA B27 and B57 have been reported to be associated with slow progression (285;286), whereas DR11 and B35 have been found to accelerate disease progression (287;288). Other alleles seem to be

protective against HIV progression including B14, C14 and C8 (289). This can be explained by the fact that some HLA molecules are better presenters of very immungenic epitopes, or bind with strong affinity, stimulating therefore T cell clones for a longer period of time. Other HLA molecules would present minor HIV epitopes, or immunodominant epitopes but with weak affinity causing a high association-dissociation rate and hence a weak or absent immune response. Moreover, it is only logical that heterozygotes in HLA molecules have the advantage of presenting twice the number of HIV peptides compared with homozygotes, a difference that should result in a more potent T cell response. The benefits of heterozygosity will depend on the allotypes they express and will diminish according to the overlap in the repertoires of peptides they present (290).

Identification of chemokine receptors as co-receptor of HIV has engendered new insights on HIV transmission and progression. Definitive evidence that CCR5 is the molecular factor that mediates HIV entry of M-tropic strains came from the identification of the mutant form of CCR5, designated CCR5Δ32. This mutant allele was associated with resistance to HIV infection due to a deletion of 32 bp in the region of the open reading frame encoding the second extracellular loop, causing a frame shift and premature stop codon in transmembrane domain 5. The truncated product is not expressed on the cell surface (291). The mutation in a homozygous form was described in two homosexual men that remained uninfected despite high-risk exposure (292). Conflicting results were reported regarding the effect of heterozygotic genotype on HIV transmission; either no effect or some protection from HIV infection are observed (293;294). Other mutations in chemokine receptors associated with delayed HIV progression are CCR2-V64I (295). Since CCR2 is not the main co-receptor for HIV entry, the protective effect against disease progression has been found to be due to a complete linkage disequilibrium with a point mutation in the CCR5 regulatory region, which probably is the cause of the delayed progression phenotype (296). Furthermore, several genetic polymorphisms in the CCR5 promoter, potentially affecting HIV transmission or disease progression, have also been identified. HIV positive individuals homozygous for the CCR5 regulatory region containing the promoter allele, CCR5P1, progress to AIDS more rapidly than those with other CCR5 promoter genotypes (297).

3.1.3. T cell activation

Several lines of evidence support the association of immune activation with pathogenesis. This association relies largely on the fact that HIV replicates predominantly in activated T cells. HIV can enter primary T cells and initiate viral DNA synthesis regardless of the state of activation of the cells, however, only incomplete form of HIV DNA synthesis is produced in resting cells (298). This is the case because transcription of HIV mediated by its long terminal repeat (LTR), depends upon host factors that are transiently activated following antigen stimulation, through binding of a cellular protein to NFκB-binding site in the LTR (299;300).

Cheynier et al. (301) have shown that in the spleen, HIV replication takes place in T cells that are driven to divide in the white pulp in an antigen-specific manner. Activation of the immune system by an on-going antigen-specific immune response to an exogenous stimulus can enhance progression to AIDS in macaques co-infected with Bacille Calmette-Guerin (BCG) and SIV (302-304). This was also reported in HIV-1 seropositive patients that progressed fast to disease after influenza vaccination (305), or tetanus toxoid immunisation (306;307).

The impact of T cell activation on HIV replication and disease progression is the result of several factors. Once activated, CD4 T cells can become target cells that support HIV replication. Eventually, few of them can become quiescent T cells, harbour proviral DNA which can escape from the host's immune defence or any drug therapy (197;198) and assuring therefore the persistence of HIV. Moreover, a network of cytokines that either assist or inhibit HIV replication usually accompanies activation of the immune system. This is well described in the SIVsmm PBJ14(6.6)/macaque model, since SIVsmm PBJ14(6.6) is known to stimulate the proliferation of macaque T lymphocytes *in vitro* and induce an acutely lethal disease in macaques. Following infection of pig tail macaques with SIVsmm PBJ14(6.6), high titers of viral RNA coincided with increases in proinflammatory cytokines like TNF-α, IL-2 and IL-6 in plasma as well as increases in the expression level of activation markers on T cells (308). In an attempt to analyse the effect of CTL secreted cytokines on HIV gene expression, upregulation of HIV-1 gene expression in chronically infected T cell lines resulted from the antigen-specific release by CTLs of tumor necrosis factor alpha (TNF-alpha) (245). On the other hand, interferons display a particularly potent antiviral effect on HIV-1 replication in primary human macrophages. Their action is observed early in the virus life cycle and result in the reduction of viral DNA synthesis (309). Chemokines such as MIP-1α, MIP-1β and RANTES, are CD8

suppressor factors that are able to block CCR5 receptors and prevent the virus from binding and entering CD4 T cells (219;310). Whether, the cytotoxic activity generated against HIV is able to produce high levels of chemokines or antiviral cytokines, the impact would result in a retarded progression to AIDS (311).

3.2. Viral Factors

3.2.1. Escape from immunological pressure

The ability of HIV to replicate despite a vigorous HIV-specific immune response is an indication that the virus has several mechanism of evading this immunological pressure. As already discussed above, antigenic variation such as those affecting recognition by T cells, or altering permanently or partially binding to MHC class I or II molecules are implicated. In both cases activation of effector cells would not occur and progression to disease would be solely dependent on the diversity of the host's immune response against HIV. A polyclonal response is usually associated with a good prognosis compared with monoclonal response where a single mutation could brake the entire host immunological defense. Other mechanisms involve the downregulation of the MHC class I molecule on the surface of HIV-infected cells, and the exhaustion of CTLs clones by continuous stimulation. Furthermore, localization of the virus in sites that are avoided by the immune system is also a way for the virus to evade recognition. These sites include the central nervous system, eyes and testis. Trapping of the virus on follicular dendritic cells is a constant source of stimulation and hence infection of CD4 T cells, and they are able to remain viable and assume production of new virions.

All of these mechanisms allow HIV to persist, and they are in constant encounter in the immune system. The outcome of this interplay determines the course of HIV infection.

3.2.2 Attenuated versus virulent viruses

Several reports have identified HIV strains that are able to slow the rate of disease progression due to deletions within the *nef* gene that renders this gene nonfunctional. The Sydney Blood Bank Cohort, is a group of eight HIV-infected persons infected from a common donor whose virus had a defective *nef* gene. All these patients showed a non-progressive phenotype at first (312). However, long term follow up revealed that some of these patients started to have detectable viral load and a drop in CD4 counts, indicating that progression is not arrested but

delayed (313). CTL responses are usually very strong in these individuals despite very low viral load (314), albeit some reports contradicting this finding. Analysis of *nef* sequences in patients harboring attenuated viruses has revealed that the most predominant deletion (118 bp) removed a highly conserved $(Pxx)_4$ motif. This deletion placed downstream sequences out of frame (182). Others have a single point mutation at position 138 that introduces a cysteine (315).

In the SIV model, Desrosiers et al. (316) have shown that the pathogenic potential of SIV in rhesus macaques can be dramatically increased by reversion of mutated *nef* gene to their full-length wild type sequence. Demonstrating the importance of a functional *nef* for maintaining high virus loads during the course of persistent infection in vivo and for full pathologic potential in SIV-infected rhesus macaques. Several SIV strains that have deletions in *nef* were generated and tested for their pathogenic potential such as SIVmacC8 which has a deletion of four amino acids and two conservative amino acids compared with the wild type strain, SIVmacJ5 (317). SIVmacC8-infected cynomolgus macaques experienced a non- or slow progression that correlated with a stable CD4 count and undetectable viral load (318).

Some other SIV strains, such as SIVsmm PBJ14(6.6) (319), show a more accelerated progression to AIDS than typical SIV strains. SIVsmm PBJ14(6.6) is unusual in the fact that in can replicate in resting PBMC with no prior antigenic activation by stimulating directly resting T lymphocytes (320). Infection of pig tailed macaques results in a rapid and severe disease and death within 10 days of infection following acute diarrhea and rash due to an extensive lymphocyte proliferation in the gut (321). Comparison of the *nef* sequences of SIVsmm PBJ14(6.6) with SIVmac239 strains has revealed the presence of an additional sequence motif, which has the characteristics of an SH3 binding domain at positions 17 and 18 (YERL). YE variant of SIVmac239 which has the SIVsmm PBJ14(6.6)sm motif, was able to infect resting PBMC in culture and induce a lethal like disease in infected macaques similarly to SIVsmm PBJ14(6.6). This variant was able to associate to Src tyrosine kinase and phosphorylate at tyrosine residue more extensively than the SIVmac239 wild type strain, indicating a role of *nef* in cellular activation and signal transduction (322). A chimeric simian-human immunodeficiency virus (SHIV89.6) was designed to be used for vaccine studies in macaques because of its highly pathogenic effect. It is composed of SIVmac239 core and HIV-1 env of a cytopathic, macrophage-tropic clone of a patient isolate of HIV-1 (89.6), as well as HIV-1 associated auxiliary genes tat, vpu, and rev. The pathogenic

form of this chimera was obtained through two rounds of *in vivo* passages in rhesus macaques, which resulted in a virus that can induce high viral load, CD4 lymphopenia and an accelerated progression to AIDS-like disease with wasting and opportunistic infections (323).

Studies investigating the role of *nef* in the maintenance of high viral load *in vivo* and progression to disease has shown that *nef* has two major but separate functional properties: Down regulation of CD4 molecules (324) and promotion of HIV growth. The former role involves a dileucine endocytosis target motif in CD4 that plays a major role in pathogenicity by preventing superinfection of cells with multiple viruses and/or facilitates the release of progeny viruses. The later role of *nef* contradicts earlier findings that reported a negative effect on HIV replication. Indeed, Spina et al. (325) and miller et al. (326) both reported that the role of *nef* in enhancing virus replication depends on the activation state of the infected cells in that Nef+ viruses replicated more efficiently compared with Nef- viruses when primary cells were infected under appropriate conditions. The infectivity potential of nef is mediated by a PxxP motif that specifically binds to biotinilated SH3 domains of tyrosine kinases Hck and Lyn but not other Src related family kinases such as Lck. Recruitment of Hck by nef results in the activation of cellular signal transduction pathway that ultimately promotes the enhancement of HIV replication (327).

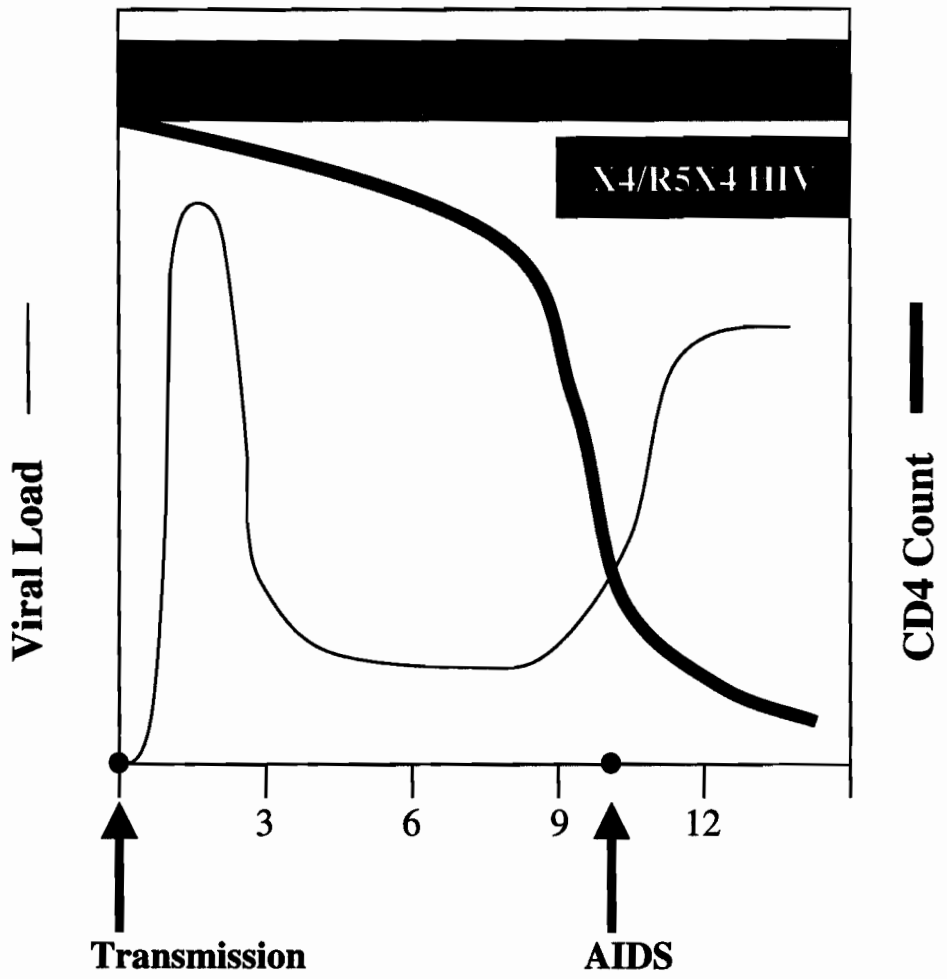
Following the discovery of the role of *nef* and its impact on course of HIV disease progression, efforts were focused on generating attenuated vaccines that have deletions in nef, as well as, other genes, with the rational that the more attenuated the virus the safer it is. However, to the disappointment of investigators, several findings have condemned or limited the use of such approach as preventive or therapeutic vaccines. First, by increasing the level of attenuation of the vaccine, viral replication decreases as well as the protection potential induced by this virus, compared with vaccination with wild type strains. This could be explained by the fact that the more attenuated the virus is the less viral output can mobilize and maintain the persistence of the humoral and cellular immune response generated against this vaccine (328). Second, attenuated SIV strains are constantly under a selective pressure to mutate back to their wild type strain (329).

3.2.3. Tropism

Two major variants of HIV-1 with respect to tropism are associated with different degrees of pathogenicity. In the early stages of HIV infection, HIV isolate tend to be non-syncytium-inducing (NSI) phenotype or M-tropic variants because of their ability to infect *in vitro* monocytes and macrophages but not established CD4+ T cell lines. As the virus mutates, the phenotype shifts to dual tropic strains (i.e. M-tropic and T-tropic) which have the ability to infect primary CD4+ T cells. At later stages of the disease, T-tropic or syncytium-inducing strains (SI) emerge. These strains are able to grow in established CD4 T cell lines. They have been associated with an increase in pathogenicity that results in a more dramatic drop in CD4 count (330-332). This transition in HIV phenotype is linked to the use of different co-receptors. CCR5 usage was restricted to NSI strains early in HIV infection; patients with progressive disease evolve to include the use of other chemokine receptors as well as CCR5 such as CCR3, CCR2b, CXCR-4. However, the emergence of viruses using exclusively CXCR-4 was associated with a switch from NSI to SI phenotype, the loss of sensitivity to chemokines, and rapid T cell depletion (333;334) (figure 6).

3.2.4. Subtype

Not all HIV subtypes have the same ability to infect and replicate in their host; they differ in their virulence and transmissibility (335). Three main HIV groups exist: M, O, and N. Among the M group, 10 genetic subtypes have been identified: A through J. In the United States and Western Europe, the most common subtype is B, whereas the rest (A, C, D, and E) predominate in developing countries. Studies of discordant couples in Thailand have suggested that the risk for heterosexual transmission is higher in patients infected with subtype E compared with subtype B (336), due to the increased tropism of subtype E for Langerhans cells (337). The latter are considered as the first line of cellular targets in heterosexual transmission because they are located at the surface of the vagina, cervix, and penile foreskin. Subtypes A and C have been also associated with higher level of transmissibility in developing countries through an increased transcriptional activation potential as well as an increase in cellular tropism (338). Reports have indicated that HIV-2-infected women have a significantly less important probability to develop an AIDS-like disease within the first 5 years after seroconversion



E.A. Berger, P.M. Murphy and J.M. Farber. *Ann. Rev. Immunol. 1999. 17:657-700*

Figure 6: Correlation between viral tropism and disease progression. Early after transmission, HIV harbors exclusively R5 variants which are usually associated with a state of asymptomatic disease. In late stages, during disease onset, HIV tropism starts to include R5 and X4 variants, or exclusively X4.

compared with HIV-1 infected patients (339). These findings and others, support the association of HIV-2 with less infectivity and less virulence than HIV-1.

4. Project rationale and research objectives

The TCR repertoire is a predominant host factor that can influence disease susceptibility and progression in animals and humans. Methodologies aimed at assessing perturbations in the TCR repertoire have become available. These methods take advantage of the relatedness between polymorphic TCR V genes, in order to structure the repertoire into a number of subfamilies corresponding to structural and functional subsets.

It has been shown that HIV-specific patients have expansions of TCRBV families within their CD8 repertoires. These expansions have been reported in adults (102), children (233;234), as well as in macaques (235-237;340). They occur early during acute infection and they are transient. However, the driving force, whether antigenic or superantigenic which shapes these perturbations in the repertoire is not yet clear due to contradicting results in both human (223) and macaque studies (237). Evidence suggested that the qualitative nature of primary immune responses against HIV correlates with the rate of disease progression (177). However, the initiator of this differential immune response is not known, whether it is the pathogenic nature of the virus or even the activation-state of the immune system due to an ongoing infection, together with HIV. Since these questions are difficult to address in humans, animal models such as SCID-Hu thy/liv mice and macaques can be used to clarify certain features of the immunopathogenesis of HIV. Therefore, the aim of this project was to use animal models as a mean to answer some of the following points:

1. To verify under controlled *in vivo* conditions whether or not HIV infection encodes a superantigen responsible for the observed perturbations in the TCR repertoire. The SCID-hu Thy/Liv mouse is a heterochimeric model of sustained, multilineage human hemolymphoid differentiation. The advantage of using such a model, is the fact that we can exclude other environmental factors that could also alter the TCR repertoire (Chapter II).
2. To study the impact of viral replication on both the CD4 TCR repertoire and disease progression in SIV infected cynomolgus macaques. Analysis of the perturbations in the CD4 TCR repertoire was performed by two color flow cytometry analysis using a panel of cross-reactive anti-human TCRBV monoclonal antibodies that covers 30% of the repertoire, as

well as a CD4 monoclonal antibody. Three SIV strains were used: 1) SIVmacJ5 induces high viral load in infected macaques and a progressive drop in CD4 count which results in AIDS-related illnesses and death within 2-3 years of infection. 2) SIVmacC8, an attenuated strain, induces low viral load and a stable CD4 count. In addition disease progression is usually arrested or retarded in infected macaques. 3) Finally SHIV89.6P, a virulent strain, causes high viral load, a drastic drop in CD4 count and death within few months (Chapter III).

3. To characterize the phenotype, clonality and persistence of CD4 T cells involved in transient high level TCRBV expansions in both fast and slow SIV-infected macaques, given the importance of helper CD4 responses in viral infections. Analyzing the composition and fate of these CD4 specific immune responses in fast versus slow progressors would allow a better understanding of the dynamics of T cell responses during persistent viral infections and a better characterization of factors that may be responsible for these outcomes (Chapter III).
4. To test the functionality and protective potential of these CD4 clonotypes *in vivo* by verifying whether or not these clonotypes are able to re-expand upon challenge with virulent SIV strains in both fast and slow progressors. This is addressed through a longitudinal follow-up of the CD4 clonotypes by heteroduplex tracking assay (HTA), cloning and sequencing, and by *in situ* hybridization (Chapter III and IV).
5. To test whether the lack of detectable CD4 responses in some slow progressors is associated with susceptibility to superinfection with virulent SIV strains such as SHIV89.6P, as compared with slow progressors with oligoclonal and persistent CD4 response. This is addressed through a longitudinal study of the diversity in the CD4 TCRBV repertoire of animals protected and unprotected by HTA (Chapter IV).

Chapter II

Superantigen-mediated deletion of specific T cell receptor V beta subsets in the SCID-hu Thy/Liv mouse is induced by staphylococcal enterotoxin B, but not HIV-1

Superantigen-Mediated Deletion of Specific T Cell Receptor Vβ Subsets in the SCID-hu Thy/Liv Mouse Is Induced by Staphylococcal Enterotoxin B, but Not HIV-1[1]

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Infection by HIV-1 has been associated with perturbations in the TCR Vβ repertoire, suggesting the involvement of a superantigen. Among the hallmarks of superantigens is the capacity to delete T cells bearing specific TCR Vβ families in the developing thymus. To verify the presence of a superantigen in HIV-1, we analyzed the SCID-hu Thy/Liv TCR Vβ repertoire within $CD4^+CD8^+$, $CD4^+CD8^-$, or $CD4^-CD8^+$ thymocytes subsets by flow cytometry using a panel of Abs recognizing about 60% of the TCR repertoire following injection of SEB or infection by two different HIV-1 isolates. Seven days following SEB injection, thymocyte subsets bearing TCR Vβ3, Vβ12, Vβ17, and Vβ20, but not Vβ5 or Vβ8, were deleted relative to mock-injected mice. In contrast, no changes were observed in the TCR Vβ repertoire in $CD4^+CD8^+$, $CD4^+CD8^-$, or $CD4^-CD8^+$ thymocyte subsets after infection with HIV-1. The T cell depletion caused by HIV-1 infection is most likely not mediated by an HIV-encoded superantigen. ***The Journal of Immunology,*** **1997, 158: 544–549.**

Superantigens are a unique class of TCR ligands that can engage T cell subpopulations bearing specific TCR variable region (Vβ) sequences (1–6). Exposure to superantigens in the periphery may lead to significant expansions of cognate TCR Vβ subsets that may then be followed by clonal exhaustion or anergy, as has been implicated in the pathogenesis of human disease states such as the toxic shock syndrome (5, 7–12). Another hallmark of superantigens is their ability to induce deletions of cognate TCR Vβ subsets among developing thymocytes (2, 13).

Prior studies have examined the peripheral blood lymphocyte TCR Vβ repertoire in HIV-1-infected subjects with either limited panels of mAbs (with specificity for TCR Vβ subsets) or quantitative PCR (utilizing primers specific for mRNA encoding TCR Vβ subsets) (14–24). These studies have not conclusively determined whether HIV-1 itself may encode a superantigen or whether perturbations evident in TCR Vβ repertoires of infected subjects could be due other factors (including co-infecting pathogens that may themselves encode superantigens, e.g., CMV) (24–26). To explore this question under defined conditions in vivo, we have studied experimental HIV-1 infection of the SCID-hu Thy/Liv mouse, a heterochimeric model of sustained, multilineage human hemolymphoid differentiation (27–31). Previous experiments have established that the thymic (Thy/Liv) organ of this mouse is permissive for HIV-1 infection and that it may be used to study the effect of antiretroviral agents on human thymocyte depletion (32–35).

We have used the SCID-hu Thy/Liv mouse and a large panel of mAbs to TCR Vβ subsets to examine the effects of a known superantigen, staphylococcal enterotoxin B (SEB),[3] and of HIV-1 on the developing human T cell repertoire. In confirmation and extension of other studies (36, 37), we observe that introduction of SEB into the Thy/Liv organ induces a profound alteration in the TCR Vβ repertoire, primarily within populations of $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes. In contrast, little or no effect on the TCR Vβ repertoire is observed after infection with two different isolates of HIV-1.

Materials and Methods

Mice

SCID-hu mice were obtained from SyStemix (Palo Alto, CA) after implantation of human fetal thymus and liver fragments into C.B-17 *scid/scid* mice as previously described (27, 28, 33).

SEB dosing

SEB (Sigma Chemical Co., St. Louis, MO) was maintained in a 50 μg/ml solution (stored at −25°C) and thawed immediately before injection. SCID-hu mice were injected i.p. with 100 μl of SEB solution (5 μg/dose) with a tuberculin syringe.

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[3] Abbreviation used in this paper: SEB, staphylococcal enterotoxin B.

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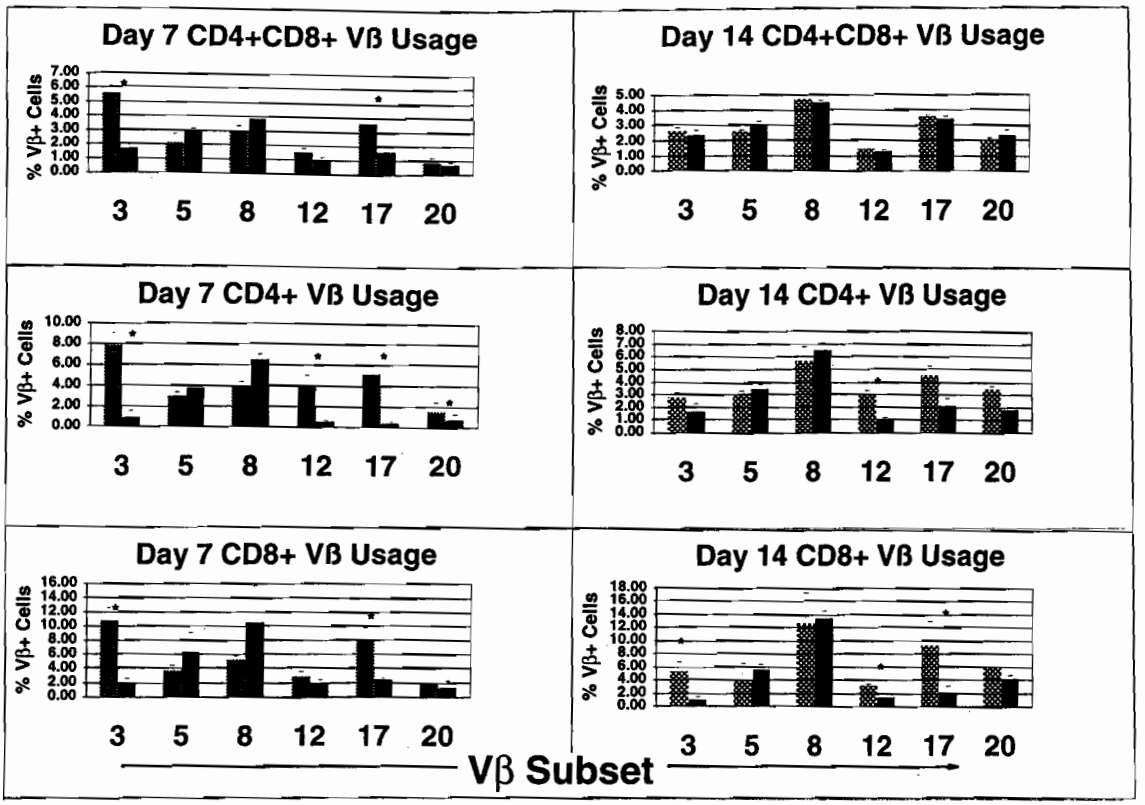

FIGURE 1. Effects of SEB on the SCID-hu Thy/Liv TCR Vβ repertoire. SCID-hu mice were injected with a single i.p. dose of 5 μg of SEB (or mock-injected with an equivalent volume of PBS). The TCR Vβ repertoire of thymocytes in the Thy/Liv was then determined 7 or 14 days after injection. Percentages of $V\beta^+$ cells are illustrated among $CD4^+CD8^+$ (*A*), $CD4^+CD8^-$ (*B*), or $CD4^-CD8^+$ (*C*) thymocytes. Gray bars represent the mean (+SD) of three mock-injected SCID-hu mice and black bars represent the mean (+SD) of three SEB-injected mice. Significant differences are marked with an asterisk (*) and are defined by a twofold or greater change in TCR Vβ expression relative to control animals. Abs used were (listed as Vβ specificity/name): Vβ3/CH92, 5/MH3–2, 8/JR2, 12/S511, 17/E17.5F3, 20/ELL1.4.

HIV-1 infection of SCID-hu Thy/Liv mice

SCID-hu Thy/Liv mice were intrathymically infected with established HIV-1 stocks (isolates NL4-3 and JD). Methods for maintenance of HIV-1 stocks, infection of Thy/Liv implants of SCID-hu mice, and harvest of thymocytes were identical with those recently published (33).

TCR Vβ mAbs

The origins and specificities of the TCR Vβ mAbs used in this report have been recently published (38).

mAb staining

Thymocytes were suspended in wash buffer (PBS-2% FCS + 0.1% sodium azide, w/v) and aliquoted into V-bottom 96-well plates at a density of 5×10^5/well. Cells were washed (centrifugation in a Beckman table top centrifuge in a 96-well plate holder at 1200 rpm for 5 min followed by resuspension of cells in 170 μl of wash buffer, then repeated). Several types of anti-TCR Vβ mAbs were used, including mAbs directly conjugated to FITC (designated as "Vβ*X* FITC") and unconjugated mAbs. Cells were blocked with human γ-globulin (100 μg/10 μl) (Gemini Bio-Products, Calabasas, CA) for 5 min at 25°C and incubated with an anti-TCR Vβ mAb for 25 min at 4°C. When unconjugated anti-TCR Vβ mAbs were used, cells were then washed and incubated with FITC goat anti-mouse $F(ab')_2$ (Caltag, South San Francisco, CA) for 25 min at 4°C. Cells were washed, blocked with normal mouse serum (Jackson ImmunoResearch, West Grove, PA) for 25 min at 4°C, washed and coincubated with PE anti-human CD4 and PerCP anti-human CD8 (Becton Dickinson, San Jose, CA) for 25 min at 4°C. When unconjugated anti-TCR Vβ mAbs were used, cells were then washed (in wash buffer, then PBS alone), resuspended in 1% paraformaldehyde and stored overnight at 4°C before FACS analysis. When using conjugated mAbs, the samples were processed in parallel to unconjugated mAb samples (with incubation in wash buffer during the primary and secondary incubations with mAb for unconjugated mAb samples). Primary incubation with TCR Vβ-FITC mAb occurred at the time of the mouse serum incubation for unconjugated mAb samples (for 25 min at 4°C). The remaining steps in the staining process (including staining with anti-CD4 and anti-CD8 mAb) were identical with those described above for unconjugated mAbs.

FACS analysis

Three-color flow cytometry was performed using a FACScan (Becton Dickinson) acquiring 30,000 events for each sample. Analysis was performed using Cell Quest software (Becton Dickinson). Frequency of TCR $V\beta^+$ cells was determined by gating thymocytes into a live-cell gate (by forward and side scatter) and sequentially gating into $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ subsets that were analyzed separately with respect to TCR Vβ expression.

Results

The TCR Vβ repertoire is conserved among SCID-hu mice established from the same fetal thymus/liver donor

Consistent with prior observations (36, 39), examination of inter- and intra-donor variability among normal SCID-hu Thy/Liv implants revealed that mice established from the same fetal thymus and liver donor had similar TCR Vβ repertoires, while clear differences were observed among mice established from different thymus and liver donors (data not shown). Further confirmation of this intra-donor consistency was observed in all subsequent experiments examining the effects of putative superantigens on the TCR Vβ repertoire (e.g., see Fig. 1).

SEB causes superantigen-mediated deletions in TCR Vβ subsets of thymocytes in the SCID-hu Thy/Liv organ

The TCR Vβ repertoire of developing thymocyte subsets in the SCID-hu Thy/Liv organ was studied seven days after injection of

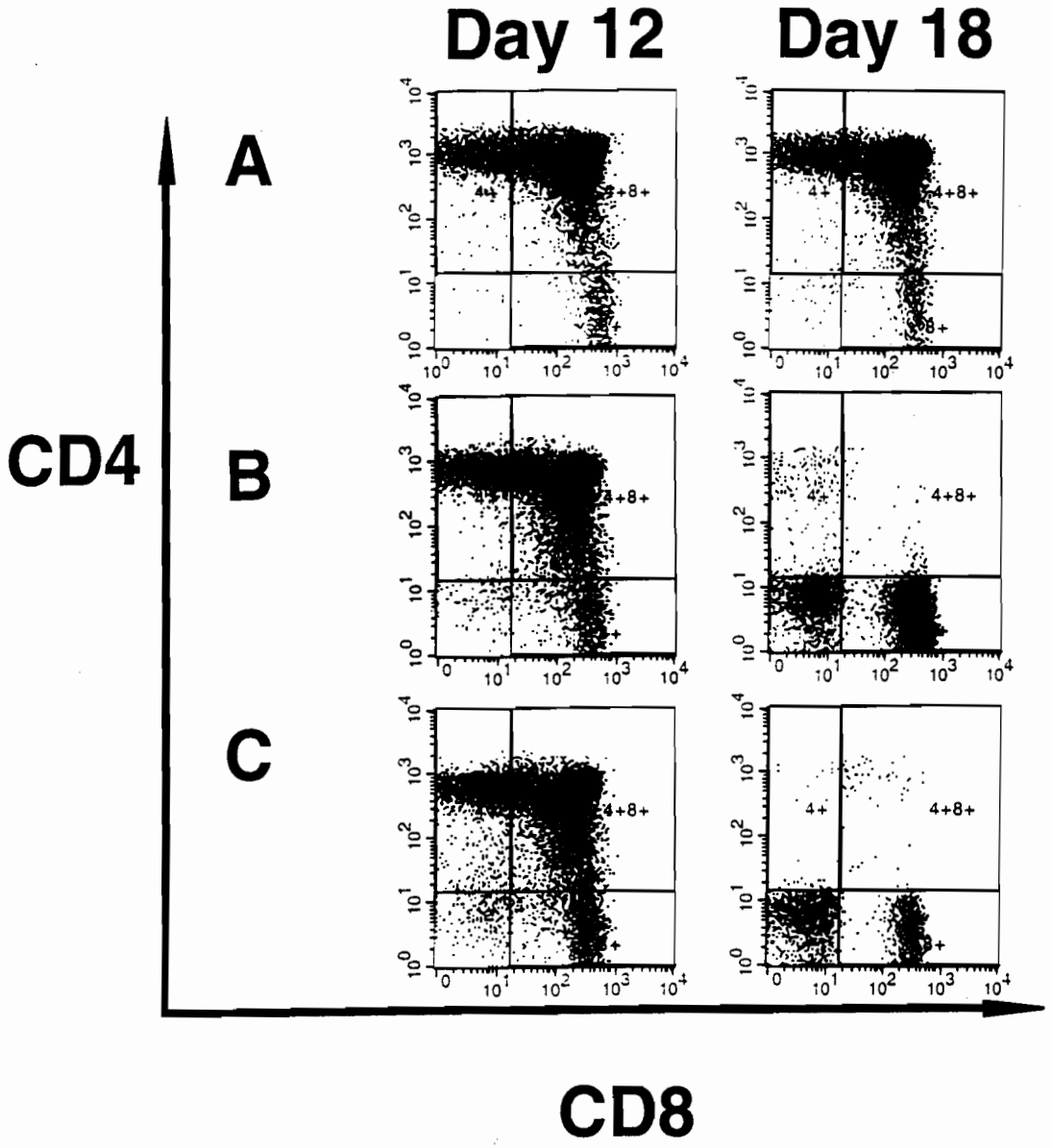

FIGURE 2. Effects of HIV-1 infection on SCID-hu thymocyte subsets. Total thymocytes harvested from Thy/Liv organs of SCID-hu mice were stained with mAbs to human CD4 and CD8. Dot plots illustrating the fraction of cells in $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ subsets are given for representative mock-infected mice (*A*) and mice infected with the HIV-1 strains NL4-3 (*B*) and JD (*C*). Subset gates were set based on parallel stainings with isotype control Abs (not shown). At day 12 after mock infection, the percentage of $CD4^+CD8^+$ in two infected animals was 90.3 and 89.3%; the percentage of $CD4^+CD8^-$ cells was 7.5 and 7.9%; the percentage of $CD4^-CD8^+$ cells was 2.2 and 2.7%. At day 12 after infection with NL4-3, the percentage of $CD4^+CD8^+$ cells in 3 infected animals was 76.1, 79.2, and 79.4%; the percentage of $CD4^+CD8^-$ cells was 15.5, 13.6, and 14.2%; the percentage of $CD4^-CD8^+$ cells was 7.4, 6.6, and 6.0%. At day 12 after infection with JD, the percentage of $CD4^+CD8^+$ cells in 3 infected animals was 85.5, 77.2, and 90.4%; the percentage of $CD4^+CD8^-$ cells was 8.9, 12.2, and 4.6%; the percentage of $CD4^-CD8^+$ cells was 4.9, 9.3, and 4.7%. At day 18 after mock-infection, the percentage of $CD4^+CD8^+$ in two infected animals was 90.1 and 87.2%; the percentage of $CD4^+CD8^-$ cells was 7.7 and 9.7%; the percentage of $CD4^-CD8^+$ cells was 2.2 and 2.8%. At day 18 after infection with NL4-3, the percentage of $CD4^+CD8^+$ cells in three infected animals was 0.8, 48.6, and 70.6%; the percentage of $CD4^+CD8^-$ cells was 3.3, 33.5, and 21.3%; the percentage of $CD4^-CD8^+$ cells was 79.5, 16.7, and 7.4%. At day 18 after infection with JD, the percentage of $CD4^+CD8^+$ cells in three infected animals was 6.7, 38.8, and 2.2%; the percentage of $CD4^+CD8^-$ cells was 10.8, 21.5, and 2.2%; the percentage of $CD4^-CD8^+$ cells was 61.6, 32.0, and 45.1%.

a single dose of SEB or PBS (mock-injected mice) using a limited panel of mAbs recognizing Vβ3, Vβ5, Vβ8, Vβ12, Vβ17, and Vβ20 (Fig. 1). Significant deletions were evident in $CD4^+CD8^+$ thymocytes expressing Vβ3 and Vβ17, while a reduced frequency of positive cells was evident in the Vβ12 and Vβ20 subsets. Concomitantly, there were relative expansions in nondeleted subsets (e.g., Vβ5 and Vβ8). Significant deletions were noted in the Vβ3, Vβ12, Vβ17, and Vβ20 subsets in $CD4^+CD8^-$ cells and in Vβ3 and Vβ17 subsets in $CD4^-CD8^+$ thymocytes, while expansions were observed in the Vβ5 and Vβ8 subsets in both subsets. In each case, the magnitude of the deletions was greater in single-positive than in double-positive thymocytes.

A second experiment (utilizing a cohort of mice established from a different fetal thymus and liver donor) examined the TCR Vβ repertoire 14 days after a single injection of SEB (Fig. 1). When $CD4^+CD8^+$ thymocytes were analyzed with respect to expression of TCR Vβ subsets, no significant differences were observed at 14 days after infection. Among more mature single-positive thymocytes

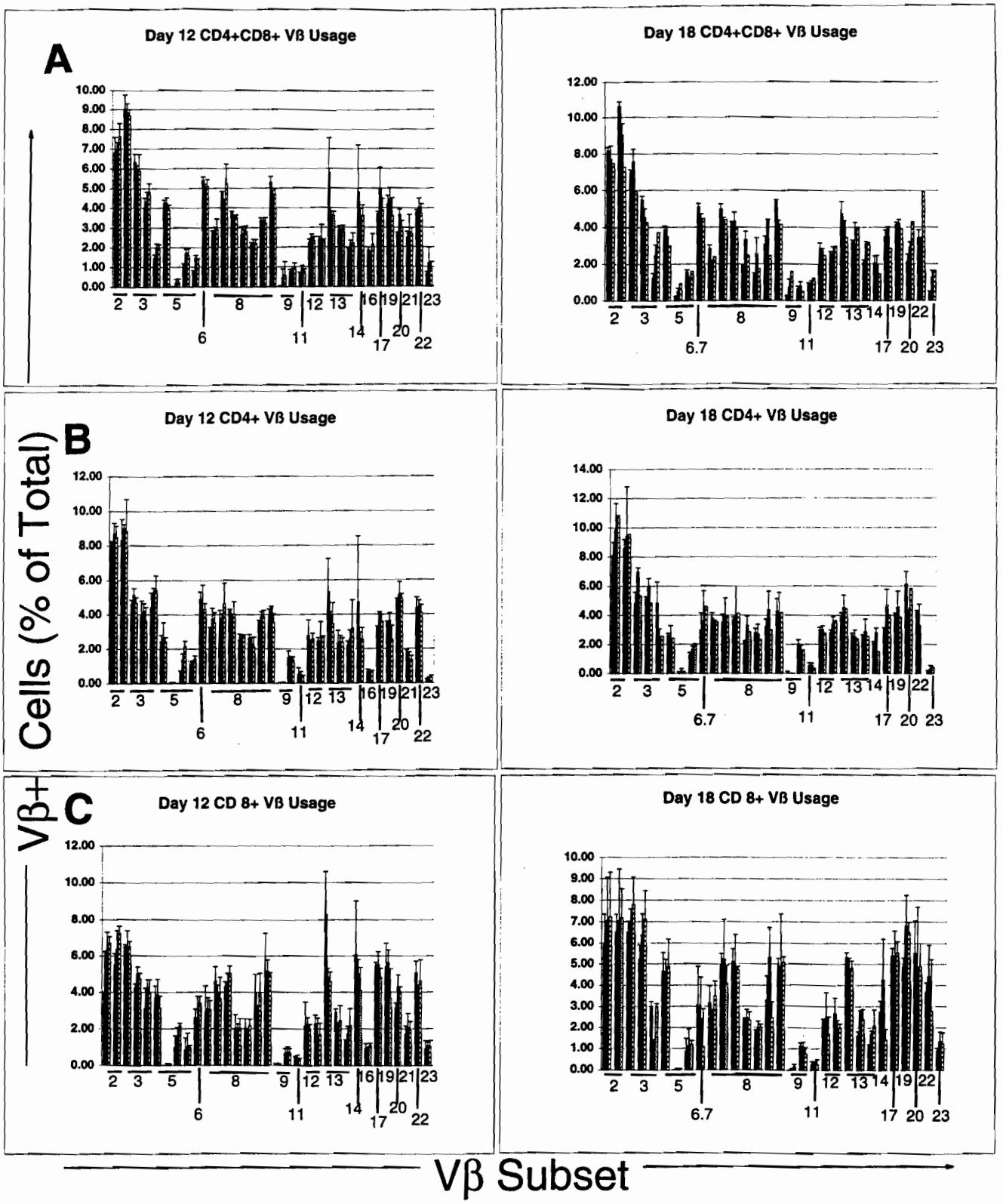

FIGURE 3. Effects of HIV-1 infection on the SCID-hu Thy/Liv TCR Vβ repertoire. SCID-hu mice were either mock-infected with PBS or infected intrathymically with the HIV-1 strains NL4-3 or JD. The TCR Vβ repertoire of thymocytes in the Thy/Liv organ was then determined at 6 (not shown), 12, or 18 days after infection. The percentage of $V\beta^+$ cells was determined amongst $CD4^+CD8^+$ (*A*), $CD4^+CD8^-$ (*B*), or $CD4^-CD8^+$ (*C*) thymocytes. Thy/Liv organs from two mock-infected mice or three HIV-1-infected mice were harvested at each time point. Hatched gray bars represent the mean (+SD) for mock-infected mice, black bars represent the mean (+SD) of SCID-hu mice infected with the NL4-3 isolate, and hatched white bars represent the mean (+SD) of mice infected with the JD isolate of HIV-1. Subset specificity of TCR Vβ mAbs is delineated on the horizontal axis, while the vertical axis reflects the percentage of $V\beta^+$ cells in each subset. The following Abs were used (listed as Vβ specificity/name, sequentially ordered as in figure): Vβ2/E2.2E7.2, 2/MPB2, 3/CH92, 3/LE89, 3, 5/MH3–2, 5.1, 5.2, 5.3/3D11, 6.7/OT145, 8/2D1, 8/MX3, 8/MX12, 8/3B9, 8/3B12, 8/4REX, 8/JR2, 9/FIN9, 9/MKB1, 11/C21, 12/VER2.32, 12/S511, 13.1, 13.2, 13.6, 14/CAS1.1.3, 16/TAMAYA (used only at days 6 and 12), 17/E17.5F3, 19/C1, 20, 21.3/IG125a, 22, 23/HUT78.

($CD4^+CD8^-$ and $CD4^-CD8^+$), however, deletions in affected subsets (Vβ3, Vβ12, Vβ17, and Vβ20) were still evident as were expansions in Vβ5 and Vβ8. The deletions or expansions observed 14 days after SEB injection were in all cases less substantial than those noted at 7 days.

HIV does not act as a superantigen in the SCID-hu Thy/Liv model

Given that the effects of a superantigen such as SEB could be monitored in the Thy/Liv model, we next evaluated whether HIV-1 may itself carry superantigens capable of modulating the TCR Vβ repertoire after intrathymic infection. Cohorts of SCID-hu Thy/Liv mice prepared from a single fetal donor were infected intrathymically with one of two HIV-1 strains or mock-infected with PBS. The two HIV-1 strains used were the molecular clone NL4-3, a T cell-tropic isolate, and JD, a macrophage-tropic primary isolate. In these experiments and as demonstrated previously (40), both NL4-3 and JD infection resulted in time-dependent depletion of thymocyte subsets in the Thy/Liv model. At day 12 after infection with either isolate, a decrease in the CD4/CD8 ratio was beginning to emerge. At day 18 after NL4-3 or JD infection, substantial depletion was noted in all animals, with an inversion in the CD4/CD8 ratio among single-positive cells and a substantial decline in the relative and absolute number of cells present in the double-positive subset (Fig. 2).

The effects of HIV-1-induced thymocyte depletion on specific TCR Vβ subsets of $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes were analyzed at each time point by multiparameter flow cytometry utilizing a large panel (≥30) of mAbs to 15 human TCR Vβ families. At day 6 (not shown) and at day 12 (Fig. 3), no significant differences were detected in the expression of any TCR Vβ subset. At day 18, the same number of animals was used for each group, but due to extreme thymocyte depletion in some of the HIV-1-infected animals (Fig. 2), samples with insufficient flow cytometric events for analysis (less than 250 gated events) were excluded from analysis. Comparison of the TCR Vβ repertoire in JD- and NL4-3-infected SCID-hu mice relative to that of control animals revealed no significant changes in individual TCR Vβ subsets (Fig. 3).

Discussion

Previous studies have suggested that HIV-1 may carry superantigens capable of interacting with and inducing expansion or deletion of specific TCR Vβ subsets of T cells (14, 15, 19–21). If such viral superantigens were to be presented in the context of the thymus, the TCR repertoire of developing thymocytes might be significantly perturbed. Indeed, superantigen-induced deletion of developing thymocytes might, in part, contribute to the thymocyte depletion often observed in lentiviral, including HIV-1, disease (35, 40–43).

We demonstrate herein that the TCR Vβ repertoire is conserved among SCID-hu mice established from the same donor, that exposure of developing human thymocytes to SEB leads to clear deletions in some TCR Vβ subsets (Vβ3, Vβ12, Vβ17, and Vβ20) and relative expansions in nondeleted subsets (Vβ5 and Vβ8), and finally, that exposure of developing thymocytes to HIV-1 Ags does not lead to deletions or expansions of TCR Vβ subsets. Given the extensive array of anti-Vβ region Abs to 15 human TCR Vβ families used in these experiments, these data suggest that HIV-1 does not carry superantigens that impact on the human TCR Vβ repertoire.

The fact that we were unable to demonstrate a role for an HIV-1-associated superantigen in the thymocyte depletion observed in SCID-hu mice should be considered with several limitations in mind. First, it is possible that the two HIV-1 isolates used in these experiments were not representative of other HIV-1 strains that could encode superantigens. Second, it is possible that the amount of HIV-1 Ags delivered was insufficient to mediate superantigen-mediated deletions in the TCR Vβ repertoire. Third, it is possible that the Ab panel utilized was insufficiently broad to detect perturbations in the repertoire that may have been present.

It is unlikely that these limitations would impact on the conclusions drawn above. First, we used two HIV isolates that differed significantly in their composition and biologic properties: NL4-3 is a molecularly cloned, T cell-tropic isolate and JD is an uncloned primary isolate tropic for both T cells and macrophages. Second, prior experiments in HIV-1-infected SCID-hu Thy/Liv mice (33, 35) have demonstrated that the quantity of p24 present at the postinfection time points used in this study is approximately 250 to 400 pg/10^6 thymocytes, an amount that is roughly equimolar to the dose of SEB demonstrated here to induce TCR Vβ-specific deletions. Third, this experiment utilized a panel of mAbs more broad in specificity than in any prior published study characterizing the human TCR Vβ repertoire, spanning at least 60% of the known human TCR Vβ repertoire. If we had failed to sample a deleted TCR Vβ subset, we would otherwise have detected (but did not observe) relative expansions in nondeleted subsets.

In summary, we have shown that SEB, a known superantigen, induces clear deletions in specific TCR Vβ subsets of developing thymocytes in the SCID-hu Thy/Liv model. Studied in parallel, HIV-1 infection of the SCID-hu Thy/Liv organ is not associated with perturbations in the TCR Vβ repertoire. We conclude that HIV-1 does not encode a superantigen that contributes to HIV-1-induced thymocyte depletion in vivo.

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Chapter III

Expansions and exhaustions of the CD4 T cell repertoire during primary infection predict disease progression in SIV infected cynomolgus macaques

Expansions and exhaustions of the CD4 T cell repertoire during primary infection predict disease progression in SIV infected cynomolgus macaques

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Keywords: SIV pathogenesis, TCRBV repertoire, CD4, disease progression, primary infection.

Abbreviations: Primary infection (PI). Simian immunodeficiency Virus (SIV)

Abstract:

The immune response during HIV/SIV primary infection (PI) bears an influence on the magnitude of viral load at set point. The evolution of the CD4 T cell repertoire diversity, as measured by analyzing the T cell receptor β chain variable region (TCRBV), was determined during PI in cynomolgus macaques infected with either SIVmacJ5, SIVmacC8, or SHIV 89.6P. The patterns of CD4+ T cell expansions were variable among animals of the same group. In each group, the number of expanded TCRBV families in the CD4 repertoire did not correlate with the relative pathogenicity of the viral isolates but were a strong prognostic factor for disease progression. In fast progressor macaques most of CD4+ T cell clonotypes in expansion during PI, as examined by molecular analysis, quickly became undetectable. In sharp contrast, TCR clonotypes identified in slow progressors persisted throughout infection and were involved in an anamnestic response to a subsequent challenge with pathogenic SIV strain. Thus, while most of the CD4 clonotypes identified during acute SIV infection are rapidly eliminated, a fraction of them persist and re-expand following heterologous challenge in slow progressors, suggesting that at least some of them are generated in response to SIV infection and may play a role in delaying disease onset and conferring protection against challenge.

Introduction:

Infection with HIV or SIV usually triggers a broad and potent antigen-specific cell-mediated immunity. The T cell receptor (TCR), a heterodimeric structure composed of α and β chains is expressed in mature T cells and constitutes the effector molecule for cellular immune responses (1). The TCR α and β chains are produced from a coding unit that stems from the rearrangement of different, noncontiguous and highly polymorphic gene segments (V-D-J for the β chain and V-J for the α chain). The TCR repertoire encompasses the total assortment of rearranged TCR molecules in a given individual (2). The TCRBV repertoire has been extensively analyzed in acute HIV infection of adults (3), HIV-infected children (4,5) and macaques acutely infected with SIV (6-9). These studies have clearly demonstrated dramatic perturbations of the TCRBV repertoire in CD8+ T cells. Moreover, qualitative differences in the patterns of TCRBV-specific expansions during PI were shown to correlate with different rates of disease progression: slow progression and low viral burden were associated with a diverse CD8 TCRBV repertoire, whereas individuals with restricted CD8 responses showed high viral load and rapidly progressed to AIDS (10).

CD4+ T-cell help is essential for the induction of humoral and the maintenance of cell-mediated immune responses (11-14). Thus, interference by HIV with helper T cell functions probably prevents the immune system from clearing the virus during infection. The fact that potent CD4 T helper responses in HIV-1 infected individuals are inversely correlated with viral load is in support of the earlier statement (15). In this study, patients with advanced disease and high viral load did not show any detectable p24-Gag-specific CD4 response, while gag-specific helper cells were present in long term survivors. Since these data were mostly gathered from chronically infected subjects, an in-depth analysis of SIV-specific helper responses during PI and a detailed understanding of the fate of these cells during the chronic phase of the infection are necessary to define the influence of CD4 immune responses on disease progression.

The CD4+ TCRBV repertoire was analyzed during PI in cynomolgus macaques infected with either pathogenic SIVmacJ5, highly pathogenic SHIV89.6P, or the attenuated SIVmacC8. Qualitative and quantitative differences in the CD4 TCRBV repertoire, as well as clonal persistence were analyzed from PI to the chronic phase. Our results demonstrate that CD4+ T cell mediated immune responses during primary infection strongly influence SIV disease progression.

Materials and Methods:

Virus

Viral isolates used in this study include: SIVmacJ5 and SIVmacC8 which originated from the molecular clones SIVmac32H(pJ5) and SIVmac32H(pC8) (16,17), respectively, SHIV89.6P (18), a pathogenic chimeric SHIV and SIVsmmPBj14(6.6) (19).

Animals

Animals used in this study were part of a large European concerted action on AIDS vaccine ((20); and Rud, unpublished data). Three groups of juvenile (approx. 3 year old) cynomolgus macaques (*Macaca fascicularis*) were infected via the intravenous route (Table 1). All animals were colony bred within the Non-human Primate Breeding Colony of Health Canada under Canadian Council of Animal Care approved conditions. They were serologically negative for Herpes B virus, STLV-1, SRV-1, 2, 5 and SIV (The Virus Reference Laboratory, Inc., San Antonio, TX).

Plasma viral RNA Load

Plasma SIV RNA concentrations were determined using a branched DNA signal amplification method developed by Dr. Peter Dailey (Chiron Corporation, Emeryville, CA) for SIV using the same approach as Quantiplex HIV RNA branched DNA method (21). For quantitating SIV viral load, target probes designed to hybridize with the *pol* region of the SIVmac strains were used. Quantification was performed by comparison with purified and *in vitro*-transcribed SIV *pol* RNA. The lower limit of detection of this assay was 10,000 copies of SIVmac RNA per ml plasma in all the animals except for C94066F and C94055F which was 1500 copies per ml before week 35 and 500 copies after.

Cytofluorometric analysis

Macaque peripheral blood mononuclear cells (PBMC) were purified from blood samples collected in preservative-free EDTA by overlaying on a ficoll gradient. Two color cytofluorometric analysis was carried out on PBMCs sampled from uninfected, SIVmacJ5 or SIVmacC8-infected cynomolgus macaques on days 0, 5, 10, 14, as well as 4, 8, 16, 24 and 32

weeks post infection. Fresh cells ($2.5x10^5$) were stained with anti-TCRBV followed by FITC (Fluoroscein isothiocyanate)-conjugated goat anti-mouse (GAM), PE (Phycoerythrin)-conjugated anti-CD4 and analyzed on a FACScan (Becton Dickinson, CA, USA). The human anti-TCRBV mAbs were: TCRBV3 (JOV13) from M. Owen (Imperial Cancer Research Fund, London, UK); TCRBV5 (MH3-2), TCRBV5S1 (IMMU157), TCRBV5S2 (36213), TCRBV7 (3G5.D5), TCRBV13S1 (IMMu222), TCRBV13S6 (JU74.3), TCRBV17 (E17.5F3.13), TCRBV19 (C1), TCRBV20 (ELL1.4) and TCRBV23 (AF23) (Beckman Coulter Inc., CA, USA). Anti-CD4-PE mAb was purchased from Becton Dickinson. In all experiments, 30,000 events were gated on live cells by forward and side scatter and then analyzed using the CELLQuest software.

Heteroduplex tracking assay

Heteroduplex tracking assays (HTA) were performed as described (22) on purified CD4 T cells for macaque C94066F and C94055F at the bleeding time points (-5, -3, 0, 1, 2, 4, 6, 8, 12, 16 weeks post infection with SIVmacJ5 and 0, 1, 2 and 4 weeks post challenge with SHIV89.6P) on CD4 purified T cells. Briefly, Macaque PBMCs ($10x10^6$ cells) collected at described time points were stained with a microbeads- conjugated CD4 antibody (Miltenyi Biotech) and CD4 T cells were purified using MACS MS columns and MACS magnetic cell sorting apparatus (Miltenyi Biotech). Purified CD4+ T cells were then resuspended in Trizol® (Life technologies, Burlington, Canada) for RNA extraction. Total RNA served for complementary DNA (cDNA) synthesis using oligo dT (12-18) primer. Nested polymerase chain reaction (nPCR) was performed on cDNA samples from sequential time points using TCRBV 1, 5 or 9out and Cβout as outer primers and TCRBV 1, 5S1 or 9in and Cβin as inner primers. All the oligonucleotides utilized in this study are listed in Table 2. The uniformity of yield and amplification efficacy of each oligonucleotide were assessed by agarose gel electrophoresis. In parallel, ^{32}P labeled probes were produced by PCR amplification of two TCRBV1, 5S1, or 9-Cβ inserts previously cloned in Bluescript (Stratagene Cloning Systems, La Jolla, Ca), using ^{32}P-labeled Cβin primer and unlabeled TCRBV1, 5S1, or 9in primers. Radiolabeled DNA heteroduplexes were obtained by mixing 4.5 µl from second round PCR with 0.5 µl of probe in annealing buffer (0.16 M NaCl, 16mM Tris [pH 8.0], 3.3 mM EDTA). DNA was denatured at 95° C for 2 min, after which they

were reannealed at 55°C for 5 min and rapidly cooled to room temperature in a thermocycler. Heteroduplexes were resolved on a 20-cm high, 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide) at 200V for 3h. HTA gels were vacuum-dried and a PhosphorImager (Amersham Pharmacia Biotech, NJ, USA) plate was used for signal detection.

Molecular cloning and sequencing of the CDR3 regions

cDNA from sequential time points (table 4) from macaques C92032M and C94066F were used as templates in PCR reactions using 3'Cβin primer and 5' TCRBV3, TCRBV5S1in and TCRBV7 primers (table 2). The PCR conditions were of 94°C for 30 sec, 56°C for 45 sec and 72°C for 1 min, for 30 cycles. Amplification products were cloned into Bluescript vector. After transformation of *E.coli.* (DH5α), plaques containing TCR inserts were sequenced using T7 DNA polymerase (Amersham Pharmacia Biotech, NJ, USA) or automated sequencing (Sheldon Biotechnology Center, McGill University, Montreal).

Screening for CD4 clonotypes in the fast progressor macaque

TCRBV3 and TCRBV7 PCR amplified products from time point 4 weeks post challenge with SIVsmm PBJ14(6.6) of macaque C92032M were cloned into M13mp18. The M13mp18 colonies were hybridized *in situ* using BV3 or BV7 probes (Table 2). 600 to 1100 M13 colonies were then hybridized in situ with clonotype-specific probes (TWQ, VPG or SPR) (Table 4) in order to identify recombinants containing the CDR3 sequence of the specific T cell clones. *In situ* hybridization was performed at low stringency (hybridization in 20% formamide, 4xSSC 0.5 SDS, 5x Denhardt, washing in 2xSSC, 0.1 SDS at 42°C). All positive colonies were amplified and sequenced as described.

Results:

Viral load during acute infection does not predict the rate of disease progression.

To address the effect of both virulence and magnitude of viral load on disease progression, cynomolgus macaques were infected with three different SIV strains: two inducing high viral load (>10^8 copies per ml) and AIDS-like disease after 6 month to 3 years for SIVmacJ5 or SHIV89.6P respectively. (23), and the attenuated SIVmacC8 (16) (Table 1).

Macaques infected with either SIVmacJ5 or SHIV89.6P experienced an initial drop in CD4 count at two weeks post infection. Following PI, five macaques from SIVmacJ5 group and one macaque from SHIV89.6P group (C92008F) had a rebound in their CD4 count to normal values (figure 1A) or showed an increase during the following 28 weeks (figure 1C). In all these animals, viral load peaked 2 weeks after infection (range: $0.4x10^7$ to $1x10^8$ copies in SIVmacJ5 group or up to $1x10^9$ copies per ml in the SHIV89.6P group) and reached a viral set point below detectable levels by week 6, with occasional detectable viral load measurements (i.e. C94066F at 20 weeks) and co-cultures remained positive (data not shown).

On the other hand, one SIVmacJ5 infected macaque (C92032M) and two SHIV89.6P infected macaques (C91080F, C92056M) failed to recover with their CD4 counts dropping significantly when compared with other animals within their groups (62% drop 6 month after infection in case of C92032M and below 200 cells per µl in case of C91080F and C92056M). Viral load was not resolved in these macaques, in the case of C92032M with viremia remaining below detectable levels for a period of 2 weeks after which a progressive increase followed to reach a plateau at about 24 weeks (Figure 1A). As for the SHIV89.6P macaques, viral set point remained between 10^5 and 10^7 copies per ml (Figure 1C).

All SIVmacC8 infected macaques (n=3) showed stable CD4 counts (between 0 to 7% CD4 drop after 6 months of infection). One macaque showed detectable plasma viral load ($0.7x10^4$ copies per ml) during PI that was resolved thereafter, while others remained under detection limit (Figure 1B). However, in these animals, virus could be isolated in co-culture experiments (data not shown), confirming the presence of ongoing infection.

These results demonstrate that, although important, the intrinsic virulence of the infecting SIV strain is not the unique factor determining disease progression, indicating that host factors may also play a role in this process.

Expansions in the CD4+ TCRBV repertoire occur in both slow and fast progressor macaques.

Flow cytometric analysis was performed on PBMC samples of the different macaques from 6 weeks prior to infection to 32 weeks thereafter (Figure 2 and data not shown), using a panel of cross-reactive anti-human TCRBV monoclonal antibodies covering approximately 30% of the cynomolgus macaque TCR repertoire (24) and a CD4 specific monoclonal antibody. Transient expansions, defined as at least a two-fold increase in the frequency of cells expressing a particular TCRBV, were observed in the CD4+ population of several of the infected animals during the first month following SIV infection. While expansions of TCRBV families were observed in all the SIVmacJ5-infected macaques, SIVmacC8-infected macaques showed very little perturbations, if any, (one macaque out of three showed a single expansion of TCRBV13S6) (Figure 2). Similarly, TCRBV expansions were observed in all SHIV89.6P infected animals (Table 3). In all models tested, expansions in the CD4+ TCRBV repertoire occurred during PI and in most cases when CD4 counts had still normal values (Figure 2 and Table 3). This indicates that, at least in SIVmacJ5 and SIVmacC8 infected macaques, all the expansions constitute absolute increases in cells expressing these TCRBVs and are not due to the selective depletion of cells expressing other TCRBVs. During the same period, uninfected control animals had stable TCRBV repertoires (Table 3). Similarly, the CD4+ TCRBV repertoire remained unchanged in the 12 animals involved in the study from 6 weeks prior to 2 weeks after infection.

The magnitude of the expansions in the CD4 TCRBV repertoire is more important in fast progressors and correlates with disease progression

While the amplitude of TCRBV expansions observed in the different macaques was independent of viral strain pathogenicity or disease progression (Figure 2 and 3), the magnitude of the observed perturbations, defined as the number of expanded TCRBV families at a given time point, was directly associated with disease progression, irrespective of the viral strains used (Table 3). Slopes of delta (Δ) scores, that are proportional to T cell repertoire destabilization, were calculated as described (25,26) for each macaque during a 10 week period in order to examine the statistical significance of the observed differences between the magnitude of CD4 expansions in slow and fast progressors. Accordingly, fast progressors showed several (4 to 6) expansions in their TCRBV repertoire and a positive slope of Δ score indicating repertoire

destabilization, C91080F had a particularly high slope of Δ score indicating a potent CD4+ T cell response during that period. While animals that subsequently did not rapidly progress to disease had a more constant repertoire (2 expanded TCRBV or less). As uninfected animals, slow progressors showed slopes of Δ scores close to zero, indicating that their repertoire was stable. The difference between fast and slow progressors was significant (p= 0.025, Mann-Witney U test), indicating that fast progressors have, during PI, a more perturbed CD4+ TCRBV repertoire, as compared with slow progressors.

In all the above-described cases, TCRBV expansions within the CD4+ population were transient following peak viremia. The CD4 T cell repertoire remained stable thereafter (Figure 3). The delay between peak of viremia and TCRBV expansions was different between slow and fast progressors. Viremia was always detectable in fast progressors (n=3) during the expansion of CD4+ T cells. In contrast, in slow progressors (n=5), reduction of the viral load to undetectable levels systematically preceded CD4+ T cell proliferation (Figure 3).

These results show that the rate of disease progression can be inversely correlated with the extent of CD4 TCRBV repertoire perturbations during PI, indicating that the risk of developing disease rapidly, increases with the number of expanding CD4 T cell clones during acute infection.

PI CD4 clonotypes are exhausted in fast progressor macaques and reactivated upon heterologous challenge in slow progressors.

Macaques used in this study were part of experiments involving challenge of SIVmacJ5 and SIVmacC8 infected macaques with heterologous (SIVsmm PBJ14(6.6) and SHIV89.6P) viruses (Table 1). In an initial set of experiments, we analyzed the clonality of TCRBV13S6 T cells since they were found to be amplified in both fast (C32032M) and slow (C92034M and C92017F) progressors. HTA analysis could not reveal any oligoclonal expansion in these animals. Three other macaques, one with a fast progressor phenotype (C92032M) and two that exhibited a slow progressor phenotype (C94066F, C94055F) were analyzed for the clonality of the expansions observed during PI by either HTA or sequencing. At week 20, SIVmacJ5 infected macaques C94066F and C94055F were challenged with SHIV89.6P. The persistence and reactivation of the CD4 clonotypes detected during PI were examined after challenge. In the case of C94066F, two HTA bands were observed at week 8 post infection, one was detected again at 2 weeks post challenge (Figure 4A). Identically, for macaque C94055F the TCRBV1

and TCRBV9 clonotypes that were detected during PI, now dominate the repertoire at 1, 2 and 4 weeks and 2 and 4 weeks post challenge respectively (Figure 4B and 4C). These results were confirmed in an independent HTA using series of other TCRBV1, 5S1 or 9 probes (data not shown). To further confirm the persistence of the expanded CD4+ T cell clones in these animals, the PCR products corresponding to TCRBV5S1 amplifications in macaque C94066F were cloned and 25, 30 or 22 clones were sequenced for time point 4 and 8 weeks post SIVmacJ5 infection, and 2 weeks post challenge respectively. Two dominant clones (NRG and LGS) were identified at high frequency at both 8 weeks post infection and 2 weeks post challenge (Table 4A).

Parallel experiments were carried out to determine the clonality and persistence of TCRBV3 and TCRBV7 expanded T cells during PI in fast progressor macaque C92032M. Molecular cloning of these TCRBV chains were performed before (2 weeks) and after (4, 8, 32 weeks post SIVmacJ5 infection) the expansions detected by flow cytometry. One TCRBV3 (TWQ) and three TCRBV7 (VPG, SPR, EGD) clonotypes were predominant at time of expansions (4 weeks). Interestingly, these CD4+ clonotypes were not detected by sequencing at later time points or were drastically reduced. These results indicate that the immune responses observed at PI quickly disappear when the viral load remains high. The potential reactivation of these clonotypes following challenge with SIVsmm PBJ14(6.6) was assessed by cloning TCRs isolated from PBMCs sampled at 4 week post challenge in M13mp18 and subjecting these recombinants to *in situ* hybridization with TWQ or VPG, EGD and SPR probes. This highly sensitive assay allows detecting infrequent clones as large number of colonies can be screened. These probes hybridized with 6, 3, none or 2 recombinant M13mp18 colonies out of the 614 TCRBV3, and 1190, 596 or 590 TCRBV7 plaques screened respectively. Sequencing of the hybridizing colonies showed that none of those recombinant M13mp18 contained a CDR3 sequence identical to the probes (Table 4B). Thus, the CD4 clonotypes detected during PI were not reactivated upon challenge in fast progressor animal.

Altogether, these results show that some of the CD4 clones activated during PI do indeed persist in slow progressor animals and can be reactivated upon re-exposure to similar viral epitopes. In contrast, in fast progressor macaque, CD4+ T cells clonally expanded during PI are not subsequently reactivated in response to viral replication (27).

Discussion:

In this report, we provide one of the first evidence for CD4+ T cell turnover at the clonotype level in a longitudinal follow-up, from the earliest phase of SIV infection to the chronic phase of the disease. TCRBV repertoire modifications occurred just after the initial peak in viral load and control uninfected macaques repertoires remained constant (figure 2), suggesting that the expansions in the CD4 compartment are probably virus induced.

The observed heterogeneity in the capacity of different macaques to generate different types of responses although they have been infected with the same virus is striking. Host factors thus appear to modulate the immune response to viral infection. Accumulating evidence supports the role of MHC class I and II in modulating the course of SIV and HIV infection by playing a direct role in the presentation of quantitatively different sets of peptides to the immune system (28-31). Moreover, it is possible that the size of the T cell pool bearing TCRs capable of recognizing a set of SIV peptides could significantly vary from one individual to another. In that regard, it was recently shown that the size of the TCR precursor pool could predict the magnitude of the immune response to a nominal antigen (32). Consequently, the different quantitative and qualitative patterns of CD4 responses reported here could be due to the size of the initial, innate T cell precursor pool. Furthermore, negative consequences of immune activation have clearly been documented during the chronic phase of the infection. Indeed, activated T cells triggered during PI either by SIV or by other antigens could also play a major role in enhancing viral replication *in vivo* (33-36). In agreement with this notion, immune activation has been demonstrated to be the driving force behind viral replication during the chronic phase of the disease in both humans and macaques (37-40).

Two different but not exclusive hypotheses can thus explain the influence of the size of the CD4+ T cell pool mobilized during PI. Indeed it is well known that the pool of latently infected T cells is established very early during PI (41). A large mobilization that can be achieved through a very potent CD4 response will lead to the infection of the majority of available specific CD4+ T cells. Such infection will have as consequence on one hand the destruction of most of the existing SIV specific CD4+ T cells, leading to the failure to efficiently initiate B cell responses and maintain pre-established primary CD8 responses. On the other hand, infection of these cells will influence the size of the initial pool of infected cells in periphery (42,43). Thus,

the larger the size of the pool is, the higher viral proliferation during the chronic phase will be. In contrast, the small initial recruitment of anti-SIV specific CD4+ T cells will generate a limited pool of latently infected cells leaving uninfected CD4+ T cells for the maintenance of cellular immunity and thereby leading to a better prognosis.

We have also shown that CD4 clonotypes expanded during PI were quickly eliminated in fast progressors whereas such clones persisted in slow progressors. Lack of persistence was demonstrated using two independent methods i.e. molecular analysis and functional challenge. Indeed, clones that were expanded during PI were not identified in the fast progressor macaque following challenge with heterologous virus despite screening between 600 and 1000 clones with specific probes. It is highly unlikely that a lack of cross-reactivity has occurred between the SIVmacJ5 isolates and the heterologous challenge with SIVsmm PBJ14(6.6), these viruses showing a high degree of conservation in most of the proteins (18,44,45). In contrast CD4 clonotypes expanded during PI in slow progressor macaques C94066F and C94055F persisted during the chronic phase and were re-expanded upon challenge with SHIV89.6P as confirmed by HTA and sequencing (Figure 4 and Table 4A). The frequency of the amplified TCRBV5S1 clonotypes in macaque C94066F was similar to what was observed during PI showing that this recall immune response was at least as potent as the one observed during the initial exposure to the antigen. Considering that SHIV89.6P infection was concomitant to a transient increase of SIVmacJ5 replication in that macaque, re-expansion of these clones could be attributed to either SIVmacJ5 or shared epitopes between SIVmacJ5 and SHIV89.6P (18,45). Indicating first that these clonotypes are probably SIV-specific, and second that the persistence of SIV specific helper immune responses can indeed be a consequence of low viral load.

These findings provide novel insights regarding the fate of HIV-specific CD4 helper T cells during the transition from the acute to the chronic phase of HIV-1 infection. Importantly they suggest that similarly to the murine lymphocytic choriomeningitis virus (LCMV) model, the persistence of the CD4 helper function can be essential for the maintenance of memory CD8 responses during chronic infection for the generation of neutralizing antibodies to viral escape mutants and for the control of viremia (12,14,46-49). Hence, in SIV infection in a similar way to other viral infections, the loss of SIV-specific helper function during the progression into the chronic phase may restrict the efficiency of anti-SIV CTLs and other immune mechanisms that can restrict viral dissemination.

Looking at the turnover of HIV-specific CD4+ T cells during the course of infection, Pitcher et al. (50) have demonstrated the presence of a CD4 HIV-specific response albeit at very low frequency during the chronic phase of the disease. These authors have not addressed the longitudinal evolution and the clonality of the response. Recently, Chen et al.(51) have demonstrated that CD4 clonotypes can persist and even become dominant at later stages of SIV infection, further confirming the holes in the CD4 repertoire reported by Connors et al. (52). Our study has looked at the evolution of the response all the way from PI to the chronic phase of the disease. In that context, our results clearly suggest that the observed dominance of the CD4 clones (51) during the chronic phase of the disease in fast progressors could result from the depletion of all other T cells expanded during PI. In slow progressors, persistence of these clones is part of the normal course of anti-viral immune responses that lead to the establishment of long-term memory T cells. Evidence for this is clearly provided by their capacity to participate in an anamnestic response following subsequent bursts in viral replication (figure 4). Altogether our results show unequivocally that the CD4 response observed in PI is quickly blunted, either by clonal exhaustion ((53) and our own data), by anergy (48), by cytopathic properties of the virus itself or by CTL responses directed against SIV proteins. This leaves the infected host with a weak virus specific CD4 response leading to the important negative consequences observed in the immune system of such infected individuals. Recent results have clearly established the benefits of early treatment in containing viral replication upon cessation of antiviral therapy (9,54). Thus, early therapeutic intervention helps preserve the elimination of a large segment of the helper T cell compartment, which, as shown in our results, is a critical factor in modulating the course of the disease.

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Legends

Figure 1: Virological and immunological status of the macaques

CD4 absolute count (upper panels) and plasma SIV RNA detected by branched DNA (lower panels) from groups **A**-SIVmacJ5, **B**-SIVmacC8, and **C**- SHIV89.6P infected macaques. Animals infected with SIVmacJ5 or SHIV89.6P show either slow or fast progressor patterns. SIV viral load target probes, designed to hybridize with the *pol* region of the SIVmac groups of strains were used to quantify SIV viral load. Results were quantified by comparison with purified and quantified *in vitro*-transcribed SIV *pol* RNA and were plotted on a log(10) scale. The detection limit of this assay was 10,000 copies of SIVmac RNA per ml plasma in the case of all macaques except for C94066F and C94055F (1500 copies per ml prior to week 35, 500 thereafter). White blood cell counts were obtained from a hematology workstation and were used to calculate the absolute CD4 counts.

Figure 2: Transient TCRBV13S6 expansions in the CD4+ T cell subset

Macaque TCRBV repertoires were analyzed by flow cytometry using a panel of cross-reactive anti-human CD4 and TCRBVs monoclonal antibodies on freshly isolated PBMCs. In all experiments, 30,000 events were acquired as gated on live cells, and the percentage of TCRBV+ T cell subsets were calculated in the CD4 T cell subset. A representative sequential analysis of the TCRBV13S6 population is shown for macaques infected with: **A**- SIVmacJ5, **B**- SIVmacC8 or **C**- SHIV89.6P. **D**- uninfected macaques. Arrowheads highlight significant expansions ≥ 2-fold increase. TCRBV specific expansions occurred early after infection and are transient in all SIV infected animals.

Figure 3: Viral titers and TCRBV expansions in CD4+ T cells during PI

Viral loads and TCRBV expansions are shown on the same time scale from six weeks prior to infection until 16 weeks after infection. Panel **A** describes data from fast progressors C92056M and C92032M while panel **B** describes results obtained from slow progressors C92034M and C92017F. Viral titers were obtained as described in Material and Methods and plotted on a log(10) scale. TCRBV were analyzed by flow cytometry. Results encompass all

of TCRBVs which were expanded during PI. The vertical lines represent the time at which peak viral load occurred.

Figure 4: Heteroduplex tracking assay on CD4+ TCRBV T cells throughout the course of infection of slow progressor macaques C94066F and C94055F

cDNAs encoding for TCRBV5S1 (panel **A**), TCRBV1 (panel **B**) or TCRBV9 (panel **C**) were PCR amplified from purified CD4+ T cells at indicated time points. Each column on the gel corresponds to an individual time point. The number of discrete bands reflects the diversity of the TCRBV populations, i.e. each band represents one clonotype. The same TCRBV1, and TCRBV9 in case of C94055F and TCRBV5S1 in case of C94066F T cell clonotypes detected during primary infection with SIVmacJ5 are also present following SHIV89.6P challenge, at time points indicated by arrows. The persistence of bands at the indicated time points was confirmed using two probes that have distinct CDR3 and TCRBJ segments for each TCRBV family.

Figure1:

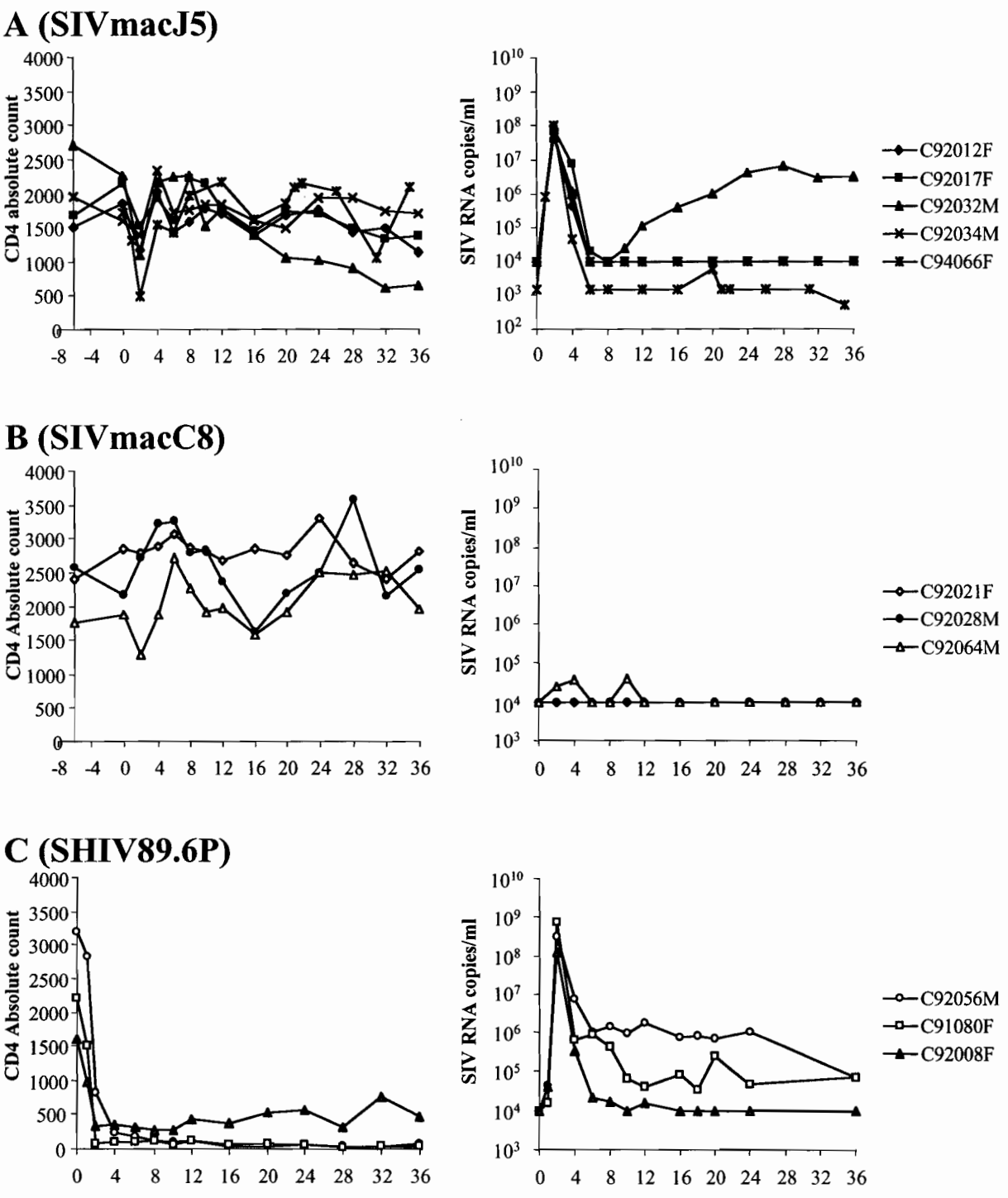
A (SIVmacJ5)
CD4 absolute count
SIV RNA copies/ml
C92012F
C92017F
C92032M
C92034M
C94066F
B (SIVmacC8)
CD4 Absolute count
SIV RNA copies/ml
C92021F
C92028M
C92064M
C (SHIV89.6P)
CD4 Absolute count
SIV RNA copies/ml
C92056M
C91080F
C92008F
Weeks P.I.

Figure 2:

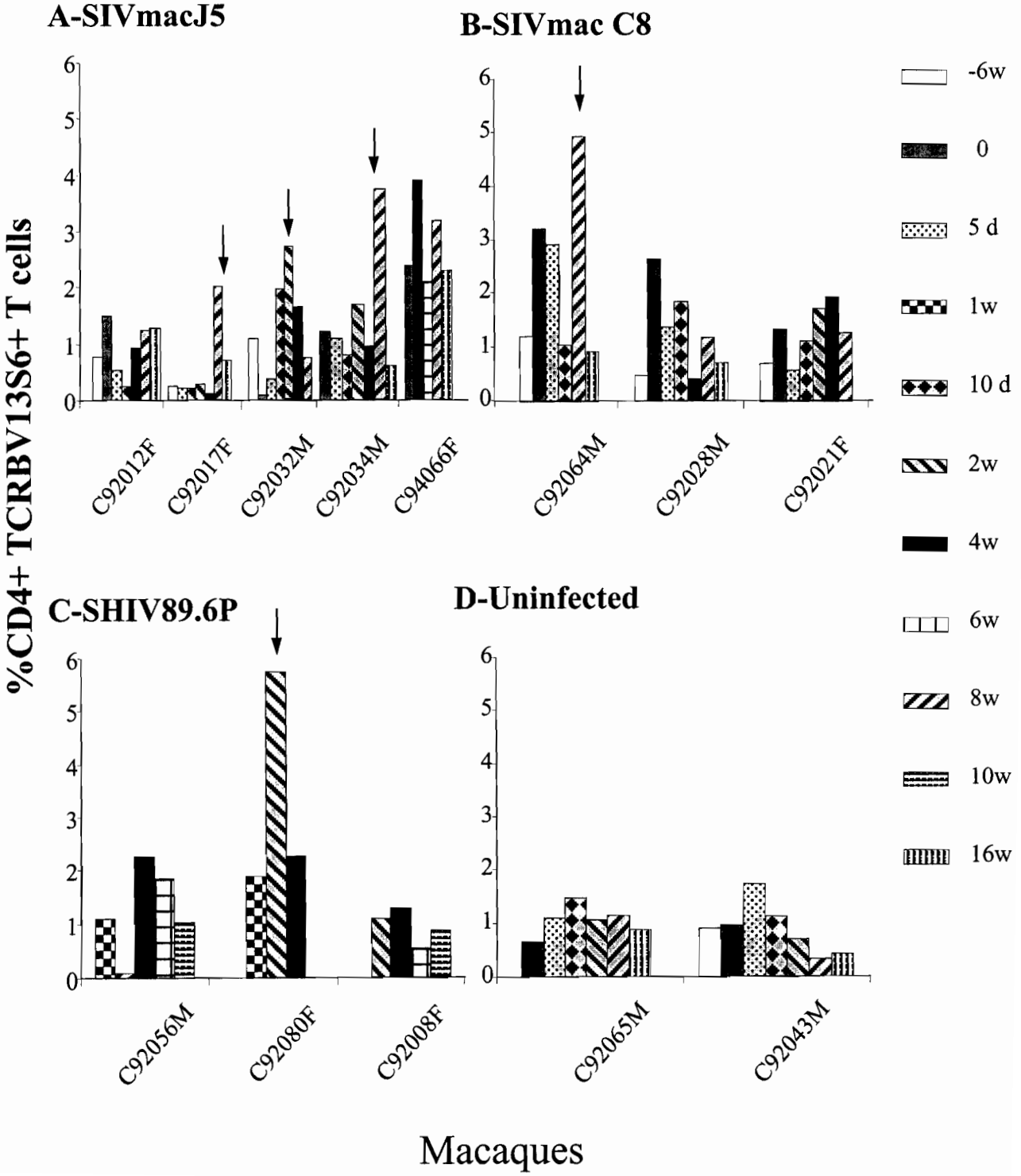
A-SIVmacJ5
B-SIVmac C8
C-SHIV89.6P
D-Uninfected
%CD4+ TCRBV13S6+ T cells
0
1
2
3
4
5
6
C92012F
C92017F
C92032M
C92034M
C94066F
C92064M
C92028M
C92021F
C92056M
C92080F
C92008F
C92065M
C92043M
-6w
0
5 d
1w
10 d
2w
4w
6w
8w
10w
16w
Macaques

Figure 3

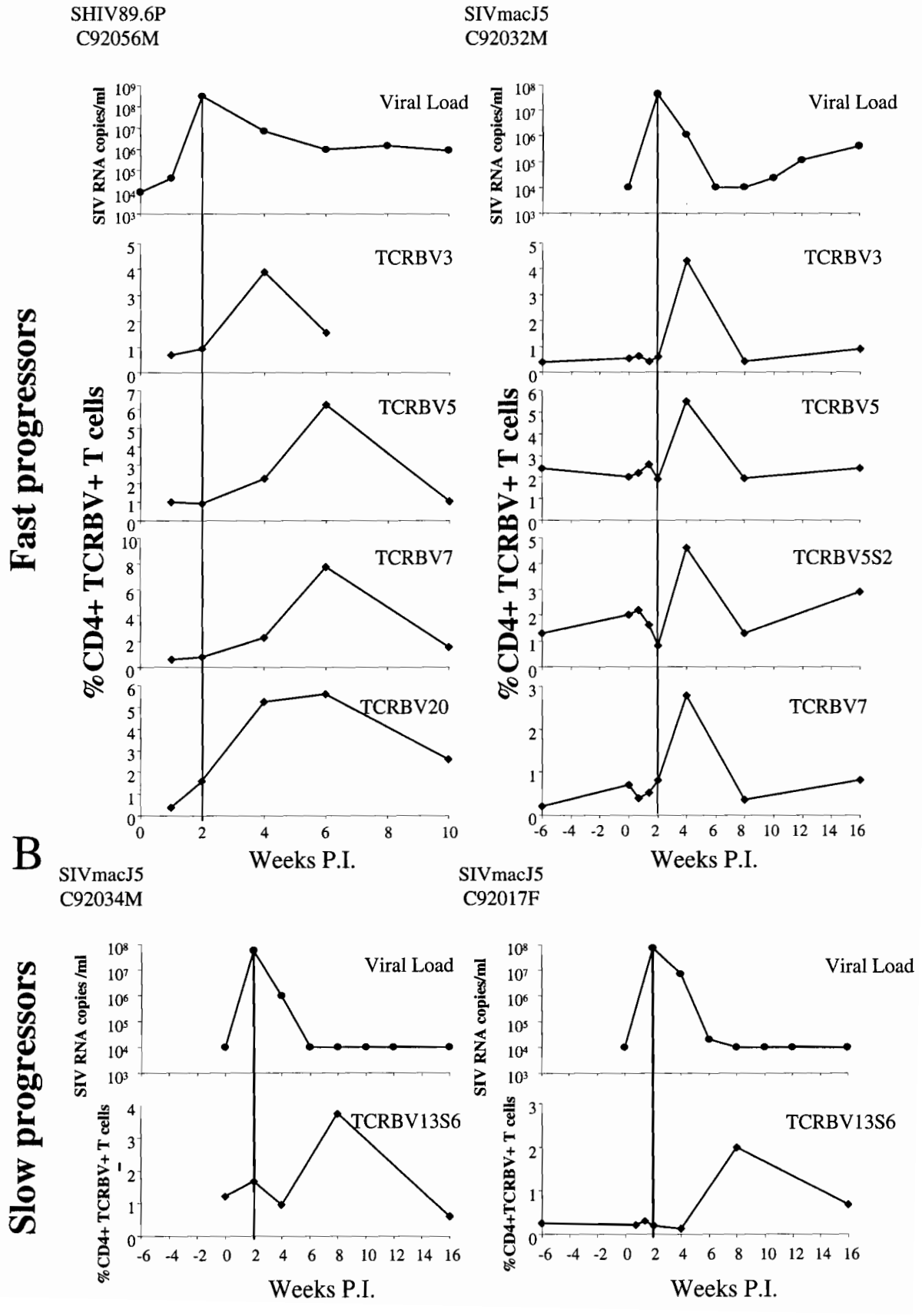
SHIV89.6P
C92056M
SIVmacJ5
C92032M
Fast progressors
Viral Load
TCRBV3
TCRBV5
TCRBV7
TCRBV20
TCRBV5S2
SIV RNA copies/ml
%CD4+ TCRBV+ T cells
Weeks P.I.
B
SIVmacJ5
C92034M
SIVmacJ5
C92017F
Slow progressors
TCRBV13S6
SIV RNA copies /ml
%CD4+ TCRBV+ T cells

Figure 4:

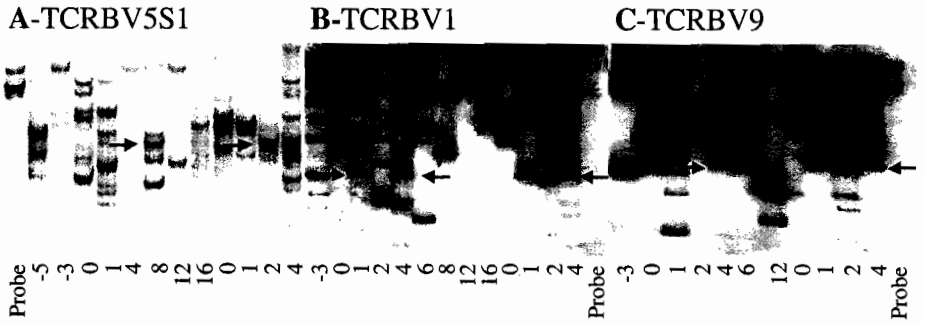
A-TCRBV5S1
B-TCRBV1
C-TCRBV9
Probe -5 -3 0 1 4 8 12 16 0 1 2 4
Probe -3 0 1 2 4 6 8 12 16 0 1 2 4
Probe -3 0 1 2 4 6 12 0 1 2 4 Probe

Table 1: Infection protocol

Macaques	Average CD4 count [a] before infection (SD) [b]	Virus	Challenge[c]
C92065M	2245 (349)	na	na
C92043M	2373 (469)	na	na
C92034M	2265 (607)	SIVmacJ5[d]	PBj14
C92017F	2046 (426)		PBj14
C92012F	1896 (286)		PBj14
C94066F	1614 (247)		SHIV89.6P
C94055F	1870 (412)		SHIV89.6P
C92032M	2713 (565)		PBj14
C92064M	2375 (401)	SIVmacC8[e]	PBj14
C92021F	2740 (437)		PBj14
C92028M	2709 (623)		PBj14
C92056M	3273 (665)	SHIV 89.6P[f]	none
C91080F	2434 (576)		none
C92008F	1521 (358)		none

[a]Average CD4 counts as well as SD were calculated from 6 months before infection until day 0 of infection.
na: not applicable.
[b]SD: standard deviation.
[c] Macaques were challenged with PBj14 (32 MID_{50}) or SHIV89.6P (10 MID_{50}) at 69 or 20 weeks post infection, respectively.
[d]Macaques were infected with $10^{4.7}$ $TCID_{50}$of SIVmacJ5 (9/90 stock).
[e]Macaques were infected with 10^5 $TCID_{50}$ of SIVmacC8 (9/90 stock).
[f]Macaques were infected with 10 MID_{50} of SHIV89.6P.

Table 2 : Oligonucleotides used as primers for TCRBV PCR analysis or probes for in situ hybridisation.

	Names of oligonucleotides	oligonucleotides sequence
Primers[a]	Cbout	5'-GGTCGGGGAGAAGCCTGTGGCCAGGC -3 '
	CBin	5'-GTGGGAGATCTCTGCTTCTGATGGCTCAAAC -3 '
	TCRBV1out	5'-GCTCCCCTAGGTCTGGAGACCGCT -3'
	TCRBV1in	5'-AGACCGCTCCGTGTACTGGTACCAA-3'
	TCRBV3	5'-GGATGTGAAAGTAACCCAGAGCTCAAGATAT -3 '
	TCRBV5out	5'- CTGATCAAAACGAGAGGACA-3 '
	TCRBV5S1in	5'- CTCCCCTATCTCTGGGCATAGAG -3 '
	TCRBV7	5'-CCCGAATTCTCACCTGAATGCTCCAAGAGCT-3 '
	TCRBV9out	5'-TATAAGCAGGACTCTAAGAAATTGC-3'
	TCRBV9in	5'-CAGGACTCTAAGAAATTGCTGAA -3'
Probes	BV3[a]	5'-GTCTCTAGAGAGAAGAAGGAG -3 '
	BV7[a]	5'-GCCAGAAGACTCAGCCCTGTATC -3 '
	TWQ[b]	5'-CAGTACCTGGCCGGGACAGGTTAA-3'
	VPG[b]	5'-TCATGTCCCGGGACAGGATTACGG-3'
	SPR[b]	5'-CCAAGTCCCCGGGGGAGCCAGGGT-3'
	EGD[b]	5'-CAAGAAGGGGACAATACG -3'

[a] TCRBV oligonucleotide sequences were determined according to the accession number M60537 for TCRBV1, AF143618 for TCRBV3, AF354294 for TCRBV5S1, M60543 for TCRBV7 and M60549 for TCRBV9 that were deduced from their homology to the human sequences.
[b] Oligonucleotide sequences corresponding to the CDR3 region of each TCRBV specific clones.

Table 3: Disease progression correlates with expansions in the CD4 TCRBV repertoires

Progression status	Macaques	CD4 count at time of expansion	Expansion in CD4+ TCRBV families[b]	Slope of delta score overtime [c]
Uninfected	C92043M	na [a]	no expansion	0,1330
	C92065M	na	no expansion	- 0,2402
Fast progressors	C92032M	2157 (1084)	3; 5; 5S2; 7; (13S6; 19)	2,1395
	C92056M	241	5; 3; 7; 20 ; 23	1,3193
	C91080F	98 (77)	3; 5S1; (13S6); 20	15,8350
Slow progressors	C92034M	1759	13S6	0,8411
	C92012F	2028	17; 23	1,4413
	C92017F	2231	13S6	0,6525
	C94066F	1533	5S1	- 1,0225
	C92064M	2059	13S6	0,1003
	C92028M	na	no expansion	- 0,1527
	C92021F	na	no expansion	- 0,0159
	C92008F	356	3	- 0,6825

[a] na: not applicable. * Data into brackets correspond to expansions occuring at a second time point.
[b]TCRBVs in which at least 2 fold expansions were observed during PI (between 0 and 10 weeks) by gating on TCRBVs and CD4+ T cells.
[c]The slopes of delta score were calculated for the period of acute infection between 0 and 10 weeks as described in material and methods.
The p calculated between fast and slow progressors was of 0.025 by using Mann-Whitney *U* test.

Table 4: Analysis of the TCRBV CDR3 sequences of the expanded CD4+ T cell clones in slow and fast progressors

A-Slow progressor C94066F

TCRBV type	Name of clone	CDR3 <N-D-N> [a]	Jβ	Clone Frequency		
				4W	8W	2WPC [b]
TCRBV5S1	NRG	NRGSF	2.5	0/25	10/30	9/22
	LGS	LGSD	2.2	0/25	9/30	3/22
	LGQ	LGQGD	1.4	0/25	5/30	0/22
	YLG	YLGQGFYG	2.1	0/25	0/30	7/22

B-Fast progressor C92032M

TCRBV type	Name of clone	CDR3 <N-D-N> [a]	Jβ	Clone Frequency				
				2W	4W	8W	32W	4WPC[b]
TCRBV3	TWQ	TWPGQV	1.4	0/11	5/25	1/17	1/27	0/614
TCRBV7	EGD	EGDNT	1.2	0/21	3/29	0/25	nd[c]	0/596
	VPG	HVPGQDYG	2.1	0/21	3/29	0/25	nd	0/1190
	SPR	PSPRGSQG	2.1	0/21	3/29	0/25	nd	0/590

[a] Aminoacid sequences were deduced from nucleotide sequences
[b] PC: Post Challenge
[c] nd: Not done

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Chapter IV

Persistence of oligoclonal CD4 T cell responses in SIV infected macaques leads to protection from SHIV89.6P superinfection

Persistence of oligoclonal CD4 T cell responses in SIV infected macaques leads to protection from SHIV89.6P superinfection

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Abstract:

Attempts to evaluate the protective effect of live attenuated SIV vaccine strains have yielded variable results depending on the route of immunization, the level of attenuation, the level of divergence between the vaccine candidate and the challenge. The protective mechanisms induced by these vaccines are still not well characterized. In an effort to address whether CD4 helper responses play a role in immunological protection induced by the SIVmacC8 attenuated strain, a longitudinal follow-up of the CD4 repertoire was performed by heteroduplex tracking assay in macaques immunized with wild type SIVmacJ5 or SIVmacC8 and challenged with simian-human immunodeficiency virus (SHIV89.6P). Virus load and CD4 absolute counts were determined in these animals and the presence of SHIV89.6P virus in challenged animals was evaluated by PBMC-co-culture and PCR. An association between the ability to generate, maintain and reactivate the primary CD4 immune responses following infection with SIV and protection from SHIV89.6P superinfection was shown, suggesting that CD4 helper responses play an important role in the immunological protection induced by the attenuated strain. These findings provide important insight into the qualitative and quantitative features of protecting immune responses.

Introduction:

Immunological responses generated against persistent viral infections are characterized by the activation and expansion of CD4 and CD8 antigen-specific T lymphocytes. Although cytotoxic T cell responses are responsible for clearance of most viral infections (1), CD4 T cells play a major role in supporting antibody production, initiating and maintaining CTL activity as well as performing direct effector activity through the production of specific cytokines (1-5). Following viral suppression through immunological control, these T cells decline in activity and number mostly through apoptosis (6), leaving the host with a sufficient number of pathogen-specific memory T cells. Memory T cells have the advantage of being able to respond more efficiently than naïve T cells do, probably due to T cell frequency as well as qualitative changes that they acquire through their differentiation process (7-9). Therefore, upon re-exposure to the same antigen, memory T cells go through a second round of proliferation, leading to a more robust and rapidly generated progeny. This has been demonstrated in both CD8 (10-14) and CD4 positive T cells (15). However, the time, magnitude, and composition of primary and secondary responses and their consequences on disease outcome have not been well characterized. Several factors including the stimuli, the host and environmental factors can contribute to create variations between antigen-selected repertoires, whether a potent antigen-specific repertoire is selected, is restricted or broad.

Attempts to generate a vaccine against HIV have led to the evaluation of attenuated strains as candidates. The attenuated strains isolated from humans (16) and macaques (17) were shown to cause little or no pathogenicity in the infected hosts, however, reversions to wild type pathogenic strains can occur (18;19). The attenuated virus vaccine has been tested in adult macaques and contradicting results were obtained from protection from homologous or heterologous challenge (20-22). The lack of protection in some animals was associated with the extent of attenuation of the viral strains (23). Indeed, the greater the number of deletions in accessory genes such as Nef, Vpx, Vif, Vpr (24), the less efficiently the virus grows *in vivo* and *in vitro*. In parallel, evaluation of immunological responses have indicated that some attenuated strains can induce potent immunity characterized by strong SIV-specific CTL and humoral responses (25-27). In contrast, other studies have reported a lack of correlation between the level of neutralizing antibodies and subsequent protection (20). The correlates and mechanisms of protection induced by attenuated

viruses are not well characterized. Furthermore, the implication of CD4 helper immune responses in protective immunity against SIV is poorly understood (28).

The present study was designed to focus on the CD4 repertoire following immunization with attenuated SIVmacC8 and pathogenic SIVmacJ5 strains. We evaluated the role and breath of the protective CD4 responses generated during primary SIV infection by challenging with a very pathogenic SHIV89.6P that contains an HIV-1 derived envelope (29). An association between protection from SHIV89.6P superinfection and persistence and re-expansions of CD4 clonotypes generated during primary SIV infection was observed.

Materials and methods:

Virus

Viral isolates include: SIVmacJ5 and SIVmacC8 originating from SIVmac32H(pJ5) and SIVmac32H(pC8) molecular clones respectively and SHIV89.6P, a pathogenic chimeric SHIV. The molecular clones, SIVmac32H(pJ5) and SIVmac32H(pC8), were isolated from cells infected with the 11/88 pool of SIVmac32H, an isolate derived by *in vivo* passage of SIVmac251 in rhesus macaque 32H (30), as previously described (31). In contrast to SIVmacJ5, which induces an AIDS-like pathology in infected macaques, SIVmacC8 shows an attenuated phenotype in monkeys; described ((31) and G. Hall, personal communication). SHIV89.6P infection results in a rapid decline in CD4+ T cell counts in rhesus and cynomolgus macaques within a couple of months of infection (32). The SIVmacJ5 and SIVmacC8 were challenged with SHIV89.6P at week 20.

Animals

Animals used in this study were part of a large European concerted action on AIDS vaccine (33) and Rud, unpublished data). Three groups of juvenile (approx. 3 year old) cynomolgus macaques (*Macaca fascicularis*) were infected via the intravenous route. All animals used in this study were colony bred within the Non-human Primate Breeding Colony of Health Canada under Canadian Council of Animal Care approved conditions. All animals were serologically negative for Herpes B virus, STLV-1, SRV-1, 2, 5 and SIV (The Virus Reference Laboratory, Inc., San Antonio, TX). The percentage of CD4-positive T-lymphocytes was determined using a FACScan® (Becton Dickinson, CA, USA) flow cytometer and CELLQuest software. Whole blood collected in EDTA was analyzed for lymphocyte subsets by incubation with anti-human CD2 (Fluorescein Isothiocyanate (FITC)-labeled, Becton Dickinson), anti-human CD4 (Phycoerythrin (PE)-labeled, Becton Dickinson). White blood cell counts were obtained from a hematology workstation (Coulter Counter S-PLUS IV, FL, USA) and were used to calculate the lymphocyte subset absolute counts.

Plasma viral Load

Plasma SIV RNA concentrations were determined using a branched DNA signal amplification method developed by Dr. Peter Dailey (Chiron Corporation, Emeryville, CA) for SIV using the same approach as Quantiplex HIV RNA branched DNA method (34). For quantitating SIV viral load, target probes designed to hybridize with the *pol* region of the SIVmac strains were used. Results were quantified by comparison with purified and *in vitro*-transcribed SIV *pol* RNA. The lower limit of detection of this assay was 1500 copies of SIVmac RNA per ml plasma in all the animal, which goes down to 500 copies at 35 weeks.

CD4 T cell purification

Macaque PBMCs (10^7 cells) collected at described time points were purified using microbeads-conjugated CD4 antibodies (Miltenyi Biotech) and MACS MS columns and MACS magnetic cell sorting apparatus (Miltenyi Biotech) as recommended by the manufacturer. Purified CD4+ T cells were then washed in PBS and resuspended in Trizol® (Life technologies, Burlington, Canada) for RNA extraction. CD4 T cell purity was verified for each sample by staining with PE- conjugated anti-CD4 and PERCP- conjugated anti-CD8 antibodies Software (Becton Dickinson, CA, USA) and analyzed on a FACScan® using the Cell Quest® Software.

Heteroduplex tracking assay

Heteroduplex tracking assays (HTA) were performed as described (35) for all macaques in this study at the bleeding time points (-3, 0, 1, 2, 4, 6, 8, 12, 16, 20, 21, 22 and 24 weeks post infection with SIVmacJ5 or SIVmacC8. Briefly, total RNA was purified from PBMC using Trizol® and was used to synthesize complementary DNA (cDNA) using oligo dT (12-18) primers. Nested polymerase chain reaction (nPCR) was performed on cDNA samples from sequential time points using TCRBVout and Cβout as outer primers and TCRBVin and Cβin as inner primers. The uniformity of yield and amplification efficacy of each oligonucleotide was assessed by agarose gel electrophoresis. In parallel, ^{32}P labeled probes were produced by PCR amplification of two TCRBV-Cβ inserts previously cloned in Bluescript (Stratagene Cloning Systems, La Jolla, Ca), using ^{32}P-labeled Cβin primer and unlabeled TCRBVin primers. Radiolabeled DNA heteroduplexes were obtained by mixing 4.5 µl from second round PCR with

0.5 µl of probe in annealing buffer (0.16 M NaCl, 16mM Tris [pH 8.0], 3.3 mM EDTA). DNA was denatured at 95° C for 2 min, after which they were reannealed at 55°C for 5 min and rapidly cooled to room temperature in a thermocycler. Heteroduplexes were resolved on a 20-cm high, 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide) at 200V for 3h. HTA gels were vacuum-dried and a PhosphorImager (Amersham Pharmacia Biotech, NJ, USA) plate was used for signal detection. The persistence of CD4 clonotypes overtime was evaluated through the use of two probes for each TCRBV family that differ by the CDR3 region and the BJ segment.

Results:

Macaques infected with either SIVmacJ5 or SIVmacC8 show a slow disease progression rate

To address the effect of both virulence and magnitude of viral load on disease progression, cynomolgus macaques were infected with two different SIV strains: SIVmacJ5 inducing high viral load (>10^8 copies per ml) and AIDS-like disease after 6 month to 3 years. SIVmacC8 an attenuated strain (30), induces low viral load, with no significant drop in CD4 count and the absence of disease progression.

In all four SIVmacJ5 infected macaques the CD4 count transiently dropped 2 weeks post infection and returned to normal values at subsequent time points. In those animals virus load peaked 2 weeks post infection (~10^8 copies per ml) and settled at week 6 below the limit of detection, with some transient viral load measurements. In the SIVmacC8 group, macaques C93054F and C94042F did not experience any significant perturbations in their CD4 counts, both macaques had low CD4 count at day 0 of infection which remained unchanged at later time points. The CD4 count in macaque C94011M initially dropped one week post infection followed by a transient increase until week 6 post infection. Following week 8 post infection, the CD4 count in that animal returned to normal levels. Macaque C94036F had a transient peak in CD4 count at week 6 post infection and stabilized at following time points. All SIVmacC8 infected macaques had a peak in viral load ($5x10^5$-$8x10^6$ copies per ml) at week 2 and the virus settled by week 6 below the limit of detection. (figure 1)

Following infection with SIVmacC8 or SIVmacJ5 all macaques exhibited a slow progression phenotype evidenced by a stable CD4 count overtime similar to uninfected animals and viral load remained at the limit of detection following acute infection.

The diversity of the CD4 TCRBV repertoire was evaluated by heteroduplex mobility assay in uninfected and SIVmacC8 or SIVmacJ5 infected macaques, prior to infection and until 8 weeks post infection. The clonality of each TCRBV family at each time point and in each macaque was analyzed using the NIH image software (Figure 3). A repertoire composed of more than 13 clones was considered as polyclonal, whereas 12 clones or less was considered oligoclonal. Although the SIVmacC8 group has a 2 log Less SIV RNA copies compared with SIVmacJ5 group, this difference in the level of viral load did not translate into a variation of the diversity of the CD4 TCR repertoire. Furthermore, the diversity of the CD4 repertoire does not seam to correlate with the disease status in the infected animals. All SIV infected macaques have a slow

progressor phenotype characterized by stable CD4 counts overtime and undetectable viral load following acute infection. However, both groups are comprised of macaques with very oligoclonal repertoires, C94066F and C94055F in the case of SIVmacJ5 group and C94036F a SIVmacC8 infected macaques. Others have oligclonal and polyclonal TCRBV families such as C94073F, C95011F and C94011M from the SIVmacJ5 or SIVmacC8 group respectively. On the other hand uninfected macaques and two SIVmacC8 infected macaques have very polyclonal repertoires (figure 2 and 4).

Protection status following challenge with SHIV89.6P

In order to investigate whether infection with the attenuated SIV strain SIVmacC8 induces protection against heterologous challenge with SHIV89.6P, macaques were either not infected or infected with SIVmacC8, or the wild type pathogenic strain SIVmacJ5. At 20 weeks post infection all three groups were challenged with SHIV89.6P.

Following challenge with SHIV89.6P at week 20, disease outcome was variable among animals. In the SIVmacC8 group two macaques (C93054F and C94042F) were superinfected with SHIV89.6P, while the two others (C94036F and C94011M) together with the SIVmacJ5 group were not and can be considered as protected (Figure 1B) and data not shown. The former experienced a slight decrease in CD4 counts. Macaque C93054F showed a rebound in viral load at week 26 (10^6 copies per ml) and remained high during the follow-up period, whereas macaque C94042F had two expansions in viral load at week 24 (10^6 copies per ml) and at week 31 ($8x10^4$ copies per ml). All protected animals had normal CD4 counts following challenge and their virus load remained undetectable overtime, except for macaque C94066F that showed a blip in viremia at week 20 post infection (figure 1).

Control uninfected animals were also infected with SHIV89.6P at week 20. All four macaques within this group showed a drastic drop in CD4 counts by week 21 below 200 cells per µl. While CD4 counts in all three animals within this group remained very low, macaque C95024M showed a transient increase in CD4 count to normal values, however by week 74 the CD4 count dropped again below 200 cells per µl. All these animals experienced very high viral load (10^7-10^9 copies per ml) by week 22. Viral set point was reached by week 24 and remained between 10^4 and 10^7 copies per ml.

SIV infected macaques with oligoclonal CD4 repertoires during PI are protected from SHIV89.6P challenge, whereas macaques with polyclonal repertoire during PI are not

The correlation between protection from SHIV89.6P superinfection, and diversity in the CD4+ TCR repertoires in these animals was evaluated. Macaques with very polyclonal repertoire during primary SIVmacC8 infection, were not protected from SHIV89.6P challenge (C93054F and C94042F), whereas the two others as well as SIVmacJ5 group that showed several oligoclonal TCRBV families were. Interestingly, both unprotected and uninfected macaques had comparable very polyclonal CD4 repertoires (Figure 4). Furthermore, several TCRBV families from protected animals have evolved overtime following SIV infection, such as TCRBV1 for macaques C94055F, C94066F, C95011F as well as TCRBV2 for macaques C94073F and C94011M (figure 4). These TCRBV families progressed from a polyclonal repertoire prior to infection to a very oligoclonal repertoire composed of less than 6 clones during PI. Other TCRBV families were either always oligoclonal (such as TCRBV3 for macaque C94055F) or evolved further to include less CD4 clonotypes (such as TCRBV3 and TCRBV5S1 for macaque C94066F). In contrast the TCR repertoire of unprotected animals remained polyclonal during acute SIVmacC8 infection.

Correlation between persistence of CD4 clonotypes during PI and protection to challenge

In protected animals, several TCRBV families containing dominant CD4+ T cells bearing clonotypes are conserved at later time points during PI (figure 5 and table 1). Interestingly, most of these TCRBV families have contracted during the progression to chronic infection, suggesting a maturation of the immune response with time (such as TCRBV1 and TCRBV8 in macaque C94055F). On the other hand unprotected and uninfected animals show little if no persistence of CD4+ T cells bearing clonotypes, although some TCRBV families in infected macaques are contracted at 2 weeks post infection but remain very polyclonal (TCRBV5S1 for macaque C93054F). This is also the case in a some TCRBV families in protected animals (TCRBV3 in macaque C95011F), indicating that elimination of CD4+ T cells during PI is a common denominator in all SIV infected macaques. However, the ability to conserve and re-expand some of these cells is only observed in animals that are resistant to superinfection with SHIV89.6P (Figure 5).

Evolution of the CD4 repertoire in unprotected and protected macaques following challenge

In order to evaluate the re-expansion of the CD4 clonotypes subsequent to challenge in protected and unprotected animals, a longitudinal study of the CD4 TCR repertoire was performed by HTA from –3 weeks prior to SIV infection to 4 weeks post challenge with SHIV89.6P. Figure 6 clearly shows an oligoclonal repertoire in protected animals following challenge. In some cases, such as TCRBV5S1 for macaque C95011F, the repertoire is constantly oligoclonal throughout the study; however, some CD4 clonotypes detected during PI persist and dominate the repertoire following challenge as early as 2 weeks post challenge. In most cases, the CD4 repertoire is contracted overtime and, by week 8, up to 6 CD4 clonotypes are present (for-example: TCRBV1 for macaque C94055F, TCRBV9 for macaque C94055F). Following challenge at week 1 through 4, some clones that were present during PI dominate the repertoire. In some TCRBV subsets following challenge, the repertoire returns to polyclonality such as TCRBV5S1 at week 4 post challenge in macaque C94066F.

CD4 repertoires of unprotected and uninfected animals are very similar. In both cases, they remain polyclonal before and after infection with SIVmacC8 or throughout the period of follow-up in case of uninfected animals, indicating a lack of CD4 response following SIV infection in unprotected animals. At week 16, macaque C94042F had an oligoclonal CD4 repertoire in TCRBV1 and 2, however, these clones do not correspond to earlier CD4 clonotypes, nor did they persist at later time points, or re-expand following challenge. Indeed, subsequent to SHIV challenge, unprotected and control animals have polyclonal CD4 repertoires at week 1 post challenge. At week 2, CD4 counts in control animals drop below 200 cells per µl, therefore the contraction of the CD4 repertoire at this time point, which includes very few clones, could represent the limited CD4 clones still remaining in these animals. In unprotected animals, the CD4 repertoire remains polyclonal during the period of follow-up.

Discussion:

In this study we provide evidence that the maintenance of CD4 clonotypes generated early following SIV infection is associated with the ability to be protected against subsequent challenge with virulent SHIV89.6P.

First we have shown through a longitudinal follow-up that macaques with a slow progression rate can have three types of CD4 repertoires during primary SIV infection: an oligoclonal repertoire in almost all TCRBV subsets, a repertoire with both oligoclonal and polyclonal TCRBV families or a completely polyclonal CD4 compartment. Whereas uninfected macaques have polyclonal CD4 TCRBV subsets overtime indicating stable repertoires, oligoclonality usually indicates antigen-driven expansion of T cell clones bearing distinct CDR3 segments (36-40). The findings that we report here indicate that the use of different viral isolates with variable pathogenic potential, able to induce different levels of viral load in the infected hosts, do not seem to have an effect on the level of clonality in the CD4 compartment. Indeed, macaques infected with SIVmacJ5 or SIVmacC8 have oligoclonal and polyclonal repertoires, suggesting that above a certain threshold of viral replication, the magnitude of CD4 responses is not a measure of the number of available viral epitopes, probably because availability on MHC molecules is limited. It is highly unlikely that polyclonality in the CD4 repertoire of SIVmacC8 infected macaques could be associated with a polyclonal CD4 response, because usually polyclonal CD4 responses mature with time to include only few CD4 clonotypes, which results in the contraction of the CD4 repertoire overtime. It is well documented that in persistent infections, repertoire selection is characterized by the recruitment of the highest-affinity TCRs through an affinity maturation process (6;41-44). There are no indication suggesting a narrowing of the CD4 repertoire overtime in SIV infected animals with polyclonal CD4 repertoire.

The most intriguing finding was the association between the lack of CD4 responses during PI and the susceptibility to superinfection with SHIV89.6P. Indeed, the CD4 repertoire in unprotected macaques remained polyclonal throughout the studied period pre and post challenge. The peak of response generated after reactivation of resting memory cells should occur faster and should give rise to a much more abundant progeny than following their primary encounter with the antigen (45). Therefore the lack of secondary CD4 responses in unprotected animals following challenge, further suggests that no primary responses were generated during the first encounter with the virus, thus explaining their susceptibility to SHIV infection. However,

SIVmacC8 infected macaques susceptible to SHIV89.6P superinfection did not experience the same rate of disease progression as did control animals. This suggests that in unprotected SIVmacC8-immunized animals, other factors may be involved in preventing a rapid disease progression, which is usually caused by virulent strains such as SHIV89.6P. Following SHIV89.6P infection, the pool of potentially infectable CD4 T cells is less important in SIVmacC8-immunized animals than they are in unimmunized controls, which would cause a higher SHIV89.P replication potential in the latter and as a consequence, a poor disease outcome. Although unprotected animals do not succumb to disease as rapidly as unimmunized controls, one of the two unprotected macaque (C93054F) died by week 105 post challenge and the other C94042F had two peaks in viremia following challenge.

Interestingly, animals with oligoclonal CD4 repertoires have shown a contraction of CD4 TCRBV subsets following peak viremia to include a handful of clones by week 8 post infection. Upon challenge with SHIV89.6P, the same clonotypes that were present during primary SIV infection became dominant as early as one week post challenge in some TCRBV families. These macaques were resistant to infection by SHIV challenge, suggesting that immunosurveillance against SIV infection rests with few CD4 clonotypes recognizing a limited array of SIV determinants. This further supports the idea that these CD4 clonotypes are stimulated against SIV epitopes and after encountering SHIV89.6P, which shares several homologies with SIVmac (32;46). Several studies have shown that the memory CD4 (15) or CD8 (10-14) response largely reflect the repertoire selected by the peak of the primary response (11-14). Through a maturation process, it is possible that the second immune response has the ability at this stage to prevent the establishment of a second round of viral infection upon challenge, a property that primary T cell responses do not have.

Considering our previous findings in fast progressor macaques, it is very likely that during acute infection a large pool of CD4 clonotypes generated against SIV or other pathogen get eliminated in most infected animals, whether slow or fast progressors.

What remains to be clarified however, is why CD4 immune responses in fast progressors are significantly eliminated early whereas slow progressors with detectable CD4 responses are able to preserve at least some of them. Several hypotheses can explain this outcome. The time lag between the peak of viral load and the expansions in TCRBV subsets was either shorter in some fast progressors, or the CD4 expansions coincided with a high viral set point in other fast

progressors compared with slow progressors from that group. This could explain the elimination of the CD4 clonotypes in fast progressors due to the high levels of circulating viral particles. Elimination would thus result through cytopathic effects from the virus, CTL killing, or even through clonal exhaustion due to continuous stimulation of CD4 clonotypes. As for slow progressor macaques, TCRBV expansions occur at a time when viral set point is low or undetectable, suggesting that at least part of them might have been rescued from killing. In animals protected from challenge, CD4+ TCRBV families that evolve overtime become oligoclonal and include less than 6 CD4 clonotypes by week 8 post infection, some of which are reactivated following challenge. This suggests that the peak of primary CD4 responses occur at week 8 post infection, at a time when the virus has settled below the limit of detection. Another explanation for the elimination of CD4 clonotype in fast progressor macaques is the magnitude of this response. A consequence of the activation of a large versus a small CD4 T cell pool is the establishment of large reservoirs than can facilitate even further SIV replication and ultimately accelerating disease progression (47). Ideally, the mobilization of a restricted SIV-specific CD4 response would minimize viral output and allow their maintenance for the transition into the chronic phase of the disease where they are mostly needed. What remains to be addressed is whether or not the magnitude of the early CD4 response in fast progressors reflects CD4 clonotypes generated against both SIV and other pathogens through a co-infection process which could serve as an additional reservoir for SIV spreading. It has indeed been demonstrated that during co-infections, immunological responses against the co-infecting pathogen can drive HIV replication (48-50). However, just as slow progressors in this and the previous study, fast progressors were serologically negative for Herpes B virus, STLV-1, SRV-1, 2, 5 and SIV before SIV infection. Furthermore, in all the infected animals as well as in the uninfected control group, no significant CD4 T cell repertoire perturbations were observed from 6 weeks pre-infection until 4 weeks after infection. In both slow and fast progressors the expansions in the CD4 compartment occur after the peak in viral load suggesting that these T cell proliferations are SIV specific. Therefore, although the co-infection hypothesis could explain the elimination of early CD4 clonotypes, it is highly unlikely that this process has occurred in these studies.

Vaccine strategies against HIV with the objective of enhancing CD4 helper responses should investigate whether interventions could be safer under conditions of minimal viral replication, which would prevent seeding of the reservoir pool and the concomitant elimination of the HIV-

specific CD4 T cells. This HIV-specific CD4 response should be sufficient to induce protection but not excessive to provoke virus outbreak.

Legends:

Figure 1: Disease status in SIV infected animals

CD4 absolute count (right panels) and plasma SIV RNA detected by branched DNA (left panels) from groups A-SIVmacJ5, B-SIVmacC8, and C- SHIV89.6P infected macaques. SIV viral load target probes, designed to hybridize with the *pol* region of the SIVmac groups of strains were used to quantify SIV viral load. Results were quantified by comparison with purified and quantified *in vitro*-transcribed SIV *pol* RNA and were plotted on a log(10) scale. The detection limit of this assay was 1500 copies of SIVmac RNA per ml until week 35 and 500 thereafter. White blood cell counts were obtained from a hematology workstation and were used to calculate the absolute CD4 counts.

Figure 2: TCRBV1 and TCRBV5S1 CD4+ repertoires in uninfected, SIVmacJ5 and SIVmacC8 infected macaques

The clonality of the CD4 repertoire does not depend on the infecting viral strain. SIVmacJ5 and SIVmacC8 infected macaques include macaques with polyclonal or oligoclonal CD4 repertoires, whereas uninfected macaques have always polyclonal repertoires. Heteroduplex tracking assay using a panel of TCRBV as 5'-primers that include (TCRBV1, 2, 3, 5S1, 6, 8 and 9) and BC as 3'-primers was performed on purified CD4+ T cells collected from –3 weeks prior to SIV infection until week 8 post infection.

Figure 3: Computerized densitometric analysis of the HTA autoradiography

Each band on the HTA gel is transformed into a peak using the NIH image software. Each band represents a CD4 clonotype. Arrows indicate discrete TCRBV heterocomplexes over the HTA profiles.

Figure 4: Summary of the changes in the clonality in the CD4+ TCRBV repertoire during primary SIV infection

The clonality of TCRBV1, 2, 3, 5S1, 6, 8 and 9 families at each time point and for each macaque in the study, was determined by HTA from –3 weeks prior to SIV infection until 8 weeks post infection. Black boxes represent polyclonal repertoires, grey boxes represent oligoclonal

repertoires containing between 7 and 12 clones, whereas white boxes represent less than 6 clones. Boxes with a cross represent samples that were not done.

Figure 5: The outcome of the CD4 clonotypes during PI

CD4+ T cells bearing dominant clonotypes that are generated during PI are either eliminated during or immediately following peak viremia. Arrows indicate clonotypes of interest.

Figure 6: Evolution of the CD4+ TCRBV repertoire in animals protected and unprotected from SHIV89.6P superinfection

Longitudinal analysis of the CD4 repertoire by HTA from time points prior to, following SIV and SHIV89.6P challenge at week 20 in macaques resistant or susceptible to superinfection by SHIV89.6P, as well as control uninfected animals that were also infected at week 20 with SHIV89.6P. These are representative examples of CD4+ TCRBV repertoires for each group.

Figure 1:

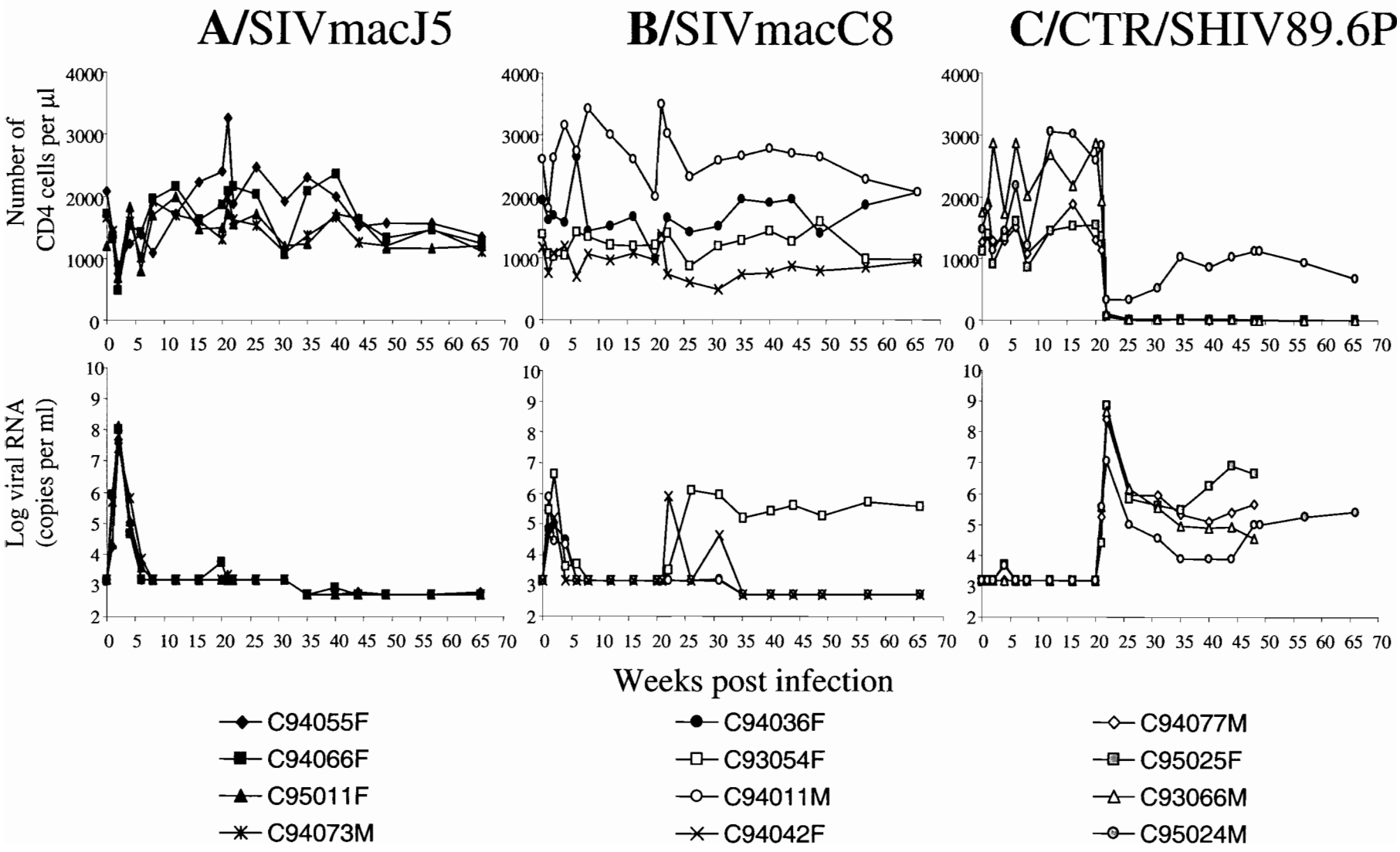

A/SIVmacJ5
B/SIVmacC8
C/CTR/SHIV89.6P
Number of CD4 cells per µl
Log viral RNA (copies per ml)
Weeks post infection
C94055F
C94066F
C95011F
C94073M
C94036F
C93054F
C94011M
C94042F
C94077M
C95025F
C93066M
C95024M

Figure 2:

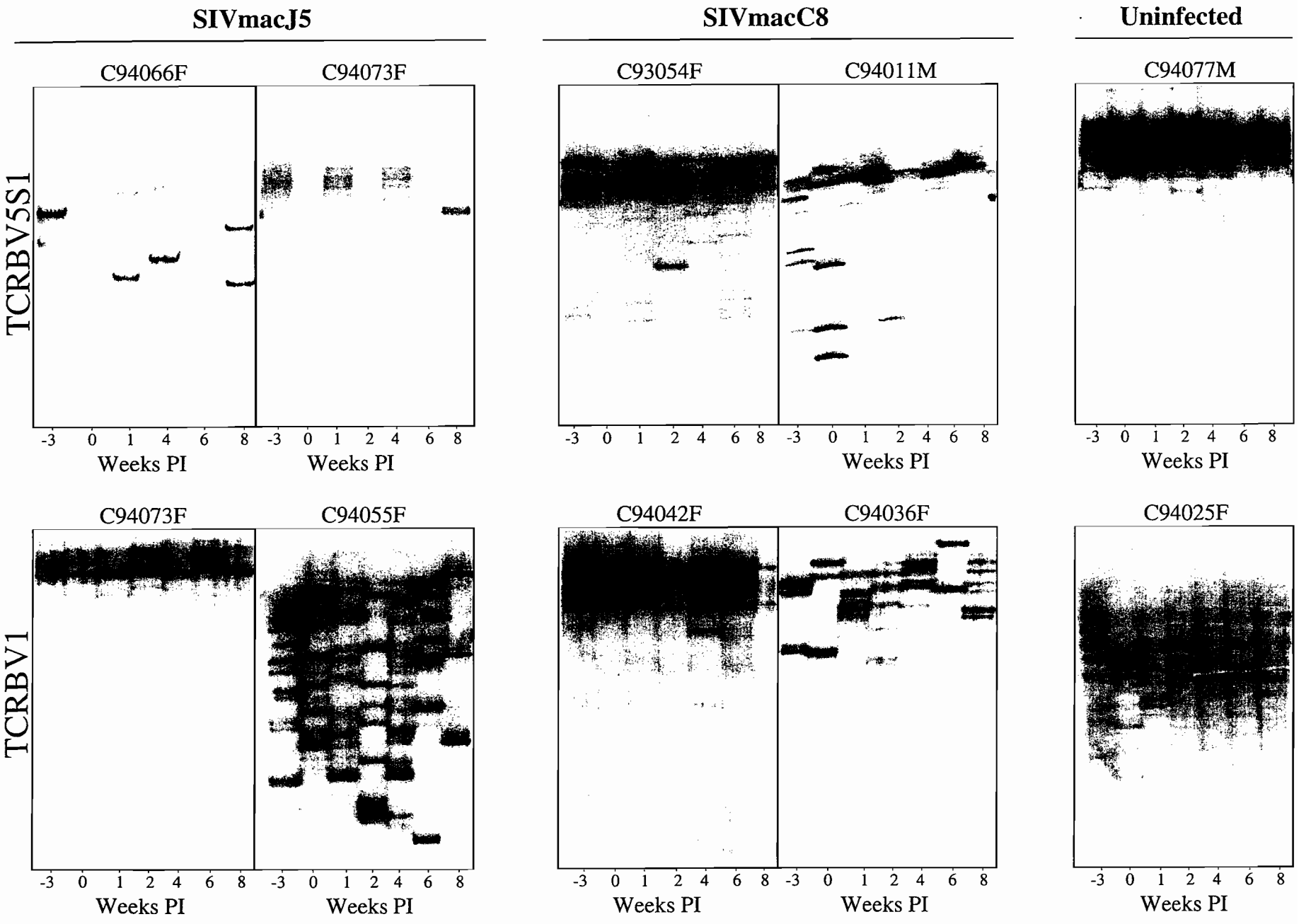
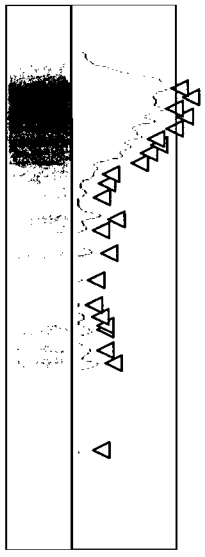
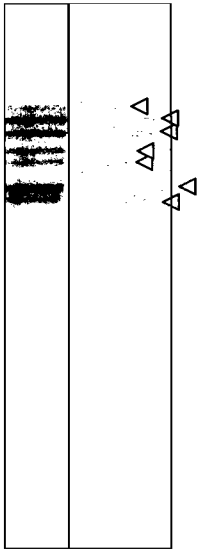

SIVmacJ5
SIVmacC8
Uninfected
C94066F
C94073F
C93054F
C94011M
C94077M
TCRBV5S1
C94073F
C94055F
C94042F
C94036F
C94025F
TCRBV1
-3 0 1 4 6 8
-3 0 1 2 4 6 8
Weeks PI

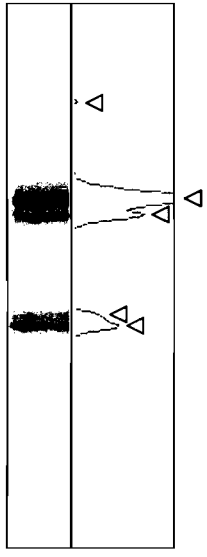
Figure 3:



Polyclonal
> 13 clones



Oligoclonal
8 clones



Oligoclonal
5 clones

Figure 4:

Macaques Resistant to challenge

SIVmacJ5

SIVmacC8

Macaques Suseptible to challenge

SIVmacC8

Unifected macaques

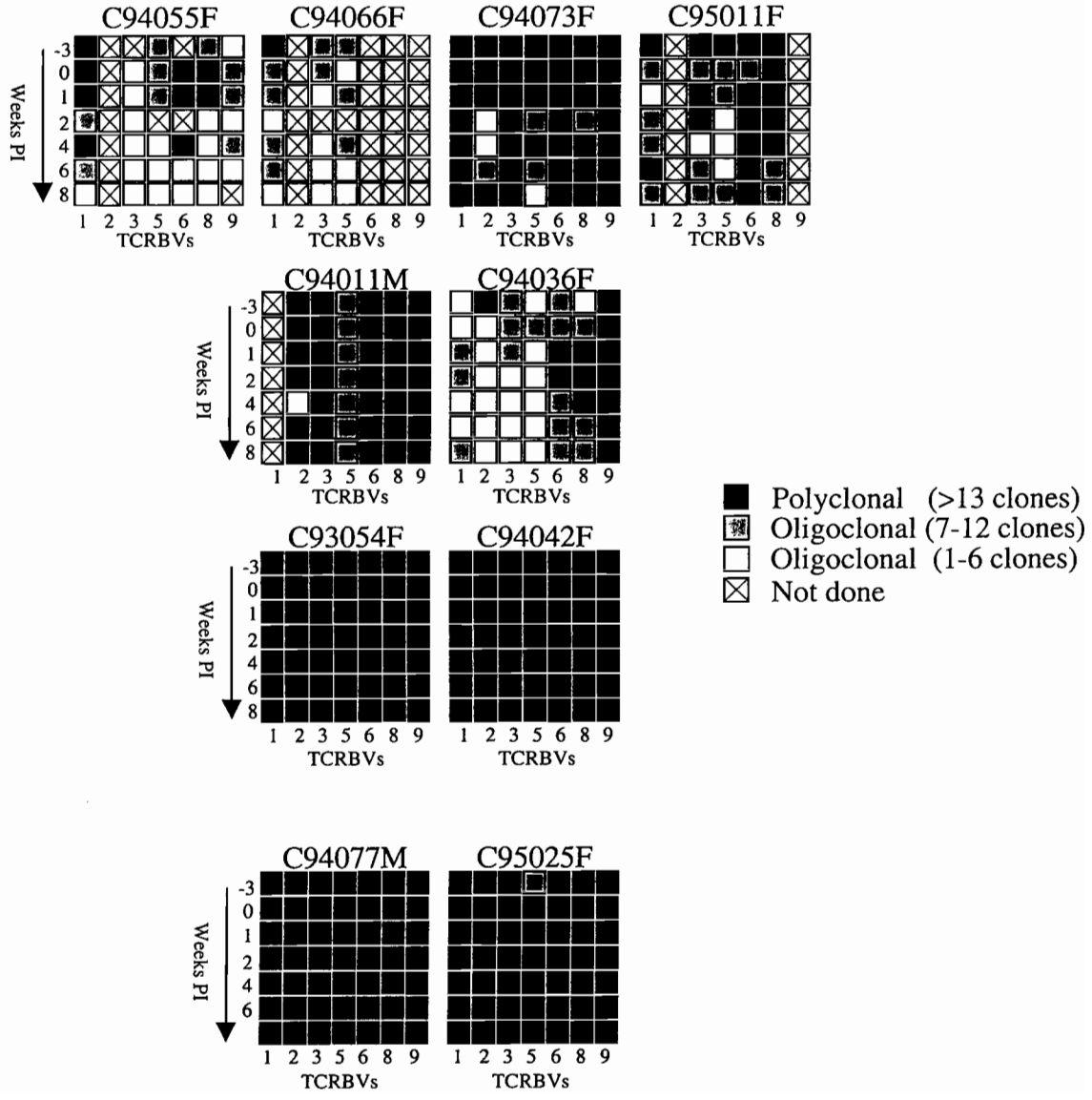


Figure 5:

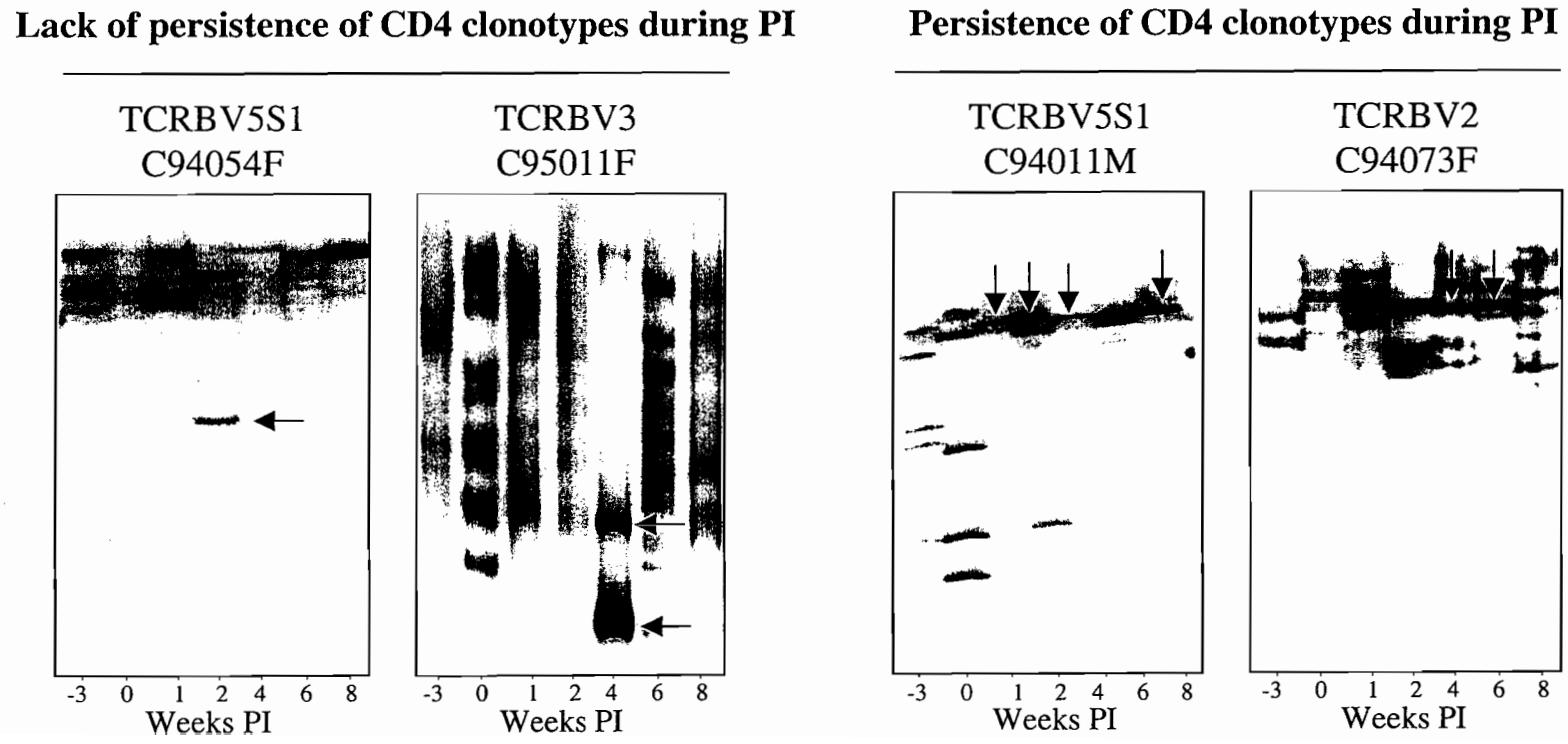

Lack of persistence of CD4 clonotypes during PI
Persistence of CD4 clonotypes during PI
TCRBV5S1
C94054F
TCRBV3
C95011F
TCRBV5S1
C94011M
TCRBV2
C94073F
-3 0 1 2 4 6 8
Weeks PI
-3 0 1 2 4 6 8
Weeks PI
-3 0 1 2 4 6 8
Weeks PI
-3 0 1 2 4 6 8
Weeks PI

Figure 6:

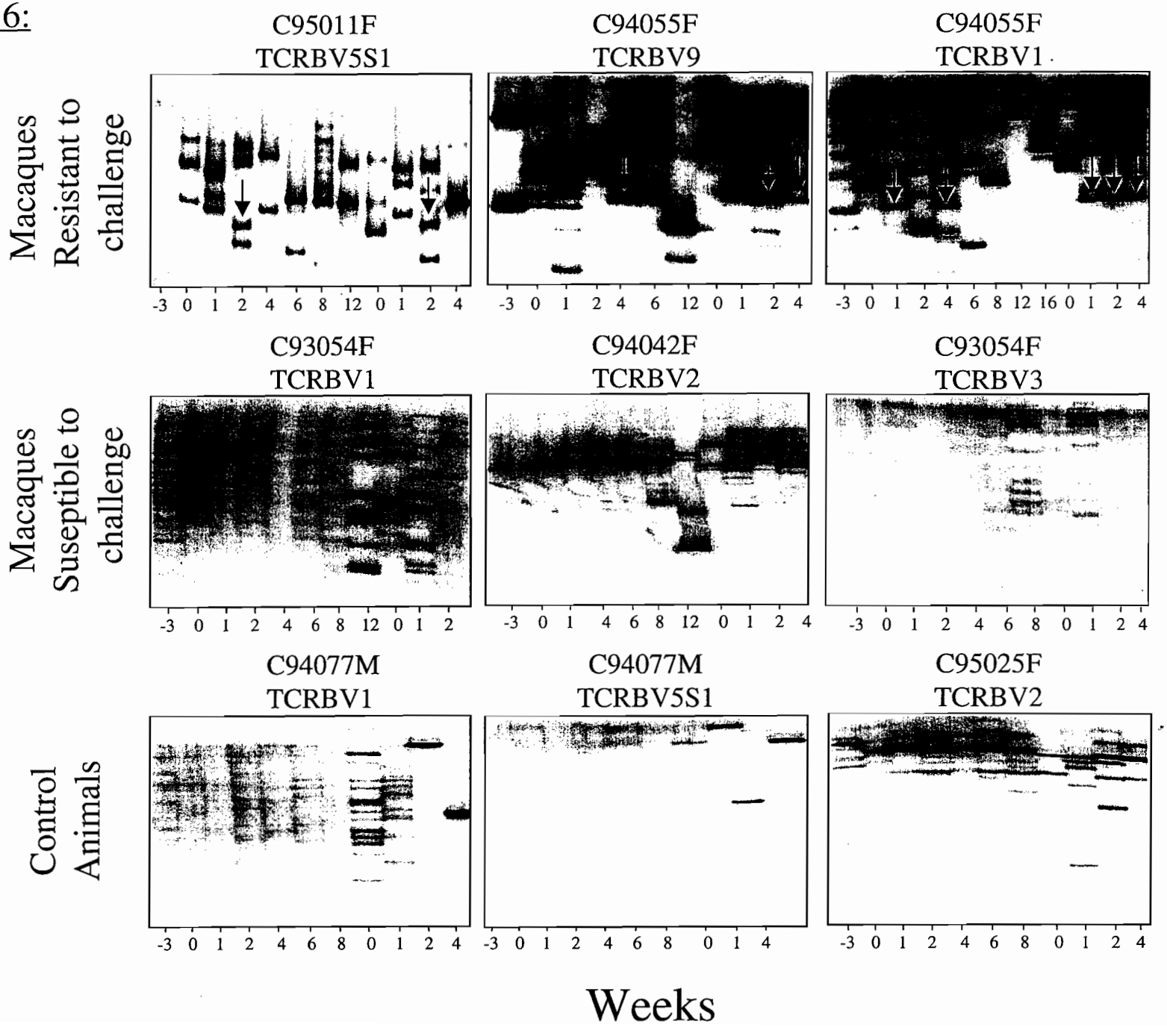

C95011F
TCRBV5S1
C94055F
TCRBV9
C94055F
TCRBV1
Macaques Resistant to challenge
-3 0 1 2 4 6 8 12 0 1 2 4
-3 0 1 2 4 6 12 0 1 2 4
-3 0 1 2 4 6 8 12 16 0 1 2 4
C93054F
TCRBV1
C94042F
TCRBV2
C93054F
TCRBV3
Macaques Suseptible to challenge
-3 0 1 2 4 6 8 12 0 1 2
-3 0 1 4 6 8 12 0 1 2 4
-3 0 1 2 4 6 8 0 1 2 4
C94077M
TCRBV1
C94077M
TCRBV5S1
C95025F
TCRBV2
Control Animals
-3 0 1 2 4 6 8 0 1 2 4
-3 0 1 2 4 6 8 0 1 4
-3 0 1 2 4 6 8 0 1 2 4
Weeks

Table 1: **Clonotype persistence during PI**

Macaque	Virus	Time points showing persistent bands during PI							Status
		TCRBV1	TCRBV2	TCRBV3	TCRBV5S1	TCRBV6	TCRBV8	TCRBV9	
C94055F	SIVmacJ5	1, 4*					4, 6		Resistant to SHIV89.6P challenge
C94066F									
C94073F			4, 6 *		1, 4 *				
C95011F		4, 6			1, 4 *				
C94036F	SIVmacC8	6, 8 *		4, 6 *		4, 6		6, 8	
C94011M			4, 8 *		1, 2, 4 *				
C94042F	SIVmacC8			4, 6					Susceptible to SHIV89.6P challenge
C93054F									
C94077M	uninfected								
C95025F									

* Persistence verified with two TCRBV probes.

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Contribution to Original Knowledge

Through my Ph.D. training in the laboratory of Dr Sekaly, I have contributed to the advancement of science in the following fields:

1. The work in which I was involved has shown that HIV-1 does not encode for a superantigen responsible for the depletions observed in the CD4+ TCR repertoire of HIV-infected individuals. We came to the conclusion that these depletions late in HIV disease must be related to an associated superantigen, such as CMV through the encounter of opportunistic infections that are very common at this stage.
2. In order to use a macaque model for the study of the immunopathogenesis of HIV, I have customized immunological techniques developed exclusively in humans to allow for investigation in cynomolgus macaques. These tools include:

- The generation of a protocol for staining macaque PBMCs with cross-reactive anti-human TCRBV monoclonal antibodies in combination with CD4 and CD8 markers designed for a longitudinal study of the TCR repertoire.
- Since very little information was available with regard to the sequences of most TCRBV families in cynomolgus macaques, I cloned and sequenced several TCRBV families in those macaques by using homologies with human TCRBV sequences and/or rhesus macaques when available. This library was then used to generate specific TCRBV primers in cynomolgus macaques, as well as probes for heteroduplex tracking assay. The sequences were submitted to Genebank.

  Ultimately these tools allowed the analysis of the TCR repertoire in cynomolgus macaques at a cellular and molecular level, these tools can be used also in future studies that involve macaques.

3. Through a longitudinal analysis of the TCR repertoire of SIV-infected macaques, we have shown that disease progression is not exclusively dependent on viral factors since slow and fast progressors were identified within the same group. However, experiments have revealed that expansions in the CD4+ TCR repertoire during primary SIV infection, correlate with disease progression. Macaques that have expansions in several TCRBV families progress faster to disease than macaques with very little perturbations in their CD4 compartment.

4. We have shown that these expansions are oligoclonal in fast as well as in slow progressor macaques by cloning and sequencing.
5. We have followed the outcome of these CD4 responses in both groups and found that expansions in fast progressors harbor CD4+ T cells bearing clonotypes that have a short life-span whereas in slow progressors, these cells persist.
6. We have shown that CD4 clonotypes were not able to re-expand in fast progressor macaque following challenge with SIVsmm PBJ14(6.6) even by using very highly sensitive assays, indicating that they are permanently eliminated. On the other hand, CD4 clonotypes from slow progressor macaques re-expanded upon challenge with SHIV89.6P between 1 to 4 weeks suggesting that they are SIV-specific.
7. We have also shown among slow progressors, an association between the generation and maintenance of the primary CD4 immune responses and protection potential from SHIV89.6P challenge.

Altogether, This is the first line of evidence that shows unequivocally that the CD4 response in PI is quickly eliminated. Second, that this early CD4 response plays an important role in the host immunological defense mechanism whether by orchestrating CTL, humoral responses or by inducing antiviral activity themselves. Ultimately preventing virus rebound during the asymptomatic phase of the disease and keeping immunological defense system in check for future exposures with the virus. These results indicate the importance of early interventions in preserving this pool of helper T cells which is a critical factor in modulating the course of HIV disease.

Contributors

Chapter II :

Superantigen-mediated deletion of specific T cell receptor V beta subsets in the SCID-hu Thy/Liv mouse is induced by staphylococcal enterotoxin B, but not HIV-1

1- Dr Krishna Komanduri injected SCID-hu Thy/liv mice i.p. with SEB, and infected the intrathymic thy/liv implants with HIV-1. Dr Krishna Komanduri stained and analyzed thymocytes from SEB infected mice with CD4, CD8 and TCRBV3, 5, 8, 12, 17 or 20 at 7 or 14 days post infection.

2- Dr Krishna Komanduri and I both have equally contributed in staining HIV-infected thymocytes with a panel of anti-human monoclonal antibodies that covers 15 human TCRBV families (~30 antibodies) together with CD8 and CD4 monoclonal antibodies. As well as FACS acquisition and analysis was performed in collaboration for the three time points: 6, 12 and 18 days post infection.

3- These experiments were performed at Dr McCune laboratory at the Gladstone Institute of Virology and Immunology, San Francisco, CA.

4- Dr Sekaly and Dr McCune both designed and supervised the study.

Chapter III :

Expansions and exhaustions of the CD4 T cell repertoire during primary infection predict disease progression in SIV infected cynomolgus macaques

Macaques involved in this study were part of a separate SIV vaccine study in Health Canada Ottawa, conducted under the supervision of Dr. Erling Rud.

1- I have performed all the processing of the blood samples, staining, cloning, sequencing as well as the *in situ* hybridization that were included in this study.

2- Dr. Rafick-Pierre Sekaly designed and with Dr. Remi Cheynier, they supervised this study.

3- Mrs Helen McGrath and Dr Taimour Langaee did the HTA experiments that are included in this study.

4- Dr Yassin-Diab was involved in the supervision of the beginning of this study before Dr Cheynier's.

5- Dr Hugo Soudeyns performed the statistical analysis of the data included in this study.

6- Dr Jocelyn Fournier was the attending Veterinarian responsible for the daily clinical follow-up, euthanasias, post-mortem exams and tissue recovery of each macaque included in this study.

7- Mrs Monique Parenteau was involved in determining the CD4 T cell count of all the macaques in this study.

8- Mrs Judy Edgar did all the Hematology on the macaques involved in this study (as data not shown).

9- Mrs Doreen Ko did all of the Taqman PCR/sequence analysis of the identity of the virus (vaccine and/or challenge virus) circulating in the macaques pre- and post-challenge involved in this study (as data not shown).

10- Mrs Alice Sherring and Mrs Dragica performed the antigen-capture p27 assays on all the culture fluids from the LDAs (as data not shown). They also collected all the plasma and processed it for viral load determination.

11- Dr Erling Rud obtained the funding for the attenuated SIV vaccine study in cynomolgus macaques and designed and supervised the virological and clinical aspect of the vaccine study.

Chapter IV:

Persistence of oligoclonal CD4 T cell responses in SIV infected macaques leads to protection from SHIV89.6P superinfection

1- I have performed all the processing of the blood samples, CD4 purificationof all the samples included in this study and the RNA extraction and nested-PCR reactions of some of the samples. I have generated all the TCRBV probes that were used in the HTA assays.

2- Dr. Taimour Langaee performed the RNA extraction and nested PCR reactions of most of the samples as well as the HTA on some of the samples.

3- Helen McGrath performed the HTA results on most of the samples.

4- Dr. Rafick-Pierre Sekaly and Dr. Remi Cheynier supervised this study.

5- Dr. Jocelyn Fournier was the attending Veterinarian responsible for the daily clinical follow-up, euthanasias, post mortem exams and tissue recovery of each macaque included in this study.

6- Mrs Monique Parenteau was involved in determining the CD4 T cell count of all the macaques in this study.

7- Mrs Judy Edgar did all the Hematology on the macaques involved in this study (as data not shown).

8- Mrs Doreen Ko did all of the Taqman PCR/sequence analysis of the identity of the virus (vaccine and/or challenge virus) circulating in the macaques pre- and post-challenge involved in this study (as data not shown).

9- Mrs Alice Sherring and Mrs Dragica performed an antigen capture p27 assays on all the culture fluids from the LDAs (as data not shown). They also collected all the plasma and processed it for viral load determination.

10- Dr Erling Rud and Dr. Rafick-Pierre Sekaly both obtained funding for the attenuated SIV vaccine study in cynomolgus macaques.

11- Dr.Rud designed and supervised the virological and clinical aspect of the vaccine study.

Discussion

Previous studies have reporting changes in TCRBV repertoire in both the CD4 and CD8 compartment such as those known to occur under the action of endogenous (MMTV) or exogenous (SEB) superantigens. These perturbations were either deletions or expansions in specific TCRBV families. Characterization of the TCRBV expansions has indicated that they can be either oligoclonal (i.e. limited to few dominant clonotypes) or polyclonal (i.e. no dominant clonotypes). The possible presentation of an HIV-1-associated superantigen driving the selective depletion of CD4 T cells in the course of HIV-1 infection was worth investigating. Analysis of the CD8 TCRBV repertoire of HIV-infected humans (101;102;231;232) or SIV infected animals (176;235-237) have implicated CD8+ T cell responses as the principle mediator of anti-HIV/SIV immunity. **However, since CD4+ T cells coordinate and regulate the effector functions of both humoral and cellular responses, a role for HIV-1-specific CD4+ T cells in anti-HIV host defense is almost implicit. In the studies reported here we have evaluated the possible implication of an HIV-related superantigen and characterized further the perturbations in the CD4 TCRBV repertoire in order to provide new insight into the participation of such cells in anti-HIV host defense.**

HIV does not encode for a superantigen

In chapter II, I presented and discussed results pertaining to the absence of an HIV-1 encoded superantigen. In our model, we use the SCID-Hu mouse which has several advantages: SCID-Hu mice transplanted with thymuses and livers derived from the same donor were either mock-infected or infected with HIV or SEB, allowing the comparison of similar TCR repertoires at base level. These mice were housed in a pathogen-free environment, which ascertain directly the effects of any HIV-related superantigen. This new implant has been shown to be permissive for HIV-1 infection and results in thymocyte depletion and increases in viral load within the implant (341). We were able to show using this model that a known superantigen, SEB, is able to induce deletions and expansions in specific TCRBV families within these implants. In order to investigate the possible implication of an HIV-related superantigen, we used two HIV-1 strains that have different tropism in case a superantigen is not encoded by all HIV strains. Second, we

used an exhaustive panel of antibodies that covers 60% of the TCR repertoire and two cohorts of mice established from different donors. Altogether, these experiments demonstrated that the TCR repertoires of HIV-infected transplants are not disrupted overtime compared with mock-infected transplant derived from the same donors.

These results contradict previous findings that have supported the presence of a superantigen, based on perturbations in the TCR repertoire. First, the reported depletion of TCRBV subsets in HIV infected individuals were random, with no concordance in findings between research groups (222;227;342). Since superantigens are TCRBV specific, these findings suggest that repertoire perturbations are caused by other factors. Second, most of these depletions were reported at late stages of HIV infection, suggesting a possible exposure to other pathogens from opportunistic infections that could carry superantigens capable of disrupting the TCR repertoire (343). Indeed, it is known that HIV infected patients are susceptible to CMV, which can induce expansions in BV12 subset (228). Further experiments illustrated that a CMV and not an HIV gene product was responsible for this superantigen-driven TCRBV12 selective HIV-1 replication *in vivo* (228). Finally, Houle et al. (344) have also confirmed our results using also the SCID-hu thy/liv mouse model, further supporting the lack of HIV encoded superantigen-like effect.

Indeed, more recently, studies focusing on the CD8 TCRBV repertoire of HIV and SIV infected subjects have later confirmed that these observed perturbations are driven by antigenic determinants (101; 102; 177; 235; 236; 261; 345).

With the intent to characterize the dynamics of SIV-specific CD4 T cell responses and their involvement in the pathogenesis of SIV, two important findings could be drawn from our study: 1) the rate of disease progression relies on the magnitude of the CD4 immune response generated during PI. 2) Protection against subsequent re-exposures to SIV or other opportunistic infections that could lead to accelerated disease progression, depends on the persistence and maturation of primary CD4 immune responses.

The magnitude of the CD4 response during primary SIV infection is a prognosticator for disease progression

Results, as described in chapter III, have shown that the extent of disruption in the CD4 repertoire did not correlate with the pathogenicity of the virus but with disease status. This original finding is demonstrated by the fact that among the SIVmacJ5 group, one fast progressor

macaque had expansions in several BV families compared with the other slow progressors that showed limited disruptions of their CD4 repertoire. The same dissimilarity in the magnitude of CD4 TCRBV expansions was observed within the SHIV89.6P group. This is the first report indicating that the presence of strong CD4 helper responses during primary infection is not beneficial to the infected host since it is associated with an accelerated disease progression rate. This has been demonstrated through the longitudinal analysis of the CD4 helper response from PI until chronic SIV infection. Indeed, all the studies performed on human or macaque samples, which have assessed the impact of CD4 helper responses on disease outcome, have indicated an inverse correlation between viral RNA copies and CD4 helper responses (174;230). Furthermore, these studies focused on CD4 responses during chronic infection only.

The composition of the primary CD4 immune response against SIV infection can be influenced by several host factors

The composition of the primary immune response generated in the CD4 compartment is determined by the combined influence of several factors that dictate whether the SIV-selected CD4 repertoire is broad or restricted. These factors, as described in figure 3B of general introduction, are as follows:

- The array of MHC alleles expressed in the infected host could influence during T cell selection in the thymus, the magnitude of the preimmune SIV-specific TCR repertoire at the periphery. The frequency of each individual CD4 T cell clone that forms the precursor repertoire for a given antigen can therefore be different among animals (109). Considering that a small fraction of the potential T cell repertoire can be used at a given time, the preimmune repertoire may be composed predominantly of one or several equally important precursor pools, giving rise to a restricted or a broad repertoire respectively upon SIV infection (first panel).
- An antigen-specific response has a limited period of time to be completed indicating that only a fraction of the antigen-specific preimmune repertoire can be engaged. The earlier T cell clones are recruited, the more advantage they would have to dominate the response. Therefore, if several CD4 clones where recruited at the same time, this would result in a wider antigen-selected repertoire as compared to one CD4 clonotype being mobilized first and giving the latter a selective advantage to dominate the response (second panel).

- The rate of expansion can also influence the diversity of the SIV-specific CD4 response. The proliferation capacity of T cells is defined by the affinity of T cell clones for their target, as well as the density of the peptide-MHC complexes on the surface of APCs (354). In that context, too high epitope density can induce hyper-activation of T cells an cell death (366). However, with reasonably high peptide density, no competition for ligand interaction is imposed and CD4 clonotypes of high and low affinity can be generated, creating a diverse CD4 repertoire. Finally, when the density of the epitope is limited, a competition for ligand would give rise to a restricted repertoire composed of T cell clones with only high affinity for their epitope (third panel).
- MHC molecules can also determine whether several or a limited set of CD4 clonotypes are recruited. They do so by shaping the panel of SIV peptides presented to the TCR repertoire, whether this panel is wide, containing few or several immunogenic epitopes, with high or low density at the surface of APCs. In that case a wide panel of SIV peptides could result in the generation of several CD4 clonotypes with variable peptide specificity which could be observed as a polyclonal CD4 response. Moreover, heterozygocity in HLA molecules and other polymorphism in the antigen processing machinery also have the advantage of presenting twice the number of SIV peptides compared with homozygocity. Indeed, recently Reusch et al. (367) have shown that gravid female fish preferred the odour of males with a large number of MHC class-IIB alleles to that of males with fewer alleles, the same matting patterns were observed in mice (372;373). Diversity in the CD4 TCRBV repertoire could arise from a better representation of SIV peptides through the use of several MHC molecules with variable affinities for their peptides.

SIV disease progression can be influenced by environmental factors

Previous exposures to certain pathogens could alter the composition of the TCR repertoire, such as exposure to superantigens related to Staphylococcus enterotoxins could induce the expansion and depletion of a whole BV subset, narrowing the CD4 repertoire and limiting the number of CD4 T cells recruited upon SIV infection (243;279-282). Co-infections with other pathogens can drive HIV replication through the activation of the immune system and the generation of a network of HIV enhancing cytokines such as TNF-α, IL-2 and IL-6 (308, 245). Moreover, the selective activation and expansion of the antigen specific CD4 T cells derived from the co-

infecting pathogen could augment the reservoirs that support HIV replication and hence accelerate disease progression. Indeed, several cases of HIV infections reported through sexual transmission as well as in injection drug users, are associated with hepatitis C infection and experience a fast disease progression rate (356;368).

Consequences of broad versus restricted SIV-specific CD4 repertoires during primary SIV infection

A consequence to the activation of a diverse versus a restricted CD4 T cell pool during PI is the establishment of larger reservoirs than can facilitate even further SIV replication and eventually accelerating disease progression. This is supported by the fact that in SIVmacJ5 fast progressor, expansions and contractions in multiple CD4+ BV subsets is followed by a rebound in viral load, as compared to slow progressors from that group that show expansions in a limited number of CD4+ BV families and maintain a a viral load at the limit of detection throughout the followup period. This suggests that mobilization of a restricted SIV-specific CD4 response would minimize viral output.

Moreover, it is important to emphasize that during acute infection circulating virus particles and CTLs are both at their peak, suggesting that activated CD4 T cells generated at this stage are susceptible to elimination by cytolytic effects of the virus or CTLs recognizing SIV-infected CD4 T cells. Therefore it is possible to speculate that the extent of expansion and elimination of CD4 T cells early in infection could cause the acceleration of disease progression through the weakening of CD4 responses during chronic infection. The lack of helper CD4 responses could compromise the efficiency of CTL and humoral responses during chronic infection and cause the virus to drive the exhaustion of the immune system and the establishment of AIDS-related illnesses (figure 1).

Moreover, the qualitative composition of the SIV-specific CD4+ T cells as defined by their capacity to expand and secrete protective Th1 cytokines that promote CTL activity as opposed to Th2 cytokines that enhance HIV replication, can have different consequences on disease outcome (369;370). A switch from a Th1 (i.e. IFN-γ and IL-2) to a Th2 (i.e. IL-4 and IL-6) response was though to occur during the course of HIV infection, suggested by the preferential secretion of Th1 cytokines during acute infection when CTL activity is optimum as opposed to chronic infection when humoral responses become evident. However, no clear evidence for a

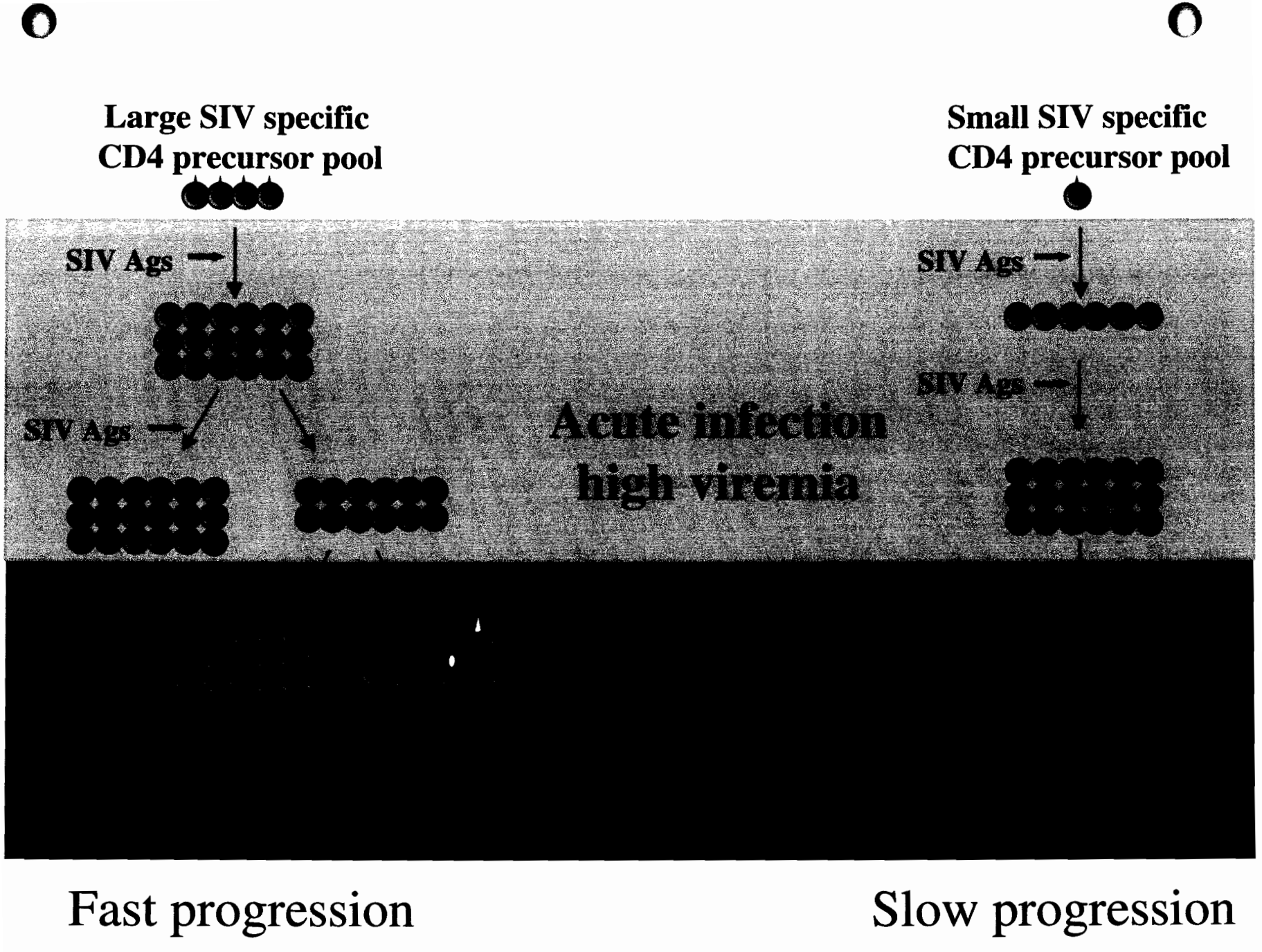

Figure 1: Proposed model for the role of CD4 responses in disease progression

definite switch in T cells that secrete Th2 type cytokines during the course of infection was attained (371).

Protection against subsequent re-exposures with the virus relies on the persistence and maturation of primary CD4 T cell responses

In chapter IV, we have addressed the protection potential of CD4 T cell responses generated during primary infection in slow progressors by examining both the susceptibility of macaques to SHIV89.6P superinfection and the diversity and persistence of the CD4 TCRBV repertoire following SIV infection and challenge with SHIV89.6P. Results have shown that macaques with no detectable CD4 responses during pre and post challenge, indicated by the persistence of a polyclonal CD4 repertoire identical to uninfected animals, are superinfected with SHIV89.6P. On the other hand, animals with oligoclonal CD4 repertoires have shown a contraction of amplified CD4 TCRBV subsets following peak viremia to include a hand-full of clones by week 8 post infection. Upon challenge with SHIV89.6P, the same clonotypes that were present during primary SIV infection became dominant as early as one week post challenge in some TCRBV families. These macaques were resistant to superinfection by SHIV89.6P, suggesting that immunosurveillance against SIV infection rests with few CD4 clonotypes specific for conserved epitopes between immunizing and challenge viruses. Through a maturation process, it is possible that the generation of the secondary immune response, observed only in protected animals, has the ability to prevent the establishment of a second round of viral infection upon challenge, a property that unprotected animals do not have.

CD4 immune responses are crucial in viral infections

In other viral infections such as LCMV, the role of CD4 help has been significantly investigated in CD4 knockout mice. Following LCMV infection of mice, normal mice have a long-lasting protective immunity mediated mainly by antiviral CTLs. Knockout mice on the other hand that lack CD4 T cell help have diminished CTL responses overtime and do not have the ability to induce protection upon subsequent challenge (149). These results indicate that the CD4 helper function allows the maintenance of CTL responses which can subsequently restrain virus from reemerging. Other studies have described a novel mechanism for silencing antiviral immune responses through the elimination of CD4 T cells (150). In the context of HIV (348) and LCMV

(254), the lack of CD4-CD8 collaboration was associated with high levels of viral load due to the lack of elaborate CD4 help to virus-specific CTLs. The mechanisms by which CD4 T cells provide help to CD8 T cells in the context of viral infections is only starting to be clarified. Two pathways have been reported, either through the secretion by CD4 T cells of cytokines such as IL-2 that would ultimately act on CD8 T cells and allow them to respond, or by the intervention of APCs through CD40-CD40L interaction (153;154). In the later, CD4 T cells may activate APCs that can then engage with CD8 T cells. Wodarz et al. (349) have recently suggested that both mechanisms might be involved depending on the level of viremia, hence, CD4-IL-2-CD8 pathway is used at high viral load such as during acute infection whereas the CD4-APC-CD8 pathway may be involved at low viral load during the period of chronic infection. As for the role in humoral immune responses in HIV and other viral infections, CD4 T cells maintain the continuous generation of neutralizing antibodies with broad specificity in the light of constantly emerging viral escape mutants (350;351).

The importance of memory recall responses in persistent viral infections

The persistence and re-expansion of memory cells subsequent to re-exposure to the virus is not new. Indeed, recall responses comprised by the same clonotypes generated during primary infection has been reported in persistent infections, such as with LCMV (358) or CMV (359), or EBV (141). Although some studies have indicated that recall responses to persistent viral infections include the selection of clonotypes with the greatest affinity to the antigen in question as compared to primary T cells through a maturation process (117;134-137). In our study, protected animals with recall responses have also shown maturation of their immune responses following primary infection, evidenced by the contraction of the TCRBV repertoire, suggesting that the CD4+ T cells bearing clonotypes re-expanding following challenge are protective due to a higher affinity for their SIV epitopes. Apart from our results, the association between long-lasting immunity and clearance of viral burden (138) has been demonstrated only in the LCMV model. The protective potential of memory T cells is most probably initiated by their capacity to get reactivated quickly and to give rise to an abundant progeny (133). Furthermore, memory T cells have distinct functions, homing and survival capacities that they acquired through a differentiation process that took place during their first encounter with the antigen. All together these properties make them more competent to control or clear viral burden as compared to

primary T cells (120;133). Future projection will characterize the phenotypic and functional properties of those memory CD4+ T cells in protected animals.

Model for the immunopathogenesis of HIV infection

Taken together, results gathered in our studies are represented in the model illustrated in figure 2. Maximal and minimal threshold in the magnitude of CD4 responses generated during primary HIV infection could be envisioned only in the case of HIV since CD4 T cells represent their principle target. Above a certain threshold, the magnitude of the CD4 response could switch the balance between viral burden and CD4 immunological control to the advantage of the virus. This is achieved by the continuous output of new virions through the activation of a large number of CD4 cells that ultimately succumb while increasing the level of viral particles. As a result, virus persistence prevails and progression to disease is precipitated. Below a certain threshold, CD4 immune responses are not sufficient to form an important reservoir for viral replication, and as a result, infected individuals would have a slow progressor phenotype. However, subsequent to opportunistic infections which can drive HIV replication, the lack of HIV-specific CD4 immune responses and the loss of a functional CTL activity through anergy or elimination as a consequence, would allow a rapid replication of HIV, and eventually an accelerated progression to disease (figure 2).

On the other hand, a diverse and potent CD4 response during PI that remains between those two limits would not be sufficient to generate important reservoirs for virus replication since they can be restrained by cellular immune responses. This is achieved through the maintenance of a broad CD8 repertoire recognizing and eliminating several CD4 reservoirs that is initiated through a CD4-CD8 interaction or also directly through CD4 cytotoxic activity. Moreover, the establishment of a potent immunological control has also the advantages of preventing the elimination of primary CD4 and CD8 immune responses. As a consequence long-term protection is then induced through the maintenance of these memory T cells during the course of the disease. Upon encounter with subsequent pathogens, the pre-established HIV-specific CD4 immune response can prevent the reemergence of the virus. For example, upon exposure to CMV, a TCRBV12-associated activation and expansion is induced, which is accompanied by the selective replication of HIV in these T cell subsets (227). The presence of cellular immune

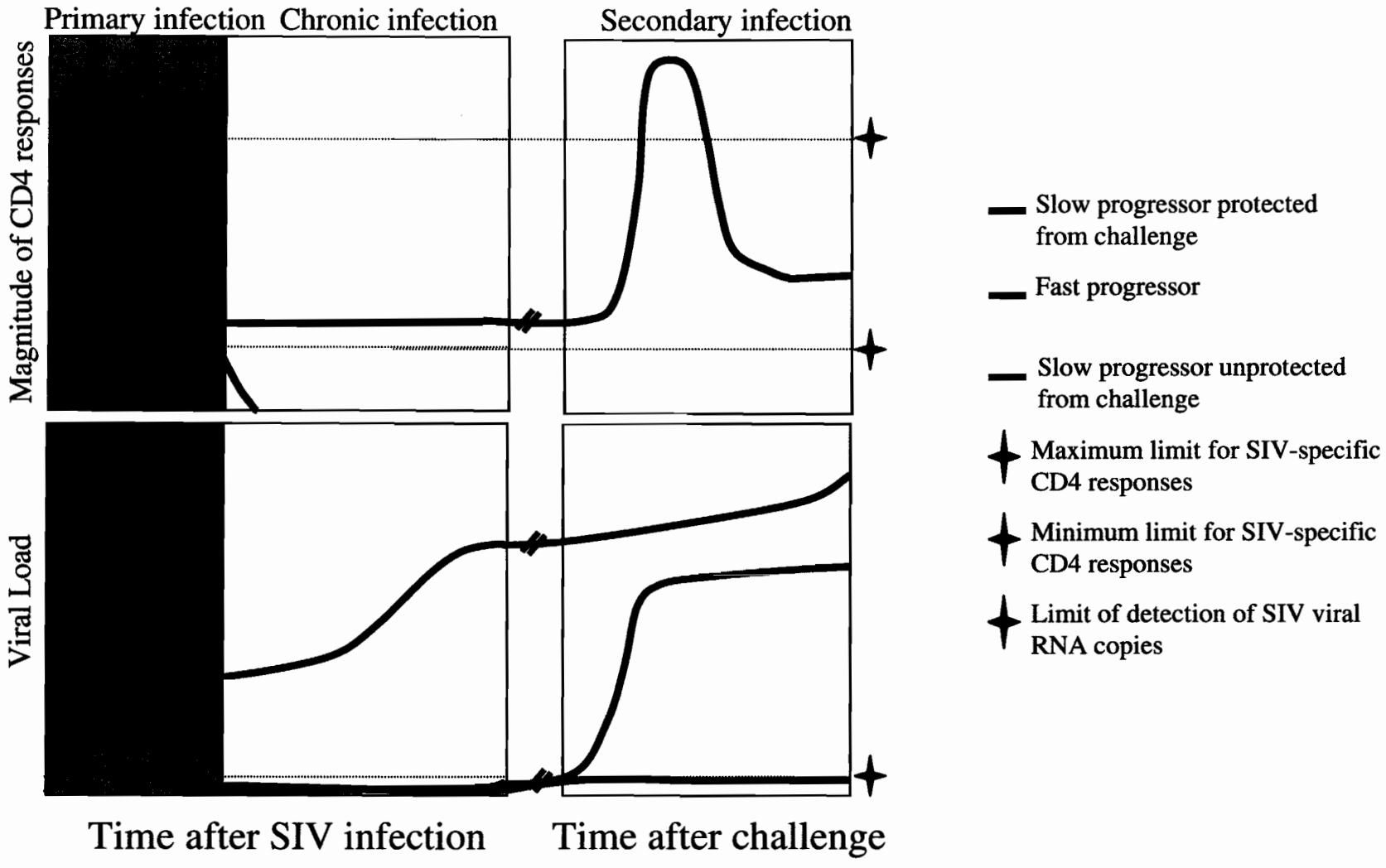

Figure 2: Model describing the effect of the magnitude of the primary CD4 responses on disease progression and protection from challenge

responses against HIV is crucial at this stage because it dictates whether the selective replication of HIV in TCRBV12 T cells would prevent the propagation of HIV to other anatomical sites.

Benefits of early antiviral treatment

The installment of a highly active anti-retroviral therapy (HAART) program has over the years shown to be beneficial in controlling viral replication and, as a consequence, rescuing the CD4 T cell population and increasing life expectancy. Furthermore, it has been reported that the earlier HIV-1 infected patients are put on HAART, the better they conserve a stable CD4 count and anti-viral T cell responses that are able to control viral rebound upon cessation of therapy (264;340;352). Recently, Berrey et al. (353) have shown that patients on HAART during primary HIV infection experience a decrease in the frequency of developing opportunistic infections and a longer rate of disease progression. According to our results, the initiation of HAART therapy as early as acute HIV infection most probably rescued primary HIV-specific CD4 T cells from being eliminated. Therefore the pre-existing CD4 clonotypes can persist through chronic infection and act as check-points for any possible reemerging viral rebound through T cell activation driven by the encounter of foreign pathogen. According to the model in figure 2, early therapeutical intervention would result in the development of strong and protective CD4 responses and slow disease status.

HIV vaccine design: where should we focus our efforts

As a general rule, vaccines are designed to induce strong immunological memory responses against the pathogen in question as a preventive measure against subsequent exposures. With several pathogens, this approach has generated sterilizing immunity, and in some cases, a total eradication of the virus was attained such as with vaccina virus or diminished significantly such as with the poliovirus. In the case of HIV, several viral and immunological factors can make the use of HIV vaccines dangerous, ineffective or not always reproducible. In the case of the use of attenuated viruses as vaccine candidates, the rate of viral mutations can cause even very attenuated HIV strains to revert back to their wild type gene and regain their ability to replicate at high levels (360-365). Furthermore, HIV has adopted several strategies to escape from immunological surveillance. Therefore, only individuals that can develop potent and broad HIV-specific CTLs may hope for a good protection from this vaccine (177). As I have discussed

earlier, not all subjects can generate this type of response, making the use of attenuated vaccine risky. More recently, DNA vaccines comprised of a library of HIV genes inserted in a viral vector such as an attenuated vaccinia virus, has gained a lot of attention mostly because of its safety. A concerted effort is focused on designing a vaccine comprised of the most immunogenic HIV determinants that can elicit strong and broad CTL responses in individuals from different ethnical backgrounds creating therefore a universal HIV vaccine. In that context, several CTL epitopes capable of eliciting cross-clade protection are emerging (374;375).

In light of the results presented here, several issues should be taken into account for the design of HIV vaccines. The generation of a diverse CD4 repertoire can be directed against either single or multiple epitopes. However, only a diverse repertoire composed of CD4+ T cells bearing clonotypes specific for several HIV epitopes is efficacious due to the fact that viral escape mutants can arise which fail to stimulate CD4 T cells (113). Prophylactic vaccination aimed at increasing the magnitude of CD4 immune responses in normal individuals could cause one of two scenarios. First, the acceleration of the disease upon infection as indicated in fast progressor macaques in this study, through the generation of reservoirs that can support HIV replication. This possible hypothesis could be verified through the immunization of macaques with vector DNA encoding for an immunodominant peptide capable of eliciting a potent CD4 immune response. Following immunization, the eventual establishment of an SIV antigen-driven CD4 T cell-selective SIV-reservoirs *in vivo* could be addressed by examining through tetramer staining the outcome and impact of the antigen-selected repertoire on viremia and disease progression subsequent to challenge with SIV. The second possible scenario, relies on the protection from HIV infection subsequent to exposure, due to the pre-establishment of strong and diverse memory CD4 and CD8 T cell responses. Upon HIV encounter, infection would not occur due to the immediate immuno-mediated eradication of the virus before it has a chance to settle.

As for therapeutical vaccines, they should be able to induce as shown in figure 2 a large enough CD4 response to insure the maturation and persistence of CD4 clonotypes after acute infection, but not too wide to cause a viral outburst and their proper elimination. Whereas, prophylactic vaccines may or may not cause the establishment of reservoirs for HIV replication, therapeutical vaccines could be harmful and useless if administered alone during acute infection when viral replication is at its peak. As a result, vaccines enhancing CD4 responses would lead to their elimination. Lori et al. (340) have recently demonstrated that SIV infected macaques on

structurally interrupted HAART treatment are able to develop CD4 responses and control viral rebound upon challenge as compared to the uninterrupted HAART treated group that do not show any virus-specific response and are susceptible to challenge by SIV. These findings suggest that viral suppression through continuous HAART treatment diminishes the immunological responses against HIV, therefore emphasizing that a minimum viral replication is required to maintain the memory pool. In light of those findings, the combined administration of antiretroviral therapy as well as therapeutic vaccine early in HIV infection should be able to eradicate the virus and maintain the delivery of HIV antigens. The only caveat is that the putative vaccine should mimic the virus by representing the same immunodominant epitopes that are expressed in the infected host in order to maintain its pre-existing and protective HIV-specific cellular immune responses. This combined strategy would be safer than the use of vaccines or antiviral therapy alone, because it would allow the possible eradication of HIV that otherwise may mutate and escape antiviral treatment, without hindering immunological responses necessary for the maintenance of protection against opportunistic infections.

Summary and conclusions

This study was aimed at analyzing the diversity and evolution of the CD4+ TCR repertoire in cynomolgus macaques following SIV infection in a longitudinal follow-up, and their impact on protection from subsequent challenge as well as disease progression. First we have addressed the nature of these perturbations in the SCID-hu thy/liv model, whether they are associated an HIV encoded superantigen or just antigen-mediated HIV determinants. Implanted thy/liv organs where injected with either two different strains of HIV or with SEB. Whereas SEB injection caused the depletion of T cells bearing specific TCRBV segments, infection of the implants has caused also a depletion of T cells, however this depletion was not TCRBV specific suggesting the absence of an HIV-encoded superantigen. As our group and other have lead to the conclusion that perturbations in the TCR repertoire are most likely mediated by HIV antigens which cause oligoclonal expansions of CD8+ TCRBV families. These T cell subsets express distinct CDR3 regions and have been shown to mediate cytotoxic activity. CD4 helper responses play a major role in the induction and maintenance of cell-mediated immune responses in several viral infections. Interference by HIV with helper T cell function might play an important role in preventing the immune system from clearing the virus during infection. The longitudinal study of the CD4+ TCR repertoire in cynomolgus macaques following SIV infection has revealed several important findings: 1) the pathogenicity of the infecting SIV strain is not the unique factor determining disease progression, host factors may also play an important role in this process. 2) The magnitude of the primary CD4 T cell responses are significantly ($p=0.025$) more important in fast progressors than they are in slow progressors. 3) Characterization of the nature of these expansion has revealed that they are oligoclonal in both fast and slow progressors. 4) In fast progressors, CD4 clonotypes involved in the primary immune response are not detectable during chronic infection, and are not re-activated subsequent to homologous challenge with SIVsmm PBJ14(6.6). 5) Slow progressors are comprised by either animals that do not develop CD4 T cell responses and are not protected against homologous or heterologous challenge; or animals that develop oligoclonal CD4 responses following the initial downregulation of viremia and are protected against superinfection with SIVsmm PBJ14(6.6) or SHIV89.6P. These clonotypes persist during chronic infection and they are involved in a secondary immune response following challenge, suggesting that they may play a role in the immunosurveillance

against SIV re-exposure is controlled by few CD4 clonotypes recognizing a limited array of SIV determinants.

These results have important implication with regard to the design of preventive or therapeutical vaccines against HIV that aim at inducing strong CD4 responses. The generation of CD4 responses during high viral load were eliminated very early in the course of SIV infection and may have contributed to the rebound in viremia and the succession of events that led to a rapid disease progression. The establishment of a potent HIV-specific CD4 T cell pool as a preventive vaccine strategy against HIV infection may in fact serve as an additional reservoir upon activation which can support viral replication and dissemination if an encounter with HIV may result. In that case unexposed seronegative individuals would be more susceptible to develop a rapid disease outcome then if they were not vaccinated. Therapeutical vaccine strategies should investigate whether interventions could be safer under conditions of minimal viral replication, which would prevent reseeding of the reservoir pool and the concomitant elimination of the HIV-specific CD4 T cells.

Taken altogether, design of HIV vaccine should aim at generating potent CD4 responses sufficient to induce protection but not excessive to provoke virus outbreak.

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