Mechanisms of decreasing HIV-1 specific CD8+ T cell activity during progression to AIDS
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T cell receptor diversity in HIV infection: Missing clones or dominant expansions

T cell receptor (TCR) diversity has been extensively analysed in various clinical settings including HIV infection. In several studies TCR repertoire analyses have been used to show incomplete TCR repertoires in which certain antigen-specific TCR's were missing from the repertoire. Other studies have investigated the TCR repertoire to detect clonal expansions within T cell subsets. Assumption of either deletion or expansion as the cause for perturbed TCR repertoires may lead to different interpretations. In this chapter we distinguish between T cell expansions and T cell deletions in repertoire analyses, and discuss the findings of T cell diversity in HIV-1 infection in light of the two viewpoints.

Diversity of T cell populations.

T cells express a receptor specialized in recognizing non-self antigen: the T cell receptor (TCR). To achieve a sufficiently broad range of antigen recognition, individual thymocytes randomly recombine germ line receptor genes to form their clone-specific T cell receptor (TCR). This process called TCR rearrangement results in a different TCR for each matured T cell (4), each TCR being specific for a more or less restricted set of antigens (73). The highest variability is found in the complementarity determining region 3 (CDR3) which is the region that recognizes non-self peptides in the context of major histocompatibility complex (MHC) molecules. The more different CDR3 regions in a T cell population, the more antigens can be recognized.

The germ line genes involved in TCR recombination are designated as variable-, diversity-, joining-, and constant-region genes. The complete sequence of the germ line TCR locus has revealed 46 functional gene segments grouped in 30 families within the variable β cluster (VB) alone (117). Randomization of the gene-segments and random insertion and deletion of basepairs during the TCR-CDR3 rearrangement results in a T cell repertoire of a highly diverse TCR nucleotide sequence diversity, each T cell comprising one of the VB families and having its own clonotypic TCR gene.

Methods in diversity assessment.

The absolute information of diversity in antigenic specificity is the nucleotide sequence of each T cell receptor present in the population. But the estimated 10^7 different T cells in humans (118) discourages extensive sequencing. A rapid estimation of the TCR diversitiy is obtained by analysing VB usage, either by quantitative PCR or by flow cytometric analysis. An overview of the extent of used VB families provides a global indication of diversity.

Due to differences in length of the germ line segments, random basepair insertions and deletions, CDR3 regions may vary in length (119). This phenomenon is exploited in the "immunoscope" technique (74) or CDR3 spectratyping. Variation of CDR3 sizes is examined by gel-electrophoresis separation of VB-specific PCR products. Random addition or deletion results in a probability distribution of different CDR3 sizes. Diverse polyclonal T cell populations display a normal distribution of CDR3 sizes; oligoclonal populations have less different CDR3 regions which is usually reflected in the detection of fewer CDR3 sizes. For each VB family (and VB-JB combination) such a CDR3 size-pattern can be generated using as many primer combinations as desired. A normal CDR3 pattern contains up to 10 sizes, that are normally distributed (Gaussian distribution) with the middle size being the most predominant, and the smaller or larger CDR3 sizes decreasing in frequency. Each CDR3 size has a distinct percentage of T cells present in the sample. Perturbation of the patterns will be reflected in changed percentages per CDR3 size, thus indicating the difference or skewing from the normal distribution of CDR3 sizes (82,120). A disadvantage is that CDR3 size expansions do not necessarily contain one unique CDR3 sequence. To confirm that VB or CDR3 perturbations are indeed caused by (oligo)clonal expansions, investigators have frequently added sequence information to show recurrent CDR3 regions. The heteroduplex mobility assay (HMA (121)) or single strand conformation polymorphism (SSCP, (102)) analysis are attractive alternatives.
Apar from gelelectrophoresis separation of VB PCR amplification products by length, it is also possible to separate PCR products by sequence. Two related methods allow for more detailed analysis of TCR sequence variation, both based on the differential mobility of CDR3 polymorphisms. Denaturation and subsequent refolding of DNA strands, as single strands or heteroduplexes, creates different conformational properties depending on basepair sequence. DNA fragments are run on a neutral gel in which they migrate in a conformation dependent fashion. Both assays create a stretched smear of DNA in case of polyclonal CDR3 populations, or distinct bands of identical CDR3 regions with identical mobility. The advantage of these techniques is that it includes the complete sample in the analysis and readily visualizes the complexity of a population. Compared to sequencing the only information lost is the actual nucleotide order, but sequencing can not match the overall view of complexity unless performed exhaustively. An exhaustive analysis of TCR sequences is however, the only way to determine the true repertoire of a T cell population, as CDR3 spectratyping and SSCP profiles only allow detection of clones with sufficiently high frequencies. Therefore, missing CDR3 sizes do not necessarily indicate total absence of T cell clones with that particular CDR3 size.

In summary, CDR3 sequencing determines the repertoire as sum of all present CDR3 regions; VB usage, CDR3 spectratyping, SSCP and HMA are methods that detect the clonal composition of the repertoire.

TCR diversity in HIV infection

T cell diversity was introduced in HIV research by a report of Imberti and colleagues who observed a skewing in VB usage in HIV infected individuals compared to healthy controls (122). This included the observation of selective deletion of specific VB families, which led to the hypothesis of an HIV encoded superantigen, but numerous following reports failed to detect similar selectivity of deletion (reviewed by Cossarizza (123)). Here the T cell diversity was used to assess the degree of T cell depletion. In a dissected spleen VB expansions were found heterogeneously distributed parallel with heterogeneous viral sequences, suggesting the expansion and clustering of HIV-specific T cells (112). Furthermore it was found that early in infection major expansions occurred in the CD8 T cell subset. Elevated Nef-specific CTL precursor frequencies, together with disturbed VB usage suggested that HIV specific clones were part of the clonal expansions (86). The role of HIV-specific clones was confirmed by TCR sequence analysis (41), and later by the use of MHC-petide tetramer complex detection of CTL clones (87). In these settings VB usage indicated the level of clonal expansions.

Roglic and colleagues first analysed the CD4 repertoire by CDR3 spectratyping and concluded that clinical progression of HIV infection was associated with increased perturbation (75). This finding supported the hypothesis that depletion of CD4 cells resulted in holes in the TCR repertoire, eventually leading to the inability to recognize foreign antigens, thus explaining opportunistic infections. Later this was further confirmed in an inverse correlation between CD4 counts and TCR CDR3 perturbations (76). The difference with Imberti’s original report is that not total VB families, but CDR3 sizes within families were undetectable and probably deleted. Here the diversity assessment was again used to demonstrate depletion of T cell clones. A plausible theory was build around the missing clones causing CDR3 pattern perturbations (115), which made it clear that antiviral therapy should result in the rebuilding of the repertoire.

T cell diversity during antiretroviral therapy

The first study to report on the influence of therapy on the CD4 TCR repertoire showed no direct improvements. A few months of treatment did not significantly alter the perturbation of several patients whose CD4 counts were nonetheless increased (81). The authors concluded that the therapy induced rise in CD4 counts was caused by expansion of pre-existing clones, and that depleted clones did not immediately reemerge. It should be noted that the patients investigated here received IL2 in addition to antiretroviral therapy, which could lead to increased expansion of T cell clones already dominating the repertoire. A second report followed which showed significant improvements, mostly at 9 months after treatment. The increase in the T cell diversity was restricted to the CD4 subset, as CD8 perturbation did not change (82). It was concluded that improvement of the repertoire reflected
regeneration of deleted CD4 clones, probably by the thymus. These seemingly contradictory results were linked by the observation that during the first months of therapy the CD4 repertoire transiently increased in clonality (57). The temporal increase in perturbation reflected the biphasic CD4 repopulation (79) consisting of early redistribution of expanded clones from lymphoid tissue to the blood, and late repertoire regeneration.

The role of the thymus in T cell regeneration has been evaluated in thymectomized patients (108). These patients were able to increase their naive CD4 counts, suggesting additional sources (like redistribution from lymphoid tissues) for early T cell increases, but the TCR repertoire showed no substantial improvements in diversity. It was argued that thymectomy prohibits the replenishment of a diverse naive repertoire, resulting in lack of new TCR specificity’s, as evidenced by unchanged CDR3 profiles. In this report and the former studies described above, CDR3 spectratyping was used to detect absence or the replenishment of depleted clones. However, expansions may be of such size that low frequency clones may not be detectable. If pre-existing expansions persist during ongoing thymic regeneration, newly formed diverse clones may not be detected, hence persistent perturbation does not necessarily indicate the lack of a functional thymus.

Not only CD4 diversity was affected by therapy, also CD8 T cell diversity showed substantial dynamics (91,92,124). A rapid improvement of the TCR repertoire was observed in the CD8 compartment and CDR3 specific hybridization showed that HIV-specific clones indeed were contracting due to therapy induced decline of antigen (92). In contrast to the CD4 repertoire expanded clones contracted upon therapy, but analogous to the CD4 repertoire most changes were observed early after start of therapy (124). For CD8+ T cells, the rapid improvement of TCR diversity was not concluded to reflect thymic regeneration of lost CDR3 sizes. It was more likely that expanded clones had perished rendering low frequency clones demonstrable again. In contrast to results on CD4 repertoire, the results on CD8 repertoire were interpreted as the level of expansion instead of depletion. We investigated CDR3 sizes in pre-treatment lymph nodes and compared these with CDR3 sizes in post-treatment blood samples. Substantial expansions present in lymph-nodes before therapy were not detected in the blood before therapy, but appeared in the peripheral blood after start of therapy (124). To investigate the kinetics of T cell clones, we utilized dominant CDR3 regions as molecular markers to track clones spatially and temporally. Here we showed that clones migrated from lymphoid tissue to blood, for which we used the CDR3 size distribution and sequences as clonotypic markers, like Pantaleo and Kalams et al. did before (86) (41).

**Interpretation of T cell diversity results**

We argue that large expansions relative to other T cell clones are the major cause of perturbations observed in CDR3 size distributions. In the event of T cell depletion, this is not evidenced directly by the loss of particular CDR3 sizes since it is unlikely that all T cells of one particular CDR3 size are preferentially depleted. By dominance of over-represented CDR3 sizes, other clones may become undetectable but are not necessarily absent. Therefore the level of CDR3 perturbations rather represents previous immune activation than the actual repertoire. Only in the event of severe T cell depletion, too few clones may be left to form the normal CDR3 size distribution, leading to perturbed repertoires, but this may well be preceded by clonal expansions that perturb the repertoire by overrepresentation.

We define the repertoire as the sum of all CDR3 sequences that are present irrespective of frequency, and diversity as the degree of equal distribution of frequencies. CDR3 spectra and SSCP profiles give an overview of the degree of diversity, however do not provide information on the complete repertoire. Clones of very low frequency may not be detected because of domination of expanded clones. In this case sequencing would be the method to detect all T cell clones and to determine the true repertoire. CDR3 spectratyping determines the diversity of the repertoire; the diversity can vary in equally complex repertoires. This can be illustrated by T cell populations during acute viral infections. Virus specific T cell clones can expand to 40% of the total CD8+ T cell population upon infection (125-128). The Total number of T cells however, also dramatically increases, and the T cells involved in the acute immune response do not
seem to affect the size of the original T cell population (126). Theoretically, the newly expanded clones do not limit the original clonal composition of the naive T cell pool. The diversity when measured by CDR3 spectratyping will be very low because of extreme oligoclonal expansions, but the total repertoire—the sum of all different TCR—is probably equal before and after the infection-induced proliferation (Although antigen-driven immune activation does have an impact on preexisting memory clones (129)).

In this respect, we do not regard HIV-induced repertoire perturbations as evidence for clonal depletion (76, 115). In addition, if CDR3 perturbations represent the clones that are left and the missing CDR3 sizes indicate holes in the repertoire, the process would be irreversible during the course of infection. We have recently investigated longitudinal CD4 memory and naive cells and found a (possibly temporal) decrease in perturbations, manifesting gaussian CDR3 size distributions (104). Missing clones reappeared in these patients. Consequently, CDR3 perturbations were not induced by deletions.

It is likely that several T cell clones and their unique CDR3 are lost during the ongoing attrition of CD4+ T cells. However, clonal expansions in principle occur much more rapid than gradual depletion of certain T cell clones. Therefore, the dramatic skewing of TCR diversity in HIV infection (in both CD4+ and CD8+ T cells) is likely to be caused by expansions, before clones are lost. HIV infection is characterized by a generally higher immune activation state than in healthy conditions, causing elevated perturbations in the TCR repertoire (106). In healthy individuals CD4+ T cell expansions also occur, but probably not as severe and frequent as in HIV infection. Furthermore HIV induces both T cell activation and depletion of T cells. In a smaller pool of CD4+ T cells, expansions of normal proportions will be readily observed. Expansions present in healthy conditions but not demonstrable in healthy T cell numbers, may be visible among decreased T cell counts, while HIV might even increase severity or frequency of clonal expansions.

Results in TCR diversity should be evaluated along with information about T cell counts. Increasing T cell numbers coinciding with increased perturbation is likely to be a process of clonal expansion. This effect does not point to the disappearance of clones, but to the submergence of unstimulated T cells in the environment of massive replicating T cell clones. Conversely, an increase in diversity in an increasing T cell population as we observed for CD4+ T cells after long-term treatment, indicates that the new CD4+ T cells are diverse in T cell receptors.

Taking into account that the TCR repertoire reflects the previous division rate of T cells involved in immune responses, it can be a useful tool for investigations characterized by distinct expansions. For instance, if HIV-infection induces a general elevated T cell turnover (130, 130), dividing cells would have diverse CDR3 regions. If on the contrary the increased turnover is caused by specific immune responses (106, 131) dividing cells will contain limited CDR3 diversity.

Finally, it is of immunological and clinical importance to investigate the degree of TCR diversity within T-cell populations that are involved in virus-specific immune responses. MHC-peptide tetrameric complexes are used to identify T cells specific for a given peptide, and these peptide-specific T cells are frequently observed as a mixture of T cells bearing different Vb families (87, 132). Recent studies have shown the development of TCR usage of epitope-specific CD8+ T cells during viral infections (133, 134). The capacity of HIV to change CTL epitopes may influence the antigen-specific TCR repertoire. Repetitive epitope mutations may lead to a narrow peptide-specific TCR repertoire (132), which may ultimately increase the chances of HIV. Therefore, the analysis of HIV-specific TCR repertoire may prove valuable information on the immune response during HIV-1 infection.