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High viral burden in the presence of major HIV-specific CD8⁺ T cell expansions: evidence for impaired CTL effector function

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To investigate the effect of HIV-specific CD8⁺ T cells on viral plasma load and disease progression, we enumerated HLA-A2-, B8- and B57-restricted CD8⁺ T cells directed against several HIV epitopes in a total of 54 patients by the use of tetrameric HLA-peptide complexes. In patients with high CD4⁺ T cell numbers, HIV-specific tetramer⁺ cells inversely correlated with viral load. Patients with CD4⁺ T cell numbers below 400/ μ l blood, however, carried high viral load despite frequently having high tetramer⁺ T cell numbers. This lack of correlation between viral load and tetramer⁺ cells did not result from viral escape variants, as in only 4 of 13 patients, low frequencies of viruses with mutated epitopes were observed. In 15 patients we measured CD8⁺ T cell antigen responsiveness to HIV peptide stimulation *in vitro*. FACS analyses showed differential IFN- γ production of the tetramer⁺ cells, and this proportion of IFN- γ -producing tetramer⁺ cells correlated with AIDS-free survival and with T cell maturation to the CD27⁺ effector stage. These data show that most HIV-infected patients have sustained HIV-specific T cell expansions but many of these cells seem not to be functional, leaving the patient with high numbers of non-functional virus-specific CD8⁺ T cells in the face of high viral burden.

Key words: HIV/AIDS / IFN- γ / CTL / MHC-peptide complex

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1 Introduction

CD8⁺ T cells are essential in suppressing viral replication in simian immunodeficiency virus infection [1, 2], and strong evidence is reported for the role of CTL in suppression of HIV-1 infection in humans [3–8]. By the use of tetrameric HLA-A2-peptide complexes, it has been shown that the percentage of HIV-specific T cells inversely correlates with viral RNA load [6]. This infers a potent suppressive effect of HLA-A2-restricted CTL on viral replication, which is further confirmed by the finding that a high frequency of A2-tetramer-binding CD8⁺ T cells is correlated with delayed disease progression [7]. However, despite the readily detectable CTL responses, most HIV-infected individuals eventually lose control of viral replication and progress to AIDS [5].

Efficacy of HIV-specific T cells to suppress virus replication and delay disease progression may depend on various parameters. Viral epitopes targeted by CTL may mutate, yielding viral escape variants that are less susceptible to CTL recognition [9–13]. In addition, CD8⁺ T cells may vary in their effector functions, such as cytotoxicity or production of anti-viral cytokines. Recent studies have demonstrated dysfunctional or anergic CD8⁺ T cells specific for viral antigens in murine [14, 15] and human viral infections ([16, 17]; Van Baarle, submitted). In several of these studies, and in HIV infection, lack of CD4⁺ T cell help is considered a cause for lack of CTL activity [18, 19]. Finally, HLA types such as HLA-B57 may play a role in protective immunity against HIV [20, 21].

To investigate the capacity of HIV-specific T cells to suppress viral load and delay disease progression, we studied a total of 54 HLA-A2, B8, or B57 typed subjects with various CD4⁺ T cell counts and AIDS-free follow-up periods, by the use of tetrameric HLA-peptide complexes.

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Phenotypic analysis of tetramer⁺ cells was performed with CD27 antibodies. Selected patients were further evaluated for viral epitope mutations and functional capacity of the tetramer⁺ cells. Peptide-specific stimulation and subsequent FACS analysis was used to demonstrate IFN- γ production by tetramer⁺ cells.

2 Results

2.1 HIV-specific T cell responses, viral load and CD4⁺ T cell numbers

To quantify HIV-specific CD8⁺ T cells in relation to viral load, we used HLA-peptide-tetramer staining to study 54 individuals. The average percentages of T cells recognizing Gag and alternative epitopes are listed in Table 1 per HLA group. As reported previously, the Gag protein was a major target for CD8⁺ T cells [21–23] although in most patients also populations of CD8⁺ T cells directed against the other epitopes tested were detected.

In the total patient group no correlations between tetramer⁺ cells and viral load or CD4⁺ T cell counts were

found (data not shown). To investigate numbers of HIV-specific T cells per HLA restriction element, we compared the HIV-specific responses for each HLA type. A2-restricted HIV-specific T cells showed an inverse correlation with viral RNA load. In contrast, no correlations were found for B8- or B57-restricted HIV-specific (Fig. 1A) and virus load. The HLA-related relative risks for disease progression were not reflected in their corresponding average tetramer⁺ cell percentages (Table 1), indicating that other factors than quantity could be involved in the protective effect of CTL.

Individuals who had less or more than 400 CD4⁺ T cells/ μ l blood are indicated in each HLA panel. This has been described as the lower limit for the asymptomatic state [24]. The group with low numbers of CD4⁺ T cells had significantly higher viral load (Mann-Whitney, $p=0.014$), but equally high tetramer⁺ percentages ($p=0.89$) compared to the group with more than 400 CD4⁺ T cells/ μ l. In Fig. 1B the tetramer⁺ percentages are plotted against viral load data clustered for the two CD4⁺ T cell strata. The sum of an individual's tetramer⁺ CD8⁺ T cell percentages directed against all epitopes included here is referred to as "HIV-specific T cells", although this may

Fig. 1. Tetramer⁺ T cell percentages in relation to viral load stratified by CD4⁺ T cell numbers and HLA type. (A) HLA-B57-, A2-, B8-restricted HIV-specific T cells in relation to viral load. Individuals with CD4⁺ T cell numbers below 400/ μ l blood are indicated with squares. (B) Relations between total HIV-tetramer⁺ T cell percentages and HIV RNA plasma load. Patients positive for more than one of the tested HLA types are indicated with open symbols, and tetramer⁺ percentages are the sum of all measured tetramer⁺ T cell numbers. Patients with CD4⁺ T cell counts below 400/ μ l have high viral plasma load despite frequently high tetramer⁺ percentages. In the group with CD4⁺ T cell counts higher than 400/ μ l blood, an inverse correlation for total HIV-tetramer⁺ cells with viral load is observed. (C) Lack of correlation between tetramer⁺ T cells and months AIDS-free survival. All statistics were computed with the Spearman's non-parametric correlation test and p values lower than 0.05 are indicated.

Table 1. Immunological data of the study population

HLA	Number of patients	Relative hazard ^{b)}	Tetramer % per epitope ^{c)}		Median viral load ^{d)}	AIDS free ^{e)}	Median ^{f)} CD4 ⁺ T cells
			Gag	Alternative			
B8	28 (2:B57, 10:A2) ^{a)}	1.35 [20]	1.0	1.0	37,000	85	403
A2	32 (8:B57, 10:B8)	0.55 [20]	0.43	0.36	23,000	113	451
B57/58	15 (2:B8, 8:A2)	0.08 [21]	1.7	0.1	11,000	108	487
Total	54		1.2	0.5	22,000	100	439

a) Numbers of individuals with combined HLA types are indicates.

b) Relative hazard for disease progression.

c) Tetramer⁺ T cell numbers are expressed as the average tetramer⁺ percentage of CD8⁺ T cells and are given for Gag-, or alternative epitope per HLA group. Gag epitopes used were derived from p17 (A2) or p24Gag (B8, B57) and alternative epitopes were derived from Pol (A2, B57) and Nef (B8) (see Sect. 4.2).

d) RNA copies/ml serum.

e) AIDS-free survival time (months) is a minimal estimation because seroconversion or AIDS diagnosis dates are not available for all patients and many patients have started therapy before diagnosis.

f) Number of CD4⁺ T cells/ μ l blood.

not be the total of an individual's anti-HIV T cell response, since more epitopes than the maximum of six tested peptides might be recognized. In the group with CD4⁺ T cell counts lower than 400/ μ l blood, no correlation was found between tetramer⁺ cells and viral load, mostly due to high tetramer⁺ cell numbers accompanied by high viral load. However, in the group with more than

400 CD4⁺ T cells/ μ l, HIV-specific T cell numbers significantly correlated with viral load. Combined measurements of more than one HLA restriction did not bias the correlation as can be noted from the distribution of the open symbols. These data suggest that efficient CTL control of viral replication, as evidenced by the inverse correlation [6], is only found in patients who still have

Table 2. Frequency of epitope mutations for HLA-B8 patients

Patient	RNA copies/ml serum	Months AIDS free	CD4 ⁺ T cells/ μ l blood	Mutated B8 EIYKRWII epitopes	Mutated A2 SLYNTVATL epitopes	Epitope mutation
642	110,000	40	290	0/18		
557	170,000	149	400	0/8		
270	81,000	105	205	0/16		
39	260,000	74	260	0/10	0/21	
187	7,000	28	200	0/15		
232	21,000	127	310	0/8		
044	1,000	173	590	0/7		
1110	730,000	37	950	0/6		
1113	66,000	78	350	0/6		
156	98,000	145	270	1/5		KIYKRWM ^{a)}
36	360,000	75	230	1/10		EIYKRRI/
656	21,000	74	350	1/8		KIYKRWI /
				1/8		EIY ER WMI/
159	140,000	33	160	3/11		EIYKRWWI/

a) Bold = mutated amino acids. Italic = anchor residues.

high CD4⁺ T cell counts. In patients with progressive disease (low CD4⁺ T cell counts, and high viral load) high numbers of HIV-specific T cells can be found but these are not capable of virus suppression.

In the total group the number of tetramer⁺ T cells did not correlate with months AIDS-free survival (Fig. 1C). When excluding the individuals who had less than 400 CD4⁺ T cells/ μ l blood a correlation for tetramer⁺ T cells and AIDS-free survival was observed ($p=0.023$, $R=0.40$). However, this result may be biased because two data points in the upper right corner of Fig. 1C are composed of summed measurements on two HLA restrictions (open symbols).

2.2 Epitope mutations are not frequently found in the B8 p24Gag epitope and do not correlate with disease progression

To investigate a possible cause for sustained viral replication in the face of high numbers of HIV-specific tetra-

mer⁺ cells (Fig. 1A, B), the possibility of development of viral escape mutants was investigated in 13 HLA-B8 patients. Viral RNA was isolated from serum and was cloned to sequence B8 p24Gag epitope "EIYKRWII" (Table 2). In 4 of 13 patients HIV clones were found that contained non-synonymous mutations at frequencies between 10 and 30%. In one patient a mutation in the anchor residue at position 4 was found ($K_4 \rightarrow E_4$). In two patients amino acid substitutions were observed which could potentially affect MHC or TCR binding, and in one patient we observed a mutation for which T cells specific for the wild type were cross-reactive (data not shown). Nonetheless, the frequency of epitope mutations did neither correlate with disease progression ($R=-0.25$; $p=0.44$), nor with viral load ($R=-0.04$; $p=0.8$, Spearman).

2.3 Limited antigen-induced IFN- γ production by HIV-specific T cells

Another explanation for the lack of effective CTL suppression of viral load could be CTL dysfunction. To test

Fig. 2. Stimulation responses of peptide-specific T cells. (A) The FACS dot-plots (left panels) are gated on CD8⁺ T cells and demonstrate tetramer⁺ cells (Y-axis), and IFN- γ production (X-axis). Percentages in the right upper corner indicate number of IFN- γ ⁺ peptide-specific T cells (right from the dotted line) of the CD8⁺ T cell population. The gates curved around the CD8⁺ T cells negative for both tetramer or IFN- γ exclude these nonspecific T cells and include peptide-specific T cells (tetramer⁺ or IFN- γ ⁺ cells). In the right histograms peptide-specific T cells are gated and percentages of IFN- γ -producing cells (X-axis) within the peptide-specific T cell population are given. (B) Stimulation response curve of EBV-specific and HIV-specific T cells. With increasing stimulation strength, peptide-specific T cells decrease and IFN- γ ⁺ T cells increase to reach a plateau. Data are the average of two separate experiments. (C) IFN- γ responses of EBV- and HIV-specific T cells are indicated as the percentage of the number of peptide-specific T cells present in the non-stimulated sample.

this, we used IFN- γ production, detected by intracellular cytokine staining, as a readout of functionality of antigen-specific T cells. To determine the fraction of tetramer-positive cells capable of IFN- γ production upon antigenic stimulation [25–27], PBMC were stimulated with the same peptides as complexed in the tetramers and stained simultaneously for IFN- γ and the corresponding tetramer complex. To establish appropriate stimulation conditions, we tested stimulation protocols varying in peptide concentration (0.1–10 μ g/ml) and stimulation duration (4–6 h). Fig. 2 shows percentages of IFN- γ -producing T cells of CD8⁺ T cells and as a fraction of tetramer⁺ T cells. In the FACS plots of Fig. 2A (0 μ g peptide), the upper left quadrant identifies tetramer⁺ IFN- γ ⁺ T cells, the upper right and lower right quadrants identify IFN- γ ⁺ T cells. These three quadrants together comprise peptide-specific T cells either by tetramer staining or by virtue of peptide-induced IFN- γ production.

Although 1 μ g and 10 μ g peptide stimulated similar numbers of CD8⁺ T cells to produce IFN- γ , the fraction of IFN- γ ⁺ T cells appears to reach 91% of peptide-specific T cells when stimulated with 10 μ g peptide. This seems to be due to a specific loss of the tetramer⁺ T cells in the upper left quadrant, which do not appear in the IFN- γ ⁺ quadrants. In Fig. 2B the stimulation response curve for peptide-stimulated EBV-specific T cells is shown. With increasing stimulus strength, a plateau in IFN- γ production is reached around 1 μ g peptide at 4 h incubation, whereas the number of peptide-specific T cells decreased with stronger and longer peptide stimulations. For the tested HIV peptides a similar response curve was observed (Fig. 2C). When adjusted for the number of tetramer⁺ T cells in the non-stimulated sample, stronger stimuli than 1 μ g did not induce substantially more IFN- γ ⁺ T cells. Stimulation resulted in CD69 up-regulation in most tetramer⁺ cells (data not shown).

Fig. 3. (A) After stimulation of PBMC with the peptide used in the tetramers, Gag-specific T cells displayed differential IFN- γ production compared to high IFN- γ production of B8-restricted EBV-specific T cells in a healthy donor. Numbers in the upper left corner indicate the peptide-specific T cells as a percentage of CD8⁺ T cells. A fraction of these are induced to produce IFN- γ as shown in the histograms. (B) For 15 patients, IFN- γ production was measured after peptide-specific stimulation. Percentages on the y-axis are IFN- γ ⁺ T cells after stimulation divided by tetramer⁺ T cells before stimulation. Patients with high levels of responsive peptide-specific T cells showed a delayed disease progression (upper panel; Spearman). The percentage of IFN- γ ⁺ T cells did not significantly correlate with viral RNA load (middle panel) or with CD4⁺ T cell numbers (lower panel).

Co-stimulation with anti-CD28 did not increase the number of IFN- γ -producing cells as detected by intracellular cytokine staining or with the ELISpot assay (data not shown). For further studies, we selected the stimulation with 1 μ g/ml peptide for 4 h because this protocol resulted in IFN- γ production in the majority of potential cells with readily detectable tetramer staining.

For 15 patients we observed percentages of IFN- γ -producing T cells which varied from 1 to 56% in peptide-specific T cells. Examples of HIV-Gag responses are shown in Fig. 3A. For comparison an EBV⁺HIV⁻ healthy control is included, who had 47% functional EBV peptide-specific T cells, which appeared to be highly reproducible in six repeated experiments ($45 \pm 2.2\%$), and ELISpot results from the same blood sample yielded similar percentages for EBV-induced IFN- γ secretion (manuscript in preparation).

Percentages of responsive peptide-specific T cells were calculated by relating the percentage of IFN- γ ⁺ among CD8⁺ T cells after peptide stimulation with the percentage of tetramer⁺ within CD8⁺ T cells in the non-stimulated sample. Correlating not percentages of tetramer⁺ cells, but the proportion of peptide-specific cells that produced IFN- γ revealed a positive association with prolonged asymptomatic survival (Fig. 3B). It would be expected that IFN- γ ⁺ T cells prolong the asymptomatic state by reducing viral load, however, such correlation was not statistically significant. This may be due to one aberrant data point skewing the correlation in this relatively small population ($n=15$). For CD4⁺ T cells, also no correlation was observed with the percentage of IFN- γ ⁺ T cells (Fig. 3B).

2.4 Nef-specific T cells display elevated effector function and phenotype

To investigate possible explanations for the observed differences in IFN- γ production excluding inter-patient variability, we compared antigen responsiveness and phenotype of p24Gag- and Nef-specific T cells in six HLA-B8 patients. Both p24Gag and Nef B8 tetramers stained significant cell numbers (Fig. 4A) in these patients. In contrast to B8 p24-specific T cells, B8 Nef-specific T cells correlated with AIDS-free survival time of HLA-B8 patients, suggesting a protective role for B8-restricted Nef-specific T cells (Fig. 4B), although high numbers of Nef-specific T cells did not correlate with low viral load (data not shown). Interestingly, peptide stimulation resulted in higher IFN- γ production by Nef-specific T cells than by p24Gag-specific T cells (Fig. 4A and C).

The tetramer⁺ T cells appeared to differ with regard to their phenotype when B8 p24Gag- and B8 Nef-specific

Fig. 4. Differential IFN- γ production and CD27 expression of B8-restricted HIV-specific T cells. (A) Difference in antigen-induced IFN- γ production within a patient's PBMC after stimulation with B8 p24Gag or B8 Nef peptides. Peptide stimulation leads to IFN- γ production (X-axis) in 1% of Gag-specific T cells, compared to 11% of Nef-specific T cells in patient 748, and 16% compared to 34% in patient 1113. (B) High numbers of B8 Nef-specific T cells correlated with delayed disease progression ($n=28$; Spearman). (C) Percentages of IFN- γ -producing cells ($n=6$) are significantly higher within B8 Nef-specific T cells than within B8 p24Gag-specific T cells (Wilcoxon). (D) Percentages of CD27⁻ T cells ($n=22$) are significantly higher in B8 Nef-specific T cells than in B8 p24Gag-specific T cells (Wilcoxon). (E) Number of CD27⁻ T cells correlates with the number of IFN- γ -producing T cells (Spearman; seven HLA-B8⁺ individuals, six individuals are plotted twice for both p24 Gag- and Nef-specific T cells).

T cells were compared. For this phenotypic analysis the combination of tetramers and CD27 antibodies was used, based on earlier reports showing that CD27⁺CD8⁺ T cells are specialized effector T cells because of their rapid effector function and high IFN- γ production [28]. Indeed, the Nef-specific T cells appeared to comprise higher percentages of IFN- γ -producing cells as well as higher percentages of CD27⁺ effector T cells (Fig. 4D). Furthermore, the percentage of CD27⁺ T cells correlated with the number of IFN- γ -producing T cells within the seven HLA-B8 individuals tested (Fig. 4E). Taken together these data show that in the same patients B8 Nef-specific T cells had differentiated more efficiently into T cells with an effector phenotype with corresponding functional capacity than B8 p24Gag-specific T cells had.

3 Discussion

CTL responses against HIV are readily detectable in most HIV-1-infected individuals, however, eventually they are incapable of suppressing virus load and preventing progression to AIDS [5]. To investigate the impact of HIV-specific CD8⁺ T cells on virus load and disease progression, we studied a total of 54 individuals who varied with respect to CD4⁺ T cell counts, HLA types and asymptomatic follow-up. HIV-specific T cells were enumerated with tetrameric HLA-peptide complexes. In the group with CD4⁺ T cell counts higher than 400 cells/ μ l blood an inverse correlation was observed between viral load and tetramer⁺ cells, indicating effective suppression of virus replication in the presence of HIV-specific T cells [6]. In contrast, in patients with lower CD4⁺ T cell counts no such correlation was found. This group contained many patients with both high numbers of tetramer⁺ T cells and high virus load, indicating impaired CTL efficacy.

We hypothesized that escape mutations had evolved in patients that had high viral load despite high numbers of HIV-specific T cells [9–13]. However, in only 4 out of 13 tested HLA-B8 patients viruses with a mutated B8-restricted p24Gag CTL epitope were found. The frequency of epitope mutations did not correlate with viral load or disease progression. Previously, Brander et al. [29] reported evidence for lack of immune selection pressure by A2-restricted CTL. Absence of escape variants of the immunodominant A2 epitope suggested low *in vivo* efficacy of the dominant CTL response [29].

An alternative explanation for the lack of T cell-mediated virus suppression would be that not all tetramer⁺ T cells are functional *per se*. In an *in vitro* stimulation assay using the same peptides as those complexed in the

tetramers we observed variable fractions of IFN- γ -producing T cells within the tetramer⁺ T cell population. We showed that differences in IFN- γ ⁺ fractions were not due to insufficient stimulation strength. The responsiveness as measured by IFN- γ production varied substantially among patients, and high proportions of IFN- γ -producing cells were correlated with delayed disease progression. Furthermore, Nef-specific T cells, which we found to be associated with delayed disease progression, showed better IFN- γ responses than the equally expanded p24Gag-specific T cells that did not correlate with disease progression. IFN- γ ⁺ T cells as percentages of CD8⁺ T cells did not show any correlation with disease parameters (data not shown), possibly because the epitopes included may not be the only antigen-specific T cells involved. The fraction of responsive IFN- γ ⁺ T cells, however, may reflect the general immune status and the quality of the total immune response against HIV, thus correlating with delayed disease progression.

Recent reports showed that a large fraction of antigen-specific T cells in murine LCMV [14] and human EBV (Van Baarle, submitted) and HCV infections [16] are dysfunctional with regard to IFN- γ production. It was hypothesized that lack of CD4⁺ T cell help rendered these T cells dysfunctional [14, 19]. Our observation that the correlation between viral load and numbers of HIV-specific T cells depended on CD4⁺ T cell counts is compatible with the need of CD4⁺ T cell help.

Our data thus provide evidence for expansions of dysfunctional HIV-specific CD8⁺ T cells. Discordant results between tetramer staining and functional HIV CTL detection assays have been reported before [30–32]. Kalams et al. [32] found a decrease in tetramer⁺ cells after initiation of therapy and a discordant temporal increase in CTL precursor frequency. This observation can be explained by the differential functionality of tetramer⁺ cells reported here: while absolute numbers of antigen-specific T cells may fall, suppression of HIV infection may allow dysfunctional T cells to regain their functional capacity, reflected in higher cytolytic activity. In addition, Gray et al. [33] found a correlation between tetramer staining and CTL activity only after *in vitro* stimulation of PBMC. It could be argued that stimulation selected for the functional tetramer⁺ cells to expand while non-functional T cells perished, similar to our control experiments described in Fig. 2. In contrast to our results, Appay et al. [34] observed that most HIV-specific tetramer⁺ T cells produced IFN- γ upon peptide stimulation, but found a deficiency in perforin content. In their study, the population consisted mainly of long-term asymptomatic or highly active antiretroviral therapy-treated patients, which differ from our untreated population that had a wide range of CD4⁺ T cell counts and progression

rates. Originally it was proposed that limiting dilution assay detects memory CTL capable of proliferating and surviving the extended culture period, whereas tetramers detect also effector CTL which do not survive *in vitro* culture, but *in vivo* actively suppress viral burden [6]. However, the lack of IFN- γ production argues against these cells being all functional effector cells. Secretion of IFN- γ is an important effector function of viral suppression in HIV infection and other viral infections [35]. IFN- γ production is typically induced shortly after antigenic stimulation [36–38], and has been shown to be a correlate for CTL function [39]. Since CD27⁺CD8⁺ T cells are known to be effector cells with high IFN- γ production and direct CTL activity [28], we investigated whether IFN- γ production correlated with the differentiation stage of the HIV-specific T cells. Indeed, the B8 Nef-specific T cells with relatively high IFN- γ production comprised significantly more CD27⁺ T cells than the B8 p24Gag-specific T cells with low IFN- γ production. Thus antigen responsiveness of T cells specific for HIV varies among patients, but also appeared to vary within patients among T cells specific for different epitopes restricted by the same HLA type. Both these differences in functionality seem to be related to differences in the degree of T cell differentiation to the CD27⁺ effector stage.

Tetramer staining shows high frequencies of HIV-specific T-cells, but at the level of antigen responsiveness only a limited fraction of tetramer⁺ cells was capable of IFN- γ production, and IFN- γ production was better correlated with asymptomatic follow-up than tetramer⁺ cells. Indeed, viral load was not suppressed in many patients with high numbers of antigen-specific T cells but low CD4⁺ T cell counts. CD8⁺ T cell differentiation to fully functional CTL depends on CD4⁺ T cell help and the functional capacity of CD8⁺ T cells may therefore diminish along with CD4⁺ T cell counts during disease progression. It is likely that in the course of HIV infection, CD4⁺ T cells and CTL function become increasingly important in prolonging the asymptomatic state. To understand CTL failure during the natural course of infection it is important to analyze the functional properties of tetramer⁺ T cells. Our data indicate a qualitative and not a quantitative problem with HIV-specific CD8⁺ T cells, which may be related to impaired T cell differentiation.

4 Materials and methods

4.1 Patients and samples

A total of 54 HIV-1-infected participants of the Amsterdam cohort were selected for HLA type A2, B8 or B57. PBMC, cryopreserved according to a standard computerized freez-

ing protocol, were selected on availability of sufficient PBMC numbers and to establish a broad range of viral load. None of the samples were drawn during antiretroviral therapy. Group characteristics are listed in Table 1. Viral load in the B57 group was significantly different from A2 and B8 group values. HLA-related differences in AIDS-free periods are indicated by the relative risks. HIV-negative, or HLA-mismatched donors served as controls for specificity of tetramers and peptide-specific IFN- γ production.

4.2 Tetrameric HLA-peptide complexes

Refolding of HLA heavy chains and tetramer formation was performed as described previously [40]. HLA heavy chains and β 2-microglobulin were constructed in pET plasmids and expressed in BL21 *Escherichia coli* strains. Heavy chain, β 2-microglobulin and peptides were refolded by dilution [41]. Peptides derived from p17 Gag (SLYNTVATL) and Pol (ILKEPVGHV) were complexed with HLA-A2; p24Gag (EIYKRWII) and Nef peptides (FLKEKGGL) were folded in HLA-B8 proteins, and Pol (IVLPEKDSW) and p24Gag (KAFSPEVPMF) were folded in HLA-B57 proteins. Monomeric complexes were concentrated, biotinylated, FPLC purified on a superdex 200 HL6/60 column (Amersham Pharmacia, Little Chalfont, GB) and bound to streptavidin-PE (Sigma, St Louis, MO). Tetrameric product was FPLC purified. For staining, PBMC were thawed, washed and stained with appropriate tetramers at 37°C [42] for 15 min, and for CD8 (PerCP; Becton Dickinson, San Jose, CA). Phenotyping of HLA-B8 tetramer⁺ cells was performed by co-staining with CD27-FITC (CLB, Amsterdam, The Netherlands).

4.3 Sequencing of viral epitopes

Serum, drawn at the same time points as the PBMC samples for tetramer staining, was used to isolate viral RNA by the method described by Boom et al. [43]. Primers covering the p24Gag fragment including the B8 epitope as described above were used for PCR. PCR fragments were cloned using the pGEM-T easy vector system (Promega, Madison, WI) and sequenced on an automated ABI 377 sequencer (Perkin Elmer, Foster City, CA).

4.4 Antigen-specific stimulation

Two million PBMC/ml were stimulated with the peptide used in the tetramer complexes at 37°C in the presence of 3 μ M monensin. Various stimulation protocols were tested varying from 0.1–10 μ g peptide/ml and 4–6 h, with or without anti-CD28 co-stimulation. HLA-mismatched peptide, matched irrelevant peptide, or medium alone were used as negative controls, and PMA/ionomycin was used as positive control. After incubation, cells were washed, stained with tetramers (PE or allophycocyanin) and CD8 (PerCP, Becton Dickinson),

fixed with 4% paraformaldehyde, permeabilized (Permeabilization kit, BD) and stained intracellularly with anti-IFN- γ (FITC, Diaclone; or FITC/PE, Becton Dickinson). Cells were analyzed using Cellquest software (BD) and gated on live lymphocytes. The IFN- γ gate was determined by each individual's negative and positive controls.

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