Cellular immunity driving HIV-1 evolution
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Citation for published version (APA):

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Persistence of HIV-1-specific CTL responses restricted by both protective and non-protective HLA alleles during HIV-1 disease progression

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Manuscript in preparation
Abstract

HLA B27 and HLA B57 are associated with relatively slow progression to AIDS, for reasons that remain largely unknown. There is accumulating evidence that the protective effect of these HLA molecules is driven by the specific HIV-1 epitopes that are present. By comparing the within-host evolution of CTL responses directed towards HIV-1 epitopes presented via protective and non-protective HLA alleles, we tested the hypothesis that HLA alleles associated with slow disease progression are protective because they specifically present the most conserved parts of the HIV-1 proteome.

Our data show that CTL responses restricted by HLA A2 are generally less well-preserved during disease progression than CTL responses restricted by protective HLA alleles, in individuals expressing either one of these alleles. This is probably a result of the faster rate of disease progression in individuals expressing none of the protective HLA alleles. In contrast, when we compared the preservation of CTL restricted by HLA A2 or one of the protective HLA molecules in individuals co-expressing these HLA alleles, CTL responses restricted by the protective HLA alleles were found to be lost at least as fast as CTL responses restricted by HLA A2. The observed loss of CTL responses was due to a combination of CTL escape mutations and functional impairment of HIV-1 specific T cells.

These data suggest that the association between HLA B27 and B57 and relatively slow HIV-1 disease progression is not due to specific presentation of the most conserved parts of the HIV-1 proteome.

Introduction

HIV-1 causes a persistent infection despite the presence of vigorous HIV-specific cytotoxic T lymphocyte (CTL) responses in most patients. These specific CTLs are thought to play an important role in initial control and, later, the containment of viral dissemination and replication 1-7. It has consistently been shown in large cohort studies that certain HLA class I alleles (e.g. HLA B27 and HLA B57) are associated with relatively slow progression to AIDS, while others (e.g. HLA B35 and HLA B53) are associated with relatively rapid rates of disease progression 8. Although the mechanism behind these associations remains largely unknown, several hypotheses have been postulated. A commonly held view is that the frequency of HLA alleles in the human population determines their protective effect 9.

Since CTL escape mutations arise rapidly in infected individuals and can be stably transmitted to a new host 10,11, individuals expressing common HLA alleles might have a selective disadvantage because the HIV-1 strains they are infected with have a larger chance to have accumulated mutations that escape CTL responses restricted by those HLA alleles. However, data concerning this hypothesis are inconclusive. While relatively small studies have shown significant negative correlations between the frequency of HLA molecules in the population and viral load 9 or the capacity to elicit CTL responses 12, no significant positive correlation was found between the relative hazard of HIV-1 disease progression and the population frequency of different HLA molecules 13.

An alternative hypothesis is that the differences in protective effect between HLA molecules are due to the specific peptides that are being presented. There is accumulating evidence that CTL responses against peptides from p24 Gag are more protective than CTL responses against other HIV-1 proteins 14,22. It remains unclear, however, why responses
against p24 peptides are beneficial. Because p24 is one of the most conserved regions of the HIV-1 genome, a straightforward explanation would be that CTL responses against p24 epitopes are better maintained throughout the course of infection than CTL responses against HIV-1 epitopes that more easily mutate.

If HLA alleles associated with slow disease progression are indeed protective because they present the most conserved HIV-1 epitopes, one would expect CTL restricted by protective HLA molecules to be better maintained throughout disease progression than those restricted by non-protective HLA molecules. To test this hypothesis we compared the within-host evolution of CTL responses directed towards HIV-1 epitopes presented via protective and non-protective HLA alleles.

**Material and methods**

**Patient selection**

Twenty-five HIV-1-infected individuals from the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS) with a known date of seroconversion were selected for this study based on the expression of HLA A2, HLA B27 or HLA B57. Six individuals expressed HLA B57 or B27 without co-expressing HLA A2 (five and one, respectively). Nineteen individuals expressed HLA A2, of which four individuals co-expressed HLA B57 and eight individuals co-expressed HLA B27. See Table I for more details. All individuals were treatment naive at the time of analysis. 2-Digit genotyping at HLA class I loci was performed by sequences specific primers (SSP) PCR as described elsewhere.

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<th>Table I Patient Characteristics per group</th>
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<th>HLA type</th>
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<th>Viral load copies/ml</th>
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<td>HLA B27 (n=1)</td>
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a. median values per group are depicted
nd, not determined

**Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay**

IFN-γ-producing antigen-specific CD8+ T cells were measured using the IFN-γ ELISPOT assay with the use of multiscrin, 96 well, membrane-bottomed plates (MSIPN4550, Millipore) and IFN-γ-specific monoclonal antibodies (MABTECH, Sweden). Cryopreserved PBMCs were thawed and suspended in RPMI 1640 medium containing 10% FCS, and incubated at a final concentration of 10^6 cells per well in triplicate in the presence or absence of 20 µg/ml peptide for 20-24 hours at 37°C. Peptides were synthesized by Mimotopes (Australia) and the peptide facility at the Netherlands Cancer Institute (The Netherlands). An overview of the peptides used in this study can be found in Chapter 8, supplementary table 1 and 25. PHA stimulation served as a positive control to test the capacity of PBMCs to produce IFN-γ, and medium without peptide or PHA served as a
negative control. IFN-γ-producing cells were detected as dark spots and counted using an AELVIS EliScan (EliAnalyse Software version 4, Germany). The number of IFN-γ-producing cells was calculated by subtracting the negative control value and was reported as number of spot-forming units (SFU) per $10^6$ PBMCs. Samples with $>$100 spots per million PBMCs, after subtraction of the negative control values were considered positive.

**Isolation of clonal HIV-1 variants**

Clonal HIV-1 variants were obtained by cocultivation of increasing numbers of patient PBMCs, that were obtained throughout the course of HIV-1 infection, with 2-3 day phytohemagglutinin (PHA) stimulated PBMCs from a healthy donor (PHA-PBMCs) as described previously. Prior to cocultivation, PBMCs from a healthy donor were cultured in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 10% Fetal Calf Serum (FCS; Hyclone), 1 μg/ml PHA (Welcome), Penicillin/Streptomycin (Pen/Strep; Gibco Brl), 5μg/ml Ciprofloxacin (Bayer) for 2-3 days in a culture flask at a cell density of $5 \times 10^6$/ml. Clonal virus variants were isolated in multiple cocultivations of 5,000-40,000 patient PBMCs with $10^5$ PHA-PBMCs in a final volume of 150 μl IMDM-IL2 medium (IMDM supplemented with 10% FCS (Hyclone), Pen/Strep (Gibco Brl), 10 U/ml rIL-2 (proleukin; Chiron Benelux BV), 5μg/ml Ciprofloxacin (Bayer) and 5μg/ml polybrene (Sigma) for 35 days in a 96-well flat-bottom microtiter plate. Every week, culture supernatants were tested for virus production in an in-house Gag p24 antigen capture enzyme-linked immunosorbent assay (ELISA). At the same time, one-third of the culture volume was transferred to new 96-well plates and fresh PHA-stimulated healthy donor PBMC were added to propagate the culture. Virus cultures were expanded by cocultivation of p24 positive microcultures with $5 \times 10^6$ PHA-PBMCs at a density of $1 \times 10^6$/ml IMDM-IL2 medium supplemented with 10% FCS (Hyclone), Pen/Strep (Gibco Brl), 10 U/ml rIL-2 (proleukin; Chiron Benelux BV), 5μg/ml Ciprofloxacin (Bayer) and 5μg/ml polybrene (Sigma) in a culture flask.

**DNA isolation, PCR amplification and sequencing of clonal HIV-1 variants**

Total DNA was isolated using the L6 isolation method. DNA was amplified using the following primer combinations: Gag: (KVL-064) 5'-GGTTGTGTGACTGCTGTAAC TAGAGATCCCTCAGA-3' and (NCRev-2) 5'-CCTTCTTTCTTCCATTTCCAACAG-3' followed by (KVL-066) 5'-TCTCTAGAGTGGCAGCCGAACAG-3' and (NCRev-3) 5'- CTTTTCTTAGGGGCTTGCAATT-3'; Nef: (5'-GFP) 5'-TTTCGTATAAGATGGGTC-3' and (3'-GFP) 5'-CTTTTTCCTTATAGGGCGC-3'; RT: (RT18) 5'-GGAAACCAAAAATGATAGGGGGAATTGGAG-3' and nested primers (RT19) 5'-GGACATAAAGCTATAGGTACAG-3' and (RT20) 5'-CGACCTTTGACGTCTTAG-3'. PCR products were subsequently purified using a High pure PCR product purification kit (Roche diagnostics) and sequenced using the ABI prism Big Dye Terminator v1.1/3.1 Cyclesequencing Kit (Applied Biosystems) using the nested PCR primers. Sequences were analyzed on an Applied Biosystems/Hitachi 3130 x1 Genetic Analyzer.

**Prediction of CTL epitopes**

Epitopes were predicted using the proteasomal cleavage/TAP transport/MHC class I combined predictor available at http://tools.immuneepitope.org. For MHC binding
predictions the most abundant four-digit HLA type of each HLA serotype was used. Cut-off values used were 1.135 for proteasomal cleavage, -0.56 for TAP transport and -2.7 for MHC binding (Schmid et al, in press).

Statistical analysis
Data were analysed using SPSS 15.0 software (SPSS, Chicago, Illinois, USA). Differences between early and chronic infection were analysed using Wilcoxon Signed Ranks or Chi Square tests. A P-value ≤ 0.05 was considered statistically significant.

Results

CTL responses restricted by protective HLA alleles are not better preserved
To test the hypothesis that CTL targeting HIV-1 peptides presented via HLA B27 or HLA B57 are better preserved during chronic HIV-1 infection than CTL targeting epitopes presented via non-protective HLA, we selected 25 HIV-1 infected individuals with a known date of seroconversion from the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS) who expressed one of the protective HLA alleles HLA B27 (RH = 0.43) or HLA B57 (RH = 0.55) and/or the HLA molecule A2 (RH = 0.91) which is not associated with slow disease progression 13. Using the IFN-γ enzyme linked immunospot (ELISPOT) assay, a total of 78 peptides derived from the entire HIV-1 genome were tested (30 HLA A2 restricted peptides, 29 HLA B57 restricted peptides and 19 HLA B27 restricted peptides, respectively). To avoid a bias in peptide selection we did not only include known HIV-1 peptides published in the Los Alamos Database, but also epitopes predicted to be presented via either one of these HLA alleles based on proteasomal cleavage, TAP transport and MHC binding. We have previously shown that such prediction programs provide powerful tools to determine CTL responses, even to HIV-11 peptides that have not previously been identified 25.

In general, the CTL response observed in chronic infection was broader than that early after seroconversion (Figure 1), in line with previous studies 29-32. Remarkably, this widening of the CTL response during the course of infection was observed for HLA A2 restricted responses (P=0.002, Wilcoxon Signed Ranks test) but not for HLA B27 and B57 restricted responses (P=0.155, Wilcoxon Signed Ranks test).

In order to compare the loss of HIV-specific CTL responses during the course of HIV-1 infection, we next focussed on CTL responses that were present during early HIV-1 infection and analysed whether those responses were still present five years later. Because the maintenance of CTL responses may not only be affected by the frequency of CTL escape mutations, but also by disease progression itself 33,34, we confined our analysis to 12 patients who co-expressed HLA A2 and one of the protective HLA alleles (either HLA B27 (n=8) or B57 (n=4)), allowing us to compare the evolution of CTL responses restricted by both types of HLA within the same host. HIV-1 specific CTL responses were analysed at two time points; within 6 months after seroconversion (early infection) and 5 years later (during asymptomatic chronic infection).
CTL responses restricted by different HLA alleles

Figure 1 Broader CTL response during chronic HIV-1 infection. CTL responses towards a maximum of 30 HLA A2, 20 HLA B27 and 30 HLA B57 restricted peptides were measured shortly after seroconversion (Primary infection) and 5 years later (Chronic infection). The y-axis represents the number of tested peptides; black bars show numbers of recognised peptides, white bars show numbers of peptides that were not recognised. Each bar on the x-axis represents the data for 1 individual, the first 7 bars represent individuals co-expressing HLA A2 and B27, the middle 4 bars represent individuals co-expressing HLA A2 and B57, and the last 6 bars are the individuals who express only HLA A2 (left panel), or only HLA B27/57 (right panel).

In co-expressors of HLA A2 and HLA B27, on average 77% (range 33-100%) of the CTL responses towards peptides presented via HLA A2 during early HIV-1 infection were still present 5 years later (Figure 2). Interestingly, this percentage was not higher for CTL responses restricted by HLA B27, where 62% (range 40-100%) of the responses measured during early HIV-1 infection were still present during chronic HIV-1 infection. In contrast, the individuals co-expressing HLA A2 and HLA B57 hardly showed responses towards peptides presented via HLA A2, neither early after infection nor during chronic infection (Figure 1). In the single individual in whom we observed a response towards HLA A2 restricted peptides, 6 out of 7 responses (86%) were still present 5 years after seroconversion, in contrast to the HLA B57 restricted response for which only 2 out of 5 responses (40%) were still present. Together, these data indicate that CTL responses towards peptides presented via protective HLA alleles are not better (if not even worse) preserved during disease progression than CTL responses restricted by the non-protective HLA A2.
To examine potential influences of protective HLA alleles on CTL responses restricted by non-protective HLA alleles, we next compared the maintenance of HLA A2 restricted CTL responses in people who did or did not co-express one of the protective HLA alleles. In the seven HLA A2 positive individuals who did not co-express HLA B27 or HLA B57 we found that on average only 30% (range 0-100%) of the CTL responses that were present during early HIV-1 infection were still present 5 years later. This suggests that the fate of CTL responses restricted via the non-protective HLA A2 is influenced by the presence of HLA alleles associated with relatively slow disease progression. In line with this, we have found that individuals expressing both HLA A2 and HLA B27 responded to significantly more HLA A2 restricted peptides and with significantly higher magnitude compared to individuals without HLA B27 (Schellens et al, manuscript in preparation).

Contribution of both CTL escape and functional impairment of the specific T cells in loss of CTL responses
Measuring the CTL response with a functional readout (such as IFN-\(\gamma\)) does not allow for the distinction between loss of the response due to CTL escape mutations or loss of the response due to functional impairment of the specific T cells. To investigate the contribution of these factors, we analysed whether the loss of CTL responses during chronic HIV-1 infection was due to CTL escape mutations within or flanking the epitopes. The proteins gag, nef and reverse transcriptase (RT) were sequenced shortly after seroconversion and 5 years later. When we focussed at the specific CTL epitopes for which we observed a CTL response early after seroconversion, which was subsequently lost during chronic HIV-1 infection, we observed examples of CTLs loss due to CTL escape mutations as well as CTLs loss in the absence of CTL escape mutations. Figure 3 shows an illustration of loss of the CTL response in the absence (QW9) or presence (TW10) of mutations within or flanking the epitope in chronic HIV-1 infection.

To analyse the within-host evolution of CTL escape mutations during chronic HIV-1 infection more broadly, we used peptide prediction programs \(^35\) (http://immuneepitope.org) to obtain the total number of potential CTL epitopes present in HIV-1 sequences derived from both time points.
This analysis confirmed the observation that CTL epitopes restricted via protective HLA alleles are lost at least as fast as CTL epitopes restricted by the non-protective allele HLA A2. On average, 91% (range 78-100%) of the HLA A2-restricted epitopes that were predicted to be present in sequences isolated during primary infection were still present in sequences isolated during chronic infection, which is comparable with (or even higher than) the 82% (range 67-100%) and 74% (range 72-76%) for HLA B27 and -B57-restricted CTL epitopes, respectively. Since the latter analysis was based solely on viral sequences, thereby disregarding the potential influence of T cell impairment, these losses should truly reflect CTL escape mutations. Together these data show that, when measured in the same individual, CTL responses restricted by protective HLA alleles are not sustained better during HIV-1 disease progression than CTL responses restricted by HLA A2, and that the loss of CTL responses that were present during early infection is due to a combination of CTL escape mutations and functional impairment of HIV-1 specific T cells.

**Discussion**

There is an ongoing debate on the mechanism via which certain HLA alleles are associated with relative protection against progression to AIDS. In the present study we found that, when measured within one and the same individual, CTL responses restricted via the protective HLA alleles HLA B27 or B57 were lost at least as fast as CTL responses restricted via the non-protective HLA allele HLA A2, suggesting that the association between HLA B27 and B57 and slow disease progression is not due to specific presentation of the most conserved parts of HIV-1. By comparing the maintenance of CTL responses restricted by protective and non-protective HLA molecules within individuals co-expressing these HLA molecules, we excluded any possible confounding effects of the rate of disease progression or host or viral factors on CTL maintenance.
An overwhelming amount of data has suggested that p24 Gag responses are most important in containing HIV-1 infection. Recently, it was shown that HLA B27 and B57 have an intrinsic preference to present epitopes derived from p24 Gag. Our current data show that the association between HLA B27 and B57 with slow disease progression is not due to better preservation of CTL responses restricted via these HLA alleles. Other potential explanations for the protective effect of HLA that specifically target gag-p24 might be that i) p24 is one of the most functionally and structurally constrained proteins of HIV-1, resulting in higher fitness cost when mutations do occur, ii) Gag-derived epitopes can be recognized by CTLs already by 2 hours post infection, before Nef can down-regulate HLA expression, and iii) the CTL responses restricted via HLA B27 and B57 that remain after primary infection might be of higher affinity compared to CTL responses restricted via HLA A2.

To investigate whether mutations disrupting CTL epitopes restricted via protective HLA alleles are indeed associated with a higher fitness cost, as suggested before, it would be very interesting to measure replication capacity of viruses with and without specific CTL escape mutations. Since our study has shown that both CTL escape mutations and functional impairment of the specific T cells can contribute to the observed loss of CTL responses, dissecting the relative contribution of CTL escape mutations to the observed loss of CTL responses during HIV-1 disease progression in protective and non-protective HLA alleles would also be intriguing.

During chronic HIV-1 infection, most individuals responded to a higher fraction of tested peptides compared to primary infection (Figure 1 and 29-32). In line with findings of Altfeld et al., we observed that in the individuals co-expressing HLA A2 and HLA B27, the HLA B27 restricted CTL response contributed relatively more to the total early response compared to HLA A2 restricted CTL (P=0.028, Wilcoxon Signed Ranks test). Interestingly, this difference was no longer present during chronic HIV-1 infection, due to an increase in responses towards HLA A2 restricted peptides. Lichterfeld et al. have recently shown that the functional avidity of HIV-1 specific CTL is higher during early HIV-1 infection compared to chronic infection, and that this reduction in avidity during chronic infection was associated with clonal deletion of the high avidity clones recruited during early HIV-1 infection. It has been suggested that those high avidity clones that are recruited during early infection are the ones significantly contributing to the dramatic decline of HIV-1 viremia commonly observed during early HIV-1 infection. Interestingly, we observed that the dominant HLA B27 restricted response KK10 (P24, residues 263-272) was already present in early HIV-1 infection in all HLA B27 positive individuals and was preserved throughout the 5 years of follow-up. Recently Almeida et al. have shown that CTL specific for KK10 are more polyfunctional and have a superior functional capacity compared to other HIV-1 specific CTL.

Although HLA B27 restricted responses were lost at least as fast as HLA A2 restricted responses in individuals co-expressing these HLA alleles, we cannot exclude the possibility that the CTL responses restricted by protective HLA alleles that are still present during chronic infection have a higher avidity compared to individuals that do not express one of the protective HLA alleles. This view is supported by our observation that the individuals expressing HLA A2 without one of the protective HLA alleles lose a significantly higher fraction of their HLA A2 restricted response that is present early after infection (30% of the early response remains during chronic HIV-1 infection in those individuals compared to
77% and 86% in individuals co-expressing HLA B27 or B57, respectively, P=0.012, Chi Square test). Together, these data suggest that both the quality and quantity of the CTL responses that persist early after HIV-1 infection might be important.

In conclusion, these data show that CTL responses restricted via HLA alleles that are associated with relative protection against progression to AIDS are not sustained better during HIV-1 disease progression than CTL responses restricted via non-protective HLA alleles when measured within the same individuals. Additionally, the loss of CTL responses between early and chronic HIV-1 infection is due to a combination of CTL escape mutations within or flanking CTL epitopes and functional impairment of HIV-1 specific T cells.

Acknowledgements
The Amsterdam Cohort Studies on HIV infection and AIDS, a collaboration between the Amsterdam Health Service, the Academic Medical Center of the University of Amsterdam, Sanquin Blood Supply Foundation and the University Medical Center Utrecht, are part of the Netherlands HIV Monitoring Foundation and financially supported by the Netherlands National Institute for Public Health and the Environment. This work was financially supported by a grant from the Landsteiner foundation for Blood transfusion research (LSBR, grant nr 0317).

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