Rapid Progressing Allele HLA-B35 Pₓ Restricted Anti-HIV-1 CD8+ T Cells Recognize Vestigial CTL Epitopes

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Abstract

Background: The HLA-B*35-Pₓ allele has been associated with rapid disease progression in HIV-1 infection, in contrast to the HLA-B*35-Pᵧ allele.

Methodology/Principal Findings: Immune responses to two HLA-B*35 restricted HIV-1 specific CTL epitopes and their variants were followed longitudinally during early HIV-1 infection in 16 HLA-B*35+ individuals. Subjects expressing HLA-B*35-Pₓ alleles showed no difference in response to the consensus epitopes compared to individuals with HLA-B*35-Pᵧ alleles. Surprisingly, all the HLA-B*35-Pₓ+ individuals responded to epitope-variants even in the absence of a consensus response. Sequencing of the viral population revealed no evidence of variant virus in any of the individuals.

Conclusions/Significance: This demonstrates a novel phenomenon that distinguishes individuals with the HLA-B*35-Pₓ rapid progressing allele and those with the HLA-B*35-Pᵧ slower progressing allele.

Introduction

Host genetics are known to have a major influence on HIV-1 disease progression [1,2,3,4,5]. For example, HLA-B*57 and -B*27 are two MHC class I alleles which have been associated with slower progression to AIDS [6,7]. At the other end of the spectrum, HLA-B*35 alleles have been shown to be associated with more rapid disease progression [8].

HLA-B*35 alleles can be segregated into B*35-Pₓ and -Pᵧ alleles, which differ by a number of amino acids located in the HLA binding groove [9]. These differences result in different peptide binding preferences. HLA-B*35-Pₓ molecules preferentially bind peptides with a proline (P) at anchor residue 2 and a tyrosine (Y) at position 9. HLA-B*35-Pᵧ molecules also bind peptides with a proline at anchor residue 2, but can accept a much broader range of residues at position 9. However, both HLA-B*35-Pₓ and -Pᵧ molecules are capable of binding epitopes that do not fit the conventional PY motif (Los Alamos Database, http://www.hiv.lanl.gov/). The most common B*35-Pᵧ alleles are B*3501 and B*3508, while the B*35-Pₓ alleles include B*3502/3503/3504/3508.

The marked difference in HIV-1 disease progression of individuals expressing either HLA B*35-Pᵧ or -Pₓ alleles is not understood. The overall magnitude of the HIV-1-specific CD8+ T cell immune response does not differ between individuals expressing either -Pₓ or Py alleles [10]. However, this was measured at the level of the total CD8+ T cell response, and thus specific responses to individual epitopes were not differentiated. Measuring responses to specific epitopes may be critical in understanding the differences in disease progression between individuals with these alleles. We therefore set out to analyze T cell responses to two HLA-B*35 restricted HIV-1 epitopes in both HLA-B*35 -Pₓ and -Pᵧ allele expressing individuals.

Methods

This study was approved by the UCSF Institutional Review Board.

Human Study Subjects and Blood Samples

Peripheral blood mononuclear cells (PBMC) samples were obtained from HIV-1 infected subjects, from the University of California San Francisco (UCSF) OPTIONS project (Table 1). HLA-A type, CD4 count and HIV-1 viral load were determined for each subject (Table 1). All samples were processed with Ficol-Paque PLUS (Amersham Biosciences, Pittsburgh, PA) and PBMC were stored frozen in 10% DMSO in fetal bovine serum prior to subsequent analysis. Written informed consent and approval for
this study was obtained in accordance with the guidelines of the Institutional Review Board.

ELISPOT assay

Quantification of HIV-specific T-cell responses using thawed viable PBMC was performed using the IFN-γ ELISPOT assay. Briefly, each well of a 96-well plate (Millipore MAHAS4510, Bedford, MA) was coated with 50 μl of anti-IFN-γ mAb (Mabtech, Stockholm, Sweden) at 5 μg/ml. After incubation, each well was washed and blocked with 10% FCS in RPMI (Cellgro). PBMC (1x10^5–2x10^6) were added to duplicate wells and peptides were added at varying concentrations to the cells. As a positive control, the mitogen phytohemagglutinin (PHA) was used at 4 μg/ml, and wells with only media added were used as a negative control. After overnight incubation (14–16 hours) at 37°C, plates were washed with phosphate-buffered saline (PBS). Biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech) was added at 1 μg/ml, and incubated at 37°C for 1 hr. Plates were washed with PBS +0.1% Tween 20 and treated with streptavidin-bound alkaline phosphatase. After 1 h incubation, plates were washed with PBS +0.1% Tween 20 and developed using Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA). IFN-γ spot-forming units (SFUs) were visualized and counted using an AID EliSpot reader (Autoimmun Diagnostika GMBH, Germany). Spots were standardized to SFU/10^6 PBMC. Spots formed in the presence of media alone were considered non-specific background and subtracted from the SFU in stimulated wells. The wells where the number of SFU was greater than two times background or greater than 50 SFU/10^6 PBMCs, which ever was higher, were considered as responses.

Viral Load Assessment

Plasma HIV-1 viral load was determined by bDNA (Bayer).

Table 1. Clinical parameters, average CD4 and viral load (VL) were generated from across the entire study window.

| B*35 | Study ID | HLA-A | HLA-B | Average CD4 | Average VL |
|------|----------|-------|-------|-------------|------------|
| Px   | Subject 1 | 1     | 2407  | 3505        | 5107       |
|      | Subject 2 | 2501  | 6801  | 702         | 3503       |
|      | Subject 3 | 11    | 11    | 18          | 35         |
|      | Subject 4 | 2901  | 3101  | 3503        | 4403       |
|      | Subject 5 | 2901  | 3101  | 8101        | 616        |
|      | Subject 6 | 2901  | 3101  | 3501        | 9401       |
| Py   | Subject 11| 301   | 2601  | 3501        | 4001       |
|      | Subject 12| 201   | 2402  | 702         | 3501       |
|      | Subject 13| 201   | 1101  | 3501        | 4402       |
|      | Subject 14| 201   | 1101  | 3501        | 4402       |
|      | Subject 15| 201   | 1101  | 3501        | 4402       |
|      | Subject 16| 301   | 2402  | 3501        | 5701       |
| Py  | Subject 6 | 1101  | 6801  | 3501        | 3505       |

Figure 1. A representative example, from subject 1, of longitudinal CD8+ T cell IFN-γ responses to the TY9 epitope (solid green bars) and VY10 (solid orange bars). All subjects showed similar responses towards both epitopes that waxed and waned over the course of the study window.

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Figure 2. CD8+ T cell IFN-γ responses to both consensus epitopes (solid bars, TY9 green and VY10 orange), and variant epitopes (TY9 variants green, checkered or striped bars, and VY10 variant orange checkered bars). Representative examples of longitudinal responses are shown for HLA-B*35 -Px+ Subjects 1 (A) and 2 (B), and HLA-B*35 -Py+ Subject 8 (C). CD8+ T cell IFN-γ responses from Subject 6, who expressed both HLA-B*35 -Px and -Py alleles (D). HLA-B*35 -Px+ individuals did not display unusual CD4 counts or viral loads, a representative example shown from Subject 1 (E).

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primary infection cohort (OPTIONS) [11]. Five individuals expressed an HLA-B*35-Px allele, and 10 individuals expressed an HLA-B*35-Py allele. One individual expressed both HLA-B*35-Px and -Py alleles. No significant difference in CD4+ T cell count ($p = 0.14$) or plasma viral loads ($p = 0.7$) were observed between the two groups, as determined by two-tailed Mann Whitney test analysis (Table 1).

CD8+ T cell responses were determined by a standard IFN-γ ELISPOT assay [12,13,14], using thawed peripheral blood mononuclear cells (PBMC). All spot numbers were normalized to numbers of IFN-γ spot-forming units (SFU) per 10⁶ PBMCs. Responses were determined by subtracting the SFU values from medium control wells from the peptide induced response. All experimental assays and analyses were conducted blinded to the individual’s specific HLA-B*35 allele.

PBMC were screened against two HLA-B*35 restricted HIV-1 P1 derived epitopes: TVLDVGDAY and VPLDEDFRKY [15,16]. At one or more time points we observed a loss of an epitope specific-response in all individuals tested, an example is shown in Fig. 1. However, there was no clear difference in the size or number of epitope-specific responses between the either HLA-B*35-Px or -Py individuals (data not shown). HLA-B*35 -Px individuals did not display unusual CD4 counts or viral loads, a representative example shown from Subject 1 (E).

Unexpectedly, all the HLA-B*35-Px individuals made responses to all the peptides tested. Strikingly, they were able to recognize the variant epitope in the absence of a consensus response at one or more time points tested, as shown for 2 patients (Subject 1 and Subject 2) (Fig. 2A and 2B). However, HLA-B*35-Py individuals never recognized the variant epitope alone at any of the time points tested (Fig. 2C). Individuals who made responses solely to variant epitopes at one time point or more, showed no unexpected clinical characteristics at these or any other time points (Fig. 2E). We observed no difference in the magnitudes of responses targeting HLA-B*35 specific epitopes in either HLA-B*35 -Px+ or -Py+ individuals. Both groups were able to recognize and respond to variant epitopes. However, only the HLA-B*35 -Px+ individuals were able to respond to variants in the absence of a wild-type response.

The ability of the five HLA-B*35 -Px+ individuals to make solely variant-specific responses implied that they may harbor variant HIV-1 within their viral population. To test this, their HIV-1 reverse transcriptase, codons 40 through 247, were sequenced using the TRUGENE HIV-1 RNA genotyping kit and OpenGene system software for sequence analysis (Siemens Medical Solutions Diagnostics) [17,18]. At no time point tested, were any variants of the consensus epitopes seen (Table 2).

Interestingly, one individual from the cohort, Subject 6, expressed both HLA-B*35 -Px and -Py alleles. This individual also responded solely to the variant epitopes at one time point (Fig. 2D). Again, this individual did not display variant viruses within their viral population. This suggests that the HLA-B*35-Px effect dominates over the HLA-B*35-Py influence. Lost or weak responses have been shown to be rescued by blocking PD-1 - PD-L1 interactions. However, PD-1 - PD-L1 blockage did not improve the restore absent responses in any of the individuals tested (data not shown).

**Discussion**

HIV-1 responses associated with delayed disease progression have been shown to be efficiently cross-reactive with epitope variants [19]. We hypothesized that the individuals with HLA-B*35-Px alleles, associated with rapid disease progression, would have limited recognition of epitope variants. Thus, we predicted that HLA-B*35 -Py+ individuals would exhibit T-cell responses to variant peptides more frequently than individuals with HLA-B*35-Px alleles. Naturally occurring variants of both epitopes were tested: TVLDVGDADF, TILDVGDAY, and IPLDEDFRKY. HLA-B*35-Py+ individuals never recognized the variant epitope alone at any of the time points tested. Unexpectedly, all the HLA-B*35-Px+ individuals made responses to all the peptides tested, and only the HLA-B*35-Px+ individuals were able to respond to variants in the absence of a wild-type response, and no evidence for variant epitope viruses. We have thus named the responses to these epitopes “vestigial” CTL epitopes, as the responses are made to sequences that appear to be no longer present.

Due to the non-standard motifs of these epitopes, HLA tetramers could not be made and therefore individual CD8+ T cell populations could not be studied with this technology. This leaves open the question of whether the responses to the variant epitopes, made in the absence of a consensus epitope response, are unique responses specifically targeting the variant epitopes or cross-reactive responses that at these time points did not respond to the consensus epitope. Without variant viruses within the viral population to drive the variant-specific responses, it is intriguing to speculate on how specific responses could be generated and maintained. One possibility is that these variant epitopes are heteroclite [20]; capable of stimulating CD8+ T cells to a greater magnitude than the consensus epitope against which they were originally generated. Since the two HLA-B*35 allele subtypes differ in their binding grooves, they may present epitopes in different orientations. Thus, at the time points where only responses to the variant (vestigial) epitopes are seen, the greater stimulus provided by vestigial epitopes create a shifting field [21,22,23].

This unusual phenomenon demonstrates a distinct difference between the HLA-B*35 alleles associated with rapid progression and those that are not. What role this plays in disease progression remains to be elucidated. Here, we provide the first evidence to our knowledge, of a difference in CD8+ T cell responses restricted to the two HLA-B*35 allele subtypes.

Table 2. HIV-1 Pol Sequencing of HLA-B*35 -Px allele expressing individuals.

| Study ID | Estimate weeks Post Infection | TVLDVGDAY | VPLDEDFRKY |
|----------|-------------------------------|------------|-------------|
| Subject 1 | 653                           | ....       | K           |
| Subject 2 | 92                            | ....       | KX          |
| Subject 3 | 182                           | ....       | N           |
| Subject 4 | 10                            | 134        | N           |
| Subject 5 | 30                            | 13         | K           |
| Subject 6 | 10                            | 37         | X           |
| Subject 7 | 61                            | 61         | X           |

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Author Contributions
Conceived and designed the experiments: CW KEG RBJ DAM GS TJL MAO ACK FMH DFN. Performed the experiments: CW KEG RBJ DAM TJL MAO ACK. Analyzed the data: CW KEG RBJ DAM GS TJL MAO ACK FMH DFN. Contributed reagents/materials/analysis tools: GS.
Wrote the paper: CW KEG RBJ DAM GS TJL MAO ACK FMH DFN.

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