Functional Avidity and IL-2/Perforin Production Is Linked to the Emergence of Mutations within HLA-B*5701–Restricted Epitopes and HIV-1 Disease Progression

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Functional Avidity and IL-2/Perforin Production Is Linked to the Emergence of Mutations within HLA-B*5701–Restricted Epitopes and HIV-1 Disease Progression

Marcus Buggert,* Melissa M. Norström,* Marco Salemi,†,‡ Frederick M. Hecht,§ and Annika C. Karlsson*

Viral escape from HIV–specific CD8+ T cells has been demonstrated in numerous studies previously. However, the qualitative features driving the emergence of mutations within epitopes are still unclear. In this study, we aimed to distinguish whether specific functional characteristics of HLA-B*5701–restricted CD8+ T cells influence the emergence of mutations in high-risk progressors (HRPs) versus low-risk progressors (LRPs). Single-genome sequencing was performed to detect viral mutations (variants) within seven HLA-B*5701–restricted epitopes in Gag (n = 4) and Nef (n = 3) in six untreated HLA-B*5701 subjects followed from early infection up to 7 y. Several well-characterized effector markers (IFN-γ, IL-2, MIP-1β, TNF, CD107a, and perforin) were identified by flow cytometry following autologous (initial and emerging variant/s) epitope stimulations. This study demonstrates that specific functional attributes may facilitate the outgrowth of mutations within HLA-B*5701–restricted epitopes. A significantly lower fraction of IL-2–producing cells and a decrease in functional avidity and polyfunctional sensitivity were demonstrated in emerging epitope variants compared with the initial autologous epitopes. Interestingly, the HRPs mainly drove these differences, whereas the LRPs maintained a directed and maintained functional response against emerging epitope variants. In addition, LRPs induced improved cell-cycle progression and perforin upregulation after autologous and emerging epitope variant stimulations in contrast to HRPs. The maintained quantitative and qualitative features of the CD8+ T cell responses in LRPs toward emerging epitope variants provide insights into why HLA-B*5701 subjects have different risks of HIV-1 disease progression. The Journal of Immunology, 2014, 192: 4685–4696.

CD8+ T cells are critical in the immune control of HIV-1 infection. Epitope mutations that evade these immune responses can become fixed in the viral population as a result of the selective pressure from HIV-specific CD8+ T cells (1–5). Viral mutations can have an impact on peptide–MHC class I (MHC I; pMHC)–TCR interactions (6, 7), binding of the peptide to the MHC I molecules (5, 8–10), and the intracellular processing of the viral peptides (11–13). Several mutations completely abrogate an epitope-specific CD8+ T cell response, whereas others are cross-recognized by the TCR of available T cell clones or induce recruitment of newly developed T cell clonotypes (14). However, the exact mechanisms driving the fixation of mutations within epitopes to which CD8+ T cell responses are directed remain elusive.

The factors that constitute an effective HIV-specific CD8+ T cell response are still debated, but differences in the functional T cell characteristics and specificities are likely to influence the efficacy. It has previously been described that both cytolytic and non-cytolytic antiviral effects are associated with the rate of HIV-1 disease progression (15–19). Other studies have shown associations between polyfunctionality and viral control (20, 21). Qualitative features surely represent an important part of an effective immune response, but most of the data presented so far have been generated in cross-sectional studies that do not include responses to individual epitope variants presented by a single HLA allele.

The rate of progression in untreated HIV-1–infected subjects varies substantially among individuals. Factors that have been linked to the predicted course of the infection include clinical, virological, and immunological parameters. In HIV infection, HLA-B*57 is the most consistent host factor that has an impact on the viral load set point and associated with a better prognosis in HIV-1 infection (22–28). Nevertheless, not all subjects carrying protective HLA-B*57 alleles have a slow progression rate. The underlying mechanisms defining the rate of disease progression are not fully understood but likely involve both virological and immunological characteristics (29). As different HLA-B*57 alleles have similar peptide-binding motifs, they are frequently grouped together when studying qualitative differences of the CD8+ T cell responses and/or their association to clinical outcome. However, small genetic differences between the HLA-B*57 alleles and the closely related HLA-B*5801 allele have been proven to have an
impact on the immunogenicity, ability to select for viral mutations, and control viral replication in an HIV subtype C–infected cohort in South Africa (10). To avoid the impact of genetic differences in the analysis of functional differences between initial and emerging epitope variants, this study was restricted to HIV subtype B–infected individuals carrying the protective HLA-B*5701 allele (27).

We have previously demonstrated that subjects with HLA-B*5701 had a more robust polyfunctional Gag-specific CD8+ T cell response, coupled with higher IL-2 production in early infection if their CD4+ T cell count was >750 cells/mm3 at baseline (29). The immunological profile in these subjects was coupled with a lower genetic diversity and more constrained mutational profile in the gag p24 region compared with subjects with a lower CD4+ T cell count at baseline (median ~13 wk postinfection). In this study, we investigated whether the initial autologous versus corresponding major and minor viral variants of HLA-B*5701–restricted epitopes in the Gag and Nef regions revealed any functional differences prior to the emergence of mutations. The analysis was restricted to epitope variants recognized by the HIV-specific CD8+ T cells. By employing this design, functional features of the HLA-B*5701–restricted CD8+ T cell responses were traced to the emergence of mutations and coupled to the risk of disease progression.

**Materials and Methods**

**Study subjects**

Six HLA-B*5701 male patients infected with HIV subtype B were recruited from the Options cohort, at the University of California, San Francisco (30), and followed from early infection (10–18 wk) up to 7 y. Based on baseline CD4+ T cell count, three subjects (P1–P3) were classified as high-risk progressors (HRPs; <750 cells/mm3) and three (P4–P6) as low-risk progressors (LRPs; >750 cells/mm3) (29, 31). The University of California, San Francisco Committee on Human Research and the Regional Ethical Council in Stockholm, Sweden (2008/1099-31) approved this study, and all patients provided written informed consent.

**RNA extraction, cDNA synthesis, and PCR amplification**

RNA extraction and HIV-1 gag p24 single-genome sequencing of longitudinal plasma samples (29) were performed as previously described. The nef sequences were obtained by performing cDNA synthesis using the ThermoScript RT-PCR System (Invitrogen) with gene-specific primer 5'-CCACTACGGCRRAACG-3' (HXB2 nt position 9523–9540) (0.1 μM). Designed subtype B–specific primers were selected to amplify the HIV-1 region of nef using a nested PCR with Platinum Taq DNA Polymerase (Invitrogen). First-round PCRs were used forward primer 5'-CATACCTA-

**Statistical analysis**

All statistical tests are described in corresponding figure legends. Statistical comparisons between two groups of individuals were performed using GraphPad Prism 5.0 software (GraphPad), and pie charts were analyzed using SPICE version 5.21 (34).

**Results**

Six HLA-B*5701 subjects, three HRPs and three LRPs, based on CD4+ T cell count at baseline were followed longitudinally from early infection up to 7 y (29). Between 18 and 34 gag p24 epitope sequences (details given in Ref. 29) and 1–29 nef epitope sequences were obtained by single-genome sequencing from each time point (Table I). CD8+ T cell responses against HLA-B*5701–restricted epitopes (i.e., peptides matching both the autologous founder virus sequence and emerging sequence variants within the epitope regions) were measured at three different time points for each subject (Table I).

**Assessment of polyfunctionality and magnitude against autologous HLA-B*5701–restricted epitopes**

We first sought to determine whether several functional parameters (IFN-γ, IL-2, MIP-1β, and perforin) of CD8+ T cells were linked to HLA-B*5701–restricted epitope escape. The CD8+ T cell functionality against conserved epitopes, for which no mutations occurred between two time intervals (n = 29), was therefore compared with responses toward epitopes for which mutations emerged (n = 12) between the same time intervals. We observed trends toward elevated magnitude (p = 0.07) and greater IL-2 (p = 0.035) expression in the CD8+ T cell responses against mutating (n = 12) epitopes compared with the conserved (n = 29) epitopes (Fig. 1B). Overall however, neither mono- nor polyfunctional features of epitope-specific CD8+ T cells were significantly associated with protection against emergence of mutations in HLA-B*5701–restricted epitopes (p = 0.72; Fig. 1C).

We further plotted the magnitude of the responses against all epitopes corresponding to the initial (first time point) autologous sequence in all subjects over time. The subjects were divided into HRPs (Fig. 2A) and LRPs (Fig. 2B). The LRPs had in general an average higher magnitude of the responses against the immunodo-

**PBMC stimulation and flow cytometry analysis**

Optimal peptides (9–11-mers) corresponding to autologous and variant HLA-B*5701–restricted epitopes in the HIV-1 Gag p24 (n = 4) and Nef (n = 3) region were used to measure the immunogenicity by CD8+ T cells. The protocols for PBMC stimulation and flow cytometry staining have previously been described in detail (29, 33). Briefly, PBMCs were thawed, rested in media containing DNase (Sigma-Aldrich), and supplemented with 2 μg/ml of optimal peptides. For the peptide dilution experiments, additional peptide concentrations ranging from 10−6 to 10−8 μg/ml was used. The cells were incubated with peptides for 6–10 h in the short-term cultures together with brefeldin A (Sigma-Aldrich). When degranulation was measured, anti-CD107a PE-CF594 (clone H4A3; BD Biosciences) was added already during the stimulation period together with monensin (BD Biosciences). For the long-term culture experiments, cells were incubated with 2 μg/ml peptides for 5 d and then restimulated with the same peptide concentration together with brefeldin A, anti-CD107a, and monensin.

The PBMCs were washed and stained with the following extracellular markers for different panels: anti-CD14 V500 (clone M5E2), anti-CD19 V500 (clone B43), and anti–HLA-DR BV605 (clone G46; BD Biosciences); anti–programmed cell death-1 (PD-1) BV421 (clone EH12.21H7; BioLegend); and LIVE/DEAD Fixable Aqua or Violet dyes (Life Technologies) to discriminate dead cells.

Cells were permeabilized and fixed with the Cytofix/Cytoperm kit (BD Biosciences) for assessment of functional characteristics, whereas the FOXP3 staining kit (eBioscience) was used to detect intranuclear proteins (Ki-67). The intracellular markers that were used for different flow panels included: anti-CD3 allophycocyanin-H7 (clone SK7), anti-CD4 V500 (clone RPA-T4), anti-CD8 PerCP (clone SK1), anti–IFN-γ AF700 and FITC (clone B27), anti–IL-2 allophycocyanin (clone MQ1-17H12), anti-TNF PE-Cy7 and FITC (clone MAb11), anti–MIP-1β PE-Cy7 (clone D21-1351), and anti–Ki-67 FITC (clone b56; BD Biosciences); anti-perforin PE (clone D48; BioLegend and Tepnel); anti-TNF eFluor450 (clone MAb11; eBioscience); and anti-CD8 Qd655 (clone 3B5) and anti-CD4 PE-Cy5.5 (clone S3.5; Life Technologies).

Cells were then washed, fixated, and run on a four-laser LSR Fortessa or Canto II (BD Biosciences). Apoptotic events were identified using FlowJo 8.8.7 (Tree Star) for gating analyses. Most manual gatings were based on fluorescence-minus-one gating strategies. A typical T cell gating strategy to distinguish CD8+ T cell polyfunctionality is shown in Fig. 1A, for which a response was considered positive if the frequency of IFN-γ–producing cells was >0.05% of total CD8+ T cells after background reduction and twice the negative background.
<table>
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<tr>
<th>ID</th>
<th>WPI</th>
<th>Gag ISW9 (147-155)</th>
<th>Gag KF11 (162-172)</th>
<th>Gag TW10 (240-249)</th>
<th>Gag QW9 (308-316)</th>
<th>Nef KL10 (82-91)</th>
<th>Nef HQ10 (116-125)</th>
<th>YT9 (120-128)</th>
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HXB2 is used as the reference sequence for the HLA-B*57–restricted epitopes in Gag p24 (ISW9, KF11, TW10, and QW9) and Nef (KL10, HQ10, and YT9).

*aPatient identity.

*bWeeks postinfection (WPI). Sequences and immunological data were obtained from plasma and PBMC samples, respectively, from the same time point in the majority of the patients. The numbers in parentheses are PBMC samples taken at a different time point compared with the plasma samples.

cThe numbers of sequenced single nef genomes are indicated after each sequence.

dHRPs: P1–P3; LRP5: P4–P6. The epitopes corresponding to the major viral population at each time point are marked in boldface. Two epitopes (in boldface) at the same time point correspond to a 50/50 proportion of the respective variants. The epitopes with a positive CD8+ T cell response are shaded in gray.

eSequence data for the Nef region were not obtained for all time points.
minant HLA-B*5701–restricted epitopes (>1% of the CD8+ T cells) at all the time points tested (Fig. 2C). As previously described, the depicted early immunodominant TW10 response in P1 and P5 was associated with the development of the TW10-3N and TW10-3N-9A escape mutations (Fig. 2D, 2E, respectively) (6, 35, 36). Surprisingly, epitopes in which no mutations emerged during the entire study period showed the highest magnitudes at all tested time points for most individuals. For instance, KF11 was measurable for all patients and had the highest magnitude at numerous time points for one HRP (P3) and all three LRPs (P4, P5, and P6). A response toward KF11 has previously been shown to provide virological control (37). In this study, the KF11 epitope variant (KF11_7I) was only detected in two individuals (P1 and P6), of which one (P1) had a low response against the autologous sequence. These results in-

**FIGURE 1.** Functional characterization of HLA-B*5701–restricted epitopes developing mutations or not. (A) FACS plots illustrating the gating scheme to distinguish viable CD8+ T cells and a typical IFN-γ response together with the other functional markers. (B) Scatter plots illustrating the magnitude and average production of IFN-γ, IL-2, MIP-1β, and perforin (all time points) for all autologous epitopes developing mutations (gray) or not (black). Significant differences between the groups were analyzed using unpaired t tests, where upper and lower whiskers show SEM. (C) Pie charts demonstrating the functional diversity (for all time points) of all autologous epitope-specific responses between epitopes in which mutations emerged or not. Permutation tests were conducted to compare significant differences between the pie charts. In the bottom row, the functional combinations (for all time points) of all mutating (gray dots) or nonmutating (black dots) autologous epitope-specific responses are depicted. Mean and SEM are provided and Student t test in SPICE was used to assess significant differences between the groups.
FIGURE 2. Frequencies of autologous and emerging epitope variant HLA-B*5701–restricted responses over time. The frequencies of all Gag (n = 4) and Nef (n = 3) autologous HLA-B*5701–restricted responses are depicted over time in HRP s (A) and LRPs (B). The y-axis illustrates the epitope-specific frequencies of total CD8+ T cells, where the colors represent the magnitude of each initial autologous HLA-B*5701–restricted responses. The dashed lines depict the responses against those epitopes that eventually developed mutations. The x-axis represents the estimated weeks postinfection as the responses were measured. (C) Unpaired comparisons between the magnitude of all autologous epitope-specific responses between HRP s (red) and LRPs (blue) at time intervals 1–3. The p values from unpaired t tests are provided, and mean and SEM are depicted for the bars. The frequency of all autologous and emerging epitope-variant HLA-B*5701–restricted responses are depicted over time in HRP s (D) and LRPs (E). The dashed lines represent the HIV-1 viral load (red) and CD4 count (black) over time, where the CD4 count is depicted on the far left y-axis and HIV-1 viral load (VL) on each subjects’ right y-axis. The left y-axis for each subject represents the epitope-specific (spec) frequencies of total CD8+ T cells, where the colors demonstrate the magnitude of each autologous and corresponding emerging epitope variant–restricted response. The colored arrows under the x-axis clarify the time points for when and which autologous and emerging epitope variants were tested for immunogenicity.
dicate that the magnitude of epitope-specific responses by themselves are not a complete predictive factor for emergence of viral mutations within HLA-B*5701–restricted epitopes.

Functional diversity of the CD8+ T cell response against autologous and emerging HLA-B*5701–restricted epitope variants

We next investigated in depth the functional patterns of the CD8+ T cell responses against the epitopes in which mutations emerged during the infection. In these subsequent analyses, the epitope variants corresponding to the autologous founder virus sequence for the tested time point were defined as the “autologous epitopes” (n = 12). Epitope variants that predominated (>50% of viral variants) at the subsequent time point were entitled “emerging epitope variants” (n = 10), whereas minority variants (<50% of viral variants) at the subsequent time interval were entitled “minor epitope variants” (n = 7). All of these epitopes were tested and compared directly before the viral mutations emerged, and the magnitudes and time points for

FIGURE 3. The outgrowth of emerging epitope variants is associated with decreased pMHC avidity and IL-2 production in HLA-B*5701 subjects. (A) Paired comparisons of the magnitudes between autologous and emerging variant epitope-specific responses. The p value from a paired r test is provided. (B) pMHC avidity comparison between autologous and emerging epitope variant-specific responses at five different peptide concentrations. The y-axis depicts the fraction of the response from the first peptide concentration (1 μg/ml) and subsequent peptide concentrations. The bars illustrate the mean (and SEM) fraction of the response for autologous (gray bars) and emerging epitope variants (white bars), whereas the sigmoidal curves are overlaid for autologous (solid line) and emerging epitope variants (dashed line). The p values were calculated based on the area under the curve for each epitope-specific response and then compared using paired t tests. (C) Pie charts illustrating the functional diversity of 10 autologous and emerging variant epitope-specific responses. Permutation tests were conducted to compare significant differences between the pie charts. (D) Paired comparisons between autologous and emerging variant epitope-specific responses showing the average production of IFN-γ, IL-2, MIP-1β, and perforin. The p values from paired t tests are provided. (E) Correlation between IL-2 and perforin production for all autologous and emerging variant epitope-specific responses using the Spearman nonparametric test. (F) Paired comparisons between autologous and minor variant epitope-specific responses for the average production of IL-2. The p values from paired t tests are provided. (G) Scatter plots demonstrating the average (mean) production of IL-2 in response to emerging and minor epitope variants. Each symbol of the dots represents different epitopes and corresponding variants. The p values were obtained from unpaired t tests.
which indicate that these emerging mutations truly represent epi- 
adavidity than the emerging epitope variants (  
and we therefore conducted pMHC avidity experiments. In these 
mutations occurred (  
differed, but surprisingly found that the response was similar before 
response against the autologous and emerging epitope variants  
were performed under saturated peptide concentrations (2  
were able to preserve the response against the emerging epitope 
variants over time (Fig. 2E).

We further determined whether the magnitude of the CD8+ T cell 
response against the autologous and emerging epitope variants 
differed, but surprisingly found that the response was similar before 
mutations occurred (p = 0.15; Fig. 3A). However, these experiments 
were performed under saturated peptide concentrations (2 μg/ml), 
and we therefore conducted pMHC avidity experiments. In these 
analyses, the autologous epitopes displayed higher ex vivo pMHC 
avidity than the emerging epitope variants (p = 0.05; Fig. 3B), 
which indicate that these emerging mutations truly represent epi- 
tope escape variants from CD8+ T cell responses. Furthermore, the 
combined functional characteristics (IFN-γ, IL-2, MIP-1β, and 
perforin) of autologous and emerging epitope variant-specific CD8+ 
T cell responses were compared, but no significant differences in 
polyfunctionality were detected (p = 0.14; Fig. 3C, Supplemental 
Fig. 1A). However, the frequencies of cells with three (including 
IL-2) to four functions was surprisingly greater for the autologous 
compared with the emerging epitope variants (Table II). By deci- 
phering the frequencies of specific functional characteristics, we 
found that higher fractions of IL-2 (p = 0.016) but lower direct ex 
vivo perforin (p = 0.015) production by CD8+ T cells were present 
against the autologous epitopes (Fig. 3D). A statistically significant 
difference was also observed for IFN-γ production (p = 0.049), but 
not for MIP-1β expression (p = 0.86; Fig. 3D). In conjunction to 
these results, an inverse correlation was found between IL-2 and 
perforin production when assessing all autologous and emerging 
epitope-specific responses (r = -0.47, p = 0.035; Fig. 3E).

We next evaluated whether fluctuating IL-2 production could 
be detected between autologous and corresponding minor epitope 
variants. No significant differences for IL-2 production (p = 0.55; 
Fig. 3G) or any other marker was found (Supplemental Fig. 1B). 
However, by comparing epitope-specific emerging and minor 
variant responses, significantly lower IL-2 (p = 0.048) production 
was found for the emerging variants, despite the small number of 
available data points (Fig. 3H). No other markers showed these 
differences (Supplemental Fig. 1C).

Additional peptide dilution experiments were conducted to verify 
whether differences could be distinguished in terms of polyfunctional 
sensitivity using different peptide concentrations. In addition to IFN-γ, 
IL-2, MIP-1β, and perforin, TNF and CD107a expression were 
also measured in these analyses. The autologous epitope-specific 
CD8+ T cell responses revealed increased polyfunctional character- 
istics (Fig. 4A) and sensitivity (p = 0.009; Fig. 4B) at lower peptide 
concentrations. As previously described (38), most functional 
markers showed a decrease in median fluorescence intensity and 
percentage as a consequence of lower peptide concentrations 
(Fig. 4C). Nevertheless, all functional markers except TNF declined 
more rapidly at lower peptide concentrations in response to the 
emerging epitope variants (p < 0.05) compared with the response 
against autologous epitopes (Fig. 4D). Interestingly, the fraction of 
perforin-producing cells increased as a consequence of lower peptide 
concentrations (Fig. 4C, 4D), particularly against autologous epitopes, 
and was potentially due to the downregulation of CD8 mole- 
cules at higher peptide concentrations. We also assessed the median 
fluorescence intensity of PD-1 and HLA-DR in the peptide-dilution 
analyses to determine whether the level of exhaustion and activation 
of the CD8+ T cell repertoire against autologous and emerging 
epitope variants differed. However, the intensity of neither marker 
changed significantly (p > 0.05) after peptide dilutions or differed 
between the groups (data not shown). Despite that polyfunctionality 
declined, these functional results were primarily driven by the lack 
of response against the emerging epitope variants at lower peptide 
concentrations in specific subjects.

**Table II. Frequency of CD8+ T cells with specific functional characteristics targeting autologous versus major epitope variants**

<table>
<thead>
<tr>
<th>No. of Functions</th>
<th>Functional Combination</th>
<th>Autologous Epitope Variants</th>
<th>Major Epitope Variants</th>
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<td>4</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>1.00 (0.04–2.04)</td>
<td>0.46 (0.34–0.89)</td>
</tr>
<tr>
<td>3</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>20.63 (12.44–28.81)</td>
<td>15.72 (6.93–24.51)</td>
</tr>
<tr>
<td>3</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>0.30 (–0.06 to 0.66)</td>
<td>0.12 (–0.09 to 0.34)</td>
</tr>
<tr>
<td>3</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>8.56 (2.85–13.86)</td>
<td>12.45 (0.16–24.73)</td>
</tr>
<tr>
<td>3</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>0.14 (–0.17 to 0.44)</td>
<td>0.11 (–0.14 to 0.37)</td>
</tr>
<tr>
<td>2</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>2.31 (0.98–3.64)</td>
<td>1.88 (0.79–2.97)</td>
</tr>
<tr>
<td>2</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>49.93 (42.2–57.65)</td>
<td>40.11 (29.24–50.97)</td>
</tr>
<tr>
<td>2</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>1.36 (–0.56 to 3.29)</td>
<td>2.27 (–0.37 to 4.91)</td>
</tr>
<tr>
<td>2</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>0</td>
<td>0.11 (–0.14 to 0.35)</td>
</tr>
<tr>
<td>2</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>1.73 (0.04–3.42)</td>
<td>1.8 (–0.26 to 3.86)</td>
</tr>
<tr>
<td>2</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>1.40 (–0.75 to 3.55)</td>
<td>8.24 (1.58–14.89)</td>
</tr>
<tr>
<td>1</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>8.18 (3.56–12.8)</td>
<td>7.18 (4.80–10.76)</td>
</tr>
<tr>
<td>1</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>3.14 (0.25–6.04)</td>
<td>4.14 (–3.42 to 11.71)</td>
</tr>
<tr>
<td>1</td>
<td>IFN-γ, IL-2, MIP-1β, perforin</td>
<td>1.53 (–0.18 to 3.24)</td>
<td>4.80 (–2.97 to 12.58)</td>
</tr>
</tbody>
</table>

Data are mean (95% confidence interval).
epitope variants \( (p = 0.87) \), whereas the HRPs lost the response against these variants at lower peptide concentrations \( (p = 0.003, \text{Fig. 5D}). \)

In conclusion, these data demonstrate that LRPs maintain primarily IL-2 production, but also polyfunctionality, at lower peptide concentrations and have a higher magnitude and pMHC avidity toward emerging epitope variants compared with HRPs.

**Relationship between ex vivo IL-2 production and perforin upregulation after long-term epitope stimulations**

Individuals with protective MHC I alleles after vaccination (39) and those controlling HIV-1 replication (18) have previously been shown to induce supreme CD8\(^+\) T cell proliferation and consequently upregulation of cytolytic functions. We therefore elucidated whether autocrine (ex vivo) IL-2 expression might induce improved cell cycle progression and upregulation of perforin in long-term cultures. PBMCs were incubated with the autologous and emerging epitope variants for 3 d to assess the expression of Ki-67 together with the other functional markers (Fig. 6A). Most of the autologous and emerging epitope variant-specific cells in LRPs were efficient to upregulate Ki-67, whereas HRPs showed limited cell-cycle progression \( (p < 0.001; \text{Fig 6A, 6B}). \) Importantly, the frequency of baseline ex vivo frequencies of IL-2-producing CD8\(^+\) T cells was highly correlated with Ki-67 \( (p < 0.001; r = 0.82), \text{CD}107\alpha \) \( (p = 0.004; r = 0.64) \) and IFN-\( \gamma \) \( (p < 0.001; r = 0.84) \) expression following long-term incubations with
autologous and emerging epitope variants (Fig. 6C). The overall ex vivo magnitude of the epitope-specific responses was also correlated with the Ki-67 upregulation (p = 0.003; r = 0.64), but IL-2 was a better predictor of cell proliferation. TNF, IL-2, and MIP-1β production were poorly expressed in cells after expanding the autologous and emerging epitope variant–specific cells in cultures (Fig. 6A), indicating that the CD8+ T cell clones exhibited late effector maturity. The majority of the Ki-67+ cells also possessed enriched levels of perforin (p < 0.001; Fig. 6D), and likewise, IL-2 production ex vivo was associated with Ki-67+ perforin+ production after 3 d incubations (p < 0.001; r = 0.84; Fig. 6E). These data suggest that LRPs maintain the magnitude and autocrine IL-2 secretion against the autologous and emerging epitope variants, leading to increased T cell turnover and hence

FIGURE 5. HRPs elicit an inverse ex vivo IL-2/perforin production, lower magnitude, and functional avidity against autologous and emerging epitope variants. Scatter plots demonstrating the mean production of IFN-γ, IL-2, MIP-1β, and perforin in response to autologous epitopes and emerging epitope variants for LRPs (A) and HRPs (B). Each symbol of the dots represents different epitopes and corresponding variants. The right plots illustrate the polyfunctional sensitivity between the autologous (solid line) and emerging epitope variants (dashed line), where the symbols illustrate the average number of functions (and SEM) in each group at different peptide concentrations. (C) Magnitude comparisons (mean and SEM) between HRPs and LRPs for autologous and emerging epitope variant-specific responses. The p values from unpaired t tests are provided. (D) pMHC avidity analysis in HRPs and LRPs, where the autologous (solid line) and emerging epitope variants (dashed line) are depicted in the sigmoidal curves. The symbols illustrate the average number of functions in each group at different peptide concentrations. The p values were calculated based on the area under the curve for each epitope-specific response and then compared between the groups using paired t tests.
Discussion
HIV-1–specific CD8+ T cell responses represents a major factor predicting the outcome of HIV-1 disease progression. Although neither the STEP trial or the RV144 trial showed evidence of CD8+ T cell responses affecting set point viremia or protection in the vaccine, recent studies have demonstrated that vector-induced T cell responses can limit HIV-1 RNA level in subjects carrying protective HLA alleles (B-27, 57, and 5801) (40) as well as limit SIV replication and possibly clear the infection (41). Thus, it still remains important to identify correlates of effective CD8+ T cell responses establishing pressure on founder viral sequences of HIV-1. In the current study, HLA-B*5701–restricted CD8+ T cell responses were closely examined from early infection in subjects with different risk of disease progression. This allowed us to characterize the functional features of the CD8+ T cell response generating pressure on the autologous founder virus as identified by emerging mutations within the epitopes.

Polymorphic characteristics have been associated with viral control in the chronic phase of HIV-1 infection (20, 21). However, in cross-sectional settings, the true characteristics of efficient CD8+ T cell responses might be misleading due to other factors exerting pressure on the autologous virus (42). Similar to previous results (43, 44), no statistically associations were found between CD8+ T cell polyfunctionality and the outgrowth of HLA-B*5701–restricted epitope mutants. However, by assessing the polyfunctional sensitivity under conditions of lower peptide concentrations, clear differences were found between response against autologous and emerging variants of the virus. These differences were driven by the HRPs that completely lost the response and functional characteristics against emerging epitope variants under lower peptide concentrations. These results are in agreement with the data from Almeida et al. (21) in HLA-B27 subjects, showing that polyfunctionality is determined by Ag sensitivity and suggest that HLA-B*5701 individuals possessing a maintained functional response against emerging escape variants of HIV-1 might have a lower risk of disease progression (45). Furthermore, our findings are in line with recent data from Pohlmeyer et al. (46) illustrating that HLA-B*57 elite suppressors are able to control the replication of engineered viral escape variants. Whether the determining factor of these diverse features is driven by the TCR repertoire remains to be proven, but public clonotypes have been linked to development of MHC I–restricted escape (14, 47) and elite control in former studies (48).
MHC I–restricted epitope escape has been shown to dramatically reduce the magnitude of the CD8 T cell response (7). Previous studies have also found associations between higher magnitude of T cell responses and rapid escape (43, 49). We identified a similar trend, with higher magnitude responses against mutating epitopes compared with conserved epitopes. In agreement with earlier studies, however, it was demonstrated that HLA-B*5701–restricted epitope variants do not necessarily impact the CD8 T cell magnitude at higher peptide concentrations (50). Nevertheless, by conducting peptide-dilution experiments, it was verified that particularly HLRs had poor pMHC avidity against emerging epitope variants and the magnitude of CD8 T cell responses was significantly higher against autologous and emerging epitope variants in LRs compared with HRs. It was recently revealed that the development of high-avidity cross-reactive KK10–specific CD8 T cell clonotypes contributes to the viral control in HLA-B*2705 study subjects (14), suggesting that the plasticity of the TCR recognizing viral epitope variants may explain the different rates of disease progression in subjects carrying protective HLA alleles.

By comparing the functional profiles we found that IL-2 production declined, whereas perforin expression increased in response to the mutated epitope variants compared with the autologous epitopes for HRs, but not for LRs. This indicates that the magnitude of CD8 T cell responses, as well as maintaining a IL-2 production toward both autologous and emerging epitope variants, may be linked with lower risk to progress toward AIDS. An inverse correlation between ex vivo IL-2 and perforin production for virus-specific CD8 T cells has previously been demonstrated (51, 52), but not in the context of viral escape. Neither has it been shown that functional differences exist between emerging and minor epitope variants that may have an impact on the selection of mutations. Noncytolytic CD8 T cell responses have been associated with viral escape (53) and may be an important influence on set point viremia (54, 55). In agreement with these studies, IL-2–producing CD8 T cells might thus represent a noncytolytic mechanism that drives fixation of epitope mutations. Another potential explanation could be that autocrine IL-2 production is linked to increased proliferation and cytolytic gene expression (16, 56). In this study, we verified these characteristics and showed that LRs were able to go through more extensive cell-cycle progression and perforin upregulation after both autologous and emerging epitope variant stimulations. Therefore, the data suggest that IL-2 and perforin are linked to each other although different memory CD8 T cell subsets usually express these functions. Thus, noncytolytic and cytolytic features most probably cooperate to induce the pressure on the founder virus.

An interesting observation was that CD8 T cell responses against minor epitope variant displayed a significantly higher IL-2 production than emerging epitope variants. This indicates that some minor viral populations might not grow in size due to the pressure from IL-2–producing cells. However, these data were generated with a small number of available data points. The small study cohort is a general limitation, reflecting the restricted possibilities of obtaining unique HLA-B*5701 patient samples longitudinally from early infection. Also, the study includes no samples from acute phase (Fiebig stage I/II) of infection and only HLA-B*5701–restricted epitopes were studied. Nevertheless, significant differences were still observed between diverse variables and the two groups of patients.

In summary, these results indicate that HLA-B*5701 subjects that have a lower risk of HIV-1 disease progression maintain the functional avidity and possess higher percentage of IL-2 producing CD8 T cells toward emerging epitope variants, compared with subjects with higher risk of progression. These findings suggest that the magnitude and cooperation between noncytolytic and cytolytic CD8 T cell responses exert pressure on autologous HLA-B*5701–restricted epitopes, which might be of importance in the future design of anti-HIV-1 therapeutic Ags.

Disclosures
The authors have no financial conflicts of interest.

References
expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. PLoS Pathog. 6: e1000917.