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In Vivo Priming Of HIV-Specific CTLs Determines Selective Cross-Reactive Immune Responses Against Poorly Immunogenic HIV-Natural Variants

Alexandre Boissonnas,* Olivia Bonduelle,* Ariane Antzack,* Yu-Chun Lone,† Cécile Gache,* Patrice Debre,* Brigitte Autran,* and Behazine Combadière‡*‡

Degeneracy of the TCR repertoire might allow for cross-recognition of epitope variants. However, it is unclear how the first encounter with HIV Ags determines recognition of emerging epitope variants. This question remains crucial in the choice of HIV vaccine sequences given the virus variability. In this study, we individualized nine natural mutations within an HIV-Nef180–189 epitope selected from several HIV-infected individuals. These variants of Nef180–189 sequence display slightly different HLA-A2 binding capacities and stabilities and we have shown that only two induced a strong CTL response in vivo in HLA-A2 transgenic mice after a single injection. We demonstrated that priming with these two immunogenic variants generated a specific pattern of cross-reactive CTL repertoire directed against poorly immunogenic peptides. Thus, the range of peptide variants recognized by HIV-specific CTL depends upon the Ag encountered during primary immunization of CD8 lymphocytes. These data have practical implications in the development of cross-reactive vaccines against HIV. The Journal of Immunology, 2002, 169: 3694–3699.

One fundamental question is how the host can develop and maintain HIV-specific immune response against newly emerging viral variants after vaccination or during HIV infection. The high variability of HIV limits vaccine efficacy by allowing the virus to escape the host immune responses (1). Such a major obstacle has prompted development of vaccines capable of eliciting multiple epitope-specific CTL directed against conserved regions of the virus (2). One relative control for SIV infection has been recently obtained with a DNA-based vaccine that induced a strong but relatively narrow CD8 response directed against a few immunodominant epitopes (3). These epitope-based vaccines have represented a powerful approach for stimulating the immune response against viral Ags. These successes have been compromised by the emergence of virus variants escaping CTL recognition that eventually led to disease progression (1). Variation within an epitope may lead to total or partial loss of functional recognition by CTL. Substitutions occurring at key anchor motifs in epitope sequences can alter the peptide affinity for MHC binding and thereby epitope presentation (4). Other variations occurring in central positions can affect the affinity of the MHC-peptide complex for the TCR and alter or abrogate TCR recognition (5–7). These epitope variants can still be presented sufficiently by MHC molecules and be recognized by the host’s immune repertoire, which confers an enormous flexibility to the immune response. A major property of a normal healthy immune system to limit the danger of high virus variability is the degeneracy of the TCR that permits T cell clones to cross-react with peptide analogs. Such degeneracy has been shown in various models of influenza-A virus infection or autoimmune diseases (8–11). In HIV infection, the question thus raised is whether primary expansion of HIV-specific immune responses would confer cross-recognition and protection against newly emerging viral variants. This question remains crucial for HIV vaccine strategies.

During HIV infection, early immune response is characterized by an oligoclonal expansion of HIV-specific CD8 lymphocytes directed against a limited number of epitopes and rapidly develops into a broad and diverse HIV-specific CTL response (12). In addition, the emergence of new virus variants could be due to the selective pressure mediated by CTL (13). We have previously shown the constant capacity of adaptation of the immune repertoire of CTL specific for the emerging HIV-Nef variants (14). The amplification of viral variants is followed by an expansion of Nef variant-specific CTL, leading in some cases to the disappearance of the virus variant (14). It is unclear whether variant-specific CTL results from either the emergence of new CTL clones or the cross-reactivity of the anti-HIV CD8 repertoire. The cross-recognition of viral epitopes has been demonstrated in a macaque model of SIV infection and in children born from infected mothers (1, 15). This question becomes of central importance for vaccine strategies that should be capable of generating not only a diverse HIV-specific CD8 response, but also a broadly cross-reactive repertoire. Several groups have demonstrated the utility of HLA transgenic mice for the purpose of epitope identification (16), and immunogenicity testing, as well as vaccine development (17–19). To assess the consequence of exposure of naturally occurring HIV variants on CD8 cell reactivity, we analyzed the effect of primary CTL expansion on the subsequent response against HIV-Nef180–189 natural variants, in HLA-A2.01 transgenic mice.
Materials and Methods

Mice

The β2-microglobulin<sup>60</sup>, H2-D<sup>107</sup>, H2-K<sup>107</sup> were made transgenic for a chimeric HLA-A*0201 monochain, the HHDI molecule in which the human β<sub>2</sub>-microglobulin molecule was linked to the N terminus of a hybrid MHC class-I H chain (16). Homozygous transgenic/doublenegative HHDI, 8- to 10-wk-old female mice (HHDI strain) were used for immunization. This DNA construct has been used to generate RMAS-HHD stable transfectant cell line (16).

Peptides

The HIV-Nef<sub>180–189</sub> LAI sequence is VLEWRFDSRL. Natural variants were isolated from HIV-infected individuals as described previously (14). HIV-Nef<sub>180–189</sub> variant sequences are described in Table I. Three peptides were used as HLA-A2 restricted epitopes: influenza matrix (M) <sup>55–66</sup> (GILGFVFTL), HIV reverse transcriptase (RT) 309–317 (ILKEPVHGV), and a hepatitis virus epitope F10V (FLPSDYFPSV). All peptides were synthesized by Syntem Laboratory (Nimes, France).

RMAS-HHD cell line

The HHD DNA construct has been used to generate the RMAS-HHDI stable transfectant cell line (a gift of F. Lemennier, Pasteur Institute, Paris, France). Cells were cultured in R-10%: RPMI 1640 with 10% FCS, 2 mM glutamine, 10 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Paisley, U.K.) and 10 µg M-2E (Sigma-Aldrich, St. Louis, MO). Maximum expression of the HHD molecule was expressed on the RMAS-HHDI cell line when cells were incubated 1 h with Nef<sub>180–189</sub> variant peptides at a concentration of 10 µM. In these conditions, MHC-class I molecule expression was stable and similar to control high affinity peptides (M 55–66, RT 309–317, or F10V) as assessed by flow cytometric analysis. HLA-A201 expression was checked by flow cytometric analysis with FITC-conjugated anti-HLA-ABC mAb (Immuno-tech, Marseilles, France) before chromium assay.

Immunizations

Mice were immunized with synthetic peptides (50 µg/mouse) at the base of the tail by s.c. injections with CFA. Splenocytes were harvested 7 days after injection and cultured for 2 days in R-10%. Live cells were separated using Ficoll gradient and suspended with 3% T-STEM culture medium (Collaborative Biomedical Products, Bedford, MA) for an additional 4 days.

Analysis of peptide MHC-class I binding

Peptide binding to soluble HLA-A*0201 was assayed by competition using <sup>125</sup>I-labeled F10V (125<sup>1</sup>I-F10V) (FLPSDYFPSV) (20). The peptide was labeled using chloromine-T catalyzing iodination. Soluble HLA-A201 molecules (5 µg; Y. C. Lone, Pasteur Institute) were incubated for 1 h at room temperature followed by 1 h at 4°C with 4 µM <sup>125</sup>I-F10V and each competitor peptide (400 µM). Unbound peptide was eliminated by ultrafiltration using Microcon 30 (Amicon, Beverly, MA) and extensive washing with PBS. Radioactivity was measured in a gamma spectrophotometer (Gammamatic; Kontron, Zurich, Switzerland). Results are expressed in percent inhibition of HLA-A2/F10V binding.

RMAS-HHD (3 × 10<sup>5</sup>) cells were incubated with 1 µg/ml of β<sub>2</sub>-microglobulin and 50 µM synthetic peptide for 60 min at 37°C. Maximum binding for each peptide was previously determined to be 1 h. Cells were washed extensively and incubated at 37°C with fresh RPMI from 1 to 5 h with an additional 5 µg/ml of brefeldin A to block the cell surface expression of newly synthesized HLA-A2 molecules. Cells were washed at each time point and stained with FITC-conjugated anti-HLA ABC Ab for 20 min (Immuno-tech). The mean of HLA-A2 expression is determined by flow cytometric analysis on FACSCalibur and a CellQuest Pro software (BD Biosciences, San Diego, CA). The dissociation complex (DC<sub>50</sub>) defined as the time required for the loss of 50% of HLA-A2-peptide complexes stabilized at t = 0 was calculated as described elsewhere (19).

Chromium release assay

CTL activity was tested using standard chromium release assay (14). Briefly, target cells used were RMAS-HHDI cells. Target cells were labeled 2 h at 37°C with Na<sup>51</sup>CrO<sub>4</sub> (70 µCi) (Amersham, Les Ulis, France), washed three times in RPMI and 3 × 10<sup>5</sup> cells were incubated in 96-well plates with 50 µM HIV-Nef<sub>180–189</sub> peptides for 1 h at 37°C. Effector cells were added at 100:1 to 5:1 E:T ratios.

Intracellular staining for IFN-γ production

Short-term activated splenocytes from immunized mice were incubated at a 10:1 ratio with RMAS-HHD target cells for 1 h at 37°C with the corresponding peptide (50–0.5 µM). Chloromethylfluorescein diacetate cytoplasmic cell tracer was used to differentiate RMAS-HHD cells from splenocytes. Cytotoxic and target cells were incubated for 2 h at 37°C before the addition of 5 µl of brefeldin A. Cells were harvested after a 16-h incubation at 37°C and washed in PBS twice. Membrane staining of CD8 cells was performed using anti-CD8-Chrome Ab (BD Biosciences). Intracellular IFN-γ detection was performed using anti-IFN-γ PE-labeled Ab (BD Biosciences). Briefly, cells were fixed with paraformaldehyde 4% for 20 min, washed in PBS-FCS-2% 0.1% saponin buffer, and incubated for 20 min in the same buffer with anti-IFN-γ PE labeled Ab.
fluorescent analyses were performed on a FACSCalibur with CellQuest Pro software (BD Biosciences). A total of 3000 events gated on live chloromethylfluorescein diacetate-negative CD8⁻ T cells were acquired.

results and discussion
affinity and stability of natural variants of HLA-A2.01-restricted HIV-Nef₁₈₀–₁₈₉ epitope

We first evaluated the HLA-A2 binding capacities and peptide stabilities of each natural HIV-Nef₁₈₀–₁₈₉ variants isolated from HIV-infected individuals and previously described by our group (14). Amino-acid substitutions on this epitope were mainly observed at positions 3, 5, 6, and 8 outside the major anchor motif in positions 1, 2, and 9 (20–22) (Table I). First, binding capacities to the soluble HLA-Aα0201 molecule were evaluated by competition assays using ¹²⁵I-F₁₀V epitope (FLPSDYFPSV) (Table I). Two control peptides (M 55–66 and HIV-RT 309–317) inhibit HLA-A2/F₁₀V binding at 90–98% (Table I). Six of 10 Nef₁₈₀–₁₈₉ variants induced 85–100% inhibition (Nef₁₈₀ AMKK, MQ, BRVA, VT, M KK, and VKH), three variant peptides induced 25–80% inhibition (Nef₁₈₀ LAI, MK, and AK), and one variant peptide (Nef₉₀VKP) containing a proline residue at the COOH terminus did not inhibit HLA-A2/F₁₀V binding (Table I).

We then measured the peptide/HLA-A2 complex stability and calculated the DC₅₀ (see Materials and Methods). The DC₅₀ of four variants (Nef₁₈₀ MQ, AMKK, MKK, and AK) as well as two positive control epitopes (M 55–66 and RT 309–317) were above 5 h. These epitopes were classified as high affinity peptides. The three following peptides (Nef₁₈₀ BRVA, VT, and VKH) were highly bound to HLA-A2 although with a lower stability on the HLA-A2 molecule (DC₅₀ range from <1 to 2.5 h). These variants were classified as medium affinity peptide. Finally, two peptides (Nef₁₈₀ LAI) and had low HLA-A2 binding affinities and low stability (Table I).

The HLA-A201 binding prediction scores, calculated as described at http://bimas.dcr.nih.gov/molbio/hla_bind, were in most cases in accordance with the HLA-A2 binding capacities and stabilities of each natural variant of Nef₁₈₀–₁₈₉ peptides as estimated by previous methods (Table I).

Although, most of these mutations are localized outside the MHC anchor motives, they can considerably affect their MHC binding capacities and stabilities. It has been suggested that residues within the CTL epitope primarily affect their immune recognition and immunogenicity (23–25). Most of the mutations observed within the Nef₁₈₀–₁₈₉ epitope corresponded to conservative or semiconservative substitutions at odd-numbered positions: 3, 5, and 7. Recent studies (26) have defined heteroclitic analogs that induced stronger CTL responses than the native epitope. Natural variants Nef₁₈₀ MQ, VT, and AK contain semiconservative mutations when compared with the Nef₁₈₀ LAI sequence according to Tangri et al. (26). In addition, several groups (10, 19) have demonstrated that peptides that form stable complexes with MHC molecules elicit dominant T cell responses characterized by a diverse TCR repertoire.

Thus, these natural variants of the Nef₁₈₀–₁₈₉ epitope (MQ, VT, and AK) would be appropriate candidates for effective and dominant CTL response.

immunogenicity of HIV-Nef natural variants in HLA-A201 transgenic mice

The utility of HLA transgenic mice for the purpose of epitope identification and immunogenicity testing, as well as vaccine development, has been well defined (17–19). To examine the capacity of natural HIV-Nef variants to induce Ag-specific CTL responses in HLA-A201 transgenic mice, we performed a single injection of synthetic peptide in CFA (Table II, Fig. 1). Peptide-specific CD8 cells were tested after a short-term culture (7 days) for effector functions such as IFN-γ production and cytotoxicity (Table II). We found that most of the HLA-A2 transgenic mice (33–66%) responded to M 58–66 and RT 309–317 peptides by an increase in the number of IFN-γ-producing CD8 cells (20–76% IFN-γ CD8⁻ cells) (Fig. 1). These CD8 cells displayed cytotoxic functions because 60±8% (M 58–66) and 45±15% (RT 309–317) of chromium release was observed (Table II). Only 2 of 10 Nef₁₈₀–₁₈₉ peptides were efficient at priming CTL: a high affinity peptide, Nef₁₈₀ MQ (15–90% IFN-γ secreting CD8⁻ cells) and a medium affinity peptide Nef₁₈₀ VT (12–60% IFN-γ-secreting CD8⁻ cells). Interestingly, not all of the high affinity peptides (AMKK, MKK) were able to induce an immune response in vivo. None of the low affinity peptides could induce CTL responses in HLA-A2 transgenic mice. Therefore, most of Nef₁₈₀–₁₈₉ variants (80%) did not induce primary CD8 responses and thus might

Table II. Impact of amino acid substitution on CTL priming in HLA-A201 transgenic mice

<table>
<thead>
<tr>
<th>Peptide + CFA</th>
<th>Sequences</th>
<th>Ag-Induced IFN-γ Production in CD8⁻ Cells</th>
<th>Chromium Release (Average ± SEM; E:T = 100:1)</th>
<th>MHC/Peptide Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza</td>
<td>GILGVFTL</td>
<td>3/6 (50%)</td>
<td>61 ± 11%</td>
<td>High</td>
</tr>
<tr>
<td>RT 309–317</td>
<td>ILKKEPVHGV</td>
<td>4/6 (66%)</td>
<td>23 ± 7%</td>
<td>45 ± 15%</td>
</tr>
<tr>
<td>Nef₁₈₀ MQ</td>
<td>VLMQFDQSL</td>
<td>8/10 (80%)</td>
<td>48 ± 29%</td>
<td>35 ± 20%</td>
</tr>
<tr>
<td>Nef₁₈₀ AMKK</td>
<td>ALMKWFDQSL</td>
<td>6/0 (0%)</td>
<td>&lt;10%</td>
<td>ND</td>
</tr>
<tr>
<td>Nef₁₈₀ MKK</td>
<td>VLMQFDQSL</td>
<td>6/0 (0%)</td>
<td>&lt;5%</td>
<td>ND</td>
</tr>
<tr>
<td>Nef₁₈₀ MK</td>
<td>VLMQFDQSL</td>
<td>6/0 (0%)</td>
<td>&lt;5%</td>
<td>ND</td>
</tr>
<tr>
<td>Nef₁₈₀ BRVA</td>
<td>VLMQFDQSL</td>
<td>6/0 (0%)</td>
<td>&lt;5%</td>
<td>ND</td>
</tr>
<tr>
<td>Nef₁₈₀ VT</td>
<td>VLMQFDQSL</td>
<td>5/12 (41%)</td>
<td>45 ± 16%</td>
<td>20 ± 5%</td>
</tr>
<tr>
<td>Nef₁₈₀ VKH</td>
<td>VLMQFDQSL</td>
<td>0/3 (0%)</td>
<td>&lt;5%</td>
<td>ND</td>
</tr>
<tr>
<td>Nef₁₈₀ LAI</td>
<td>VLMQFDQSL</td>
<td>0/6 (0%)</td>
<td>&lt;5%</td>
<td>ND</td>
</tr>
<tr>
<td>Nef₁₈₀ AK</td>
<td>VLMQFDQSL</td>
<td>0/6 (0%)</td>
<td>&lt;5%</td>
<td>ND</td>
</tr>
<tr>
<td>Nef₁₈₀ VKP</td>
<td>VLMQFDQSL</td>
<td>0/6 (0%)</td>
<td>&lt;5%</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Single injection of synthetic peptide (50 µg/mouse) in CFA. Peptide-specific CD8 cells were tested after a short-term culture (7 days) for IFN-γ production by flow cytometric analyses or in a ³¹Cr release assay at a 10:1 E:T ratio and 50 µM peptide. Percent responder mice (underlined) for any given peptide are defined as >10% CD8⁻ IFN-γ⁻ cells and >10% chromium release.
participate in viral escape. These results show that the relative high affinity of peptides for MHC molecules together with high stability MHC/peptide complexes were necessary, but not sufficient, for immunogenicity. The repertoire of these HLA-A2 transgenic mice has been shown to be complete (27), suggesting that most of the Nef\textsubscript{180-189} variants were poorly immunogenic in HLA-A2 transgenic mice. Only two Nef\textsubscript{180-189} variants (MQ and VT) induced a strong primary CTL response.

The poor immunogenicity of some variants in HLA-A2 transgenic mice or in HIV-infected patients could result from the absence of proper Th cell function as suggested by others (28). Franco et al. (28) have shown that subdominant epitopes with intermediate MHC binding affinity that were studied required either a class II MHC-restricted Th cell epitope or administration of Ab to CD40 or IL-2 production to obtain significant CTL priming. Depending on the epitope, one source of help was much more efficient than the other. In addition, we had previously examined the CTL response generated in HIV-infected patients against HIV Nef\textsubscript{180-189} variants (14). In a previous work, we were able to detect peptide-specific CTL responses after in vitro cell culture of PBMC from HIV-infected patients directed against Nef\textsubscript{180-189} MQ and Nef\textsubscript{180-189} VT and also Nef\textsubscript{180-189} MK (high affinity) or Nef\textsubscript{180-189} AK (low affinity) (14). These results suggest that these peptides were processed in vivo and generated variant-specific CD8 T responses. However, one could also hypothesize that the adaptation of CTL response to newly emerging variants would result from a cross-reactive TCR repertoire.

Cross-reactivity of poorly immunogenic HIV-Nef variants is determined at CTL priming

One of the questions raised is whether a primary CTL response directed against an immunogenic peptide would lead to cross-reactive CTL responses to Nef\textsubscript{180-189} natural variants. To test that hypothesis, mice were primed in vivo with either Nef\textsubscript{180-189} VT or Nef\textsubscript{180-189} MQ peptides. Splenocytes were harvested at day 7 and cultured for 7 days with the index peptide. CTL were tested against Nef\textsubscript{180-189} variants in IFN-γ production assays. We showed that six of seven Nef\textsubscript{180-189} variants were able to induce IFN-γ production of MQ-specific CTL (Fig. 2). Among the MQ-specific CTL population, a fraction of cells (40–60%) were able to recognize Nef\textsubscript{180-189} MK, AMKK, BRVA, VT, and AK. Interestingly, most of the MQ-specific CTL (78 to 87%) cross-reacted with Nef\textsubscript{180-189} MK and M KK suggesting that these epitopes are recognized by a common TCR repertoire. In addition, MQ-specific CTL only weakly recognized the Nef\textsubscript{180-189} LAI epitope with a frequency of 10%. Summary of results are represented as the average of four mice (Fig. 3A) and show that the cross-reactivity of MQ-specific CTL was independent of MHC-peptide affinity because IFN-γ-positive cells were also found in response to Nef\textsubscript{180-189} BRVA and Nef\textsubscript{180-189} AK, medium and low affinity variants, respectively.

We performed the same experiment after primary immunization with Nef\textsubscript{180} VT. We found that VT-specific CTL generated in vivo were able to cross-react with only three of six peptides tested: Nef\textsubscript{180} MQ (high affinity), BRVA (medium affinity), and LAI (low affinity) peptides (Fig. 3B). VT-specific CTL display a different cross-reactive pattern because they recognized Nef\textsubscript{180-189} BRVA and LAI sequences but did not recognize other Nef\textsubscript{180-189} variants. In addition, MQ-specific CTLs were activated in response to peptides that were not recognized by VT-specific CTL. Thus, priming with Nef\textsubscript{180} VT induced expansion of CTLs with less cross-reactive capacities than MQ-specific CTL.

These results demonstrate that the priming of HIV-specific CTL determines selective cross-reactive immune responses against poorly immunogenic HIV natural variants. Haanen et al. (9) proposed that the repertoire of influenza A nucleoprotein-specific T cells is dependent on prior Ag encounters because the memory T cell pool created provides protection against a broad range of antigenic variants. The CTL response in SIV infection to a dominant viral SIV\textsubscript{gag} epitope can be clonally diverse and recognize potential epitope variants (29, 30). Our results demonstrate that the degeneracy of the HIV-specific CTL generated after primary mice
immunization allows for cross-recognition of poorly immunogenic variants. Different patterns of cross-reactivity are generated according to the variant originally used at priming. The specificity of the cross-reactive response should be linked to the primary structure of the peptide and suggests that the host’s response to a pathogen may be modified by its previous experience with other unrelated pathogens (31). Thus, the ability of the immune system to limit the danger of high virus variability is the degeneracy of the TCR repertoire. This degeneracy permits T lymphocytes to cross-react with several peptide analogs. Structurally related peptide analogs with lower affinity can participate in the termination of T cell tolerance to an immunodominant epitope (32). Therefore, research for the best immunogenic peptide has great importance in preventing and treating viral infections. A general vaccine strategy would be to enhance the immunogenicity by increasing the affinity of the host’s immune system to the best immunogenic peptide has great importance in preventing viral infections.

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10. Buseyne, F. A. Lemonnier, and K. Kosmatopoulos. 2000. A general strategy to enhance the immunogenicity by increasing the affinity of the host’s immune system to the best immunogenic peptide has great importance in preventing viral infections. A general vaccine strategy would be to enhance the immunogenicity by increasing the affinity and stabilization capacities of the HLA-A2-restricted peptides (19). One could hypothesize that primary polyclonal expansion of the HIV-specific CTL determines and may allow for the expansion of CTL directed against low affinity variants. We propose that careful determination of the epitope sequences used for CTL priming would generate CTL responses with broadly cross-reactive capacities against virus variants and thus would limit the risk of HIV escape.

FIGURE 3. In vivo priming of HIV-specific CTL determines selective cross-reactive immune responses against poorly immunogenic HIV natural variants. HIV-NefMQ (MQ) or HIV-Nef180 VT (B) (50 μg/mouse) in CFA were used for primary immunization of HLA-A2 transgenic mice. Peptide-specific CTLs were tested against HIV-NAf180 variants (50 μM). The average of four mice is shown for each peptide. Results are expressed in percent maximum CD8+ IFN-γ+ cells obtained with the peptide used for mice immunization. Percent maximum CD8+ IFN-γ+ for MQ-specific CTL are 97, 35, and 52% for each mouse. Percent maximum CD8+ IFN-γ+ for VT-specific CTL are 50, 35, and 30% for each mouse.


