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HIV-1-Specific CTL Responses Primed In Vitro by Blood-Derived Dendritic Cells and Th1-Biasing Cytokines

Cara C. Wilson, Walter C. Olson, Thomas Tuting, Charles R. Rinaldo, Michael T. Lotze, and Walter J. Storkus

Vaccine strategies designed to elicit strong cell-mediated immune responses to HIV Ags are likely to lead to protective immunity against HIV infection. Dendritic cells (DC) are the most potent APCs capable of priming both MHC class I- and II-restricted, Ag-specific T cell responses. Utilizing a system in which cultured DC from HIV-seronegative donors were used as APC to present HIV-1 Ags to autologous T cells in vitro, the strength and specificity of primary HIV-specific CTL responses generated to exogenous HIV-1 Nef protein as well as intracellularly expressed nef transgene product were investigated. DC expressing the nef gene were able to stimulate Nef-specific CTL, with T cells from several donors recognizing more than one epitope restricted by a single HLA molecule. Primary Nef-specific CTL responses were also generated in vitro using DC pulsed with Nef protein. T cells primed with Nef-expressing DC (via protein or transgene) were able to lyse MHC class I-matched target cells pulsed with defined Nef epitope peptides as well as newly identified peptide epitopes. The addition of Th1-biasing cytokines IL-12 or IFN-α, during priming with Nef-expressing DC, enhanced the Nef-specific CTL responses generated using either Ag-loading approach. These results suggest that this in vitro vaccine model may be useful in identifying immunogenetic epitopes as vaccine targets and in evaluating the effects of cytokines and other adjuvants on Ag-specific T cell induction. Successful approaches may provide information important to the development of prophylactic HIV vaccines and are envisioned to be readily translated into clinical DC-based therapeutic vaccines for HIV-1. The Journal of Immunology, 1999, 162: 3070–3078.

The incorporation of cytokines into vaccines as a means of enhancing or altering the resulting immune response represents an area of intense research interest. Ahlers et al. (5) evaluated murine immune responses generated against synthetic HIV peptide vaccines containing recombinant cytokines as adjuvant. The addition of granulocyte-macrophage CSF (GM-CSF)3 to the cutaneous vaccine microenvironment led to an overall enhancement of both cellular and humoral responses to the vaccine construct, whereas GM-CSF and IL-12 synergized to enhance CTL induction. A number of other studies have demonstrated that copriming with IL-12 enhances the induction of HIV Ag-specific cell-mediated immunity (6-8). Kim et al. reported a bias toward Th1-type immune responses to HIV Ags in a murine model following coinoculation of plasmids containing IL-12 and HIV DNA constructs (7). These murine studies hold promise for HIV vaccine development, but the impact of adjuvant cytokines on the type and specificity of vaccine-induced T cell responses to HIV Ags in humans remains poorly defined.

Dendritic cells (DC) are the most potent APCs capable of priming MHC class I- and II-restricted, Ag-specific T cell responses in vivo and in vitro (9). Their superior immunostimulatory capacity is felt to be due in part to the high level expression of MHC and costimulatory molecules, such as CD40, CD80, and CD86, as well as to their ability to produce Th1-biasing cytokines, such as IL-12 (10-12) and IFN-α (13-15). The capacity of DC to prime T cell responses to Ag and their presence in multiple organs and in the skin suggests a central role for DC in mediating immune responses to HIV vaccines. The mechanisms by which DC process and present both extracellular and intracellular HIV-1 Ags are likely to impact the type of T cell responses generated using a particular vaccine format.

3 Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; DC, dendritic cell; B-LCL, B-lymphoblastoid cell line; PE, phycoerythrin; MFC, mean fluorescence channel.
We previously reported that DC genetically engineered to express tumor Ags could prime tumor-specific CTL in vitro and that the induction of tumor-specific CTL was enhanced when DC were engineered to coexpress Th1-biasing cytokines (16). In the present study, we extend these observations by evaluating immunodominance of epitopes recognized by cytotoxic T cells induced by DC engineered to express an HIV Ag or loaded with exogenous HIV protein. Furthermore, we have attempted to establish the mechanisms by which Th1-biasing cytokines enhance HIV-specific T cell induction using either Ag loading approach. This in vitro human vaccination model for HIV-1 utilizes DC cultured from HIV-1-seronegative donors to stimulate primary Ag-specific autologous T cell responses in vitro. HIV-1 Nef was investigated as a model immunogen because of its proven immunogenicity during natural HIV infection (17–20) and its relatively conserved amino acid sequence among variant HIV strains (21). Prima, MHC class I-restricted CTL responses were generated by DC exposed to recombinant HIV-1 Nef protein, as well as Ag expressed in the DC as a result of bioballistic nef gene transfer. T cells stimulated in this fashion were tested for recognition of defined MHC class I-restricted Nef epitope peptides, and a correlation was made between surface of Tefzel tubing using a tube loader. The tubing was cut into 0.5-inch segments, resulting in the delivery of 0.5 mg of gold coated with 1 μg of plasmid DNA per transfection with the Accell helium pulse gun. Gold particles, tubing, tube loader, and the Accell helium pulse gun were kindly provided by Auragen/Geniva. DC were transfected in suspension in six-well plates. DC were harvested and pelleted by centrifugation, and 2 × 10^6 cells were resuspended in 20 μl of fresh medium and spread evenly in the center of each well. Cells were bombarded at a pressure of 300 psi of helium, and fresh culture medium was added immediately.

**Induction of primary T cell responses**

For protein pulsing experiments, day 5–10 DC were pulsed overnight with recombinant HIV-1 Nef (LAI strain) (AGMED, Bedford, MA) at a concentration of 20 μg/ml, washed, irradiated with 3000 rad, and cocultured with autologous nonadherent PBMC at a ratio of 1 DC:20–50 PBMC in AIM-V medium supplemented with 5% human AB serum. In some experiments, recombinant cytokine hIL-12 (final concentration, 500 pg/ml) or hIFN-α-b (final concentration, 100 U/ml) (Schering-Plough) was added to the DC-PBMC coculture on day 1 of induction. Proliferating responder T cells were restimulated weekly with irradiated, autologous DC pulsed with 5 μg/ml Nef overnight and grown in AIM-V 5% human AB serum with 10 U/ml rIL-2. For gene delivery experiments, DC cultured for 5–10 days were irradiated with 3000 rad and transfected with the pCI-Nef plasmid by particle-mediated transfer as described above. For coexpression of cytokines, DC were transfected first with pCI-Nef, then in a subsequent "shot" with pCMV-A-hIFNα-b or pCMV-A-hIL-12 plasmids, and cocultured with autologous responder cells as described above. Responder T cells were restimulated weekly with autologous DC transfected with pCI-Nef only. At the end of each restimulation period (7–10 days), T cells were tested for Nef-specific proliferation and lytic activity.

**Materials and Methods**

**DC generation**

PBMC were isolated from heparinized peripheral blood obtained by venipuncture from normal donors using density centrifugation. After four or five washes with HBSS (Life Technologies, Grand Island, NY), 3 × 10^6 cells/ml serum-free AIM-V medium (Life Technologies) were plated in flasks and incubated for 1–1.5 h at 37°C. Nonadherent cells were removed with gentle washes, and plastic-adherent cells were cultured for 5–10 days in AIM-V medium supplemented with 1000 U/ml rIL-4 and Gm-CSF. Following this culture period, nonadherent cells (DC) were harvested and further purified as necessary by discontinuous density centrifugation on a layer of Nycoprep 1.064 (Nycomed, Oslo, Sweden):LSM (Organon-Teknika, Durham, NC), 9:1, 1000 × g for 10 min. Cells generated in this fashion were determined to be >90% DC based on morphology and the expression of a CD3/CD14/CD16/CD20-negative, MHC class II+, CD80+, CD86+, phenotype assessed by direct immunofluorescence assays monitored by flow cytometry. Day 7 yields were approximately 5–15% of starting normal donor PBMC numbers.

**Plasmid DNA**

The plasmid pCMV-A-hIFNα-b was constructed by ligating a NotI-EcoRI fragment containing the hIFNα-b cDNA (kindly provided by Dr. Paul Zavedny, Schering-Plough Research Institute, Kenilworth, NJ) into CMV-A. pCMV-A-hIL-12 (p40-IRES-p35) was constructed by ligating a BamHI fragment containing the IRES sequence from EMCV followed by the 3′ fragment of p35 DNA (22) into pCMV-A-p40 (kindly provided by Dr. Will Swain, Auragen/Geniva, Madison, WI). The plasmid pcI-Nef was constructed by subcloning the ORF of HIV-1 Nef (LAI strain) into the expression plasmid pcI (Promega, Middleton, WI). Using PCR techniques, a SalI-NotI fragment was generated from a proviral construct containing HIV-Nef (kindly provided by S.-Y. Kim, Seoul, Korea) and ligated into pcI. The insert was sequenced in both directions to exclude mutations introduced by PCR. Plasmids were grown in Escherichia coli strain DH5α and purified using Qiagen Endofree Plasmid Maxi Kits (Qiagen, Chatsworth, CA).

**Particle-mediated gene transfer to DC**

Plasmid DNA was precipitated onto 2.6-μm gold particles at a density of 2 μg of DNA per mg of particles. Briefly, gold particles and DNA were resuspended in 100 μl of 0.05 M spermidine (Sigma Chemical, St. Louis, MO), and DNA was precipitated by the addition of 100 μl of 1 M CaCl2. Particles were washed in dry ethanol to remove H2O, resuspended in dry ethanol containing 0.075 mg/ml PVP (Sigma), and coated onto the inner surface of Tefzel tubing using a tube loader. The tubing was cut into 0.5-inch segments, resulting in the delivery of 0.5 mg of gold coated with 1 μg of plasmid DNA per transfection with the Accell helium pulse gun. Gold particles, tubing, tube loader, and the Accell helium pulse gun were kindly provided by Auragen/Geniva. DC were transfected in suspension in six-well plates. DC were harvested and pelleted by centrifugation, and 2 × 10^6 cells were resuspended in 20 μl of fresh medium and spread evenly in the center of each well. Cells were bombarded at a pressure of 300 psi of helium, and fresh culture medium was added immediately.

**Cytotoxicity assays**

Autologous B-lymphoblastoid cell line (B-LCL) or MHC class I-matched allogeneic B-LCL were incubated with 10–20 μg/ml of Nef peptide and 100–200 μCi of ^51^Cr in a total volume of 200 μl for 1–2 h at 37°C before use as targets. Targets were washed and added to plates at 5 × 10^3 cells/well in 100 μl. Responder T cells were plated at varying (2 or 3) E:T ratios in triplicate and assayed for cytotoxicity in a standard chromium release assay. The percentage of specific ^51^Cr release was calculated as 100 × (experimental release − spontaneous release)/maximum release − spontaneous release. Lytic activity was expressed as the percentage specific lysis, or percentage lysis of HIV Ag-expressing targets minus percentage lysis of non-HIV Ag-expressing targets.

**Peptides**

Peptides spanning the entire Nef protein (LAI strain of HIV-1) were kindly supplied by Dr. Bruce Walker (Massachusetts General Hospital, Boston, MA) and consisted of sequenced 20-aa peptide (aa) peptides overlapping by 15 aa. P1–P6 represent pools of these overlapping peptides, with 4 peptides per pool (except P6, which contains the terminal 3 peptides) with P1 containing the N-terminus and P6 containing the C-terminus of Nef. The minimal epitope peptides were synthesized at the University of Pittsburgh Cancer Institute Peptide Synthesis facility using standard F-moc chemistry and purified by reverse phase HPLC, with purity exceeding 90% based on mass spectrometry for m.w. All minimal epitope peptides are based on the LAI strain amino acid sequence of HIV-1 Nef (21).

**Hla typing**

HLA typing was performed by Dr. P. Morel (University of Pittsburgh) or Children’s Hospital HLA Laboratory (University of Pittsburgh Medical Center) using standard serotyping assays.

**Mhc class i peptide binding assay**

Binding of peptides to HLA-A2 and HLA-B7 were assessed using a class I reconstitution assay, as previously described (23). Briefly, the class I-reduced B cell line transfected expressing HLA-A2.1 (CIR-A2) or HLA-B7 (CIR-B7) was treated with a citrate-phosphate solution (pH 3.3) to denature preexisting class I complexes, as determined by loss of binding to conformation-dependent mAb (W6/32). Acid-treated cells were incubated with varying concentrations of peptides overnight in the presence of β2-microglobulin (Sigma, 5 μg/ml). Cells were washed, fixed, stained with FITC-conjugated W6/32 mAb or anti-A2 mAb (BB7.2), and evaluated by flow cytometric analysis.
Flow cytometry

For immunophenotyping, DC or T cell responders were washed in HBSS supplemented with 1% BSA and 0.1% NaN₃, and incubated (30 min at 4°C) with one of the following mAb: phycoerythrin (PE)-conjugated anti-HLA-DR (Beckton Dickinson, Mountain View, CA), FITC-conjugated anti-CD80 (Ancell, Bayport, MN), FITC-conjugated anti-CD86 (PharMingen, San Diego, CA), FITC-conjugated anti-CD40 (PharMingen), PE-conjugated anti-CD3 (Beckton Dickinson), FITC-conjugated anti-CD4 (Beckton Dickinson), PE-conjugated anti-CD8 (Beckton Dickinson), FITC-conjugated anti-CD14 (Beckton Dickinson), PE-conjugated anti-CD16 (Beckton Dickinson), and FITC-conjugated anti-CD20 (Beckton Dickinson). DC were also stained with corresponding isotype-matched control mAb (PharMingen). Surface expression was analyzed using a FACSscan flow cytometer (Beckton Dickinson) and Lysis II software, with data being collected on 5000 to 10000 viable cells.

Results

MHC class I-restricted, Nef-specific T cell responses can be primed in vitro by autologous DC from seronegative donors, engineered to coexpress HIV-1 Nef and Th1-biasing cytokines IL-12 or IFN-α

We have previously shown that DC engineered to express tumor Ags in vitro are potent stimulators of MHC class I-restricted, tumor-specific CTL (16). In the present study, we sought to determine whether DC engineered to express an HIV Ag could induce primary HIV-specific T cell responses in vitro and whether this in vitro vaccine model could serve to identify immunogenic epitopes as potential vaccine targets. Cultured DC were transfected with HIV-1 Nef plasmid cDNA by particle-mediated gene transfer as described above. Expression of HIV-1 Nef was confirmed in 1–5% of DC by immunohistochemical staining with Nef-specific mAb (data not shown). Additionally, in previous studies, transfection of cultured DC with cDNA encoding hIL-12 and/or IFN-α cDNA using a gene gun was observed to result in modest levels of cytokine production (50 – 150 pg/million DC/48 h), increased expression of DC-associated costimulatory molecules, and enhanced induction of tumor-reactive CTL in vitro (16). We investigated whether coinserterion of the plasmids encoding hIL-12 and hIFN-α with the Nef-encoding plasmid could enhance the efficiency of induction of Nef-specific CTL or alter the pattern of CTL epitopes recognized.

HIV-uninfected donors expressing the common HLA class I molecules HLA-A2, HLA-A3, or HLA-B7 were initially evaluated as responders. Using bioballistic gene transfer, cultured DC were transfected with pCI-Nef alone or with pCI-Nef plus IL-12 or IFN-α cDNA, irradiated, and used to stimulate autologous responder T cells. Responder T cells were restimulated with autologous DC expressing Nef, without coexpression of IL-12 or IFN-α, and tested for the ability to lyse HLA-matched target cells pulsed with defined Nef epitope peptides or overlapping, pooled Nef peptides. CTL responses restricted by several different HLA class I molecules and targeting to defined epitope peptides, as well as larger, overlapping peptides, were noted in multiple individuals. Fig. 1 depicts the HLA-B7-restricted Nef peptide-specific CTL responses induced by autologous DC engineered to express Nef alone or to coexpress Nef and hIL-12 or hIFN-α during priming. A response against only the P6 pool of peptides was noted following stimulation with DC-Nef alone, whereas IL-12-primed T cells responded to the P6 pool and weakly to the P2 peptide pool. IFN-α-primed T cells did not respond strongly to the P6 peptides, but instead displayed low level lysis of several other peptide pools. These results suggest that in certain cases the addition of cytokines to primary DC-T cell cocultures may result in differences in the epitope specificity of the Ag-specific T cell responses generated. These data may reflect differences in processing or presentation of peptides under differing cytokine conditions or reflect skewing of the T cell repertoire resulting from error in sampling of low frequency responses.

No HLA B7-restricted CTL epitopes had previously been identified in the C-terminal region of Nef spanned by the P6 peptides, suggesting that CTL with a novel epitope specificity were generated using this approach. A single B7-restricted epitope within the P2 pool, Nef 68–76 (FPVTPQVPL), was previously identified in HIV-infected individuals by Haas et al. (24). This peptide was synthesized, was determined to be a strong HLA-B7 binder (Fig. 2A) in an HLA-B7 reconstitution assay (23), and was used to pulse B7-expressing targets in a chromium release assay. Specific killing of this peptide was mediated by responder cells from the donor depicted in Fig. 1, which were stimulated with DC-Nef + IL-12 and to a lesser extent DC-Nef + IFN-α, but not DC-Nef alone (Fig. 2B). Interestingly, no cytotoxic response to the P3 pool of peptides, which contains another previously reported immunodominant B7-restricted epitope (Nef 128–137, TPGPGVRYPL) (24), was identified in this donor following any of the evaluated priming conditions.

In addition to evaluating CTL reactivity against pooled, overlapping peptides, DC-stimulated responder T cells were also tested directly against targets pulsed with defined peptide epitopes, in some cases. Three well-characterized HLA-A2-restricted CTL epitopes in Nef have been previously identified in HIV-infected individuals: Nef 136–145 (PLTFGWCYKL), Nef 180–189 (VLEWRFDSDL), and Nef 190–198 (AFHHVAREL) (21, 24, 25). Using the described MHC class I reconstitution assay (23), we determined the relative binding affinities of each of these peptides for HLA-A2.1, with relative A2 binding of Nef 136 > Nef 180 >> Nef 190 reproducibly detected in multiple assays (Fig. 3A). Nef 190–198 bound weakly to HLA-A2.1 in this sensitive assay, but we and others have been unable to detect binding of this peptide to HLA-A2.1 using the T2 binding assay (26). PBMC from nine HIV-1-seronegative donors expressing HLA-A2 were stimulated with DC engineered to express HIV-1 Nef with or without IL-12 or IFN-α. Following three stimulations with DC transfected with nef only, responder cells were tested for their ability to kill targets expressing only HLA-A2.1 (C1R.A2) when pulsed with the above epitope expressing Nef peptide-specific CTL responses.
10% specific lysis) were identified in five of the nine donors, with two donors responding to two different peptides (Table I). Equal numbers of responses against peptides 136–145 and 190–198 were identified, whereas only a single donor responded weakly to Nef peptide 180–189. This pattern of CTL responses would not necessarily have been predicted based only on binding affinities to HLA-A2.1 (Fig. 3A), as Nef 180–189 consistently bound to HLA-A2 better than did Nef 190–198, yet was poorly recognized by responder T cells in this system.

With only one exception, the addition of Th1-biasing cytokines during priming enhanced the induction of CTL responses (Table I and Figs. 3B and 4). Overall, IL-12 was slightly more effective than IFN-α in enhancing the induction of such responses. Figs. 3B and 4, A and B, depict the Nef peptide-specific responses of the two donors in whom responses against more than one peptide were identified (donors 1 and 5; Table I). The pattern of CTL responses in donor 1 responders (Fig. 3B) shows that the addition of either IL-12 or IFN-α during the primary stimulation led to detection of stronger CTL responses and that the pattern of epitope specificity generated under all conditions was similar. As shown in Fig. 4, Nef peptide-specific lysis was only detected in those T cell cultures coprime with IL-12, with specific lysis of Nef 190 > N180 > N136-pulsed targets mediated by CTL from donor 5.
MHC class I-restricted Nef-specific T cell responses can be primed in vitro by autologous DC from seronegative donors, pulsed with recombinant Nef protein and recombinant IL-12 or IFN-α

DCs have been reported to have the ability to process exogenous proteins for presentation by MHC class I Ags (27, 28). We therefore sought to determine whether primary class I-restricted responses to the HIV-1 Nef protein could be induced by DC pulsed with recombinant protein and whether induction in the presence of recombinant Th1-biasing cytokines would enhance or alter this process. DC cultured from HIV-seronegative donors were pulsed with recombinant HIV-1 Nef protein, irradiated, and used as APC to stimulate autologous nonadherent T cells as described in Materials and Methods. Responder cells were restimulated weekly with irradiated, Nef protein-pulsed DC and maintained in media with low dose rHL-2. HIV-specific cytotoxicity was evaluated using HLA-matched or partially matched B-LCL pulsed with defined minimal Nef epitope peptides or with pools of larger peptides spanning the entire Nef protein sequence (P1–P6). MHC class I or class II restriction of responses was determined either by using targets matched only at certain class I alleles or by blocking with anti-MHC I monomorphic (W6/32) or MHC II monomorphic (L243) mAbs. Strong MHC class II-restricted responses to the HIV Nef were noted in the majority of donors tested (data not shown; C. C. Wilson, manuscript in preparation), and Nef-specific CTL responses were detected in three of six donors tested. Significant CTL responses were generally first noted after two or three rounds of stimulation, with marked enhancement of responses noted after four or five stimulations. The majority of responder T cells stimulated in this fashion were CD3+ CD4+ (70–90% after three stimulations), but one donor generated primarily a CD3+ CD8+ T cell response in response to stimulation with DC pulsed with Nef protein (Fig. 5A). Fig. 5A illustrates HLA-A3-restricted killing of target cells pulsed with a previously identified Nef peptide (Nef 73–84, QVPLRPMTYK) by T cells from an HLA-A3+, B7− HIV-seronegative donor, after five stimulations with DC pulsed with rNef protein. The B7-restricted peptide (Nef 128–137, TPFPGVRYPL) determined to be an immunodominant epitope in HIV-infected individuals (24) was not recognized by this bulk T cell population.

The addition of either rIL-12 or IFN-α to the cultures, in concentrations similar to those expressed by gene gun-transfected DC, only during the initial stimulation, led to the subsequent enhancement of HIV peptide pool-specific CTL responses, as depicted in a representative assay in Fig. 5B. T cells from this donor (D.M., HLA-A11+, A24+, B54+) specifically recognized autologous targets pulsed with the P3 and P6 pooled Nef peptides. These responses were enhanced following priming with IL-12 or IFN-α. Although minimal epitope specificities were not mapped in this case, it is interesting to note that both previously identified A11-restricted Nef epitopes, Nef 75–82 (PLPGVRYPL) and Nef 84–92 (AVDLSHFLK) (17, 29), are located within the P3 pool of Nef epitopes tested.

Mechanism of cytokine-mediated enhancement of DC-dependent T cell induction

To determine the mechanism by which IL-12 and IFN-α enhanced the induction of HIV-specific T cell responses in this system, we

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Table I. Nef epitope peptides recognized by T cells primed in vitro with autologous DC transfected with nef and cytokine cDNA

<table>
<thead>
<tr>
<th>Donor</th>
<th>Nef</th>
<th>Nef + IL-12</th>
<th>Nef + IFN-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N136</td>
<td>N136, N190</td>
<td>N136, N190</td>
</tr>
<tr>
<td>2</td>
<td>N136</td>
<td>N136</td>
<td>N136</td>
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<td>3</td>
<td>N190</td>
<td>N190</td>
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<td>4</td>
<td>N180</td>
<td>N180, N190</td>
<td>N180, N190</td>
</tr>
<tr>
<td>5</td>
<td>6–9</td>
<td>6–9</td>
<td>6–9</td>
</tr>
</tbody>
</table>

*PBMC from nine HIV-seronegative, HLA-A2+ donors were stimulated with autologous DC transfected with HIV-1 nef cDNA with or without hIL-12 or IFN-α DNA and restimulated twice with Nef-expressing DC as described. Bulk responder cells were tested for recognition of HLA-A2+ targets (C1R.A2) pulsed with defined A2-restricted Nef epitope peptides, N136 (Nef136-145, PLTFGWCYKL), N180 (Nef 180-189, VLEWRFDSDL), and N190 (Nef 190-198, AFIHVAREL), or no peptide. Peptide-specific lysis of ≥10% is designated by the particular peptides.

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**FIGURE 4.** Nef peptide-specific, HLA-A2-restricted CTL responses augmented by priming with IL-12. Nonadherent PBMC from donor 5 (Table I) stimulated with autologous DC transfected with HIV-1 nef cDNA with or without hIL-12 or IFN-α cDNA and restimulated twice with Nef-expressing DC as described were tested for recognition of HLA-A2+ targets (C1R.A2) pulsed with defined A2-restricted Nef epitope peptides, Nef 136–145 (PLTFGWCYKL), Nef 180–189 (VLEWRFDSDL), and Nef 190–198 (AFIHVAREL), or no peptide. A, Lysis of peptide-pulsed targets by the three bulk responder populations at a single E:T ratio. B, Lysis of peptide-pulsed targets by bulk responder T cells from the same donor primed with DC transfected with nef and IL-12 cDNA at three E:T ratios.
MHC class I-restricted recognition of Nef epitope peptides after in vitro priming with DC pulsed with Nef protein and enhancement with addition of rIL-12 or IFN-α during T cell priming. A. Nonadherent PBMC from donor D.M. (HLA-A3+, B7+) were stimulated with autologous DC pulse overnight with recombinant HIV-1 Nef protein and restimulated with Nef protein-pulsed DC weekly for 4 wk. Bulk responder T cells were tested for their ability to lyse HLA-A3+ or HLA-B7+ targets pulsed with or without defined Nef epitope peptides, Nef 73–82 (A3 restricted) and Nef 128–137 (B7 restricted), in a 4-h standard chromium release assay. Responder cells were phenotypically 91% CD3+ and 9% CD3-CD8+ (80 ± 11%, 74 ± 16% respectively). As previously described, T
cell cultures induced by DC cotransfected with IL-12 or IFN-α cDNA and tumor Ag cDNA displayed cytokine-dependent phenotypic changes (16). In these experiments, the coexpression or addition of IL-12 to DC at the time of T cell priming led to a significant (95% confidence, Student’s t test) expansion of CD8+ T cells, markedly reducing the calculated CD4/CD8 ratio after three stimulations (Table II). Despite the addition of IL-12 during T cell priming, the majority of DC-stimulated cultures still contained a predominance of CD4+ T cells (absolute CD4/CD8 ratio > 1) (Table II legend). IFN-α, expressed or added during priming of T cells, had a minimal effect on subsequent T cell phenotype, only slightly increasing the CD4/CD8 ratio in most cases (Table II).

We next evaluated the impact of each cytokine on DC phenotype. We previously reported that the coexpression of IL-12 or IFN-α with tumor Ag in DC by bioballistic gene transfer led to phenotypic changes in the DC, with up-regulation of MHC and costimulatory molecules most markedly following expression of IFN-α. In these experiments, we observed that the addition of rIFN-α to DC cultures also up-regulated expression of CD40, CD80, CD86, and MHC I and II on GM-CSF/IL-4-cultured DC, whereas rIL-12 had little or no impact on DC phenotype, even at concentrations of up to 10 ng/ml cytokine (Fig. 6).

**Discussion**

In this study we established an “in vitro vaccine” model that takes advantage of the unique immunostimulatory properties of DC’s (30) to induce primary T cell responses to the well-characterized HIV-1 Nef Ag in vitro. Many investigators have reported the capacity of DC to prime MHC class I- and II-restricted T cell responses to HIV and other Ags in vitro (16, 31–42). Our study is unique in its application of a DC-based stimulation system to determine the impact of altering vaccine format (i.e., Ag loading and cytokine adjuvants) on the strength, epitope specificity, and immunodominance of the CTL responses primed in vitro against an HIV Ag by DC. Using this system, MHC class I-restricted, Nef-specific CTL responses were generated in vitro from the blood of HIV-1-seronegative donors by stimulating with autologous DC engineered to express the Nef gene or pulsed with recombinant Nef protein. We previously reported the ability of DC genetically engineered to express tumor Ags to stimulate primary tumor-specific CTL in vitro, and these Ag-specific responses were augmented by the coexpression of either IL-12 or IFN-α genes during priming (16). In this study, we evaluated the effect of IL-12 and IFN-α on the in vitro priming of Nef-specific T cell responses by DC using
changes), as we observed with IFN-
(epitope specificity. It is possible that this effect may be mediated
primary T cell induction resulted in responder T cells with altered
responses (43–46). IL-12 is known to be a potent inducer of cyto-
are known to bias toward Th1-type, or cell-mediated, immune re-
a

FIGURE 6. Effect of recombinant cytokines IL-12 and IFN-α on DC phenotype. DC were grown for 7 days in hIL-4 and GM-CSF and then treated with the addition of the indicated cytokine at 10 ng/ml for 48 h, at which time FACS analysis was performed. Fold control mean fluorescence channel (MFC) represents the ratio of cytokine-treated DC MFC to untreated DC MFC.

different Ag loading strategies. Exogenous or expressed cytokines were only present during the initial stimulation (“priming”), to ensure that the measured effects were on T cell induction and not directly on effector function (assessed after several restimulations) and also to simulate a prime-boost vaccine approach. The data show that the addition of IL-12 or IFN-α at the time of initial, or primary, T cell induction with DC + Ag increased the likelihood that an Ag-specific CTL response would be detected after multiple stimulations. This enhancement of CTL reactivity occurred regardless of the mode of Ag loading and likely resulted from either a preferential outgrowth or activation of Ag-specific CD8+ effector cells. The presence of the cytokine only during the primary stimulation would be unlikely to have a direct effect on effector function after several restimulations in the absence of exogenous cyto-
kine. There are a number of mechanisms by which this effect on CTL reactivity might be achieved, and it is likely that IL-12 and IFN-α achieve similar results by very different mechanisms. IL-12 and IFN-α, both shown to be produced by activated DCs (10–15), are known to bias toward Th1-type, or cell-mediated, immune re-
sp

mately resulting in the differential processing and MHC presenta-
tion of antigenic epitopes.

Epitope immunodominance in a vaccine setting is likely to be determined by a number of factors, including Ag format, route, Ag dose, and the MHC background of the recipient (52–54). Without a directly comparable animal model for HIV-1 vaccine develop-
ment, identification of immunodominant epitopes as potential vac-
cine targets has been difficult. A relative measure of immunoge-
nicity can be determined by measuring MHC class I binding of epitope peptides (23) or in vivo studies of potential CTL epitopes in HLA transgenic mice (55, 56), but it is clear that factors other than MHC binding determine in vivo immunogenicity (57). A large number of MHC class I-restricted CTL epitopes have been identified in HIV-infected individuals (21), yet viral variation and the associated selection pressure placed on the immune response make it difficult to know with certainty which of the defined epitopes would be recognized following a given immunization ap-
proach in uninfected individuals. DCs, potent APC located in skin
and lymphoid organs, are likely to play a major role naturally in promoting immune responses generated using most vaccine for-
mats. Therefore, an understanding of the constraints of processing and presentation of HIV Ags by DC should prove critical in de-
signing effective vaccine strategies against HIV-1. Our results sug-
gest that this DC-based system may provide a relevant means of
determining which CTL epitopes in HIV-1 are likely to be recog-
nized using a given vaccine format and may aid in determining factors that influence immunogenicity. This information may not
ecessarily be predicted using standard in vitro assays of immu-
nogenicity or peptide binding to HLA molecules (57). For in-
stance, Nef 190–198 is recognized by T cells primed by DC in vitro as well as by T cells isolated from HIV-infected individuals, despite the low binding affinity of this peptide for HLA-A2 as measured in peptide binding assays. This suggests that, in certain cases, in vitro assays of peptide binding to HLA molecules may not reflect the true HLA binding capacity or immunogenicity of naturally processed peptides. Since no in vitro system can possibly reflect the complexity of the in vivo immune response to a vaccine, it will be important to confirm that the immunogenicity of epitopes identified by this in vitro system are truly reflective of in vivo immunogenicity.

In summary, our results show that this DC in vitro stimulation system may be effectively used to evaluate the impact of altering important vaccine parameters, such as Ag format and biologic adju-
vants, on the relative strength and epitope specificity of bulk
Ag-specific CTL responses generated under each condition. Re-

sults of these studies, which incorporate DC-produced, Th1-bias-
ing cytokines as adjuvants, should aid in understanding the mech-

anisms by which DC mediate the induction of Ag-specific T cells.
Information gained using this system may also aid in the develop-
ment of prophylactic vaccines for HIV-1 by identifying the ap-
propriate antigenic format and biologic adjuvant, such as Th1-
biasing cytokines, required for the optimal activation of a pro-
teactive CTL repertoire. Successful in vitro vaccine approaches may also more directly serve as a basis for clinical DC-based ther-

peutic vaccines in HIV-1-infected individuals.

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References


