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Potent CD4⁺ T Cell Responses Elicited by a Bicistronic HIV-1 DNA Vaccine Expressing gp120 and GM-CSF

Dan H. Barouch,* Sampa Santra,* Klara Tenner-Racz,† Paul Racz,† Marcelo J. Kuroda,* Joern E. Schmitz,* Shawn S. Jackson,* Michelle A. Lifton,* Dan C. Freed,‡ Helen C. Perry,‡ Mary-Ellen Davies,‡ John W. Shiver,‡ and Norman L. Letvin*

Virus-specific CD4⁺ T cell responses have been shown to play a critical role in controlling HIV-1 replication. Candidate HIV-1 vaccines should therefore elicit potent CD4⁺ as well as CD8⁺ T cell responses. In this report we investigate the ability of plasmid GM-CSF to augment CD4⁺ T cell responses elicited by an HIV-1 gp120 DNA vaccine in mice. Co-administration of a plasmid expressing GM-CSF with the gp120 DNA vaccine led to only a marginal increase in gp120-specific splenocyte CD4⁺ T cell responses. However, immunization with a bicistronic plasmid that coexpressed gp120 and GM-CSF under control of a single promoter led to a dramatic augmentation of vaccine-elicited CD4⁺ T cell responses, as measured by both cellular proliferation and ELISPOT assays. This augmentation of CD4⁺ T cell responses was selective, since vaccine-elicited Ab and CD8⁺ T cell responses were not significantly changed by the addition of GM-CSF. A 100-fold lower dose of the gp120/GM-CSF bicistronic DNA vaccine was required to elicit detectable gp120-specific splenocyte proliferative responses compared with the monocistronic gp120 DNA vaccine. Consistent with these findings, i.m. injection of the gp120/GM-CSF bicistronic DNA vaccine evoked a more extensive cellular infiltrate at the site of inoculation than the monocistronic gp120 DNA vaccine. These results demonstrate that bicistronic DNA vaccines containing GM-CSF elicit remarkably potent CD4⁺ T cell responses and suggest that optimal Th cell priming requires the precise temporal and spatial codelivery of Ag and GM-CSF. The Journal of Immunology, 2002, 168: 562–568.

Increasing evidence over the past several years has confirmed the importance of virus-specific T cell responses in controlling HIV-1 replication. Considerable efforts have therefore recently focused on the development of HIV-1 vaccine candidates that elicit potent CD8⁺ CTL responses (1, 2). In addition to CD8⁺ CTL responses, virus-specific CD4⁺ Th lymphocyte responses also play a critical role in controlling viremia. Although typically weak in most chronically HIV-1-infected individuals, vigorous CD4⁺ T cell proliferative responses were detected in long term nonprogressors and in individuals following treatment of acute HIV-1 infection (3–5). It will therefore probably be important for HIV-1 vaccines to elicit potent virus-specific CD4⁺ as well as CD8⁺ T cell responses.

Plasmid DNA vaccines have been shown to elicit CTL, Th cell, and Ab responses in a variety of animal models (6–9). The potential utility of plasmid DNA as a candidate HIV-1 vaccine strategy has therefore been an area of active investigation (10–18). DNA vaccines augmented by IL-2/Ig cytokine fusion constructs as well as DNA/MVA prime-boost regimens have recently been shown to elicit potent virus-specific CTL responses and to provide substantial control of viral replication following a pathogenic SHIV challenge in rhesus monkeys (19, 20). Optimizing the induction of vaccine-elicited HIV-specific CD4⁺ T lymphocyte responses may further improve candidate HIV-1 vaccine strategies.

Plasmid GM-CSF has been investigated as a potential vaccine adjuvant in a number of murine disease models. Co-administration of plasmid GM-CSF was first shown to augment the Ab response elicited by a rabies-specific DNA vaccine (21). Plasmid GM-CSF has subsequently been shown to increase DNA vaccine-elicited immune responses, including cellular proliferative responses to HIV-1 (22–24), hepatitis C virus (25, 26), herpes simplex virus type 2 (27), Mycobacterium tuberculosis (28), and Plasmodium yoelii (29). The mechanism for the adjuvant properties of plasmid GM-CSF may involve increased recruitment of macrophages and dendritic cells to the site of injection (30–32).

In this report we investigate the ability of plasmid GM-CSF to augment the CD4⁺ T cell responses elicited by an HIV-1 gp120 DNA vaccine in mice. We show that the delivery of a bicistronic plasmid expressing both gp120 and GM-CSF elicits dramatic gp120-specific CD4⁺ T lymphocyte responses. Precise temporal and spatial codelivery of Ag and GM-CSF therefore appears to be critical for optimal induction of DNA vaccine-elicited immune responses.

Materials and Methods

Plasmids

The plasmid DNA vaccine pV1J-gp120 expressing HIV-1 IIB gp120 was used for these experiments (33). Construction of the monocistronic pV1J-GM-CSF and bicistronic pV1J-gp120/GM-CSF plasmids has been described previously (34). In the bicistronic pV1J-gp120/GM-CSF plasmid an internal ribosome entry site from encephalomyocarditis virus was placed between the two genes to obtain efficient internal initiation of translation (35). The sham plasmid was the empty pV1J vector. Plasmids were prepared from large scale bacterial cultures by standard alkaline lysis followed by double CsCl gradient banding (34).
Mice and immunizations

Eight- to 12-wk-old BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Groups of mice (n = 4/group) were immunized with varying concentrations of plasmid DNA in 50 μl 0.15 M sterile saline. Injections were performed i.m. in the quadriceps muscle. For experiments with recombinant vaccinia virus, 5 × 10^6 PFU vacc-HIV-1 Env BH-10-gp160 (Therion Biologics, Cambridge, MA) was injected i.p. in 200 μl sterile PBS. For both DNA and vaccinia immunizations, the seroconversion rate was 100% at 2 wk. Mice were typically sacrificed for immunological assays at 3 wk.

Antibody ELISA

Serum anti-gp120 Ab titers from immunized mice were measured by a direct ELISA as previously described (34). Ninety-six-well plates coated overnight with 100 μl/well of 1 μg/ml recombinant IIBB gp120 (Intracel, Cambridge, MA) in PBS were blocked for 2 h with PBS containing 2% BSA and 0.05% Tween 20. Plates were washed three times with PBS containing 0.05% Tween 20 and incubated for 1 h with a 1/5000 dilution of a peroxidase-conjugated affinity-purified rabbit anti-mouse secondary Ab (Jackson ImmunoResearch Laboratories, Bar Harbor, ME). The plates were then washed three times, developed with tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD), stopped with 1% HCl, and analyzed at 450 nm with a Dynatech MR9000 ELISA plate reader.

Tetramer staining assays

Tetrameric H-2D^b complexes folded around the HIV-1 IIBB V3 loop optimal P18 epitope peptide (P18-110 or RGPGRAFVTI) (36) were prepared and used to stain P18-specific CD8^+ T cells as essentially described (37, 38). Mouse blood was collected in RPMI 1640 containing 40 U/ml heparin. A lysis of the RBCs, 0.1 μg PE-labeled D7P18 tetramer in conjunction with APC-labeled anti-mouse CD8α mAb (Ly-2, Caltag, San Francisco, CA) were used to stain P18-specific CD8^+ T cells. The cells were washed in PBS containing 2% FBS and fixed in 0.5 ml PBS containing 1.5% paraformaldehyde. Samples were analyzed by two-color flow cytometry on a FACSCalibur (BD Biosciences, Mountain View, CA). Gated CD8^+ T lymphocytes were examined for staining with the D7P18 tetramer. All tetramer staining experiments were confirmed by standard functional chromium release CTL assays using P18 peptide-stimulated splenocytes as effector cells as previously described (34, 38).

Proliferation assays

Standard [3H]thymidine incorporation assays were performed to assess CD4^+ T cell proliferative responses. Splenocytes from immunized mice were resuspended at 4 × 10^6 cells/ml in RPMI 1640 containing 5% FBS. One hundred microliters were added to each well in 96-well plates with 1:0.2, 0.04, or 0 μg/ml recombinant HIV-1 IIBB gp120 (Intracel, Cambridge, MA). After 4 days of culture 100 μl of 1 μCi [3H]thymidine (ICN Biochemicals, Costa Mesa, CA) was added to each well. Following a 16-h incubation, cells were harvested on glass filter paper, and radioactivity was measured in a Wallac 1450 Microbeta liquid scintillation counter (Wallac, Gaithersburg, MD). The stimulation index (SI) was calculated as: (cpm with Ag stimulation) / (background cpm without Ag). For experiments involving depletion of CD4^+ or CD8^+ T cells, splenocytes were incubated with magnetic microbeads coated with mAbs specific for murine CD4 (L3T4) or CD8 (Ly-2; Miltenyi Biotec, Auburn, CA). Separation using MilimACS separation columns was performed according to the manufacturer’s instructions. Cell depletions were 95–100% efficient.

ELISPOT assays

ELISPOT assays were used to assess IFN-γ production by unfractionated splenocytes or splenocytes depleted of CD4^+ T cells or CD8^+ T cells. IFN-γ responses were measured using the optimal CTL epitope peptide P18 (36) or a pool of 47 overlapping 15-mer peptides derived from HIV-1 IIBB Env gp120 (Centralized Facility for AIDS Reagents, Potters Bar, U.K.). Ninety-six-well multiscan plates (Millipore, Bedford, MA) coated overnight with 100 μl/well of 10 μg/ml rat anti-mouse IFN-γ mAb (BD Pharmingen, San Diego, CA) in PBS were washed with endotoxin-free Dulbecco’s PBS (Life Technologies, Gaithersburg, MD) containing 0.25% Tween 20 and blocked with PBS containing 5% FBS for 2 h at 37°C. The plates were washed three times with Dulbecco’s PBS containing 0.25% Tween 20, rinsed with RPMI 1640 containing 10% FBS, and incubated in triplicate with 5 × 10^5 splenocytes/well in a 100-μl reaction volume containing 8 μg/ml peptide. For studies using the Env peptide pool, each peptide in the pool was present at 8 μg/ml. Following an 18-h incubation, the plates were washed nine times with Dulbecco’s PBS containing 0.25% Tween 20 and once with distilled water. The plates were then incubated for 16 h with 75 μl/well 5 μg/ml biotinylated rat anti-mouse IFN-γ, washed six times with Coulter wash (Coulter, Miami, FL), and incubated for 2.5 h with a 1/500 dilution of streptavidin-AP (Southern Biotechnology Associates, Birmingham, AL). Following five washes with Coulter wash and once with PBS, the plates were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate chromogen (Pierce, Rockford, IL), stopped by washing with tap water, air-dried, and read using an ELISPOT reader (Hitech Instruments, Edgemont, PA).

Cytokine secretion assays

Splenocytes (4 × 10^6) in 1 ml RPMI 1640 containing 5% FBS were cultured with 1 μg/ml recombinant HIV-1 IIBB gp120 (Intracel, Cambridge, MA). After 72 h supernatants were harvested and analyzed for the presence of cytokines using commercial ELISA kits according to the manufacturer’s protocols (Endogen, Cambridge, MA).

Pathological analysis

The quadriceps muscles from mice were excised 7 days after plasmid DNA immunizations and frozen in Tissue Freezing Medium (Jung, Nussloch, Germany) in a dry ice/methanol bath. Five-micrometer-thick sections were cut with a cryostat, air-dried, and stained with H&E.

In vivo protein expression assays

To quantitate GM-CSF expressed in vivo, quadriceps muscles from vaccinated mice were excised 48 h after plasmid DNA immunizations. Muscles were then homogenized with a No. 10 Medicon homogenizers (BD Biosciences) in 1 ml PBS containing 0.05% Tween 20. Muscle homogenates were incubated on ice for 30 min, cell debris was removed by centrifugation, and supernatants were analyzed for GM-CSF by ELISA.

Results

Plasmid GM-CSF delivered concurrently with the gp120 DNA vaccine marginally augments cellular proliferative responses

We initiated studies by coimmunizing mice with the DNA vaccine pV1J-gp120 expressing HIV-1 IIBB gp120 plus the monocistronic pV1J-GM-CSF plasmid (34). Groups of mice were immunized i.m. with 50 μg sham plasmid, 50 μg pV1J-GM-CSF alone, 50 μg pV1J-gp120 alone, or 50 μg pV1J-gp120 plus 50 μg pV1J-GM-CSF on day −2, 0, or +2 relative to the pV1J-gp120 immunization. Ab, tetramer staining, cytotoxicity, and proliferative responses were measured 3 wk following initial immunization. As shown in Fig. 1, A and B, comparable anti-gp120 Ab responses and P18-specific CD8^+ T lymphocyte tetramer responses were measured in all the gp120-vaccinated groups. P18-specific chromium-release CTL cytotoxicity assays using P18-stimulated splenocytes as effector cells mirrored the results of the tetramer staining studies (data not shown). As shown in Fig. 1C, p120-specific splenocyte proliferative responses were augmented approximately 2-fold in the animals that were inoculated concurrently with pV1J-gp120 and pV1J-GM-CSF. The mean SI of splenocytes using 0.2 μg/ml gp120 stimulation was 6 in mice immunized with pV1J-gp120 alone, but was 14 in mice that received pV1J-GM-CSF on day 0 concurrent with pV1J-gp120 vaccination. Interestingly, no augmentation of this response was observed when pV1J-GM-CSF was injected on day −2 or day 2. In addition, no adjuvant effect was detected when 50 μg pV1J-gp120 was delivered with 50 μg pV1J sham plasmid control, and no adjuvant effect was observed when 50 μg pV1J-gp120 and 50 μg pV1J-GM-CSF were inoculated at different muscle sites (data not shown). These experiments suggested that augmentation of vaccine-elicited proliferative responses by plasmid GM-CSF required the precise temporal and spatial codelivery of GM-CSF with Ag.
The bicistronic gp120/GM-CSF vaccine augments CD4+ T cell proliferative responses

We reasoned that the most precise codelivery of GM-CSF with Ag would be achieved with a bicistronic pV1J-gp120/GM-CSF plasmid that coexpressed gp120 and GM-CSF under control of a single promoter. The gp120/GM-CSF bicistronic plasmid (34) included a single promoter with the two genes separated by the internal ribosome entry site from encephalomyocarditis virus to obtain efficient internal initiation of translation (35).

The immune responses elicited by pV1J-gp120/GM-CSF were compared with those elicited by the combination of the two monocistronic plasmids and with those elicited by recombinant vaccinia-env. Groups of mice were immunized with 50 μg pV1J sham plasmid, 50 μg pV1J-gp120, 50 μg pV1J-gp120 plus 50 μg pV1J-GM-CSF, 50 μg pV1J-gp120/GM-CSF, or 5 × 10^7 PFU vaccinia-env. As shown in Fig. 2, A and B, comparable anti-gp120 Ab responses and P18-specific CD8+ T cell tetramer responses were elicited in the three groups of DNA-vaccinated mice, although significantly higher responses were observed in the mice immunized with vaccinia-env. Functional CTL assays performed concurrently corroborated these tetramer staining results (data not shown).

Fig. 2C shows the CD4+ T lymphocyte proliferative responses of splenocytes from these animals. No gp120-specific proliferative responses were detectable in the mice that received the pV1J sham vaccine. As before, proliferative responses were readily measured in the mice that received pV1J-gp120, and 2-fold higher proliferative responses were observed in the animals that were injected concurrently with pV1J-gp120 plus pV1J-GM-CSF. Strikingly, the proliferative responses from mice immunized with the bicistronic pV1J-gp120/GM-CSF vaccine were approximately 10-fold higher than the proliferative responses from mice immunized with the monocistronic pV1J-gp120 (mean SI of 118 compared with 9 using 0.2 μg/ml gp120 stimulation). The proliferative responses primed by pV1J-gp120/GM-CSF were also markedly higher than those primed by high dose vaccinia-env immunization. Moreover, the increased proliferative responses elicited by pV1J-gp120/GM-CSF persisted for >3 mo (data not shown). As shown in Fig. 2D, depletion of CD4+ T cells, but not CD8+ T cells, before performing the proliferation assays abrogated these responses, confirming that the proliferative responses primed by pV1J-gp120 and pV1J-gp120/GM-CSF were CD4+ T cell responses.

As shown in Table I, splenocytes from these animals were then examined for their ability to secrete cytokines following stimulation with recombinant gp120. Cultured splenocytes from all the plasmid DNA- and recombinant vaccinia-immunized mice had high levels of IL-2 and IFN-γ production and low levels of IL-4 and IL-10 production, consistent with Th1-type immune responses. Compared with mice immunized with only pV1J-gp120, the addition of the monocistronic GM-CSF plasmid resulted in marginally higher IL-2 production. In contrast, splenocytes from mice immunized with the bicistronic gp120/GM-CSF plasmid demonstrated 3-fold increases in IFN-γ, IL-2, IL-4, IL-10, and GM-CSF production, consistent with the increased proliferative activity observed in the splenocytes of these animals.

We next investigated whether different expression levels of GM-CSF in vivo could account for the immunologic differences between the monocistronic and bicistronic GM-CSF plasmids. Groups of mice were immunized with 50 μg sham plasmid; 50 μg pV1J-gp120; 50 μg pV1J-gp120 plus 30, 150, or 300 μg pV1J-GM-CSF; or 50 μg pV1J-gp120/GM-CSF. As shown in Table II, the monocistronic GM-CSF plasmid had lower GM-CSF expression in vivo in mouse muscle compared with the bicistronic gp120/GM-CSF plasmid. Increasing the dose of the monocistronic GM-CSF plasmid, however, led to increased cytokine expression and
increased proliferative responses. A high dose (500 μg) of the monocistronic GM-CSF plasmid was required to elicit proliferative responses comparable with a standard dose (50 μg) of the bicistronic gp120 GM-CSF plasmid. Moreover, comparable levels of gp120 were detected from COS cells transiently transfected with pV1J-gp120 or pV1J-gp120/GM-CSF (data not shown). These data suggest that the immunogenicity of the bicistronic gp120/GM-CSF vaccine reflected both efficient cytokine expression as well as coordinate expression of GM-CSF with gp120.

A dose reduction study was then performed to determine the minimal dose of pV1J-gp120 and pV1J-gp120/GM-CSF required to elicit detectable gp120-specific proliferative responses. Groups of mice were immunized with 5, 0.5, or 0.05 μg pV1J-gp120 or pV1J-gp120/GM-CSF. As shown in Fig. 3, the dose of plasmid required to elicit detectable proliferative responses (defined as SI ≥ 3) was 5 μg for pV1J-gp120, but was only 0.05 μg for pV1J-gp120/GM-CSF. Thus, the bicistronic gp120/GMCSF vaccine was approximately 100-fold more potent than the monocistronic gp120 DNA vaccine in eliciting detectable proliferative responses.

We next investigated whether bicistronic vaccines expressing other cytokines (34) also had the ability to augment gp120-specific splenocyte proliferative responses. Mice were immunized with 5 μg pV1J-gp120 plus 50 μg pV1J-GM-CSF or 5 × 10^7 PFU vac-env. Immune responses were measured 3 wk after immunization. A, Anti-gp120 Ab titers were determined by ELISA. Geometric mean titers (GMT) with SEs are shown for each group. B, P18-specific CD8^+ T lymphocyte responses were determined by staining lymphocytes isolated from whole blood with a D7/P18 tetramer and gating on CD8^+ T lymphocytes. Mean tetramer responses are shown for each group. C, The gp120-specific proliferative responses of splenocytes following stimulation with 1, 0.2, 0.04, or 0 μg/ml recombinant gp120 were determined by thymidine incorporation assays. Mean SIs are shown for each group. D, The gp120-specific proliferative responses of total splenocytes or splenocytes depleted of CD4^+ T cells or CD8^+ T cells were also assessed using thymidine incorporation assays.

Table 1. Cytokine secretion of splenocytes from vaccinated mice after stimulation with recombinant gp120 as determined by ELISA^a^

<table>
<thead>
<tr>
<th>Cytokine Secretion (pg/ml)</th>
<th>Sham</th>
<th>gp120</th>
<th>gp120 + GM-CSF</th>
<th>gp120/GM-CSF bicistron</th>
<th>vac-env</th>
</tr>
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<tbody>
<tr>
<td>IFN-γ</td>
<td>&lt;100</td>
<td>6,717 ± 1,224</td>
<td>6,870 ± 1,648</td>
<td>17,821 ± 1,603</td>
<td>1,073 ± 440</td>
</tr>
<tr>
<td>IL-2</td>
<td>254 ± 33</td>
<td>3,712 ± 779</td>
<td>5,788 ± 1,562</td>
<td>10,284 ± 2,365</td>
<td>1,667 ± 500</td>
</tr>
<tr>
<td>IL-4</td>
<td>&lt;10</td>
<td>12 ± 1</td>
<td>13 ± 2</td>
<td>57 ± 12</td>
<td>&lt;10</td>
</tr>
<tr>
<td>IL-10</td>
<td>&lt;25</td>
<td>90 ± 8</td>
<td>84 ± 6</td>
<td>314 ± 78</td>
<td>78 ± 7</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>&lt;10</td>
<td>430 ± 214</td>
<td>426 ± 183</td>
<td>1,348 ± 148</td>
<td>102 ± 41</td>
</tr>
<tr>
<td>TNF-α</td>
<td>&lt;25</td>
<td>&lt;25</td>
<td>&lt;25</td>
<td>&lt;25</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>

^a^ Groups of mice were immunized with 50 μg pV1J sham plasmid, 50 μg pV1J-gp120, 50 μg pV1J-gp120 plus 50 μg pV1J-GM-CSF, 50 μg pV1J-gp120/GM-CSF, or 5 × 10^7 PFU vac-env.
or 0.5 μg pV1J-gp120, pV1J-gp120/GM-CSF, pV1J-gp120/IL-2, or pV1J-gp120/IL-4. As shown in Fig. 4, only pV1J-gp120/GM-CSF primed for markedly increased gp120-specific splenocyte proliferative responses. Thus, this adjuvant effect was specific for GM-CSF.

The bicistronic gp120/GM-CSF vaccine augments CD4+ T cell IFN-γ responses

To confirm the results obtained with the proliferation assays, we used IFN-γ ELISPOT assays to measure gp120-specific T cell responses of splenocytes from vaccinated mice. We measured CD4+ T cell and CD8+ T cell IFN-γ ELISPOT responses by depleting splenocytes of lymphocyte subsets before peptide stimulation. Mice were immunized with 5 μg pV1J sham plasmid, 50 μg pV1J-gp120, or 50 μg pV1J-gp120/GM-CSF. Fig. 5A shows the results of ELISPOT assays in which splenocytes were stimulated with a pool of 47 overlapping 15-mer peptides spanning the entire gp120 IIIB protein. Splenocytes from sham-vaccinated mice had no detectable IFN-γ responses (zero to five spots per 10^6 cells). Potent IFN-γ responses were detected in splenocytes from mice immunized with pV1J-gp120, and higher total IFN-γ responses were observed in mice immunized with pV1J-gp120/GM-CSF. Following depletion of CD4+ T cells, comparable IFN-γ responses were observed in both groups of mice, suggesting that both vaccines elicited comparable CD4+ T cell responses. However, following depletion of CD8+ T cells, IFN-γ responses from mice immunized with pV1J-gp120/GM-CSF were >7-fold higher than from mice immunized with pV1J-gp120, confirming that the gp120/GM-CSF bicistronic vaccine elicited markedly augmented CD4+ T cell responses. Fig. 5B shows the results of ELISPOT assays in which splenocytes were stimulated with the single optimal H-2D^d-restricted CTL epitope peptide P18 (RGPGRAFVITI) (36). Similar P18-specific IFN-γ responses were detected in splenocytes from mice immunized with pV1J-gp120 and pV1J-gp120/GM-CSF. Depletion of CD8+ T cells abrogated these responses, confirming that both vaccines elicited comparable P18-specific CD8+ T cell responses.

The bicistronic gp120/GM-CSF vaccine increases cellular infiltrates at the site of vaccination

We next examined the cellular infiltrates at the site of DNA inoculation. Mice were injected i.m. with 50 μg pV1J sham plasmid, 50 μg pV1J-gp120, or 50 μg pV1J-gp120/GM-CSF and were sacrificed after 7 days for histologic analysis of the injected quadriceps muscles. Numerous sequential sections were analyzed. Fig. 6 shows representative H&E-stained sections. Whereas the pV1J sham plasmid evoked little or no inflammatory response, inoculation with pV1J-gp120 evoked moderate cellular infiltrates consisting of 40–100 cells/μm^2. In contrast, injection with pV1J-gp120/GM-CSF evoked dense inflammatory responses consisting of 300–400 cells/μm^2, with large clusters of inflammatory cells as well as scattered cells throughout the interstitium. These cellular infiltrates were comprised predominantly of macrophages, CD4+ T cells, and neutrophils, as determined by immunohistochemistry (data not shown).

Discussion

The importance of virus-specific CD4+ T cell responses in controlling HIV-1 replication (3, 4) suggests that prophylactic and therapeutic HIV-1 immunization strategies should elicit these immune responses. In this report we demonstrate that immunization of mice with a bicistronic DNA vaccine expressing both HIV-1 gp120 and GM-CSF elicits remarkably potent CD4+ T cell responses in addition to Ab and CD8+ T cell responses.

A modest augmentation of CD4+ T lymphocyte responses was observed by coinjecting the monocistronic pV1J-GM-CSF plasmid together with the pV1J-gp120 DNA vaccine. This effect could be amplified by increasing the dose of pV1J-GM-CSF, but it required

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**Table II.** Splenocyte proliferative responses and in vivo expression levels of GM-CSF<sup>α</sup>

<table>
<thead>
<tr>
<th>Proliferative responses (SI)</th>
<th>50 μg Sham</th>
<th>50 μg gp120</th>
<th>50 μg gp120 + 50 μg GM-CSF</th>
<th>50 μg gp120 + 150 μg GM-CSF</th>
<th>50 μg gp120 + 500 μg GM-CSF</th>
<th>50 μg gp120/GM-CSF Bicistron</th>
</tr>
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<tbody>
<tr>
<td>Proliferation responses of splenocytes following stimulation with 1 μg/ml recombinant gp120 were determined by thymidine incorporation assays. Mean SIs are shown for each group.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF expression (pg/muscle)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>10 ± 3</td>
<td>121 ± 20</td>
<td>186 ± 51</td>
</tr>
</tbody>
</table>

<sup>α</sup> Groups of mice were vaccinated with 50 μg pV1J sham plasmid, 50 μg pV1J-gp120 with varying concentrations of pV1J-GM-CSF, or 50 μg pV1J-gp120/GM-CSF. Proliferation assays were performed using splenocytes stimulated with 1 μg/ml recombinant gp120. Homogenized muscle samples harvested 48 h after injection were assessed for GM-CSF by ELISA.

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**FIGURE 3.** Splenocyte proliferative responses elicited by various doses of pV1J-gp120 and pV1J-gp120/GM-CSF. Groups of mice were immunized with 5, 0.5, or 0.05 μg pV1J-gp120 or pV1J-gp120/GM-CSF. The gp120-specific proliferative responses of splenocytes following stimulation with 1, 0.2, 0.04, or 0 μg/ml recombinant gp120 were determined by thymidine incorporation assays. Mean SIs are shown for each group.

**FIGURE 4.** Splenocyte proliferative responses elicited by various bicistronic pV1J-gp120/cytokine constructs. Groups of mice were immunized with 5 or 0.5 μg pV1J-gp120, pV1J-gp120/GM-CSF, pV1J-gp120/IL-2, or pV1J-gp120/IL-4. The gp120-specific proliferative responses of splenocytes following stimulation with 1 or 0 μg/ml recombinant gp120 were determined by thymidine incorporation assays. Mean SIs are shown for each group.
simultaneous temporal and spatial delivery of both plasmids. A previous study has similarly shown that separating the delivery of plasmid GM-CSF from the plasmid Ag abrogated the adjuvanticity of GM-CSF (32). These observations, together with the dramatic augmentation of CD4⁺ T cell responses elicited by the bicistronic pV1J-gp120/GM-CSF vaccine, suggest that precise temporal and spatial codelivery of Ag and GM-CSF is required for optimally harnessing the adjuvant properties of GM-CSF.

Histologic analysis revealed strikingly dense cellular infiltrates in muscles injected with pV1J-gp120/GM-CSF. Smaller infiltrates were detected in muscles inoculated with pV1J-gp120. These infiltrates consisted predominantly of macrophages, CD4⁺ T cells, and neutrophils (data not shown). Thus, GM-CSF may act by recruiting inflammatory cells to the site of inoculation. These results extend prior studies that have described cellular infiltrates in muscles injected with monocistronic plasmids expressing GM-CSF (30–32). It is likely that the coordinate expression of Ag and GM-CSF achieved using the bicistronic plasmid approach optimally harnesses these recruited inflammatory cells for maximizing Ag presentation and triggering of CD4⁺ T cells.

A number of laboratories have reported the ability of plasmid GM-CSF to augment the immunogenicity of DNA vaccines in a variety of experimental models. These reports include increased Ab responses (21, 23, 25–27, 29), CTL responses (22, 39), and T cell proliferative responses (23, 25–29). A recent study also showed that the timing of plasmid GM-CSF administration determined the type of immune responses that were augmented (31).

The differences in the findings among these studies are significant and may reflect the differences in Ags, expression vectors, and specific assays used. In the system used in the present study, plasmid GM-CSF had a largely selective effect on augmenting CD4⁺ T cell responses, with little effect on Ab and CTL responses. It is possible that the Ab and CTL responses were not limited by T cell help in our system. A threshold level of T cell help may be required for maximizing Ab and CTL responses, above which little benefit is gained.

The ability of GM-CSF bicistronic DNA vaccines to elicit remarkably potent HIV-specific CD4⁺ T cell responses in addition to CD8⁺ T cell responses may be particularly useful in improving the efficacy of prophylactic and therapeutic HIV-1 DNA vaccines. Moreover, Ag/GM-CSF bicistronic constructs could be readily incorporated into recombinant live vector vaccines to augment their immunogenicity as well. During primary HIV-1 infection, the early loss of HIV-specific CD4⁺ T cells is probably due to their particular susceptibility to infection, and the subsequent deficiency of HIV-specific T cell help may be central to the ultimate failure of the immune system to control viremia (3, 4, 40). Augmented vaccine-elicited CD4⁺ T cell responses may enhance the control of viremia following HIV-1 infection through a number of immunologic mechanisms, including increasing the proliferation, maturation, and functional activity of CD8⁺ CTL, providing increased help for B cells, and directly producing antiviral cytokines (40). However, it is theoretically possible that increased numbers of vaccine-elicited CD4⁺ T cells may instead merely provide a larger number of cellular targets for the virus and accordingly worsen the clinical outcome of HIV-1 infection. Vaccine studies in nonhuman...
primates will therefore be required to evaluate the efficacy of this GM-CSF bicistron strategy in controlling a pathogenic viral challenge.

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