M Cell DNA Vaccination for CTL Immunity to HIV

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M Cell DNA Vaccination for CTL Immunity to HIV

Xinhai Wang,* David M. Hone,† Asmahan Haddad,* Mohamed T. Shata,† and David W. Pascual2*

To facilitate invasion, reovirus has evolved to attach to M cells, a specialized epithelium residing within the follicle-associated epithelium that covers mucosal inductive tissues. Thus, we questioned adapting reovirus protein σ1 to ferry DNA vaccines to the mucosa to immunize against HIV. Three expression plasmids encoding HIV(Ba-L) gp160, cytoplasmic gp140, and secreted gp140 were tested in mice as protein σ1-poly-L-lysine-DNA complexes (formulated vaccine) via the intranasal route. Evaluation of cell-mediated immunity showed that the formulated gp160 DNA vaccine was more effective for stimulating envelope (Env)-specific CTL responses in lungs, lower respiratory lymph nodes (LN), cervical LN, submaxillary gland LN, and spleens. Three doses of vaccine were required for CTL responses, and intranasal naked DNA immunizations were ineffective. The greatest CTL activity was observed between weeks 8 and 10 for gp160-vaccinated mice, and activity remained detectable by week 16. These Env-specific CTL responses were perforin dependent in peripheral tissues, but mostly Fas dependent in the lungs. These Env-specific CTLs also produced IFN-γ. Mice vaccinated with the formulated gp160 DNA vaccine showed potent antiviral immunity against vaccinia virus-env replication in ovaries. Thus, compared with live vectors, protein σ1-mediated DNA delivery represents an alternative mucosal formulation for inducing cellular immunity against HIV-1. The Journal of Immunology, 2003, 171: 4717–4725.

The mucosal surfaces are the primary sites of transmission for most infectious diseases requiring the induction of mucosal immunity for preventing pathogen entry at these surfaces. More recently, vaccines, particularly attenuated viruses (1, 2), have been designed for their mucosal application. Although these vaccines are effective for stimulating appropriate host immune responses, alternative vaccines and vaccine delivery strategies are warranted to avoid the potential side effects of live viral vectors. Such alternatives may lie with subunit vaccines or DNA vaccines, the latter being relatively easy to prepare and administer. Recent evidence shows that DNA vaccination can confer protective immunity against a number of infectious agents including influenza (3, 4), herpes simplex virus (5), HIV (6, 7), and Borrelia burgdorferi (8) infections. However, naked DNA immunizations have been proved less than optimal for stimulating mucosal immunity (7, 9, 10) when they are administered peripherally. In contrast, microencapsulated DNA vaccines effectively elicit protective mucosal responses in a murine rotavirus challenge model (11, 12). Although it is unclear whether such protection was conferred by the protective properties of microencapsulation, the ability of microspheres to interact with Peyer’s patches, M cells (13, 14), or a combination of both suggests that more efficient uptake of DNA by mucosal inductive tissues favors enhanced mucosal immunity.

Receptor-mediated gene transfer is effective for delivering transgenes into tissues by specific cellular receptors. One such method is to conjugate to a DNA-binding domain a ligand specific for a cell surface receptor. This can be facilitated using poly-L-lysine (PL). This DNA-ligand complex is internalized by targeted cells when the ligand binds to its respective cell surface receptor. Such receptor-mediated gene transfer has been accomplished by targeting a variety of receptors: asialo-orosomucoid (15, 16); transferrin (17–19); lectins (20); and insulin (21).

Reovirus is an enteric pathogen that infects the host following attachment to intestinal Peyer’s patch M cells (22). Thus, as with other enteric pathogens, reovirus exploits M cells as a means to gain entry into the host. Mediating reovirus attachment is its adsorbin, protein σ1, which is expressed and located at the 12 vertices of the viral icosahedron (22). Protein σ1 is a 45-kDa protein that polymerizes via its N terminus (23) to form either a trimer (24) or a tetramer (25) depending on how it is isolated.

Our results show that protein σ1-mediated transgene vaccination can be used for immunization against HIV. Three DNA vaccines were tested, and the data for the mice given the gp160 DNA as protein σ1-PL-gp160 DNA (formulated vaccine), but not as naked DNA, showed long-lasting Env-specific CTLs with potent antiviral immunity.

Materials and Methods

Production of recombinant reovirus σ1 protein

The recombinant protein σ1 from reovirus serotype 3 as a maltose-binding protein was generated and expressed in Escherichia coli, as previously published (1).

1Abbreviations used in this paper: PL, poly-L-lysine; i.n., intranasal; Env, envelope; LN, lymph nodes; LRLN, lower respiratory LN; CLN, cervical LN; SMLN, submaxillary gland LN; CM, complete medium; CMA, concanavalin A; FAE, follicle-associated epithelium; βgal, β-galactosidase; FasL, Fas ligand.

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described (26). This fusion protein was purified by affinity chromatography using amyllose resin according to the manufacturer’s directions (New England Biolabs, Beverly, MA). The MBP::protein or fusion protein is referred to as recombinant protein or1.

Mice
Specific pathogen-free male and female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 5–6 wk of age and maintained in the Animal Resources Center at Brown University (Providence, RI) at 90% of the cells lysed. Culture supernatants were collected by centrifugation at 4000 × g for 10 min, and aliquots of the supernatants were stored in liquid nitrogen until use. These supernatants typically yielded ~5 × 10⁵ pfu/ml as determined by a direct plaque assay on BSC-1 cells. To assess antiviral protection, groups of BALB/c mice were vaccinated i.n. or i.m. with naked gp160 DNA vaccine or i.n. with the formulated gp160 DNA vaccine as described above. All mice were vaccinated at the same time intervals. Mice were given 10⁹ pfu of pV1174 i.p. 16 wk after primary vaccination. The ovaries from the challenged mice were harvested, homogenized, and clarified by centrifugation at 4000 × g for 10 min. Serial 10-fold dilutions were made for infection of BSC-1 cell monolayers, and plaques were enumerated 2 days after in vitro culture.

Statistical analysis
Statistical differences between experimental parameters were determined significant if was <0.05 using Student’s t test and one-way ANOVA. For the challenge study, Wilcoxon’s two sample test was performed.

Results
Mode of HIV-1 Env expression impacts induction of CTL responses
Previous results have shown that M cell-targeted DNA delivery represented an effective means to mucosally vaccinate for the stimulation of CTL responses (27). To adapt this vaccine vehicle to potentially immunize against HIV, M cell-targeted DNA delivery using recombinant rev protein or1 was used for i.n. immunization of BALB/c mice. Three groups of BALB/c mice (five per group) each received an Env (Ba-L) expression plasmid in conjuction with protein or1 conjugated to PL and were given 20 µg of plasmid per dose on days 0, 7, and 14. Each group received a DNA vaccine that expressed full-length gp160, which is located in the plasma membrane, a truncated derivative of Env that lacks the transmembrane and cytoplasmic domains and is secreted (gp140ect), or a leader peptide-deficient derivative of gp140 that is expressed cytoplastically (gp140cyt). After 6 wk post-primary immunization, various respiratory and splenic tissues were isolated, and mononuclear cell fractions were subjected to cytolytic assays by ⁵¹Cr release to determine specific reactivity for Env. Both freshly isolated lymphocytes and in vitro restimulated lymphocytes were assessed for Env. Env-specific CTL activity using BC-Env, a derivative of the mastocytoma cell lines P815, stably transduced with the full-length env gene of HIV-1 and BC-βgal as negative control target cells, which are P815 cells, stably transduced with the E. coli lacZ gene (32). Targets were treated with mitomycin C (50 µg/ml, Sigma-Aldrich, St. Louis, MO) and extensively washed with complete medium (CM; RPMI 1640 plus 10% FBS (HyClone, Logan, UT) plus 10 mM HEPES buffer plus 10 mM nonessential amino acids plus 10 mM sodium pyruvate plus 10 U/ml penicillin/streptomycin). Targets were subsequently labeled with Na₂⁵³CrO₄ (DuPont/NEN, Wilmington, DE) for 1 h at 37°C. The cells were washed three times in CM. Effector cells were incubated with various ratios of labeled targets in bottom-96-well microtiter dishes (Corning-Costar, Oneonta, NY) for 4 h at 37°C. After incubation, cells were spun, and supernatants were assessed for released ⁵¹Cr. Specific cytolyosis was determined as the amount of ⁵¹Cr released in the presence of effector cells corrected for spontaneous ⁵¹Cr release divided by maximal (detergent-induced) release corrected for spontaneous ⁵¹Cr release. For Ag restimulation, splenic and LN mononuclear cells were restimulated with mitomycin C-treated BC-Env cells at 5:1 in CM for 5 days at 37°C. To assess cytolytic mechanisms, washed ex vivo or Ag-restimulated lymphocytes were treated with anti-FasL mAb or 10 mM concanamycin A (CMA; Wako Bioproducts, Richmond, VA) for 2 h at 37°C, similar to that previously described (33). Likewise, some ex vivo or Ag-restimulated lymphocytes were treated with 10 µg/ml anti-FasL mAb (clone MFL3, BD PharMingen, San Diego, CA). Cells were assessed for CTL activity, as described above.

Cytokine detection essays
Supernatants collected from Env (Ba-Env)-restimulated cultures were analyzed for expression of IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10. Commercially produced cytokine-specific mAbs (BD PharMingen) were used, as previously described (31, 34).
ever, on in vitro restimulation, cytolytic activity was greatly enhanced by LRLN, CLN, SMLN, and splenic lymphocytes (Fig. 1, D–F, and Table I). Again, the Ag-restimulated lymphocytes from mice vaccinated with formulated gp160 vaccine at E:T 50:1 showed specific lysis between 42 and 61% (Table I and Fig. 1, D–F). In contrast, Ag-restimulated lymphocytes from formulated gp140 cyt or gp140 sec vaccines at E:T 50:1 showed specific lysis between 31 and 45%, and 27 and 38%, respectively (Table I and Fig. 1, D–F). Protein α1-DNA immunization is more effective than naked DNA delivery

To discern the effectiveness of protein α1-PL-DNA immunization, groups of BALB/c mice were immunized i.n. with HIV expression plasmids either as formulated or naked DNA vaccines. As depicted in Fig. 2 and Table II, the formulated vaccine was clearly more effective for stimulating CTL responses against HIV Env than mice immunized with naked DNA. Activity of freshly isolated lung lymphocytes at E:T

Table I. Mice vaccinated with formulated gp160 DNA showed enhanced lysis of Env-expressing target cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells</th>
<th>BC-Env</th>
<th>% lysis</th>
<th>SE</th>
<th>BC-βgal</th>
<th>% lysis</th>
<th>SE</th>
<th>gp140 cyt</th>
<th>BC-Env</th>
<th>% lysis</th>
<th>SE</th>
<th>gp140 sec</th>
<th>BC-Env</th>
<th>% lysis</th>
<th>SE</th>
<th>gp140 sec</th>
<th>BC-βgal</th>
<th>% lysis</th>
<th>SE</th>
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</thead>
<tbody>
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<td>Ex vivo</td>
<td>Lung</td>
<td>47.10</td>
<td>4.48</td>
<td>4.76</td>
<td>1.60</td>
<td>35.58</td>
<td>3.25</td>
<td>4.72</td>
<td>1.60</td>
<td>35.58</td>
<td>3.25</td>
<td>&lt;0.001</td>
<td>26.36</td>
<td>3.33</td>
<td>&lt;0.001</td>
<td>5.72</td>
<td>2.15</td>
<td>5.72</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>29.74</td>
<td>4.71</td>
<td>3.93</td>
<td>1.17</td>
<td>20.97</td>
<td>2.37</td>
<td>3.58</td>
<td>1.17</td>
<td>20.97</td>
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<td>NS</td>
<td>17.93</td>
<td>3.58</td>
<td>NS</td>
<td>5.25</td>
<td>0.88</td>
<td>5.25</td>
<td>0.88</td>
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<tr>
<td></td>
<td>LRLN</td>
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<td>3.68</td>
<td>8.54</td>
<td>2.78</td>
<td>18.63</td>
<td>3.20</td>
<td>3.88</td>
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<td>18.63</td>
<td>3.20</td>
<td>NS</td>
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<td>2.15</td>
<td>&lt;0.001</td>
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<td>1.00</td>
<td>7.06</td>
<td>1.00</td>
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<tr>
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<td>5.46</td>
<td>3.16</td>
<td>16.78</td>
<td>1.14</td>
<td>7.63</td>
<td>3.16</td>
<td>16.78</td>
<td>1.14</td>
<td>NS</td>
<td>18.50</td>
<td>3.07</td>
<td>NS</td>
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<td>1.60</td>
<td>2.65</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td>SMLN</td>
<td>16.88</td>
<td>2.03</td>
<td>5.41</td>
<td>1.99</td>
<td>16.62</td>
<td>1.83</td>
<td>6.38</td>
<td>1.99</td>
<td>16.62</td>
<td>1.83</td>
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<td>NS</td>
<td>5.10</td>
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<td>5.10</td>
<td>0.19</td>
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<td>2.16</td>
<td>38.27</td>
<td>2.36</td>
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<td>&lt;0.001</td>
<td>5.66</td>
<td>1.41</td>
<td>5.66</td>
<td>1.41</td>
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<tr>
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<td>4.72</td>
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<td>43.14</td>
<td>3.54</td>
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<td>1.26</td>
<td>43.14</td>
<td>3.54</td>
<td>&lt;0.001</td>
<td>27.11</td>
<td>1.99</td>
<td>&lt;0.001</td>
<td>3.87</td>
<td>3.10</td>
<td>3.87</td>
<td>3.10</td>
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<tr>
<td></td>
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<td>4.16</td>
<td>4.58</td>
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<td>2.75</td>
<td>&lt;0.001</td>
<td>0.65</td>
<td>32.44</td>
<td>2.75</td>
<td>&lt;0.001</td>
<td>38.35</td>
<td>3.47</td>
<td>&lt;0.001</td>
<td>7.93</td>
<td>1.90</td>
<td>7.93</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>SMLN</td>
<td>43.51</td>
<td>2.94</td>
<td>8.36</td>
<td>3.26</td>
<td>43.17</td>
<td>3.61</td>
<td>5.11</td>
<td>3.26</td>
<td>43.17</td>
<td>3.61</td>
<td>NS</td>
<td>27.54</td>
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<td>NS</td>
<td>3.96</td>
<td>1.24</td>
<td>3.96</td>
<td>1.24</td>
</tr>
</tbody>
</table>

* Lymphocytes from BALB/c mice immunized with formulated gp160, gp140 cyt, or gp140 sec DNA were isolated 6 wk into the experiment and 51Cr release assays were conducted as described in Fig. 1. Freshly isolated (ex vivo) or 5-day Ag-restimulated cells were measured.

* Lymphocytes were isolated from lungs, spleen, LRLN, CLN, and SMLN.

* The specificity of lysis of targets expressing Env vs. βgal was significant (p < 0.001).

* Mean ± SE of three experiments at E:T 50:1.
50:1 from formulated DNA varied between 25 and 55% lysis, depending on the DNA vaccine used (Table II and Fig. 2, A–C), but those immunized i.n. with naked DNA varied only from no lysis to 19%. For the other lymphoid tissues, only the i.n.-formulated DNA-vaccinated mice showed lytic activity, whereas the lymphocytes from naked DNA-immunized mice showed no lytic activity (Table II and Fig. 2, A–C). Although in vitro Ag- restimulation of respiratory LN and splenic lymphocytes did enhance cytolytic activity from naked DNA-immunized mice, the magnitude of enhancement was not nearly as great for mice vaccinated with formulated DNA (Table II and Fig. 2, D–F). Thus, the addition of protein H9268 1-PL to DNA expression plasmids clearly facilitates immunization, whereas naked DNA does not. Likewise, in the absence of protein σ1, i.n. immunization with PL-DNA also fails to enhance CTL responses as naked DNA immunization (data not shown).

**Induced CTL activity is dose dependent**

To determine whether three doses of vaccine were required to induce the CTL responses, groups of mice were immunized with a single, two, or three doses of formulated vaccine. CTL responses were assessed 6 wk post-primary immunization. As shown in Fig.

---

**Table II. Protein σ1-PL facilitates i.n. immunization**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells</th>
<th>gp160 Formulated % lysis</th>
<th>DNA only% lysis</th>
<th>gp140 cyt Formulated % lysis</th>
<th>DNA Only% lysis</th>
<th>gp140 sec Formulated % lysis</th>
<th>DNA Only% lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex vivo</td>
<td>Lung</td>
<td>54.48 5.12</td>
<td>19.08 2.26</td>
<td>38.97 3.00</td>
<td>9.91 3.48</td>
<td>24.38 3.70</td>
<td>9.51 1.36</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>21.51 3.30</td>
<td>10.35 2.89</td>
<td>16.39 2.43</td>
<td>7.41 2.59</td>
<td>19.16 3.18</td>
<td>7.34 1.15</td>
</tr>
<tr>
<td></td>
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<td>22.28 2.44</td>
<td>7.17 3.66</td>
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<td>9.28 1.81</td>
<td>14.24 4.15</td>
<td>6.43 2.32</td>
</tr>
<tr>
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<td>19.67 3.34</td>
<td>11.92 2.89</td>
<td>21.67 2.07</td>
<td>2.20 1.45</td>
<td>17.42 4.19</td>
<td>5.62 2.51</td>
</tr>
<tr>
<td></td>
<td>SMLN</td>
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<td>5.47 3.43</td>
<td>15.01 3.12</td>
<td>8.19 1.49</td>
<td>19.64 4.88</td>
<td>7.51 2.09</td>
</tr>
<tr>
<td>In vitro</td>
<td>Spleen</td>
<td>58.86 7.44</td>
<td>17.23 1.85</td>
<td>42.69 4.12</td>
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<td>37.66 3.48</td>
<td>14.13 2.55</td>
</tr>
<tr>
<td>restimulated</td>
<td>LRLN</td>
<td>50.66 4.56</td>
<td>21.07 4.05</td>
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<td>15.15 3.95</td>
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</tr>
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<td>57.72 3.75</td>
<td>15.76 4.11</td>
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<td>14.78 4.57</td>
<td>27.16 2.86</td>
<td>11.19 3.14</td>
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<tr>
<td></td>
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<td>42.37 3.72</td>
<td>9.38 1.89</td>
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<td>16.00 1.34</td>
<td>17.94 3.45</td>
<td>6.92 1.78</td>
</tr>
</tbody>
</table>

* Lymphocytes from BALB/c mice immunized with formulated or naked gp160, gp140 cyt, or gp140 sec were isolated 6 wk into the experiment, and 51Cr release assays were conducted as described in Fig. 1. Freshly isolated (ex vivo) or 5-day Ag-restimulated cells were measured.

* Lymphocytes were isolated from lungs, spleen, LRLN, CLN, and SMLN.

* Mean ± SE of three experiments at E:T 50:1.

* p was < 0.001 between formulated and naked DNA-vaccinated groups.
Three doses of gp160 were necessary to effect anti-Env CTL responses in the lungs, spleens, LRLN, and SMLN (p < 0.013). The gp140 cyt DNA vaccines induced Env-specific CTL activity in the lungs, draining respiratory LN and spleens (p < 0.014), whereas the gp140 sec DNA vaccine was poorly immunogenic in these sites where only the spleen (p = 0.010), LRLN (p = 0.035), and SMLN (p = 0.013) showed significant enhancement by the three doses.

Kinetic analysis of induced CTL activity

To ascertain the longevity of the induced CTL responses against Env, groups of mice were immunized i.n. with one of the three DNA vaccines using protein 1-PL-DNA vaccine. Beginning at wk 6 through wk 16, lymphocytes from lungs, draining LN, and splenocytes were isolated and immediately evaluated for CTL activity or were subsequently restimulated with Ag in vitro. Evidence in this study (Fig. 4) demonstrated that lung CTL responses peaked between wk 6 and 10 in the mice vaccinated with the gp160 DNA vaccine, whereas mice vaccinated with gp140 cyt and gp140 sec DNA vaccines displayed Env-specific CTL responses that peaked between 8 and 10 wk after vaccination. Similarly, LN and splenic CTL responses peaked at ~10 wk after vaccination. By 16 wk after vaccination, mice vaccinated with the gp160 DNA vaccine still exhibited measurable Env-specific CTL activity in all tissues, whereas lymphocytes from mice vaccinated with gp140 cyt required in vitro Ag restimulation before measurable cytolytic activity was evident (Fig. 4, B and E). In contrast, 16 wk after immunization, neither freshly isolated nor in vitro-restimulated lymphocytes from mice vaccinated with the gp140 sec DNA vaccine displayed cytolytic activity (Fig. 4, C and F).

Anti-Env CTL responses are perforin dependent

To assess the lytic mechanism of the observed CTL responses to HIV Env, mice were immunized i.n. with the three doses of the formulated vaccine. Ex vivo lung and splenic lymphocyte cultures were treated with 10 nM CMA to block perforin-mediated killing or with 10 μg/ml anti-FasL mAb to block Fas-dependent killing. The lung responses were only modestly impacted by CMA treatment (p = 0.003). In contrast, the cytolsis was completely inhibited with the anti-FasL mAb (Fig. 5; p < 0.001). Unlike the...
lung, the splenic CTL were mostly inhibited by the CMA treatment (Fig. 5B; p > 0.001). Ag-restimulated splenocytes and LRLN lymphocytes showed the expected increased cytolytic activity, which was inhibited entirely by CMA treatment (p < 0.001), whereas the anti-FasL treatment was partially inhibitory for splenic responses (p = 0.012; Fig. 5, C and D). Collectively, these data demonstrate that perforin is important for immunity induced by formulated gp160 vaccination in the draining respiratory LN.

IFN-γ is important for initial CTL response to Env

To assess the contribution of IFN-γ to cytolytic activity induced by protein α1-PL-gp160 vaccination, both BALB/c and IFN-γ−/− mice were vaccinated i.n. Freshly isolated lymphocytes from IFN-γ−/− lungs, respiratory LN, and spleen failed to exhibit cytolytic activity when compared with BALB/c responses (p < 0.01; Fig. 6, A and B). In contrast, in vitro Ag restimulation showed vigorous killing by IFN-γ−/− lymphocytes, indicating that the Env-specific CTLs did not require IFN-γ for lytic activity.

To assess the cytokine profiles subsequent to Ag restimulation, supernatants from Ag-restimulated lymphoid cultures from spleen, LRLN, CLN, and SMLN were assayed by cytokine-specific ELISAs. The three vaccines showed preferential stimulation of IFN-γ and IL-2 production when compared with IL-4, IL-5, IL-6, or IL-10 levels (Fig. 7).

Vaccinia-Env challenge of mice vaccinated with formulated gp160 DNA vaccine

The vaccinia virus challenge bearing an HIV protein is generally used to assess levels of cell-mediated immunity (35). To assess the potential efficacy of our formulated gp160 DNA vaccine, BALB/c mice were given the formulated or naked gp160 DNA vaccine via the i.n. route as previously described. As a positive control, one group of mice were immunized i.m. with the same gp160 DNA vaccine at the same time intervals and with the same doses. The last group of mice were left unimmunized to serve as a naive control. Approximately 16 wk after primary immunization, each group was challenged with vaccinia-env, and ovaries were assessed for levels of vaccinia PFU. It was quite evident that the group of mice given the formulated gp160 DNA vaccine showed potent antiviral immunity when compared with challenged naive mice or other immunized groups (Fig. 8). Thus, these data show

![FIGURE 6](image-url)  ![FIGURE 7](image-url)  ![FIGURE 8](image-url)
that protein σ1 targeting is an effective means to stimulate host responses via the i.n. route.

Discussion
A unique attribute of mucosal inductive tissues is the specialized epithelium covering these organized mucosal lymphoid tissues, termed follicle-associated epithelium (FAE). Within the FAE is a differentiated epithelial cell subset referred to as M cells. M cells are adept at uptake and transport of luminal Ags, including soluble proteins and infectious agents. It is also believed that certain pathogens, such as *Salmonella typhimurium* (37), *Yersinia enterocolitica* (38), and reoviruses (22), invade the host tissues by binding to M cells. To take advantage of this specialized niche, we hypothesize that targeting M cells using proteins derived from such infectious agents would serve as M cell ligands. One such ligand, reovirus protein σ1, was previously shown in its recombinant form to bind M cells and facilitate i.n. immunization using expression plasmid reporter gene constructs (26). Both transfection (26) and immunization (27) required covalent attachment of protein σ1 to PL to enable complexing to DNA vaccine. It was evident in this previous study (27) that targeting was necessary to generate mucosal responses because naked or PL-complexed DNA failed to stimulate mucosal Ab or CTL responses.

To expand on the earlier observation that the protein σ1-PL-DNA delivery system can stimulate CTL responses, work described here focused on the adaptation of this technique to stimulate cell-mediated anti-HIV Env responses. The best results were obtained using the expression plasmid encoding gp160 rather than gp140syt or gp140sec. The reason that differences were obtained was not resolved, although the mode of expression may have impacted the immunogenicity of Env in these constructs. Minimally, we know that it was not attributed to differences in protein production because transfection assays with the three DNA vaccines yielded similar levels of expressed protein (data not shown). We had expected the secreted gp140 to induce CTL responses resembling those induced by membrane-bound gp160, but the magnitude of CTL responses between the two vaccines was notably different. Although some improvement in host CTL responses was observed between 8 and 10 wk post-primary immunization, the gp160 vaccine was clearly the most effective to stimulate enhanced CTL responses when given i.n., and these CTL responses continued to be evident for at least 16 wk after primary immunization. Perhaps one possibility to account for the increased immunogenicity may be related to the increased size of gp160 making it more immunogenic or providing additional target peptides. In contrast, CTL responses induced by gp140sec waned 16 wk after vaccination and were undetectable even after in vitro Ag re-stimulation. Finally, these results showed that at least three doses of vaccine were required to obtain the observed magnitude in CTL responses.

The potency and longevity of these CTL responses were also assessed using the vaccinia-env challenge similar to that previously described (35, 36). As our in vitro analyses suggested, the formulated gp160 DNA vaccine induced potent antiviral immunity as evidenced by the reduced vaccinia virus PFUs when compared with mice given the same vaccine by the naked i.n. or i.m. routes. Such data support our contention that targeting vaccines can enhance immune responses. It is also important that only one previous study (36) presented a similar extent of cell-mediated responses when a DNA vaccine was given i.n. However, this required a live vector to carry the DNA vaccine but again suggests that targeting mucosal inductive tissues may be important for successful vaccination (39). Thus, a DNA subunit vaccine is capable of eliciting strong cell-mediated immunity if it can be effectively targeted using our approach or by live vectors.

The CTL responses acquired using the gp160 vaccine were accomplished in the absence of adjuvant. Typically, successful i.n. DNA vaccination against HIV immunogens has required the use of a live vector, as in the case for adenovirus (40, 41), vesicular stomatitis virus (42), or a bacterial vector (30). Otherwise, adjuvant is coadministered (43) or incorporated into the DNA (44). In addition, the enthusiasm for administering adjuvants i.n. has been dampened by recent data showing olfactory bulb apoptosis (45, 46). Our data suggest that effective targeting to mucosal inductive tissues may circumvent the need for additional adjuvants and still be able to stimulate long-lasting CTL responses; thus, effective vaccine targeting potentially can supplant the need for additional adjuvants. Current studies are assessing these possibilities. Collectively, from this study, as well as those described for live vector vaccines (30, 40–42) and incorporation to liposomes, it is evident that for i.n. delivery of DNA vaccines, the DNA must be protected or targeted to mucosal inductive sites to facilitate vaccination. Otherwise, as shown here, application of naked DNA to the nasal passages fails to implement effective transgene vaccination.

CD8+ T cells do appear to play an active role in the control of lentivirus infections, either by cytolytic mechanisms or production of β-chemokines. In experimental models, depletion of CD8+ T cells in SIV-infected rhesus monkeys (47) or HIV infection in mice (48) implicates the role of CD8+ T cells in the control viremia. Perforin, one of the cytotoxins released by immune CTLs, mediates lysis of infected target cells by facilitating entry of proteases or granzymes into target cells. Recent data have suggested that during chronic HIV infection, HIV-specific CTLs are induced but express low levels of perforin (49–52), despite the ability to express granzyme A (51, 53). Given these findings, a vaccine that stimulates perforin-dependent, HIV-specific CTL responses may provide a benefit to the vaccinee. Evidence reported above shows that one attribute of the protein σ1-PL-DNA delivery is the capacity to stimulate perforin-dependent CTL responses following i.n. vaccination.

Coupled to the ability of gp160 DNA vaccine to induce CTL responses was the capacity of immune T cells to produce of IFN-γ. Ag-restimulated T cell cultures showed enhanced production of IFN-γ and IL-2, whereas only low levels of cytokines associated with Th2/Tc2 cells were detected in these cultures. In a previous study (27), Th2 cytokines were detected after Ag restimulation of CD4+ T cells; however, cytokine responses by immune CD8+ T cells were not evaluated at that time. Future studies will evaluate the ability of the gp160, as well as other HIV DNA vaccines to stimulate systemic and mucosal Abs against Env, and determine which Th cell subsets support these responses. Such evaluations were beyond the scope of these current studies.

In summary, a method is described for DNA vaccination via the i.n. route. Thus, DNA vaccines induce perforin-dependent CTL responses after i.n. administration in formulations containing the σ1-PL-DNA delivery fusion protein. Of three DNA vaccines tested, the plasma membrane expressed gp160 DNA vaccine was the most immunogenic for CTL responses, suggesting that the mode of expression influences the immunogenicity of DNA vaccine-expressed immunogens delivered via the i.n. route. The ability to target respiratory mucosal inductive sites in the respiratory tract aided by the ability to elicit CTL responses in the lungs, as
well as in the draining respiratory LN, will provide a useful tool to develop vaccines against pathogens that target mucosal surfaces, such as HIV-1 and influenza.

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References


