Plasmid Chemokines and Colony-Stimulating Factors Enhance the Immunogenicity of DNA Priming-Viral Vector Boosting Human Immunodeficiency Virus Type 1 Vaccines


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Heterologous “prime-boost” regimens that involve priming with plasmid DNA vaccines and boosting with recombinant viral vectors have been shown to elicit potent virus-specific cytotoxic T-lymphocyte responses. Increasing evidence, however, suggests that the utility of recombinant viral vectors in human populations will be significantly limited by preexisting antivector immunity. Here we demonstrate that the coadministration of plasmid chemokines and colony-stimulating factors with plasmid DNA vaccines markedly increases the immunogenicity of DNA prime-recombinant adenovirus serotype 5 (rAd5) boost and DNA prime-recombinant vaccinia virus (rVac) boost vaccine regimens in BALB/c mice. In mice with preexisting anti-Ad5 immunity, priming with the DNA vaccine alone followed by rAd5 boosting elicited only marginal immune responses. In contrast, cytokine-augmented DNA vaccine priming followed by rAd5 vector boosting was able to generate potent immune responses in mice with preexisting anti-Ad5 immunity. These data demonstrate that plasmid cytokines can markedly improve the immunogenicity of DNA prime-viral vector boost vaccine strategies and can partially compensate for antivector immunity.

Priming with plasmid DNA vaccines and boosting with recombinant viral vectors such as replication-defective adenoviruses and poxviruses have been shown to generate potent virus-specific cytotoxic T-lymphocyte (CTL) responses (2, 9, 11, 17, 19). Since virus-specific CTL responses are critical for the control of human immunodeficiency virus type 1 (HIV-1) (6, 10, 13, 15), these “prime-boost” strategies are being assessed as candidate AIDS vaccines (12). Recent studies have demonstrated that DNA prime-replication-defective adenovirus serotype 5 (rAd5) boost regimens (17) as well as DNA prime-recombinant-modified vaccinia virus Ankara (rMVA) boost regimens (2) afford significant control of pathogenic simian-human immunodeficiency virus (SHIV) challenges in rhesus monkeys. Similarly, DNA prime-rAd5 boost regimens have protected nonhuman primates against lethal challenge with Ebola virus (19).

The clinical utility of such prime-boost regimens, however, will likely be limited by preexisting immunity to the viral vector. Antivector immunity may result in the rapid elimination of the vaccine vector and thus could substantially reduce its immunogenicity. This problem is expected to be a major limitation for rAd5 vectors, since a large fraction of the human population has preexisting anti-Ad5 immunity as a result of natural exposure to Ad5. Strategies to improve these vaccine regimens are therefore needed.

One potential strategy for improving the immunogenicity of DNA prime-viral vector boost vaccine regimens is to improve the efficiency of the DNA prime by the coadministration of adjuvants. Our laboratory and others have previously demonstrated that plasmid cytokines can augment DNA vaccine-specific humoral and cellular immune responses in both mice and rhesus monkeys (4–6, 16, 21, 23). Here we investigate the utility of chemoattractant plasmid cytokines in augmenting the immunogenicity of DNA prime-viral vector boost vaccine regimens in mice, both in the absence and in the presence of antivector immunity.

MATERIALS AND METHODS

Mice and immunizations. Six- to eight-week-old BALB/c mice were purchased from Charles River Laboratories (Wilmington, Mass.). For DNA immunizations using pVRC expression plasmids, 50 μg of pVRC-HIV-1 Env IIIB gp120 plasmid DNA vaccine (5) was first mixed with various amounts of sham plasmid or plasmid cytokines and then injected intramuscularly (i.m.) in 100 μl of sterile saline divided between the right and left quadriceps muscles. For rAd5 immunizations, mice were injected i.m. with various quantities of E1-deleted replication-incompetent rAd5-HIV-1 Env IIIB gp140ΔCFI in 100 μl of sterile phosphate-buffered saline (PBS). To prepare this rAd5 vector, a plasmid expressing the CXCR4-tropic HIV-1 HIV2 Env IIIB (GenBank accession no. K03455) was made synthetically by using codons typically found in human cells. To express a truncated mutant Env protein, a stop codon was introduced after position 680 to produce gp140. The Env protein was further changed by deleting amino acids 503 to 537 and 593 to 619, which removed the cleavage site sequence, the fusion domain, and a part of the spacer between the two heptad repeats to produce gp140ΔCFI. All mutations were confirmed by sequencing both strands of the cDNAs. Sequence analysis indicated that the codon-modified gp140ΔCFI vaccine was correct, except for the minor point substitutions previously described (7). The cDNAs were cloned into the XbaI and BamHI sites of the Ad5 vector, which contained a deletion in E1 that rendered the vector replication-defective and a partial deletion or substitution in E3 that disrupted the coding sequences for the E3 proteins (14). The rAd5-gp140ΔCFI vaccine was then prepared as previously described (3, 19). For rVac immunizations, mice were injected intraperitoneally by guest http://jvi.asm.org/Downloaded from
serum anti-gp120 antibody titers from immunized mice were measured by a similar direct ELISA in which plates were coated overnight with 10^6 Ad5-ΔE1 particles per well.

Statistical analyses. Statistical analyses were performed with GraphPad Prism version 2.01 (GraphPad Software, Inc., 1996). Comparisons of cellular immune responses among groups of mice were performed by two-tailed t tests for two groups of animals or by analysis of variance (ANOVA) for more than two groups. Bonferroni adjustments were included when appropriate to account for multiple comparisons. In all cases, P values of <0.05 were considered significant.

RESULTS

Augmentation of DNA vaccine priming by plasmid GM-CSF and plasmid MIP-1α. We have demonstrated that the coadministration of plasmid granulocyte-macrophage colony stimulating factor (GM-CSF) and plasmid MIP-1α increased recruitment of dendritic cells to the site of inoculation and augmented DNA vaccine-elicited immune responses in mice (submitted). In the present study, we sought to determine whether DNA vaccines augmented by plasmid chemokines and GM-CSF would prime for improved immune responses following viral vector boost immunizations. Given the variability in the magnitude of immune responses observed in experimental animals vaccinated with DNA vaccines and certain plasmid cytokine adjuvants (4, 5, 16, 21), we first assessed the reproducibility of CD8⁺ T-cell responses primed by GM-CSF/MIP-1α-augmented DNA vaccines in large groups of BALB/c mice (n = 20 per group). Mice were immunized with either 50 μg of gp120 DNA vaccine (5) plus 100 μg of sham plasmid or 50 μg of gp120 DNA vaccine plus 50 μg of plasmid murine GM-CSF and 50 μg of plasmid murine MIP-1α. Vaccine-elicited CD8⁺ T-cell responses specific for the immunodominant H-2D^d-restricted P18 tetramer per group. Mice were immunized with either 50 μg of gp120 DNA vaccine (5) plus 100 μg of sham plasmid or 50 μg of gp120 DNA vaccine plus 50 μg of plasmid murine GM-CSF and 50 μg of plasmid murine MIP-1α. Vaccine-elicited CD8⁺ T-cell responses specific for the immunodominant H-2D^d-restricted P18 epitope (20) were assessed following tetramer binding to CD8⁺ T lymphocytes.

Mice primed with a single injection of the unadjuvanted gp120 DNA vaccine generated mean peak P18-specific responses of 1.14% of peripheral blood CD8⁺ T lymphocytes on day 14 following primary immunization (Fig. 1). These responses declined to 0.34% by day 28 and were stably maintained thereafter. In contrast, mice primed with a single injection of the gp120/GM-CSF/MIP-1α DNA vaccine generated approximately eightfold-higher mean peak P18-specific responses of 8.76% of CD8⁺ T lymphocytes several days earlier on day 10 following primary immunization. These responses declined to 1.03% on day 28 and were stably maintained thereafter. In both groups of mice, the standard errors were approximately 15% of the magnitude of the responses. Coadministration of plasmid GM-CSF and plasmid MIP-1α thus resulted in reproducible and robust increases in peak and memory P18-specific responses (P < 0.0001 comparing responses between groups on day 10 or day 49 by using two-tailed t tests).

Augmentation of DNA prime-viral vector boost regimens by plasmid cytokines. We next investigated the ability of rAd5 or rVac vectors expressing HIV-1 Env IIB to boost cellular immune responses primed by the DNA vaccine with or without plasmid cytokine adjuvants. Mice primed with the gp120 DNA
vaccine alone were boosted 3 months later with a dose titration of replication-defective rAd5-gp140ΔCFI (Fig. 2A) or replication-competent rVac-gp160 (Fig. 3A). Even at low doses, both vectors efficiently expanded P18-specific tetramer-positive CD8\(^+\) T lymphocytes. A high dose of \(10^9\) particles of rAd5-gp140ΔCFI expanded P18-specific responses to 40 to 50% of peripheral blood CD8\(^+\) T lymphocytes on day 7 following the boost, and these responses were stably maintained through day 21 (Fig. 2A). A low dose of \(10^6\) particles of rAd5-gp140ΔCFI also boosted P18-specific responses to 4 to 5% of CD8\(^+\) T lymphocytes. Different kinetics of expansion of CD8\(^+\) T-cell responses were observed after boosting with rVac-gp160 vectors. P18-specific responses peaked on day 7 following the rVac boost and declined between days 7 and 21 (Fig. 3A). A low dose of \(10^4\) PFU of rVac-gp160 efficiently expanded P18-specific responses to 33% of CD8\(^+\) T lymphocytes on day 7, and these responses declined to 12 to 14% of CD8\(^+\) T lymphocytes by days 14 to 21.

We next assessed the ability of these rAd5 and rVac vectors to boost immune responses primed by cytokine-augmented DNA vaccines. Groups of mice (\(n = 4\) per group) were primed with the gp120 DNA vaccine alone, or the gp120 DNA vaccine with the plasmid cytokines GM-CSF, MIP-1\(\alpha\), GM-CSF plus MIP-1\(\alpha\), MCP-1, or GM-CSF plus MCP-1 and then boosted on day 45 with \(10^6\) PFU of rVac-gp160. Vaccine-elicited cellular immune responses were measured by tetramer binding to CD8\(^+\) T lymphocytes. Arrows indicate immunizations.

These animals were boosted with low doses of rAd5-gp140ΔCFI (\(10^6\) particles) or rVac-gp160 (\(10^6\) PFU) on day 45 following primary immunization. The rAd5-gp140ΔCFI boost resulted in an efficient expansion of the DNA-primed memory responses in all animals (Fig. 2B). Interestingly, the mice that received the gp120/GM-CSF/MIP-1\(\alpha\) DNA vaccine prime maintained the highest responses following the boost. In fact, the relative hierarchy of responses among groups of mice that received the different priming regimens was unchanged by the
rAd5 boost. In mice primed with the gp120 DNA vaccine alone, P18-specific responses expanded to 4 to 5% of CD8⁺ T lymphocytes following the rAd5-gp140ΔCFI boost. In mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine, responses expanded to 20 to 25% of CD8⁺ T lymphocytes. Intermediate responses were observed in the groups that received the other plasmid cytokine adjuvants. These data demonstrate that the augmentation of DNA vaccine priming by a variety of plasmid cytokine adjuvants resulted in increased efficiency of the rAd5 boost. Similarly, mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine developed higher responses than mice primed with the gp120 DNA vaccine alone following the rVac-gp160 boost (Fig. 3B). However, differences among groups were less clear following the rVac boost than following the rAd5 boost.

Functional analysis of vaccine-elicited immune responses. We next sought to investigate the functional characteristics of the immune responses elicited by the cytokine-augmented DNA prime-rAd5 boost regimens. Groups of mice (n = 4 per group) were primed with sham plasmid, the gp120 DNA vaccine alone, or the gp120 DNA vaccine with the plasmid cytokine adjuvant GM-CSF, MIP-1α, or GM-CSF plus MIP-1α. Mice received 50 µg of each plasmid with sufficient sham plasmid to make the total inoculum 150 µg of DNA. With the exception of the sham-vaccinated mice, all groups were boosted with 50 µg of gp120 DNA vaccine alone at week 8. All groups then received a third injection of 10⁶ particles of rAd5-gp140ΔCFI at week 24. The rAd5 boost efficiently expanded P18-specific tetramer-positive CD8⁺ T lymphocytes in all groups, and the hierarchy of responses among groups was preserved (Fig. 4A). Trends toward higher responses following the rAd5 boost were observed in mice primed with the gp120/GM-CSF DNA vaccine or the gp120/MIP-1α DNA vaccine than in mice primed with the gp120 DNA vaccine alone. In contrast, significantly higher responses were elicited in mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine than in mice primed with the gp120 DNA vaccine alone (P < 0.01), the gp120/GM-CSF DNA vaccine (P < 0.01), or the gp120 DNA vaccine alone (P < 0.001 comparing responses among groups on day 14 following the rAd5-gp140ΔCFI boost by ANOVA with Bonferroni adjustments to account for multiple comparisons). Remarkably potent and durable P18-specific responses of 45 to 52% of CD8⁺ T lymphocytes were elicited in mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine.

FIG. 4. Functional analysis of vaccine-elicited immune responses. Groups of mice (n = 4 per group) were primed with sham plasmid, the gp120 DNA vaccine alone, or the gp120 DNA vaccine with the plasmid cytokine adjuvant GM-CSF, MIP-1α, or GM-CSF plus MIP-1α. These mice were then boosted at week 8 with the gp120 DNA vaccine alone and at week 24 with 10⁶ particles of rAd5-gp140ΔCFI. Vaccine-elicited cellular immune responses were assessed by (A) tetramer binding to CD8⁺ T lymphocytes, (B) fresh ex vivo cytotoxicity assays, and pooled peptide and P18-specific IFN-γ ELISPOT assays using either unfractionated (C), CD4-depleted (D), or CD8-depleted (E) splenocytes. SFC, spot-forming cells. (F) Vaccine-elicited humoral immune responses were assessed by a gp120 ELISA prior to immunization (white bars) and 28 days after the rAd5-gp140ΔCFI boost (black bars).
and boosted with the gp120 DNA vaccine and low-dose rAd5-gp140ΔCFI.

In order to determine whether these high-frequency CD8+ T-lymphocyte responses in fact represented functional CTLs, these mice were sacrificed on day 42 following the rAd5 boost. Splenocytes were isolated and evaluated in fresh ex vivo cytotoxicity assays. Unstimulated splenocytes of mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine and boosted with rAd5 exhibited potent fresh cytotoxicity (Fig. 4B). IFN-γ secretion by splenocytes of these mice was also assessed by ELISPOT assays using the Env IIIB peptide pool or the P18 epitope peptide. Trends toward higher ELISPOT responses were observed in mice primed with the gp120/GM-CSF DNA vaccine or the gp120/MIP-1α DNA vaccine as compared to those in mice primed with the gp120 DNA vaccine alone (Fig. 4C). In contrast, splenocytes of mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine exhibited high-frequency ELISPOT responses of over 5,000 spot-forming cells per 10⁶ splenocytes following exposure to pooled peptides or the P18 peptide. These responses were significantly higher than the responses observed in mice primed with the gp120/MIP-1α DNA vaccine (P < 0.05), the gp120/GM-CSF DNA vaccine (P < 0.01), or the gp120 DNA vaccine alone (P < 0.01 comparing ELISPOT responses among groups by ANOVA with Bonferroni adjustments to account for multiple comparisons). Similar results were also observed in ELISPOT assays using splenocytes depleted of CD4+ T lymphocytes or CD8+ T lymphocytes (Fig. 4D and E).

Table 1 shows cytokine secretion profiles of antigen-stimulated splenocytes from these groups of mice. Remarkably high levels of IFN-γ and interleukin-2 (IL-2) secretion were observed in the mice primed with cytokine-augmented DNA vaccines, particularly the animals that received the gp120/GM-CSF/MIP-1α DNA vaccine. Moreover, following the rAd5-gp140ΔCFI boost, 10-fold-higher anti-gp120 antibody titers were elicited in mice that were primed with the gp120/GM-CSF/MIP-1α DNA vaccine than in mice that were primed with the gp120 DNA vaccine alone following the rAd5-gp140ΔCFI boost (Fig. 4F). These data demonstrate that coadministration of these plasmid cytokines with DNA vaccine priming resulted in a marked enhancement of vaccine-elicited humoral and cellular immune responses following the rAd5 boost.

**Inhibitory effects of preexisting anti-Ad5 immunity.** An important practical limitation of rAd5 vectors is that a large percentage of the human population has preexisting immunity to Ad5 that may blunt the immunogenicity of rAd5 vectors. To model this phenomenon, we evaluated the effects of preexisting anti-Ad5 immunity on immune responses elicited by DNA prime-rAd5 boost vaccine regimens in mice. Groups of mice (n = 4 per group) were preimmunized 28 days prior to vaccination with either saline or empty Ad5 (ADV-ΔE1 [5 × 10⁸ particles]), to induce active anti-Ad5 immunity. Mice preimmunized with saline and primed with the gp120 DNA vaccine alone exhibited potent expansions of tetramer-positive CD8+ T-cell responses following the rAd5-gp140ΔCFI boost as expected (Fig. 5A). In these groups of animals, P18-specific responses expanded to 25 to 30% of CD8+ T lymphocytes following a boost with 10⁹ particles of rAd5-gp140ΔCFI and to 3 to 5% of CD8+ T lymphocytes following a boost with 10⁶ particles of rAd5-gp140ΔCFI. In contrast, mice preimmunized with empty Ad5 and similarly primed with the gp120 DNA vaccine alone exhibited a dramatic blunting of tetramer-positive CD8+ T-cell responses following the rAd5-gp140ΔCFI boost (Fig. 5B). P18-specific responses expanded only to 3 to 5% of CD8+ T lymphocytes following a boost with 10⁶ particles of rAd5-gp140ΔCFI, and minimal boosting effects were observed with lower doses. Thus, preexisting anti-Ad5 immunity induced by this regimen resulted in a 3-log reduction of the effective rAd5-gp140ΔCFI dose and a 90% reduction in the absolute response. Anti-Ad5 antibody titers in mice preimmunized with Ad5 were confirmed by ELISA (Fig. 5D).

We reasoned that mice that received a more potent DNA vaccine priming regimen may be capable of overcoming in part the inhibitory effects of preexisting anti-Ad5 immunity. Mice preimmunized with empty Ad5 and primed with the gp120/GM-CSF/MIP-1α DNA vaccine exhibited a partial recovery of tetramer-positive CD8+ T-cell responses following rAd5-gp140ΔCFI boost. P18-specific responses expanded to 12 to 15% of CD8+ T lymphocytes following a boost with 10⁹ particles of rAd5 and to 3 to 5% of CD8+ T lymphocytes following a boost with 10⁷ particles of rAd5. Thus, the coadministration of these plasmid cytokine adjuvants with DNA vaccine priming restored approximately 2 of the 3 logs of the effective rAd5 dose and 50% of the absolute response. Partial restoration of gp120-specific antibody responses was also evident following the rAd5 boost in Ad5 preimmunized mice that were primed with the gp120/GM-CSF/MIP-1α DNA vaccine compared to those given the gp120 DNA vaccine alone (Fig. 5E).
DISCUSSION

Plasmid DNA prime-viral vector boost vaccine regimens have been shown to elicit potent virus-specific cellular immune responses in animal models and are therefore being developed as candidate vaccines for HIV-1 and other pathogens (2, 9, 11, 17, 19). These prime-boost strategies, however, may be considerably less immunogenic in humans than in animal models, particularly in individuals with preexisting immunity to the viral vector. Strategies to improve these vaccine regimens are therefore needed. Here we show that coadministration of plasmid cytokines that improved the efficiency of DNA vaccine priming also resulted in potent and durable augmentation of the overall immunogenicity of DNA prime-viral vector boost vaccine regimens in BALB/c mice. Moreover, priming with cytokine-augmented DNA vaccines was more effective than priming with DNA vaccines alone in overcoming the inhibitory effects of preexisting anti-Ad5 immunity following rAd5-gp140ΔCFI boosts.

These results demonstrate that the efficiency of DNA vaccine priming is an important factor in determining the magnitude of immune responses following viral vector boosts. It is likely that mice primed with cytokine-augmented DNA vaccines were more effectively boosted with rAd5 than mice primed with DNA vaccines alone because they had larger numbers of circulating antigen-specific memory T lymphocytes available to expand following a second exposure to antigen. We cannot exclude the possibility that the plasmid cytokines may have also directly altered or improved the function of the vaccine-elicited T lymphocytes. However, we believe this to be unlikely, since we were unable to detect differences in the immunophenotypes of the tetramer-positive CD8\(^+\) T lymphocytes elicited by the cytokine-augmented DNA vaccines as compared with the DNA vaccines alone (data not shown).

Preexisting anti-Ad5 immunity in human populations may limit the utility of the DNA prime-rAd5 boost vaccines under development. The results of this study show that cytokine-augmented DNA vaccine priming is more effective than DNA vaccine priming alone (24) as a strategy to overcome the inhibitory effects of preexisting anti-vector immunity. We investigated the hypothesis that a larger pool of antigen-specific memory lymphocytes induced by cytokine-augmented DNA vaccines as compared with DNA vaccines alone may require a lower “threshold dose” of rAd5 to achieve a defined boosting effect. The threshold dose of rAd5 necessary to elicit a response of 3 to 5% of tetramer-positive CD8\(^+\) T lymphocytes following the rAd5 boost, may require a lower “threshold dose” of rAd5 to achieve a defined boosting effect. The threshold dose of rAd5 necessary to elicit a response of 3 to 5% of tetramer-positive CD8\(^+\) T lymphocytes following the rAd5 boost, may require a lower “threshold dose” of rAd5 to achieve a defined boosting effect. The threshold dose of rAd5 necessary to elicit a response of 3 to 5% of tetramer-positive CD8\(^+\) T lymphocytes following the rAd5 boost, may require a lower “threshold dose” of rAd5 to achieve a defined boosting effect.

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dose required to achieve this response was increased to 10^9 particles of rAd5 (Fig. 5B). In mice with preexisting immunity that were primed with the gp120/GM-CSF/MIP-1α DNA vaccine, however, this threshold dose was reduced to 10^7 particles of rAd5 (Fig. 5C). These data demonstrate that increasing the efficiency of DNA vaccine priming using plasmid cytokine adjuvants effectively lowered the threshold dose of rAd5 vector required to achieve this defined boosting effect, thus partially overcoming the suppressive effects of preexisting anti-Ad5 immunity.

These data suggest that priming with cytokine-augmented DNA vaccines and boosting with a viral vector warrants further investigation as a potential candidate HIV-1 vaccine strategy. Augmenting the efficiency of DNA vaccine priming may be particularly important in the setting of preexisting antivector immunity. Other strategies are also being investigated to mitigate the effects of preexisting antivector immune responses on the immunogenicity of rAd vectors, including developing vectors from rare adenovirus serotypes (18), utilizing adenoviruses to further improve DNA prime-viral vector boost vaccine regimens.

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