Interleukin-12 (IL-12) Enhancement of the Cellular Immune Response against Human Immunodeficiency Virus Type 1 Env Antigen in a DNA Prime/Vaccinia Virus Boost Vaccine Regimen Is Time and Dose Dependent: Suppressive Effects of IL-12 Boost Are Mediated by Nitric Oxide

M. MAGDALENA GHERARDI,† JUAN C. RAMÍREZ, AND MARIANO ESTEBAN*

Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología, CSIC,
Campus Universidad Autónoma, E-28049 Madrid, Spain

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We previously demonstrated that codelivery of interleukin-12 (IL-12) with the human immunodeficiency virus type 1 (HIV-1) Env antigen from a recombinant vaccinia virus (rVV) can enhance the specific anti-Env cell-mediated immune (CMI) response. In the present study, we have investigated the effects of IL-12 in mice when it is expressed in a DNA prime/rVV boost vaccine regimen. The delivery of IL-12 and Env product during priming with a DNA vector, followed by a booster with VV expressing the Env gene (rVVenv), was found to trigger the optimal CMI response compared with other immunization schedules studied. Significantly, if IL-12 is also delivered as a booster from the viral vector, an impairment of the effects of IL-12 was observed involving nitric oxide (NO), since it was overcome by specific inhibitors of inducible NO synthase. NO caused transient immunosuppression rather than impairment of viral replication. Moreover, at certain viral doses, coadministration of the NO inhibitor during the booster resulted in IL-12-mediated enhancement of the specific CD8* T-cell response. In addition, the dose of the IL-12-encoding plasmid (pIL-12) and the route of administration of both vectors were relevant factors for optimal CMI responses. Maximal numbers of Env-specific CD8* gamma interferon-secreting cells were obtained when 50 μg of pIL-12 was administered intramuscularly at priming, followed by an intravenous rVVenv boost. Our results demonstrate, in a murine model, critical parameters affecting the success of vaccination schedules based on a combination of DNA and VV vectors in conjunction with immunomodulators.

Cell-mediated immunity (CMI), especially that due to cytotoxic T lymphocytes (CTL), is an essential component of immune surveillance. The aim of vaccines for many infectious diseases is therefore to induce CTL populations that recognize specific pathogen-derived epitopes involved in protection. Current understanding of the immune response induced against human immunodeficiency virus type 1 (HIV-1) infection and the results of different vaccination studies emphasize the importance of CTL in combating this infection and controlling the development of AIDS (48). Similarly, in simian immunodeficiency virus (SIV) infection, CD8* T-cell cytotoxic subsets are essential in controlling viremia (50), as suggested during vaccination of macaques (16). To develop an effective vaccine against AIDS, it therefore seems essential to follow strategies that could enhance the specific immune response as well as to steer it toward the desired cell type. Development of effective CMI after vaccination rests on an extensive array of factors, among which cytokines present during immune response induction play a critical role. Several lines of evidence show that the early choice of a Th1 (cellular) or a Th2 (humoral) immune response is dependent mainly on the balance between interleukin-12 (IL-12) (which favors a Th1 response) and IL-4 (which favors a Th2 response) (55). The use of vectors delivering cytokines able to trigger a Th1 response, in conjunction with appropriate antigens, is an encouraging approach for induction of strong, stable CMI responses to HIV-1 infection.

DNA vaccines and recombinant vaccinia virus (rVV) vectors are both attractive anti-HIV-1 vaccine delivery systems due to their ability to elicit CMI as well as humoral immune responses; both vectors nonetheless have limitations in practical applications. For DNA vaccines, weak responses are often elicited by single immunizing doses (5, 13), and rVV-based vaccines elicit strong immune responses to the virus, which diminish the efficacy of repeated booster immunizations with the same vector (32). To circumvent these difficulties, vaccination schedules based on combined prime-boost regimens using different vector systems to deliver the desired antigen appear to be a successful alternative. Indeed, the efficacy of rVV vectors in booster injections, after priming with specific peptides or unrelated recombinant viruses, has been demonstrated (37). Recent approaches (7, 20, 22, 23, 27, 43, 49) in HIV-1, SIV, and malaria models found that a DNA-rVV prime-boost regimen is a very efficient procedure by which to elicit an enhanced CMI response to the specific antigens and that it might be further improved by the use of immunomodulators.

Cytokines (57) and other molecules involved in costimulation signaling (29) as adjuvants during DNA immunization modulate specific immune responses. For example, enhancement of the antigen-specific antibody response has been demonstrated by coexpressing IL-2 or granulocyte-macrophage colony-stimulating factor with the antigen in a DNA vector (8, 52). In studies using HIV-1 antigens in DNA immunization...
strategies (28, 38, 53), granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha synergism with IL-12 enhances the induction of specific CTL (1). Indeed, other Th1 cytokines such as IL-15 and IL-18 have also been coadministrated with HIV-1 antigens from DNA vectors in mouse models and have proven to be efficient adjuvants to modulate specific CMI (30, 31, 58).

The cytokine IL-12 is involved in the generation of CTLs and the activation of cytotoxicity in both CD8+ T and NK cells, especially potentiating gamma interferon (IFN-γ) production by T lymphocytes and NK cells; it also plays a prominent role in the generation of Th1 cells and the optimal differentiation of CTL (55). These functions have been exploited successfully by both DNA and rVV immunization schedules, promoting the generation of specific CMI in several models. We recently demonstrated enhancement of CMI to the HIV-1 Env product by expressing IL-12 and env genes from rVVs, with attenuation of the vector and no loss of the desired properties of live-virus-based vaccines (18). These effects were dose dependent, and the highest specific CMI was obtained in mice coimmunized with a low dose (2 × 10^7 PFU) of rVV expressing murine IL-12 (rVVIL-12) and 1 × 10^7 PFU of rVVenv (expressing the env gene).

To our knowledge, there are no reports in the literature exploring the proven beneficial effects of IL-12 in a combined immunization regimen based on DNA and rVV delivery vectors. This led us to evaluate whether the enhancement of CMI against the Env product observed in our previous study (18) could be improved using combined DNA and rVV immunization together with IL-12 expression. We also optimized conditions for administration of the cytokine delivery vector, since IL-12-associated dose- and schedule-dependent toxicity has been described, which can reverse the desired immunological effects; therapeutic use of IL-12 in clinical trials (9) and in mice (10) has thus been accompanied by dose-dependent toxicity under certain circumstances. Indeed, transient IL-12 suppression of the immune response was observed in several murine models when it was administered exogenously as a soluble product (34) or when it was expressed from adenovirus vectors (35); this appears to be mediated by the NO generated by activated macrophages (35).

The findings reported here demonstrate that IL-12 administration during priming from a DNA vector in conjunction with a DNA vector expressing HIV-1 Env antigen, followed by an rVVenv boost, improves the CMI enhancement observed when IL-12 was delivered from rVV. We established that both the dose and time of cytokine administration are critical factors for positive CMI effects. Moreover, we found that rVV-mediated expression of IL-12 during the booster can impair the beneficial effects observed when it was administered only during priming and that NO production is involved in the immunosuppressive action of the cytokine. Our findings demonstrate the role of variables important for the future design of efficient vaccines aimed to strengthen the CMI to a specific antigen through immunomodulator coexpression.

**MATERIALS AND METHODS**

**Viruses and cells.** The rVVs used in this study were derived from the laboratory Western Reserve (WR) strain. rVVlac (expressing the luciferase and β-galactosidase genes), rVVenv (expressing the entire env gene of HIV-1 strain IIIB and the β-galactosidase gene with an insertional inactivated hemagglutinin gene), and rVVIL-12 (expressing the p35 and p40 murine IL-12 subunits) have been described previously (18). Viruses were grown in HeLa cells, subjected to titer determination in BSC-40 African green monkey kidney cells, and purified as described previously (11).

**Immunization of mice and serum sample collection.** BALB/c mice (H-2b) (6 to 8 weeks old) were immunized intraperitoneally (i.p.) or intravenously (i.v.) in the tail vein with various doses of the different rVVs in 200 or 100 μl of sterile phosphate-buffered saline (PBS), respectively. At 14 days after virus inoculation, blood was obtained from the retroorbital plexus by using a heparinized capillary tube connected in an Eppendorf tube, and centrifuged, and serum was isolated and stored at −20°C.

**Plasmids and DNA immunization.** DNA plasmids carrying the HIV-1 strain IIIB Env gene (penv) or the murine IL-12 p35 and p40 genes (pIL-12) were expressed under the control of cytomegalovirus immediate-early (IE) promoter. The DNA vector penv was a generous gift of A. Böttler (Munich, Germany) and contains gpl20 modified for optimized codon usage (syn(gpl20)) cloned in PCRI, as described previously (2). Both murine IL-12 subunits are expressed from synthetic mRNA, similar to human cytomegalovirus promoters. The expression cassette of pIL-12 was inserted into the pGH vector. The luciferase gene was inserted into the pGL3 Basic vector. The drug was dissolved in sterile PBS at 2 mg/ml and 100 or 200 μl was administered i.p.

**Measurement of luciferase activity in mouse tissues.** rVV replication in different mouse tissues was monitored using highly sensitive luciferase assay as previously described (44). Different groups of mice received an i.p. inoculation with a low dose of the rVVlac virus. At various times postinoculation, animals were sacrificed and spleens were removed, washed with sterile PBS, and stored at −70°C. Tissues from individual mice were homogenized in luciferase extraction buffer (300 μl/spleen) (Promega Corp., Madison, Wis.), and luciferase activity was measured in the presence of luciferin and ATP in a Lumat LB 9501 luminometer (Berthold, N continuum, Calif., N.H.). Activity was expressed as relative luciferase units (RLU) per milligram of protein. The protein content in tissue extracts was measured by using the biochinnchomic acid protein assay reagent kit Pierce, Rockford, Ill.).

**T-cell restimulation assays.** Lymphocytes were isolated from spleens by passing tissues through a sterile mesh. Cells were suspended in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mm-l-glutamine, and 10 mM 2-mercaptoethanol). Erythrocytes in spleen cell preparations were lysed with 0.1% ammonium chloride. Splenocytes were cultured in triplicate (10^6 cells/well) in 96-well flat-bottom microtiter plates, stimulated with purified gp160 protein (1 μg/ml; Intracel Corp., Cambridge, Mass.) or concanavalin A (ConA) (1 μg/ml; Sigma), and incubated at 37°C under 5% CO2. Cytokine levels (IFN-γ and IL-4) in culture supernatants were determined after a 72-hr incubation. Supernatants from triplicate cultures were pooled and stored at −70°C until used for the assay.

**Evaluation of cytokine levels by ELISA.** Cytokine levels in culture supernatants and sera were determined by enzyme-linked immunosorbent assay (ELISA) using the appropriate combination of antibodies from Genzyme Diagnostics (Cambridge, Mass.). Briefly, 96-well flat-bottom plates were coated with 100 μl of anticytokine antibodies diluted in buffer, as specified by the manufacturer. Erythrocytes were incubated for 15 min. The wells were washed, incubated with horseradish peroxidase-conjugated streptavidin at 37°C for 15 min and developed with TMB substrate. The absorbance values were measured at 450 nm.

**Evaluation of CD8+ T cells by the ELISPOT assay.** The enzyme-linked immunospot (ELISPOT) assay to detect antigen-specific CD8+ T cells was performed as described previously (17). Briefly, 96-well nitrocellulose plates were coated with 8 μg of anti-mouse IFN-γ monoclonal antibody R4-6A2 (PharMin- gen, San Diego, Calif.) per ml in 75 μl of PBS. After overnight incubation at room temperature, the wells were washed three times with RPMI 1640, 100 μl of complete medium supplemented with 10% fetal calf serum was added to each well, and the plate was incubated at 37°C for 1 h. Triplicate spots of erythrocyte-depleted spleen cells were plated in twofold dilutions from 1 × 10^6 to 1.25 × 10^6 cells/well. P815 cells (a mastocytoma cell line that expresses only major histocompatibility complex class I molecules) were used as antigen-presenting cells. The number of CD8+ IFN-γ secreting cells specific for the V3 loop epitope of the HIV-1 Env protein was evaluated by using P815 cells with a 10^−4 M concentration of the synthetic peptide RGIQRAFVTI (10 env) and treating with anti-CD8 (1 μg/ml) or control reagent (20 μg/ml). After 20 min. At the time of the addition of medium, 10^5 P815 cells were added to each well. As a control, P815 cells not pulsed with peptide or treated with an unrelated peptide (SYVPSAEOL; H-2d restricted product of the Plasmodium yoelii CS protein) and reacted under similar conditions were used. When results were used from the ELISPOT P815 wells, an average of 20 to 50 spots were found.
RESULTS

Priming with DNA vectors expressing murine IL-12 and HIV-1 Env, followed by an rVVenv booster, induced the highest CMI against HIV-1 Env. To optimize the benefits of IL-12 in HIV-1 vaccine strategies, we first established whether delivery of IL-12 from DNA in a DNA prime-rVV boost vaccine regimen improved the specific CMI enhancement previously observed by coadministration of two rVV (rVVIL-12 plus rVVenv) in a single immunization dose (18).

(i) Enhancement of CD8<sup>+</sup> IFN-γ-secreting T cells against HIV-1 Env protein. Groups of mice were immunized with DNA plasmids expressing HIV-1 Env (penv) or in combination with DNA plasmids expressing IL-12 (penv + pIL-12) and, when required, boosted 14 days later with penv (Fig. 1, groups II or IV) or with rVV expressing gp160 (groups VII and VIII). The responses elicited by a single dose of DNA vaccine (group I) or rVVenv (group V) were compared with the action of IL-12 delivered by DNA (group III) or rVV (group VI) under previously defined conditions (18). At 14 days after immunization, the number of IFN-γ-secreting T cells against the HIV-1 Env IIIIB epitope was evaluated by the ELISPOT assay (Fig. 1). Under these experimental conditions, a weak specific cellular response was found in the groups that received only a DNA immunization regimen (groups I to IV), whereas the animals that received rVV in any of the immunizing doses (groups V to VIII) developed a significantly higher response. As previously described, coadministration of rVV-rVVIL-12 led to a Th1 response, whereas rVV-immunized mice developed a significantly lower response (Fig. 1). Under these experimental conditions, a very weak specific cellular response was found in the groups that received only a DNA immunization regimen (groups I to IV), whereas the animals that received rVV in any of the immunizing doses (groups V to VIII) developed a significantly higher response. As previously described, coadministration of rVV-rVVIL-12 led to a Th1 response, whereas rVV-immunized mice developed a significantly lower response.

(ii) Splenocyte Th1/Th2 cytokine secretion pattern after gp160 protein restimulation. We next investigated the effect of IL-12 expression on the Th1/Th2 response induced after application of the immunization regimens described above. At 14 days after the last immunization, splenocytes from mice of the groups in Fig. 1 were used in specific restimulation experiments with purified gp160 protein. After 72 h of culture, IFN-γ (Th1 cytokine) and IL-4 (Th2 cytokine) levels were measured. Data from one representative experiment that was repeated twice are summarized in Table 1. IL-4 levels were low but significant differences (P < 0.01) compared with the group indicated in the text.

![Image](http://jvi.asm.org/)
FIG. 2. Effects of the time of pIL-12 administration and the route of VV vector inoculation on the specific anti-HIV-1 Env CMI. Groups of four 6- to 8-week-old BALB/c mice were primed i.d. with 50 μg of the indicated plasmids; 14 days later, they were boosted i.p. (A) or i.v. (B) with 1 × 10^7 PFU of rVVenv given alone or mixed with 2 × 10^6 PFU of rVVL-12. Two weeks after the second immunization, the number of HIV-1 gp160-specific IFN-γ-secreting cells was determined by an ELISPOT assay. Mean values and standard deviations from triplicate pooled splenocyte cultures are represented. *, significant differences (P < 0.01) with respect to all other experimental groups.

Effects of IL-12 administration time and the route of the VV vector inoculation on the specific anti-HIV-1 Env CMI. The type of response induced (Th1/Th2) by the different immunization regimens was analyzed by measuring the pattern of splenocyte-secreted cytokines (IFN-γ and IL-4) in different groups after in vitro restimulation with the specific antigen (gp160) or in culture medium alone. Figure 3 shows the results from mice boosted by the i.v. route. Comparable results were observed in splenocytes from mice boosted i.p. (data not shown). IL-12 administration only during priming reduced IL-4 levels but increased IFN-γ levels compared to those in the control group (mice that did not receive cytokine), producing a 10-fold increase in the Th1/Th2 ratio. When IL-12 was present during priming, the second delivery of IL-12 in the booster inhibited both Th1 (IFN-γ) and Th2 (IL-4) cytokines compared to the group in which IL-12 was present only at priming. Nevertheless, the mice in which IL-12 was delivered only at boost showed a diminished Th2 response with respect to control mice (without IL-12).

FIG. 3. Pattern of cytokine secretion after specific restimulation of splenocytes from mice immunized i.d. with a DNA vector followed by an rVV boost. Splenocytes from mice used in the same experiment as in Fig. 2B (DNA primed and i.v. rVV boosted) were restimulated in vitro with 1 μg of purified HIV-1 gp160 per ml; 72 h later, cell supernatants were collected and the levels of IFN-γ (solid bars) and IL-4 (striped bars) were determined by ELISA. Bars represent data from pooled samples of triplicate cultures. Background levels (100 pg of IFN-γ per ml; 15 pg of IL-4 per ml) obtained with RPMI have been subtracted. Data are representative of two ELISA determinations from one experiment.
response than did control mice (without rVVIL-12) (Fig. 4A, left panel; \( P \leq 0.01 \)), as expected from previous experiments (Fig. 1). This inhibition increased in parallel with the dose of rVVIL-12 used. We noted nearly a fourfold enhancement over control values \( (P < 0.01 \) and \( P < 0.005 \) in the number of specific CTLs was observed when higher doses of rVVIL-12 were used. There was nearly a fourfold enhancement over control values \( (P < 0.005 \) when animals were inoculated with \( 2 \times 10^5 \) PFU of rVVIL-12 in the presence of L-NAME, demonstrating the benefits of IL-12 in the booster when applied at this dose. To further analyze whether the immunosuppressive effects of IL-12 were temporary, mice were inoculated with pENV + pIL-12 and boosted with either \( 1 \times 10^7 \) PFU of rVVenv alone or \( 1 \times 10^5 \) PFU of rVVenv + \( 2 \times 10^5 \) of rVVIL-12 and the specific CMI was evaluated 40 days after the boost. The number of specific cytotoxic cells was nearly equal in the two groups (720 and 850 specific IFN-\( \gamma \)-secreting cells/10^6 splenocytes) (Fig. 4A, right panel), indicating that the immunosuppressive effect of IL-12 was temporary and reversible. There was nonetheless an effect due to waning of the immune response with time, since the number of specific IFN-\( \gamma \)-secreting cells diminished at 40 days after the boost compared to the number of 14 days after the boost.

We next assessed whether NO had an effect on viral replication that would account for the observed impairment of the IL-12 effects when delivered at booster via rVV. Mice were inoculated with \( 10^7 \) PFU of rVVluc, a vector expressing the luciferase reporter gene to assay for virus replication in tissue homogenates, together with \( 2 \times 10^5 \) PFU of rVVIL-12. One group of animals was treated with L-NAME, and the other was mock treated. Then, virus replication was monitored in tissue homogenates by measuring luciferase activity levels from 1 to 14 days postinoculation. The two groups of mice showed similar luciferase activity levels on days 1 to 4 after rVV administration (Fig. 4B). Given the specificity of the inhibitor and the assay conditions, we can rule out significant NO inhibition of virus replication, indicating that the undesired effects of IL-12, when present at boost, are modulated by the iNOS activity.

**Optimization of IL-12 dosage and route of DNA vector inoculation at priming**. The experiments in Fig. 1 and 2 revealed that in a DNA-VV immunization schedule, IL-12 inoculation was most effective when delivered at priming followed by an rVVenv boost. To optimize the IL-12-induced enhancement of anti-gp160-specific CMI, we next characterized the effects of IL-12 dosage and route of inoculation of DNA vector during priming. Groups of mice were primed with graded doses of pIL-12 (0 to 100 \( \mu \)g/mouse) by the i.d. or i.m. route and boosted with rVVenv i.v. The magnitude of the specific CD8^+ T-cell response at 14 days after the booster immunization was affected both by the dosage and by the route of administration of pIL-12 DNA (Fig. 5). At all doses, the i.m. route was most efficient, eliciting a two- to threefold-higher CMI response to Env, and the differences between the DNA routes were signif-
anti-HIV-1 Env CMI. Groups of four mice were injected i.d. or i.m. with a boosted i.v. 2 weeks later with 10^7 PFU of rVVenv. At 14 days later, spleen cells was between 10 and 50 for both DNA routes; for the i.d. route, the optimal pIL-12 dose at intermediate doses (Fig. 5). This effect was observed for IC50 significant (p < 0.01). Moreover, the effect of IL-12 on HIV-1 Env CMI enhancement was dose dependent, with maximal values at intermediate doses (Fig. 5). This effect was observed for both DNA routes; for the i.d. route, the optimal pIL-12 dose was between 10 and 50 μg/mouse, whereas the i.m. route gave the largest number of specific IFN-γ-secreting cells at 50 μg/mouse.

To examine the type of Th populations generated, we measured the cytokine levels secreted after nonspecific ConA re-stimulation of splenocytes from mice immunized i.m. with DNA and boosted i.v. with rVV, as above. IL-12 administration at the higher dose produced a cytokine pattern (Th1/Th2) similar to that found when it was absent, whereas at intermediate IL-12 doses, IL-4 levels were lower, resulting in a higher Th1/Th2 value (Fig. 5, insert).

The experiments in Fig. 5 demonstrated that immunization schedules consisting of penv + pIL-12 priming and an rVVenv boost can be improved by varying the dose of routes of pIL-12 at priming.

**DISCUSSION**

There is growing evidence that CTLs play an important role in protection against viruses such as HIV-1 (48). Although cytolytic activity cannot prevent incoming cell-free virus from infecting host cells, in the laboratory specific CTLs can kill HIV-1-infected cells before the production of new virions; this effect is enhanced by the release of chemokines (40, 56, 59).

Thus, detection of HIV-1-specific CTL responses in exposed but uninfected sex workers and health care workers (39, 46) and in uninfected infants born of HIV-1-infected mothers (6, 47) may be explained by the capacity of CTLs to clear the initial small number of infected cells before HIV-1 establishes a generalized infection. This concurs with the finding that rapid progressors show low HIV-1-specific CTL activity (19). All of these observations suggest that HIV-1 infection and the progression to AIDS may rely on the inability to establish and/or maintain an adequate anti-HIV-1 specific CMI.

Combined vaccination protocols, involving different vaccine vehicles, routes, or means of antigen presentation to the immune system, can be used to induce specific immune responses more efficiently than single vectors (37). DNA prime-VV boost vaccine regimens have proved to be an efficient approach to enhancing specific CTL responses. It has been shown that in the murine model, DNA priming followed by a boost with the highly attenuated modified VV Ankara (MVA) efficiently induced CTL responses to HIV-1 (21) and malaria (51) antigens, correlating with complete protection in the latter system. Moreover, recent studies in the macaque model showed induction of SIV-specific CTLs using a multiepitope gene and DNA prime-MVA boost vaccination regimen (23). We previously demonstrated that it is possible to enhance the CTL response to the HIV-1 Env antigen by expression of IL-12 and env genes from rVV and that the effects of the cytokine were dose dependent. The aim of this study was to define the extent of this enhancement on the specific CMI when the IL-12 cytokine was delivered in bimodal immunization, based on DNA priming and boosting with rVV.

In this study using DNA and VV vectors expressing IL-12 and HIV-1 Env, we found that the largest numbers of anti-HIV-1 IFN-γ-secreting CD8+ T cells were induced when IL-12 and the antigen were delivered via a DNA vector at priming, followed by an rVVenv boost. Direct evaluation of specific IFN-γ-secreting CD8+ T cells by the immediate ELISPOT assay did not demonstrate a significant response in mice immunized with only a DNA vector. The first DNA immunization resulted in priming a specific CTL response, as demonstrated by the fact that mice deprived of this immunization, which received only one dose of rVV, showed a minor response. These findings concur with those of Hanke et al. (23) for macaques, where they showed that DNA immunization primed the response, since no significant CTL activity was found after DNA gene gun immunization, although this response was enhanced after MVA boosting. Our inability to detect a specific CMI in the DNA-immunized mice groups contrasts with the results of others (28, 53), who showed enhancement of the specific CTL response when IL-12 and the HIV-1 Env product were delivered via DNA vectors. Those differences may be explained by the experimental approach used, since in those cases CTL activity was evaluated after in vitro restimulation of immune splenocytes whereas we performed an immediate ELISPOT assay without restimulation.

The cytokine pattern of CD4+ T cells after specific gp160 in vitro restimulation of splenocytes revealed that IL-12 administration via either rVV or plasmid vectors diminished the Th2 response (lower IL-4 levels [Table 1, groups VI to VIII]). Nonetheless, this reduction was not followed by enhancement of the Th1 response when the IL-12 was administered at booster only or in both priming and booster inoculations (Fig. 3). This contrasts with the potent skew toward a Th1 response when IL-12 was delivered only at priming. The higher Th1 response observed following in vitro restimulation was thus associated with the larger numbers of IFN-γ-secreting CD8+ T cells in mice immunized with penv + pIL-12 followed by...
rVVenv. Our findings resemble those of other studies (1) in which enhancement of the CTL response due to synergism between IL-12 and tumor necrosis factor alpha was associated with an increase in IFN-γ production and thus a Th-to-Th1 response shift in mice.

We also found that the i.v. route of rVV administration was more effective than the i.p. route with regard to the ability to enhance the CD8+ T-cell response. During i.m. DNA priming, rMVA boosting in a malaria-infected mouse model (51), the influence of the route of MVA administration had a major impact on the CMI elicited, since significantly higher immunogenicity was observed for the i.v. and i.d. routes than for i.p. or subcutaneous routes. Moreover, studies in which immunity against VV antigens was analyzed in a murine model also indicated that i.v. immunization stimulated higher antibody and CTL responses than did immunization via other routes (3).

IL-12 expression during immunization strategies has been associated with immunosuppressive effects, as described by others (33–35) and confirmed by our present observations. To explain this, several interactions between iNOS and IL-12 have been reported, including the inhibition of macrophage IL-12 production by NO, the possible induction of the IL-12 antagonist (homodimeric p40) by NO, and the iNOS-dependent suppression of T-cell responses by IL-12 (25, 54). This last effect has been demonstrated in studies in mice, and it has been shown that the events leading to immune suppression by high IL-12 doses are initiated by induction of IFN-γ production by lymphocytes. Sufficiently high IFN-γ levels promote the induction of iNOS activity by activated macrophages, generating levels of NO that impair T-cell proliferation. This impairment may result from a NO-specific inhibition of JAK2 and JAK3 kinases or by a disruption of the JAK/Stat5 signaling pathway (4, 14). These negative regulatory roles of NO contrast with recently described positive regulatory functions (12, 35). These previous observations led us to propose that the immunosuppressive action of IL-12 when administered via rVV in the booster may be NO mediated. The immunosuppression increased with the rVVIL-12 dose administered at boosting; however, if the iNOS inhibitor L-NAME was inoculated simultaneously with rVVS, an enhancement in the number of anti-HIV-1 Env protein CD8+ T cells was observed, restoring the response observed in the control. Moreover, mice primed with penv + pIL-12 and boosted with rVVenv + 1 × 106 PFU of rVVIL-12 in the presence of L-NAME showed a cellular response nearly fourfold higher than that in the control group (not receiving rVVIL-12 at boosting). After the booster inoculation in the presence of IL-12, analysis of the CMI after 40 days shows that the immunosuppressive effects of the cytokine disappear, indicating the transient nature of these effects. Nevertheless, we cannot rule out a specific action of IL-12 in the long-term survival of the effector cells, which may account for the observations obtained 40 days after the booster. These results concur with previous studies (34) showing transient immunosuppression mediated by soluble IL-12 in a murine model of vaccination against tumor cells. Those effects were dose dependent and NO mediated, since they were reversed upon administration of an iNOS inhibitor (33).

In other reports, when IL-12 was delivered via adenovirus vectors (35), L-NAME produces no apparent toxicity in mice receiving low doses of the IL-12-delivering virus but killed all the animals treated with higher doses of IL-12. The discrepancies with our data, in which L-NAME causes no toxic effects in any group of animals, may be due to differences in the amounts of IL-12 produced by the vectors used and in the L-NAME doses applied.

In recent years, a number of reports have shown an association between NO and antiviral effects, both in vivo and in vitro. In cell cultures of several DNA and RNA viruses, the induction of iNOS activity before infection is associated with an inhibition of virus replication (42). It has been shown that IFN-γ-treated RAW cells produced NO, which inhibited VV replication (26), and that rVV-expressing iNOS induced inhibition of VV replication at the level of late proteins (36) and suppression of viral DNA synthesis mediated by inhibition of viral ribonucleotide reductase (24). In vivo studies have nevertheless demonstrated that iNOS is activated during VV infection in mice, but treatment of animals with an iNOS inhibitor did not alter the course of infection (45). Coinciding with these results, we also found that NO does not affect VV replication in mice, even in the presence of IL-12. The differences between the results obtained in cell cultures and in animals can be explained by the level of NO produced. High NO levels lead to VV inhibition, while NO low levels have little effect on VV, as previously shown in cell cultures (36).

The better results obtained with i.m. injection than with i.d. injection (Fig. 5) are in accordance with the results of other studies that systematically characterized the effects of the route of DNA immunization on protective immunity (15). More recently, it has been shown that repeated gene gun injections are required to achieve CTL responses comparable to a single i.m. injection (23), although opposite results have been obtained in other systems (27). However, it is noticeable that MVA boosting on gene gun or i.m. DNA-primed mice abolished the differences caused by the route of DNA immunization. Our results were obtained using the virulent laboratory WR strain, which, in comparison with attenuated VV strains such as MVA or NYVAC, elicits a minor recombinant antigen-specific immune response (41). These findings may thus be an underestimation of the extent of the CMI that would be obtained if MVA were used in bimodal DNA-VV immunizations combined with cytokines such as IL-12. The immunization protocols described are relevant when bimodal immunizations are applied in combination with immunomodulators. The time of administration of the cytokine-expressing vector and the inoculation route of both rVV and DNA vectors may be critical factors in the extent of the CMI response elicited. For combinations of vectors, antigens, and cytokines, these variables must be taken into account to optimize the desired final results for a successful vaccine.
IL-12 ENHANCEMENT OF CMI AGAINST HIV-1

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