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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

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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/VV 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

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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 coadministered 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 (2×10^4 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 (33).

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. rVVluc (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-2*^{d}) (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, collected 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. Bültmann (Munich, Germany) and contains gp120 modified for optimized codon usage (syngp120) cloned in PCR3, as described previously (2). Both murine IL-12 subunits are expressed from a polycistronic mRNA, since encephalomyocarditis virus internal ribosome entry site was introduced between the two genes in the PI19 plasmid (the gift of J. A. Melero, Madrid, Spain). Plasmids were purified on Qiagen columns using pyrogen-free material and eluted in pyrogen-free deionized water.

DNA was used for immunization by injecting the appropriate dilution in 200 μl of sterile PBS into shaved abdominal skin (intradermal [i.d.] route) or in 100 μl of sterile PBS into the trigeminal muscle (intramuscular [i.m.] route) or mice anesthetized with halothane (Fluothane; Zeneca Farmesa, Pontevedra, Spain).

When animals were treated with the inducible nitric oxide synthetase (iNOS) inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME; Sigma, St. Louis, Mo.), 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 distinct doses of the rVVluc virus. At various times postinoculation, animals were sacrificed and spleens were resected, 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, Nashua, 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 bicinchonicic 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 M 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% CO_2. Cytokine levels (IFN-γ and IL-4) in culture supernatants were determined after a 72-h 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, and incubated overnight at 4°C. The wells were washed with PBS plus 0.05% Tween 20 (PBS-T) and blocked at 37°C for 2 h with PBS containing 1% bovine serum albumin. Serial twofold dilutions of supernatants or sera and appropriate dilutions of standard cytokines were added in duplicate and incubated at 37°C for 1 to 2 h. The wells were washed with PBS-T and incubated with the specific biotinylated anticytokine antibody diluted in PBS-T plus 1% BSA for 1 to 2 h. After three or four washes, the wells were incubated with horseradish peroxidase-conjugated streptavidin at 37°C for 15 min and developed with TMB reagent (Sigma), the reaction was terminated with 2 N H_2SO_4, and 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 (PharMingen, 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 samples of erythrocyte-depleted spleen cells were plated in twofold dilutions from 1×10^6 to 1.25×10^5 cells/well. P815 cells (a mastocytoma cell line that expresses only major histocompatiblity 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 pulsing P815 cells with a 10^{-6} M concentration of the synthetic peptide RGPGRAFVTI (10 env) and treating with mitomycin C (30 μg/ml; Sigma) for 20 min. After three washes with culture 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 (SYVPSAEQI, an *H-2*^{d} restricted peptide of the *Plasmodium yoelii* CS protein) and reacted under similar conditions were used. When 10^6 splenocytes/well from either experimental group were seeded in the presence of these control P815 cells, an average of 20 to 50 spots were found.

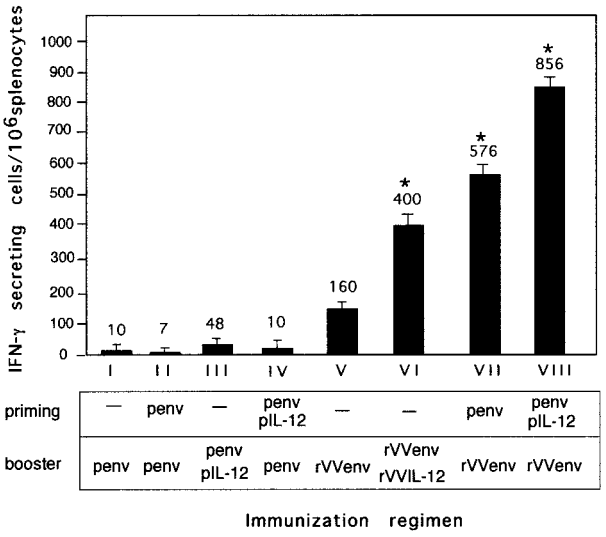

FIG. 1. A high CMI response to HIV-1 Env is obtained in a DNA-VV immunization regimen in the presence of IL-12 at priming. Groups of four 6- to 8-week-old BALB/c mice were immunized i.d. with the indicated plasmids at a dose of 50 μg of plasmid per mouse. Fourteen days later, they were boosted either with the same dose of the indicated plasmid or i.p. with 1×10^7 PFU of rVVenv given alone or in combination with 2×10^4 PFU of rVVIL-12. Two weeks later, spleen cells were used to count the gp160-specific IFN-γ-secreting cells by the ELISPOT assay. For accurate comparisons, control animals receiving only one dose of DNA vaccine or rVV were injected when their counterparts were boosted and the immune response was analyzed at the same time postinoculation. The immunized groups are indicated in Roman numerals. Bars represent mean and standard deviation for triplicate pooled splenocytes. *, significant differences ($P < 0.01$) compared with the group indicated in the text.

Plates were incubated (at 37°C for 24 h under 5% CO_2), washed extensively with PBS-T, and incubated for 2 h at room temperature with 2 μg of biotinylated anti-mouse IFN-γ monoclonal antibody XMG1.2 (PharMingen) per ml in PBS-T. The plates were then washed with PBS-T, 100 μl of peroxidase-labeled avidin (1/800 dilution in PBS-T) (Sigma) was added to each well, and the plates were incubated at room temperature. One hour later, the wells were washed with PBS-T and PBS. Spots were developed by adding 1 μg of the substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma) per ml in 50 mM Tris-HCl (pH 7.5) containing 0.015% hydrogen peroxide. Spots were counted using a Leica MZ122 APO stereomicroscope and Imaging System QWIN software (Leica, Cambridge, United Kingdom).

Statistical analysis. Data were analyzed using the unpaired Student *t* test and the Stat View 4.5 statistical program.

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 $CD8^+$ 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 IIIB epitope was evaluated by the ELISPOT assay (Fig. 1). Under these experimental conditions, a very weak specific cellular response was found in the groups that received only a DNA immunization regime (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-delivered IL-12 and HIV-1 Env in one immunization dose gave an approximately threefold increase in the CMI response over that obtained when only rVVenv was administered (group V versus group VI; $P < 0.01$). A response of comparable magnitude was induced when a DNA-prime/rVV boost regimen was applied in the absence of IL-12, since the number of specific IFN-γ-secreting $CD8^+$ T cells was increased 3.6-fold with respect to that in mice receiving rVVenv in one dose (groups VII versus group V; $P < 0.01$); differences between groups VI and VII were not significant ($P = 0.06$). When IL-12 and antigen were delivered by DNA in the priming, followed by an rVVenv boost (group VIII), the number of specific IFN-γ-secreting $CD8^+$ T cells was 1.5 times larger than in mice that did not receive the cytokine (group VII) ($P < 0.01$). This combination elicited the highest CMI response observed among all groups under these experimental conditions. These findings establish that in a DNA primer-rVV boost vaccine regimen, optimal CMI results are obtained when IL-12 is coexpressed with antigen during the generation of the primary immune response, allowing specific enhancement of the CMI response to the HIV-1 Env antigen compared to that in other immunization schedules assayed.

TABLE 1. Levels of IFN-γ and IL-4 in supernatants of splenocytes from immunized mice after specific restimulation with gp160[a]

Cytokine	Amt (pg/ml) of cytokine after immunization regimen[b]:						
	I	II	III	IV	VI	VII	VIII
IFN-γ	150	110	ND[c]	250	1,070	1,024	1,350
IL-4	20	24	8	30	24	76	16

[a] Splenocyte supernatants were collected after 72 h of culture in the presence of 1 μg of HIV-1 gp160 per ml.

[b] The immunization schedule was as indicated in the legend to Fig. 1. A 50-μg portion of each plasmid was injected i.d. into each mouse. Viruses were inoculated i.p. at a dose of 1×10^7 PFU of rVVenv and 2×10^4 PFU of rVVIL-12 per mouse. Background levels obtained after culture of splenocytes from each group with RPMI have been subtracted (15 pg/ml for IFN-γ determination; negative values were obtained for control IL-4 determinations). The sensitivity of the ELISAs was 5 pg/ml for both cytokines.

[c] ND, not determined.

(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 above the assay sensitivity threshold and were similar among the immunization groups; we therefore focused our analysis on IFN-γ levels secreted upon restimulation. After immunization with DNA vectors alone, comparable but relatively low cytokine levels were found in the different groups, with higher levels of IFN-γ than of IL-4 (Table 1). This suggests the predominance of a Th1 response, whereas rVV-immunized mice produced higher IFN-γ levels. In groups VI to VIII, adminis-

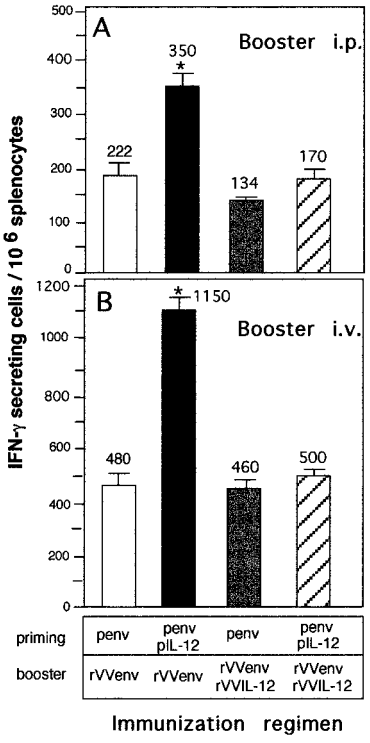

FIG. 2. Effects of the time of pIL-12 administration and the route of VV vector inoculation on the specific anti-HIV-1 Env CMI. Groups of four 6- to 8-week-old BALB/c mice were primed i.d. with 50 μg of the indicated plasmids; 14 days later, they were boosted i.p. (A) or i.v. (B) with 1×10^7 PFU of rVVenv given alone or mixed with 2×10^4 PFU of rVVIL-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.

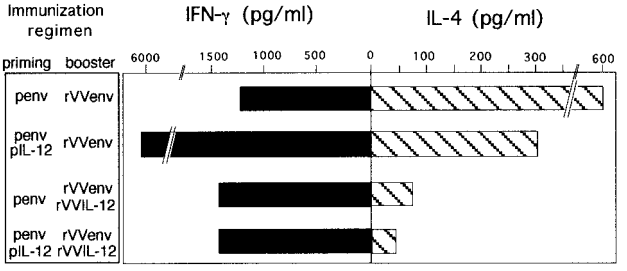

FIG. 3. Pattern of cytokine secretion after specific restimulation of splenocytes from mice immunized i.d. with a DNA vector followed by an rVV boost. Splenocytes from mice used in the same experiment as in Fig. 2B (DNA primed and i.v. rVV boosted) were restimulated in vitro with 1 μg of purified HIV-1 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.

tration of IL-12 via either an rVV or a DNA vector diminished the Th2 response (IL-4), leading to a higher Th1/Th2 ratio. Group VII, which received antigen from DNA in the priming and antigen from rVV in the booster, showed IL-4 levels five- or threefold higher than did groups in which IL-12 was applied via DNA (group VIII) or rVV (group VI), respectively. Since IFN-γ levels are similar between groups VI and VII but lower than in group VIII, a stronger Th1 immune response was elicited in the group VIII mice, in accordance with the higher CMI response determined by ELISPOT in this group (Fig. 1).

Effects of IL-12 administration time and the route of the VV vector inoculation on the specific anti-HIV-1 Env CMI. We next analyzed the appropriate time of IL-12 administration in a DNA-VV immunization schedule and the route of virus inoculation (i.p. versus i.v.) in each immunization. The influence of these parameters on the specific $CD8^+$ IFN-γ-secreting T cells to HIV-1 Env was evaluated. Figure 2 shows the immunization schedules applied, in which IL-12 was present either at priming or in the booster dose or in both inoculations. Comparison of the two virus inoculation routes shows that when rVVs were inoculated by the i.v. route (Fig. 2B), the specific CMI anti-Env product was approximately three times as potent for all groups with respect to the response when the i.p. route was used (Fig. 2A). The combination of penv + pIL-12, followed by rVVenv in the booster, generated an enhancement of 1.5- to 2.4-fold (i.p. and i.v. routes, respectively; $P < 0.01$) in the response obtained compared to that in the group in which pIL-12 was absent (Fig. 1). This enhancement of the specific CMI was not observed, however, if the cytokine was administered only in the booster or in both priming and booster inoculations, regardless of the virus inoculation route used (Fig. 2). These data suggest that if the cytokine was administered only in the second immunization, it did not redirect the type of Th response induced during priming of the immune response, since the levels are comparable to those observed in the absence of IL-12 in the immunization regimen. In addition, IL-12 given in the booster displayed an apparent suppressive effect on the cytokine-directed CMI enhancement during the primary immune response.

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).

IL-12-induced immune suppression of HIV-1 Env is mediated by NO. It has been found that IL-12, administered as a soluble recombinant product or delivered via a viral vector, can be ineffective at certain doses and that this effect is NO mediated (33). To determine whether this phenomena occurred during the rVVIL-12 booster in these experiments, we studied the effects on the CMI response to HIV-1 Env when the specific reversible inhibitor of iNOS, L-NAME, was administered. Mice were primed i.d. with a mixture of penv and pIL-12 DNA vectors, and 14 days later they were boosted i.v. with 10^7 PFU of rVVenv together with increasing doses (ranging from 2×10^2 to 2×10^6 PFU) of rVVIL-12. At this time and for the next 3 days, four groups of mice received L-NAME and another four were mock treated. Two weeks after the booster, an ELISPOT assay was performed to measure the number of IFN-γ-secreting cells specific for the gp160 V3 loop. Animals which did not receive the inhibitor and which were boosted with rVVIL-12 developed a 1.5- to 2-fold lower cellular immune

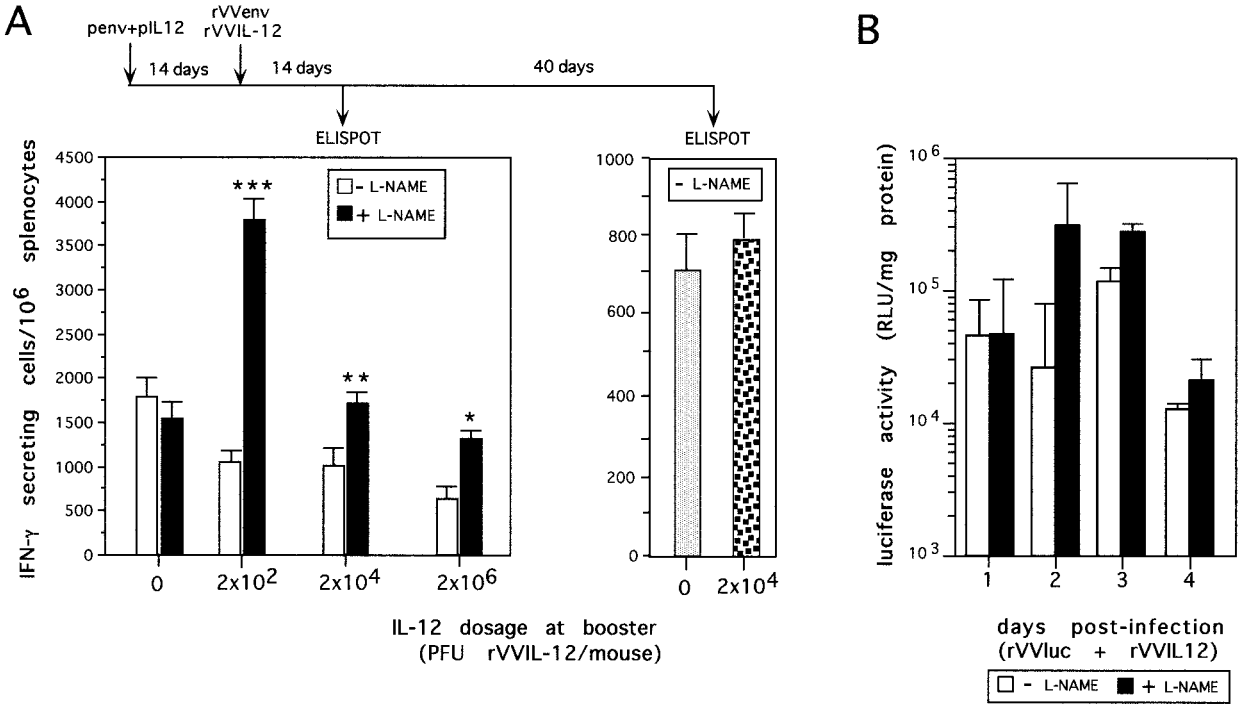

FIG. 4. The inhibitory effects of IL-12 delivered at booster are reversed by the iNOS inhibitor L-NAME. (A) A scheme summarizing the immunization procedure is shown. (Left) Three mice per group were primed i.d. with a mixture of 50 μg of penv + pIL-12 per mouse and boosted i.v. 14 days later with 10^7 PFU of rVVenv and the indicated amount of rVVIL-12. At this time and for the next 3 days, the animals received a dose of 200 μg (days 0, 1, and 2) or 400 μg (day 3) of the iNOS inhibitor L-NAME i.p. (solid bars) in 200 μl of PBS or were left untreated (open bars). At 14 days after the boost, an ELISPOT assay was performed to determine the number of HIV-1 gp160-specific IFN-γ-secreting cells. Significant differences: ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.01$ with respect to the corresponding group not treated with inhibitor. (Right) Groups of three mice were primed and boosted with rVVenv and rVVIL-12 at the indicated doses, as in left panel, in the absence of the inhibitor L-NAME; 40 days after the boost, an ELISPOT assay was performed. Bars indicate the mean and standard deviation for triplicate cultures. (B) Mice were inoculated i.v. with 1×10^7 PFU of rVVluc plus 2×10^2 PFU of rVVIL-12; one group was treated with L-NAME as described in panel A, and the other was mock treated. Luciferase activity was determined in spleen homogenates at the indicated days postinoculation. Bars represent the mean RLU per milligram of protein and standard deviation for three individual mouse samples.

response than did control mice (without rVVIL-12) (Fig. 4A, left panel; $P \leq 0.01$), as expected from previous experiments (Fig. 2). This inhibition increased in parallel with the dose of rVVIL-12 inoculated. When L-NAME was administered, however, an enhancement similar to 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 ($P < 0.0005$) when animals were inoculated with 2×10^2 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 primed with penv + pIL-12 and boosted with either 1×10^7 PFU of rVVenv alone or 1×10^7 PFU of rVVenv + 2×10^4 of rVVIL-12 and the specific CMI was evaluated 40 days after the boost. The number of specific cytotoxic cells were nearly equal in the two groups (720 and 850 specific IFN-γ-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-γ-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 2×10^2 PFU of rVVIL-12. One group of animals was treated with L-NAME, and the other was mock treated. Thereafter, virus replication was monitored in spleen 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 (differences between the groups were not statistically significant [$P \geq 0.06$]) (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 μg/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-

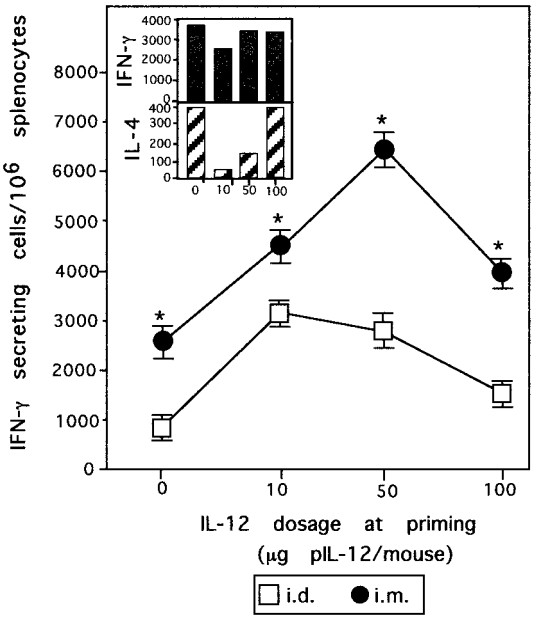

FIG. 5. Effects of pIL-12 dosage and route of inoculation on the specific anti-HIV-1 Env CMI. Groups of four mice were injected i.d. or i.m. with a mixture of 50 μg of penv and pIL-12 at the indicated amounts (0 to 100 μg) and boosted i.v. 2 weeks later with 10^7 PFU of rVVenv. At 14 days later, spleen cells were used to determine the number of HIV-1 gp160-specific IFN-γ-secreting cells by an ELISPOT assay. Each point represents mean and standard deviation for triplicate cultures. *, significant differences ($P < 0.01$) with respect to the corresponding group in which DNA was inoculated i.d. The inset shows the effect of pIL-12 dose on the proportion of Th1 and Th2 cells. Spleen cells from mice primed and boosted i.v. were restimulated nonspecifically with 1 μg of ConA per ml in triplicate cell cultures; 72 h later, the supernatants were pooled. The cytokine effect was measured in ELISA as the amount (picograms per milliliter) of IFN-γ and IL-4 present in supernatants. Background levels obtained from cell cultures in the presence of RPMI alone (300 pg of IFN-γ per ml; 25 pg of IL-4 per ml) have been subtracted.

icant ($P < 0.01$). 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 restimulation 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 rVVs 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 + 1×10^2 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 day 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 produced 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 cause 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 any combination of vectors, antigens, and cytokines, these variables must be taken into account to optimize the desired final results for a successful vaccine.

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