IL-12 Delivery from Recombinant Vaccinia Virus Attenuates the Vector and Enhances the Cellular Immune Response Against HIV-1 Env in a Dose-Dependent Manner

M. Magdalena Gherardi, Juan C. Ramirez, Dolores Rodríguez, Juan R. Rodríguez, Gen-Ichiro Sano, Fidel Zavala and Mariano Esteban

*J Immunol* 1999; 162:6724-6733; 
http://www.jimmunol.org/content/162/11/6724
IL-12 Delivery from Recombinant Vaccinia Virus Attenuates the Vector and Enhances the Cellular Immune Response Against HIV-1 Env in a Dose-Dependent Manner

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To develop vaccination strategies against HIV-1 infection aimed to specifically enhance the cell-mediated immunity (CMI), we have engineered vaccinia virus (VV), the prototype member of the poxvirus family, that was successfully used as a live vaccine to eradicate smallpox (1). This virus represents a good candidate for vaccination purposes because of its broad host range and the ability to generate recombinant viruses (rVV) that express a variety of foreign Ags (2). Moreover, rVV have been proven effective in field animal vaccination programs (3). When rVV containing HIV-1 and SIV genes were tested in different vaccination schedules on simian hosts, specific cellular and humoral immunity, both systemic (4) and mucosal (5), were elicited. The above findings suggest that VV-based vaccination approaches using highly attenuated strains might be a promising prophylactic strategy against HIV-1 infection.

The development of an effective CMI response after vaccination rests on an extensive scope of factors, among which cytokines present during the priming could play a critical role. Two different subsets of CD4+ T lymphocytes, Th1 and Th2, differing in the pattern of cytokines produced, have been described to be crucial in the generation of a cellular or an Ab immune response, respectively. Different lines of evidence showed that the early decision toward Th1- or Th2-type immune response is mainly dependent on the balance between IL-12 (which favors a Th1 response) and IL-4 (which favors a Th2 response). Among the main functional features of IL-12 are 1) it potentiates cytokine production, particularly IFN-γ, in T lymphocytes and NK cells; 2) it acts as a growth factor for preactivated T and NK cells; and 3) it is involved in the stimulation of preactivated T and NK cells; and 3) it is involved in the

Received for publication December 22, 1998. Accepted for publication March 16, 1999.

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generation of CTLs and in the activation of cytotoxicity in both CD8\(^+\) T and NK cells. In addition, IL-12 has a prominent role in the generation of Th1 cells and the optimal differentiation of CTLs (14). Thus, to induce strong and stable CMI responses against HIV-1 infection, the use of vectors delivering cytokines capable of triggering a Th1 response in conjunction with appropriate Ags is a encouraging approach. In this regard, different vaccination strategies with IL-12 delivered as a soluble product, expressed from DNA vectors or viral vectors, have provided evidence for enhancement of CTL responses that correlated with regression of tumors (15), resolution of autoimmune diseases (16), and protection against various intracellular pathogens (17) and against the development of murine AIDS (18).

In addition to the promising prophylactic and therapeutic advantages of the use of live vectors delivering immunomodulators, the potential capability of the encoded cytokine to modulate the vector pathogenicity might be a desirable property, especially in the case of live virus-based vaccines by decreasing the risk of side-effects during virus infections. Indeed, different recombinant virus-encoding cytokines have been described as immunological tools for dissecting the in vivo functions of cytokines in antiviral immunity. Expression of cytokines from rVV have been reported to have a profound effect on viral infection (19), but their induction and their involvement in promoting specific immune responses to Ags are poorly characterized events. Hence, analysis of the effect of cytokines on the virus vector itself should be explored to understand the implications for the Ag-specific immune responses elicited when both Ag and cytokine are delivered from a live-based vector.

In this investigation we have defined the antiviral and immunological roles of IL-12 when expressed from VV in the absence and the presence of HIV-1 Env. Our findings demonstrate that rVV expressing IL-12 genes are safe vectors, since VV replication is severely compromised through the induction of IFN-\(\gamma\). In immunized mice IL-12 expression drives a Th1-type response against both the vector and the env gene product, leading to an enhanced cellular immune response and a biased serum Ab response in favor of IgG2a subclass. Moreover, we show that the dosage of the rVV expressing IL-12 and env genes plays an essential role in the enhancement of the cellular immune response against the HIV-1 gp160 protein, and that by coexpressing IL-12 and env genes in different ratios it is possible to trigger a desirable cellular immune response to the HIV-1 Ag.

Materials and Methods

Viruses and cells

The VV recombinants employed in this study derived from the wild-type WR strain, rVVluc (expressing the luciferase and \(\beta\)-galactosidase genes) and rVVenv (expressing the entire env gene of HIV-1 strain IIIB and \(\beta\)-galactosidase gene), have been described previously (20, 21). The recombinant viruses rVVlucIL-12 (expressing the luciferase gene and p35 and p40 IL-12 subunits), its control rVVlucHA- (expressing the luciferase gene and insertional inactivated in the HA gene), as well as rVVenvIL-12 and rVVenvHA- were generated for this study, and their construction is described below. Viruses were grown in HeLa cells, titrated in African green monkey kidney BSC-40 cells, and purified as described previously (22).

Engineering of the VV recombinant viruses

The cDNAs coding for both IL-12 subunits (p35 and p40) linked by an internal ribosomal entry site sequence (IRES) were isolated from plasmid pBS-IL-12 by digestion with the restriction enzymes EcoRI and BamHI. The DNA fragment containing the complete IL-12 sequence (p35-IRES-p40) was blunt ended by treatment with the large fragment of the Escherichia coli DNA polymerase I (Klenow) and cloned into the Smal site of the VV insertion vector pJR101. As a result of this cloning strategy, we isolated a plasmid, pJR101-IL-12, that contains the IL-12 (p35-IRES-p40 cassette) genes under the control of a VV synthetic early/late promoter e/l (23), the E. coli \(\beta\)-glucuronidase marker gene under the control of the VV early/late promoter p7.5, and all these sequences flanked by regions from VV homograftin (HA) gene. Double rVV were prepared by infecting BSC-40 cells with either the VVenv or the VVluc recombinant virus and transfecting them with the plasmid pLR101-IL-12. Cell cultures were harvested at 48 h postinoculation (hpi), and the double-recombinant viruses were selected after plaque assay by the addition of X-Gluc to the agar overlay (24). By this procedure, rVV containing the HIV-1 env gene (VVenvIL-12) or the luciferase gene (VVlucIL-12) into the TK region and the IL-12 cassette into the HA locus were isolated. After three rounds of selection, viruses were purified following standard procedures. A similar strategy was followed to generate control viruses VVlucHA- or VVenvHA-, but in this case, transfection was performed with empty VV insertion plasmid pLR101. In Fig. 1A is shown a schematic representation of the different rVV constructed for this study. In Fig. 1B is shown IL-12 expression in extracts from rVV BSC-40-infected cells by Western blot analysis. An IL-12 bioassay was also performed with the same samples, indicating that IL-12 expressed from rVV was bioactive (data not shown).

**Immunizations of mice and serum sample collection**

BALB/c mice (H-2\(^d\); 6–8 wk old) were immunized i.p. with different doses (indicated as PFU) of the different rVV in 200 \(\mu\)l of sterile PBS. Fourteen days after virus inoculation, blood was obtained from the retro-orbital plexus by a heparinized capillary tube, collected in an Eppendorf tube, and centrifuged, and serum was obtained and stored at \(-20^\circ\)C.

**Measurement of luciferase activity in mice tissues**

Replication of rVV in different mouse tissues was followed by a highly sensitive luciferase assay, previously described (20). Different groups of BALB/c mice (H-2\(^d\)) were injected i.p. with rVV containing luciferase and informative doses of each virus. At different times after infection, bioluminescence in different organs was measured using a LB 9507 luminometer. The results are expressed as counts per minute (cpm) per organ. One representative experiment of three performed is shown.

**FIGURE 1.** Characterization of the different rVV generated. A, Scheme of the VV genomes. The genes coding for luciferase (Luc) and HIV-1 Env (Env) proteins were inserted into the thymidine kinase (TK) locus of the VV genome (\(\square\)). The DNA cassette containing the genes coding for IL-12 was inserted into the HA locus (\(\Box\)) to generate the double recombinant rVVlucIL-12 and rVVenvIL-12. p11, p7.5, and e/l represent different VV promoters. B, Western blot analysis of extracts from cells infected with the different rVV. Monolayers of BSC-40 cells were infected with the indicated rVV. The proteins were fractionated by SDS-PAGE under reducing conditions, transferred to nitrocellulose paper, and reacted with an anti-gp120 rabbit polyclonal Ab (left panel) or an anti-IL-12 p40 rat mAb (right panel). Ab reactivity was detected by immunoperoxidase staining using standard procedures.
mice received an i.p. inoculation with 5 × 10^7 PFU/mouse of the recombining viruses: rVVhuc, rVVhucA1, or rVVhuc-I12. At various times postinoculation animals were sacrificed, and spleens, livers, and ovaries were dissected, washed with sterile PBS, weighed, and stored at −70°C.

Then, tissues from individual mice were homogenized in luciferase extraction buffer (300 μl/spleen extract and 100 μl/volary extract) containing 1% Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM MgSO4, 4 mM EGTA, 1 mM DTT, 1 mM PMSF, 100 μg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin. The luciferase activity was measured in the presence of luciferase reagent using a Lumat LB 9501 Berthold luminometer (Berthold, Nashua, NH), and it was expressed as relative luciferase units per milligram of protein. Protein content in tissue extracts was measured employing the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL).

**RNA extraction**

Total mRNA was purified from aseptically removed spleens. Relatively identical small pieces of spleens from three mice per group were pooled and homogenized in extraction buffer using an Ultraturrax T8 mechanical homogenizer (Janke & Kunkel, Staufen, Germany). Clear lysates were source for mRNA purification using QuickPrep Micro mRNA purification kit (Pharmacia, Uppsala, Sweden) following the instructions of the manufacturer.

**Amplification of mRNA by RT-PCR**

Semiqualitative RT-PCR was performed on mRNA using the SuperScript One-Step RT-PCR System (Life Technologies, Gaithersburg, MD). The RT conditions used were 30 min at 40°C followed by a denaturing step at 94°C for 2 min followed by 30 (for hypoxanthine phosphoribosyltransferase, HPRT), 40 (for IFN-γ and IL-12) cycles. The number of cycles was adjusted for every pair of primers to get a linear range between the reactions. Cycling conditions were 94°C for 30 s, 60°C (58°C for IL-12) for 30 s, and 68°C for 1.5 min, followed by a final extension step at 68°C for 5 min. Primers sequences were: for HPRT, 5'-GTTGGATACAGGC CAGACTTTGGT-3' (sense) and 5'-GATCACTTGGCGCATCTC TAGGC-3' (antisense); for IFN-γ, 5'-TGAACGCTACACA CATGTCATCTTG-3' (sense) and 5'-CGACTCTTTTCTGGCTTCTGAG-3' (antisense); and for IL-12 p40 subunit, 5'-CTCAATCATGCTGGTCCTGA AA-3' (sense) and 5'-CTCCTGATCATCTTTCTTCTT-3' (antisense). PCR products were analyzed by ethidium bromide staining after electrophoresis on 1.2% agarose gels.

**Ab measurements by ELISA**

ELISA was used to determine the presence of Abs against VV Ags in serum samples. The VV Ags employed to coat 96-well flat-bottom plates at a concentration of 1 μg/ml consisted of envelope proteins extracted from purified virions, as described previously (25). VV Ags were suspended in carbonate buffer, pH 9.6, plated at 50 μl/well, and incubated overnight at 4°C. Afterward, the contents of the wells were discarded and washed three times with PBS plus 0.05% Tween-20 (PBS-T), and blocking buffer (bothe the wells were washed and blocked with 1% BSA, 1 mM EDTA, and 0.05% Tween-20) was added at 100 μl/well and incubated for 1 h at 37°C. The plates were washed once with PBS-T, and samples diluted in blocking buffer were added in a volume of 100 μl/well and incubated for 1 h at 37°C. Then, plates were washed three times before the detection Ab was added. Peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Southern Bio-technology Associates, Birmingham, AL) Abs were diluted 1/1000, 1/500, or 1/200 respectively in blocking buffer and incubated for 1 h at 37°C. After washing the plates three times with PBS-T, the wells were reacted with the peroxidase substrate O-phenylendiamine dihydrochloride (Sigma, St. Louis, MO). After 10–15 min of incubation at room temperature, the reaction was stopped by adding 2 N H2SO4, and the absorbance values were measured at 492 nm on a Labsystems Multiskan Plus plate reader (Chicago, IL).

**T cell proliferation assays**

Lymphocytes were removed from spleens by passing tissues through a sterile mesh to obtain cell suspensions. Cells were suspended in complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 10 mM 2-ME). RBC in preparations of spleen cells were lysed with 0.1 M ammonium chloride buffer. Splenocytes were cultured in triplicate (10^6 cells/well) in 96-well microtiter flat-bottom plates and stimulated with purified VV previously inactivated by UV light at 1 μg/ml, purified gp160 protein (Intracel, Cambridge, MA; 1 μg/ml), or Con A (1 μg/ml). Plates were incubated for 3 days at 37°C in 5% CO2. After this incubation period, proliferation assays were conducted by labeling the cells with [3H]thymidine (1 μCi/well) for 18 h. Following automated harvesting, [3H]thymidine incorporation was measured by liquid scintillation counting.

**CD8+ T cell purification**

CD8+ cells were purified using MACS High Gradient Magnetic Separation Columns V9 (Miltenyi Biotec, Bergisch Gladbach, Germany) for positive selection from whole splenic populations following the manufacturer’s
procedure. Cells were labeled with CD8α (Ly2.53–6.7) Micro beads (Miltenyi Biotec), and 10^6 cells/ml were loaded in the column. A fraction of the whole population or positive and negative eluates were formaldehyde fixed and labeled with fluorescence-specific anti-CD4 and anti-CD8 Abs for cell sorting by flow cytometry (FACS, Becton Dickinson, Mountain View, CA). The sorted profiles were used to evaluate the accuracy of the purification and quantitate the ELISPOT assay performed with those populations. Less than 3% of CD8⁺ cells were present in the CD8⁻-depleted fraction.

**Results**

Effects of IL-12 expression on VV replication and virus persistence in mice

Little information is available on the effect of IL-12 on VV infection; hence, our first goal was to evaluate the effect of IL-12 on the capability of the viral vector to infect and persist in target tissues. Thus, we studied the replication of a WR-based rVV that expresses the IL-12 gene (rVVlucIL-12) by measuring the activity of the coexpressed luciferase reporter gene. As the IL-12 expression cassette was inserted into the HA gene, and as it is known that inactivation of this gene reduces VV infectivity in vivo (28), we employed as controls two luciferase expression viruses rVVlucHA⁻ and rVVlucHA⁺ with an inactivated or an intact HA gene, respectively. To evaluate in vitro whether IL-12 expression could have any effect on VV replication, BSC40 cells were infected (10 PFU/cell) with the three different VV recombinants (rVVlucIL-12, rVVlucHA⁻, and rVVluc) and at different times postinfection (5, 18, and 24 hpi) luciferase activity was measured in cell extracts. No significant differences in luciferase activity were observed among the different rVV (data not shown), indicating that IL-12 expression had no effect on viral replication. To evaluate in vivo the effect of IL-12 expression on VV replication, groups of mice were i.p. inoculated with a single dose of 5 × 10⁷ PFU/mice of rVVluc, rVVlucHA⁻, or rVVlucIL-12, and three animals per day and group were sacrificed on days 1, 2, 3, 4, and 7 postinoculation (dpi). Luciferase activity in spleen and ovaries of individual samples were measured, and results are depicted in Fig. 2. At 1 dpi, similar luciferase values were detected in spleen samples from mice inoculated with either rVVlucIL-12 or rVVlucHA⁻, and these were about 50-fold lower than those found after infection with rVVluc. However, at 2 dpi luciferase levels fell 100-fold in animals given the IL-12-expressing virus, and no infectious virus was detected by plaque assay in this group (not shown). By this time, levels of replication of rVVlucHA⁻ or rVVluc control viruses remained essentially stable. At 3 dpi only background luciferase activity was measured in all mice given the rVVlucIL-12, whereas low, but still measurable, luciferase activity was detected in mice inoculated with control viruses. The kinetics of the rVVlucHA⁻ virus were parallel to those of control rVVluc virus, but infection in the spleen was resolved later in animals inoculated with rVVluc virus.

Ovaries are target tissues of VV infection on the mouse, and viral clearance from this organ is delayed with respect to that from spleen or liver (29). As observed in Fig. 2 multiplication of rVV in ovaries was extended for the three viruses analyzed compared with that in the spleen. In this organ, differences between rVVlucHA⁻ and rVVlucIL-12 were more pronounced than those in spleen. At 1 dpi, luciferase activity in ovaries was 50-fold lower in animals inoculated with rVV expressing IL-12 than in animals inoculated with the control virus (rVVlucHA⁻). By 2 dpi, luciferase activity reached a peak in both rVVlucHA⁻ and rVVluc-inoculated groups, whereas 100-fold lower activity was detected in mice infected with rVVlucIL-12. In all groups, luciferase expression in ovaries declined by day 3 and was greatly reduced by 4 dpi in animals receiving IL-12. A slower decrease in luciferase levels was observed in mice given control viruses, especially in those receiving rVVluc.

The results shown in Fig. 2 demonstrate that IL-12 expression during VV infection impairs virus multiplication and promotes a rapid clearance of the virus from infected tissues, suggesting that delivery of IL-12 ensures attenuation of VV along the infectious process in mice.

Induction of IL-12 and IFN-γ in vivo after inoculation of mice with a rVV expressing IL-12

To determine whether the decreased infectivity observed for rVVlucIL-12 in mice could be correlated with induction of IFN-γ expression, we next analyzed the levels of IL-12 and IFN-γ in serum samples from mice inoculated with rVVlucIL-12 or rVVlucHA⁻. Due to the rapid clearance observed for VV after IL-12 expression (Fig. 2), we examined the levels of these cytokines at early times postinfection. Thus, serum samples were collected every 6 h during the first dpi and daily thereafter until 7 dpi. IL-12 was measured both by ELISA against the p40 subunit (data not shown) and by determination of bioactive p70 heterodimeric form (Fig. 3A, upper panel), rendering comparable results. The maximum amount of IL-12 was found at 6 hpi in the inoculated groups. Nevertheless, at least 5-fold higher levels were found in samples from rVVlucIL-12-immunized mice than in animals inoculated with the control virus. Levels of IL-12 in the control group fell into the detection limit of the assay (<0.64 U/ml) beyond 12 hpi and were the same as those found in naive mice (not shown). The higher levels of IL-12 (ranging from 480–240 U/ml) were found during the first dpi in samples from mice inoculated with rVVlucIL-12, but IL-12 was still detected at 3 and 4 dpi. This result clearly demonstrates that there is a rapid induction of IL-12 upon infection with rVV expressing this cytokine, and significant levels are still present during the clearance period of the virus (see Fig. 2).

High levels of IFN-γ, evaluated by ELISA, were detected in serum from mice inoculated with IL-12 expressing rVV. The maximum level of IFN-γ appeared, with a lag of 12 h with respect to IL-12 production, around 18 hpi in rVVlucIL-12-immunized mice. High levels were still present at 2 dpi, falling to background levels.
by 4 dpi, while in the control group detectable levels were only found at 6 and 12 hpi (Fig. 3A, lower panel).

The kinetics of mRNA expression for the inducible p40 subunit of IL-12 and for IFN-γ in the spleen were analyzed by semiquantitative RT-PCR (Fig. 3B). Induction of IL-12 mRNA was observed at 6 hpi in mice inoculated with rVVlucIL-12, increased about 10 times by 18 hpi and declined by 1 dpi, whereas in mice inoculated with the control rVVlucHA virus, low but consistent amounts of p40 mRNA were present at 18 hpi. Levels of IFN-γ mRNA in the spleens of mice inoculated with the rVVlucIL-12 peaked at 24 hpi and were detectable until 2 dpi (Fig. 3B and data not shown). The induction of IFN-γ mRNA was clearly detected at 18 hpi in mice inoculated with the control rVV, and the levels were >10 times lower than those in mice inoculated with rVVlucIL-12. Under the conditions of the assay, spleens from naive noninoculated mice did not reveal detectable mRNAs for the two cytokines.

Evaluation of the anti-VV immune response elicited in mice inoculated with rVVlucIL-12

The findings presented in Figs. 2 and 3 clearly reveal that IL-12 has a profound effect on the replication of VV and that this cytokine potentiates IFN-γ production. As both cytokines could have a major impact on the modulation of host immune responses, our next approach was to analyze the role of IL-12 in specific immune responses against the VV vector.

CD8+ IFN-γ-secreting T cells against VV

As IL-12 has the capacity to augment cell-mediated immune responses (30, 31), and as CD8+ CTL responses are involved in the resolution of infection by poxviruses (32, 33), we first evaluated the CD8+ T cell immune response elicited against VV following expression of IL-12 in mice inoculated with rVVlucIL-12. We employed a modification (34) of the ELISPOT assay that quantifies the number of specific anti-VV MHC class I-restricted IFN-γ-secreting cells. Groups of three or four mice were i.p. inoculated with 5 × 10^7 PFU/mice of rVVlucIL-12 or rVVlucHA−, and at 7 or 14 dpi the number of anti-VV IFN-γ-secreting CD8+ T cells was determined. As shown in Fig. 4A, at 7 dpi the numbers of specific IFN-γ-secreting CD8+ T cells in spleens from animals inoculated with rVVlucIL-12 (1641 ± 104/10^6 cells) were significantly different (p < 0.05) with respect to those in mice inoculated with the control virus (964 ± 64/10^6 cells). This represents a 1.7-fold more MHC I class-restricted anti-VV IFN-γ-secreting cells in animals inoculated with rVVlucIL-12 virus than in the control group. As expected, by 14 dpi the number of specific anti-VV CD8+ T cells IFN-γ-secreting cells decreased in the two groups of animals (Fig. 4A), but differences between the groups were maintained. The number of specific CD8+ T cells quantified after purification revealed no differences when the total population was compared with the CD8+−selected fraction (Fig. 4B). These observations confirm that in the ELISPOT assay shown in Fig. 4A the MHC class I-restricted cell population responsible for IFN-γ secretion was mainly CD8+ T cells.

Pattern of cytokine secretion in splenocytes after VV Ag restimulation

We next investigated the influence of IL-12 expressed from VV on the pattern of cytokines expressed by T cells in vitro after Ag restimulation. Two groups of mice were immunized i.p. with 5 × 10^7 PFU/mice of rVVlucIL-12 or rVVlucHA−, and 14 days later splenocytes from both groups of mice were restimulated in vitro with UV-inactivated VV. As shown in Fig. 4C, high levels of IFN-γ (Th1-type cytokine) were found in splenocyte cultures restimulated with VV in both groups of animals, with a slight increase in rVVlucIL-12 with respect to control virus. However, levels of IL-10 (Th2-type cytokine) were significantly decreased in supernatants of splenocytes from rVVlucIL-12 compared with controls, suggesting that IL-12 was suppressing an antiviral Th2 type of response, rather than enhancing the CD4+ Th1 response.

Effect of IL-12 delivered by rVV on systemic Ab response to VV Ags

In murine systems it has been shown that Th2 cytokines favor the induction of IgG1 subclass Abs, whereas IgG2a subclass Abs are induced in a context of Th1 cytokines (35). Thus, we next evaluated anti-VV IgG subclasses in sera, 2 wk after inoculation of mice with rVVlucIL-12 or rVVlucHA−. As shown in Fig. 5, there were no major differences in levels of specific IgG or IgG2a Abs between the groups, while in rVVlucIL-12-inoculated mice, anti-VV IgG1 subclass Abs were greatly reduced compared with the levels found in control mice. Thus, the ratio of IgG2a/IgG1 Abs (Fig. 5,
IFN-γ (g). Fourteen days later, splenocytes were cultured in vitro with VV Ag (UV inactivated) at 1°C (A). Anti-vaccinia IgG, IgG1, and IgG2a Ab titers are referred to as the described in Fig. 3, blood was obtained, and sera were tested for specific Abs. Anti-vaccinia IgG1, and IgG2a Ab titers are referred to as the mean number ± SD for individual samples (at 7 days) or the average of three pooled samples ± SD (at 14 days) from triplicate cultures. Data are representative of at least two independent experiments performed at 7 and 14 days after immunization. Data from mice inoculated with the different rVV were significant (*, p < 0.01). B. Number of IFN-γ-secreting CD8+ T cells specific for VV Ags in total splenocytes, in CD8+ selected T cells, or in CD8+ -depleted T cells 14 days after immunization with rVVlucHA+. The mean ± SD were calculated from data from triplicate cultures of pooled samples. C. Pattern of cytokine secretion in supernatants of spleen cells after Ag restimulation. Mice were inoculated as in A with rVVlucHA+ (■) or rVVlucIL-12 (■). Fourteen days later splenocytes were cultured in vitro with VV Ag (UV inactivated) at 1 μg/ml, and supernatants were collected and cytokines determined at 48 h (IL-10) or 72 h (IFN-γ) by ELISA. Three different experiments were conducted, and the results of one representative experiment are shown.

**FIGURE 4.** Cellular immune response elicited against VV upon expression of IL-12. A. Determination by the ELISpot assay of the number of IFN-γ-secreting CD8+ T cells specific for VV Ags. Groups of four mice were immunized i.p. with 5 × 10^7 PFU/animal of rVVlucHA+ (■) or rVVlucIL-12 (■), and 7 or 14 days later spleen cells were employed as responder cells in the ELISpot assay using P815 cells infected with VV as targets. Bars represent the mean number ± SD for individual samples (at 7 days) or the average of three pooled samples ± SD (at 14 days) from triplicate cultures. Data are representative of at least two independent experiments performed at 7 and 14 days after immunization. Data from mice inoculated with the different rVV were significant (*, p < 0.01). B. Number of IFN-γ-secreting CD8+ T cells specific for VV Ags in total splenocytes, in CD8+ selected T cells, or in CD8+ -depleted T cells 14 days after immunization with rVVlucHA+. The mean ± SD were calculated from data from triplicate cultures of pooled samples. C. Pattern of cytokine secretion in supernatants of spleen cells after Ag restimulation. Mice were inoculated as in A with rVVlucHA+ (■) or rVVlucIL-12 (■). Fourteen days later splenocytes were cultured in vitro with VV Ag (UV inactivated) at 1 μg/ml, and supernatants were collected and cytokines determined at 48 h (IL-10) or 72 h (IFN-γ) by ELISA. Three different experiments were conducted, and the results of one representative experiment are shown.

**FIGURE 5.** Humoral immune response against VV Ags following immunization with rVVlucHA+ or rVVlucIL-12. Fourteen days after mice were inoculated i.p. with 5 × 10^7 PFU/mouse of the different rVV as described in Fig. 3, blood was obtained, and sera were tested for specific Abs. Anti-vaccinia IgG, IgG1, and IgG2a Ab titers are referred to as the inverse log, dilution of sera that gave an absorbance at 492 nm of >0.1. Values represent the mean ± SD for individual samples corresponding to four or five mice per group.

**right panel** in mice inoculated with rVVlucHA+ was 1.7, and this ratio was increased 13.5 times in sera from mice inoculated with rVVlucIL-12. These findings indicate that IL-12 expressed from rVV modulates the humoral immune response by down-regulating specific IgG1 subclass Ab (Th2 cytokine) production, rather than by promoting up-regulation of the IgG2a subclass (Th1 cytokine), a process resemble in vitro restimulation assays (see Fig. 4C).

Coexpression of IL-12 and HIV-1 Env by rVV increases the cellular immune response against gp160 in a dose-dependent manner

To assess whether IL-12 delivered by VV might be effective in potentiating the cellular immune response against the Env protein of HIV-1, we first evaluated the IL-12 action when the Ag and the cytokine were coexpressed from the same virus vector. To this aim, groups of mice were immunized i.p. with either 5 × 10^7 or 1 × 10^7 PFU/mouse of rVVenvHA+ (that expresses the complete env of HIV-1 strain IIIB) or rVVenvIL-12 (coexpressing env and IL-12 genes). Fourteen days after immunization, we evaluated the numbers of specific splenic IFN-γ-secreting CD8+ T cells, using P815 cells pulsed with a CD8+ T cell peptide specific for the V3 loop of env. Immunization with 1 × 10^7 PFU of rVVenvIL-12 induced about 2-fold higher number of splenic Env-specific IFN-γ-secreting CD8+ T cells with respect to spleen cells from mice inoculated with control virus (p < 0.01; Fig. 6A). However, this immune response was 3-fold lower than that in control when mice were inoculated with the higher dose (5 × 10^7 PFU) of rVVs (Fig. 6A). Indeed, levels of IFN-γ and IL-10 measured 14 dpi after Ag in vitro restimulation of splenocytes from mice inoculated with 1 × 10^7 PFU of rVVenvIL-12 were 3-fold higher and 10-fold lower, respectively, than those found in control samples, indicating that an increase in the Th1-type immune response was occurring at the low dose (1 × 10^7 PFU) of infection with the IL-12-expressing rVV (data not shown).

To understand the dose-dependent action of IL-12 delivered from rVV we attempted to establish a relationship among the virus dose, serum levels of IL-12 and IFN-γ, and the extent of viral clearance during short times after rVV inoculation. To this aim, groups of four mice were inoculated i.p. with either 5 × 10^7 or 1 × 10^7 PFU/mouse of rVVlucHA+ or rVVlucIL-12, which as live vectors are phenotypically undistinguishable from rVV expressing Env, but allowed measurement of VV replication following luciferase activity in tissues. Levels of IL-12 in serum at 12 hpi were 10- or 50-fold higher in mice inoculated with 1 × 10^7 or 5 × 10^7 PFU of rVVlucIL-12, respectively, than in the corresponding control groups (Fig. 6B). However, levels of IFN-γ induced were 10-fold higher in mice inoculated with 5 × 10^7 PFU of rVVlucIL-12 than in control immunized mice, but lower levels of IFN-γ (20–120 pg/ml) were present in mice inoculated with 1 × 10^7 PFU of either control or IL-12-expressing rVV viruses. Interestingly, by the second dpi 50-fold lower luciferase activity was found in mice inoculated with 5 × 10^7 PFU of rVVlucIL-12 compared with that in control infected mice (Fig. 2), while at this time luciferase levels were identical in mice given 1 × 10^7 PFU of either rVVlucHA+ or rVVlucIL-12 (data not shown).
The results presented in Fig. 6 showed that IL-12 levels can be controlled by the dose of rVV expressing IL-12 inoculated, which seems to be critical for the extent of cellular immune responses to Env.

Enhancement of the immune response to HIV-1 Env by delivering Env and IL-12 from two different rVV vectors

To more accurately explore the dose-dependent action of IL-12 on HIV-1 Env, we tried to modulate the immune response by delivering Env and IL-12 from two different vectors. Groups of four mice were inoculated i.p. with the indicated dose of rVV env or rVV envIL-12, which were pooled 14 days after immunization, and the number of specific IFN-γ-secreting CD8+ T cells was determined after coculture with P815 cells coated with the specific peptide (9 Env) by ELISPOT assay. Bars represent the mean ± SD for triplicate cultures. A, Data represent the numbers of IFN-γ-secreting CD8+ T cells specific for the V3 loop epitope of the HIV-1 Env protein. Splenocytes from four mice immunized i.p. with the indicated dose of the rVV envHA- or rVV envIL-12 were pooled 14 days after immunization, and the number of specific IFN-γ-secreting CD8+ T cells was determined after coculture with P815 cells coated with the specific peptide (9 Env) by ELISPOT assay. Bars represent the mean ± SD for triplicate cultures. B, Data represent IL-12 and IFN-γ serum levels at 12 h postinoculation at the indicated doses. Pooled sera from four mice inoculated in each case were used to measure IL-12 or IFN-γ by bioassay or ELISA, respectively. Results are the mean ± SD of triplicate measurements.

The results shown in Fig. 7A revealed that coadministration of 2 × 10^5 PFU of the rVV expressing IL-12 with 1 × 10^7 PFU of rVV envHA- increased by 3 times (p < 0.001) the number of specific IFN-γ-secreting CD8+ T cells. In contrast, groups of mice inoculated with higher doses of the rVV expressing IL-12 (2 × 10^6 and 2 × 10^7 PFU) showed no significant differences (p > 0.2) with respect to the control group inoculated only with rVV envHA-. To further investigate the IL-12 enhancement of cellular activity we performed Th cell proliferation assays 2 wk after immunization with splenocytes from immunized mice. Fig. 7B (left panel) shows similar stimulation indexes in spleen cells from mice immunized with rVV env alone (1 × 10^7 PFU) or with the same dose of rVV env and 2 × 10^5 PFU of rVV lucIL-12. However, a nearly 3-fold increase in specific T cell proliferation activity appeared in the mice receiving the lower dose of rVV lucIL-12 virus (2 × 10^5 PFU). Since IFN-γ production by CD4+ T cells is the most reliable indicator of a Th1 phenotype, we also measured the levels of IFN-γ secreted in stimulated spleen cells. As shown in Fig. 7B (right panel) higher levels of IFN-γ expression correlated with the higher T cell proliferation. The findings shown in...
Fig. 7 established that the dose of $2 \times 10^4$ PFU of rVVlucIL-12 significantly increased the cellular immune response against the gp160 Ag delivered by $1 \times 10^7$ PFU of rVVenv HA, augmenting the numbers of specific CD8 T cells and specific Th1 cell response.

**Discussion**

The potential of VV as a vector for vaccine purposes and the promising previous observations in different vaccination schedules with rVV expressing HIV-1 and SIV genes (4, 13, 36, 37), encourage further studies to improve the vaccination strategies against HIV-1 based on VV vectors. In this regard, a critical point related to the use of live viral vaccines is to develop approaches to attenuate the vector without losing the immunogenicity of the expressed Ag. One way to circumvent this problem and increase the immunogenicity of the recombinant product is the local coexpression of the Ag and immunostimulating cytokines. In this study we show in the mouse model that delivery of IL-12 from rVV has a marked effect on clearance of the virus from tissues, as previously described for other cytokines expressed from VV (19, 38). However, by modulating the levels of expression of IL-12 and Ags it is possible to achieve enhanced cellular immune responses against VV and a foreign Ag such as HIV-1 Env. In addition, the immune response can be modulated to a Th1 type, as observed by the ratio of IgG2a/ IgG1 subtype and elevated levels of IFN-γ in serum. To our knowledge this is the first study of IL-12 induction during VV infection in mice. We have shown that IL-12 is produced after VV infection as early as 6 hpi, and this is followed by an increase in IFN-γ at 12 hpi (Fig. 3). This finding, observed at the protein level in serum samples and at the mRNA accumulation in spleen cells, clearly shows that VV per se induces a Th1-type immune response. Furthermore, when IL-12 is expressed from the rVV, both the levels and time course of IL-12 and IFN-γ accumulation are greatly enhanced following viral infection.

The rVV expressing the IL-12 was attenuated, and it was eliminated from target tissues at earlier times than control virus. By the second and third dpi the levels of replication of rVV-IL-12 in spleen and ovaries were 100-fold lower than those in mice inoculated with the corresponding control virus. However, minor differences at the anatomical level were observed in mice inoculated with the rVV expressing IL-12. Splenomegaly was apparent in 70–80% of the mice inoculated with $5 \times 10^7$ PFU of rVVlucIL-12, and this was associated with a marked increase in levels of IFN-γ in serum (data not shown), with no consequences on mouse survival during the course of the experiment. These results agree with previous findings after in vivo administration of rIL-12 (39).

Data obtained with in vitro restimulation of splenocytes from immunized mice revealed that a Th1-type immune response is elicited upon infection with VV. Moreover, after delivery of IL-12 from rVV, a down-regulation of a Th2-type response is triggered (Fig. 4C). In this regard, we have found that VV infection elicits an Ab response biased toward the isotype IgG2a, and the IgG2a/ IgG1 ratio is increased 13.5 times relative to that for control VV when the IL-12 gene is expressed from rVV. Hence, our findings clearly demonstrate that IL-12 expression from rVV steers a potent Th1 response following inoculation in mice.

Th1-type immune responses characterized by production of IFN-γ are documented to occur during viral infections, pointing out the important antiviral role played by this cytokine as a first-line defense mechanism of the organism. A number of studies have observed IL-12 induction in mice upon viral infection. Expression of IL-12 has been demonstrated in mice early after infection (12–24 hpi) with RNA (murine hepatitis virus, lactate dehydrogenase-elevating virus, influenza virus) and DNA (adenovirus, HSV-1, and murine CMV) viruses, at both mRNA and protein levels (40–42). Studies on experimental infection in mice with different viruses correlated the IL-12 effect on antiviral immunity with the IFN-γ induction (43, 44) and activation of the CTL response (45). However, alternative pathways of IFN-γ induction during viral infection independent of IL-12 have been reported (46). In mice inoculated with VV, it has been shown that the anti-vV activity of IL-12 is abolished in IFN-γ−/− mice (19). Moreover, a drastic impairment of VV control results from neutralization of IFN-γ or from the functional deficiency of the IFN-γ gene in knockout mice (47). Thus, it is well established that IFN-γ is involved in the clearance of VV infection. In view of our findings, we propose that the rapid elimination of rVV expressing IL-12 in mice, compared with control virus infection, is mediated by the induction of IFN-γ, acting as a first antiviral nonspecific immune response of the host. Inhibition of VV replication by IFN-γ is probably mediated by the production of nitric oxide, as we have previously reported that treatment of macrophages with this cytokine potently induce nitric oxide, and this correlates with inhibition of VV replication (48). Furthermore, we have shown that inducible expression of nitric oxide synthase by rVV leads to inhibition of VV DNA replication and induction of apoptosis (49, 50).

As documented here, IL-12 expression by VV has the capacity to modulate the antiviral immune response of the host, which was revealed by the nearly 2-fold increase in the number of anti-VV CD8+ IFN-γ-secretng cells at 7 and 14 days after inoculation compared with that in controls. Although studies in other viral model systems showed that IL-12 can promote unspecific expansion of CD8+ lymphocytes that can control viral infection (51), here we demonstrate that a reduction in VV titers after expression of IL-12 correlated with an increased number of specific CD8+ T cells. In concordance with our results, expression from VV of IL-4, a cytokine that down-regulates Th1 responses, inhibits the development of mature antiviral CTL (52). Furthermore, mucosal delivery of IL-12 from rVV was effective in restoring the antiviral CTL activity in a murine model of allergic airway disease (28).

Different lines of evidence support the current opinion that induction of CMI may be an important requirement for any candidate vaccine for HIV-1. In this investigation we have examined the potential enhancement of cellular immunity against HIV-1 by immunomodulating the specific immune response to an HIV-1 Ag through the codelivery of IL-12 and the HIV-1 Env protein. We found that immunization of mice with rVV coexpressing both genes enhanced the cellular immune response against Env when the rVV was administered at a dose of $1 \times 10^7$ PFU/animal, while a higher dose reversed the effect. We found that the level of IFN-γ produced compromises the effectiveness of the CMI elicited, as that parameter is critical in the velocity of the resolution of the VV infection. However, expression of IL-12 to levels above those induced as a consequence of the VV infection is required to trigger a strong cellular anti-Env immune response. These findings suggested that to achieve an enhancement of the cellular immune response to HIV-1 Env, critical Ag and IL-12 expression levels should exist. Indeed, in the SIV macaque model a direct correlation has been shown between the ability of attenuated SIV to replicate in the host and the degree of protection that was conferred (53). In this regard, Orange et al. (51) showed that IL-12 doses required to promote protective, but not detrimental, responses might vary extremely in the context of different infections or immune responses.

We have also optimized the immunization procedure using the rVV expressing IL-12. Thus, we found that delivering Env Ag and...
IL-12 genes from individual VV vectors can lead to an enhancement of the specific CMI to Env by using different doses of the two rVV. For this concern it is noticeable that the dose-response effect of the IL-12 on the CMI elicited is observed either using double rVV (Fig. 6) or delivering the Ag and the IL-12 from separate vectors (see Fig. 7). However the optimal dosages required are different in each case; thus, an IL-12-mediated enhancement of the CMI anti-gp120 is observed at 10^6 PFU of the double rVV (expressing IL-12 and env genes; Fig. 6, left panel), while in the mixing experiment this effect was observed with 2 x 10^6 PFU of the IL-12-expressing rVV but not when 2 x 10^6 PFU was used (Fig. 7A). These empirical data cannot be attributable to differences in IL-12 expression from the different rVV (see Fig. 1B), but probably reflect the fact that nonidentical infectious processes are taking place in each case, involving mechanisms that have a critical role in the balance between the amount of IL-12 and the Ag. The finding that the dosage of rVVIL-12 plays a critical role in the generation of an effective Th1-type immune response against the recombinant Ag is important, especially when considering the implementation of vaccination strategies based on rVV. In a recent vaccine trial with NYVAC-SIV recombinants in macaques, it was found that although the addition of rNYVAC-IL-12 enhanced the CMI, it did not appear to influence the outcome of SIV challenge (54). It is possible that in this particular experimental animal model, the levels of IL-12 expressed by rVV were not optimal for providing the qualitative and/or quantitative modulation of the immune response to influence the vaccine efficacy, as only one dose of the cytokine delivering recombinant virus was assayed. Attempts to enhance the CMI response against HIV-1 Ags by coadministration of IL-12 and plasmids encoding HIV-1 genes have been described in various immunization strategies based on DNA vaccines (55–57), and increases in both specific CTL and Th1 proliferative activities were obtained (58). In our study we demonstrate similar IL-12 effects when the cytokine is delivered from VV vector, with the advantage that IL-12 is produced only transiently, thus diminishing the undesirable side effects derived from the expression of the cytokine for long periods of time, as expected after DNA immunization. In conclusion, in this investigation we have designed protocols of immunization based on rVV that increased significantly the cellular immune response to HIV-1 Env. We have also characterized the replication of rVV in tissues and how the IL-12 cytokine modulates the immune response to VV Ags. The feasibility of practical strategies able to enhance the immune response to an Ag delivered by rVV together with IL-12 and to steer the response toward a desired arm of the immune system, cellular or humoral, should provide a practical means to improve vaccination against pathogens, such as HIV-1, in which some immune responses may be protective and others detrimental.

Acknowledgments

We thank Victoria Jimenez for excellent technical assistance.

References


