Review

Towards a new generation of vaccines: the cytokine IL-12 as an adjuvant to enhance cellular immune responses to pathogens during prime-booster vaccination regimens

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Summary. A main goal of the industrialized world is the development of effective vaccines to control infectious diseases with major health and socio-economic impact. Current understanding of the immune response triggered during infection with pathogens causing malaria, hepatitis C and AIDS emphasizes the importance of cytotoxic T lymphocytes (CTLs) in combating these infections. This has led to the development of new vaccination strategies, some of which are in phase I/II clinical trials. Promising strategies of vaccination are based on highly attenuated viral vectors, such as Vaccinia virus (VV) in combination with heterologous like vectors naked DNA, referred to as priming/booster vaccination. While these immunization schedules increased the production of specific CTLs, there is a need to further expand the CD8+T cell population to control an infection. Among molecules that play a significant role in the modulation of the CTL response is the cytokine IL-12. Immunoregulation by IL-12 is of central importance in cell-mediated immunity (CMI) against those pathogens and tumors that are controlled by cell-mediated mechanisms, supported by Th1 cells. The use of this cytokine in combination with highly immunogenic VV-derived vectors is a promising system for development of future vaccination schedules. In this review, we summarize recent data on the use of IL-12 in vaccination procedures, as well as undesired side-effects of the cytokine that can be overcome by accurate use of dose, route and time-window administration of IL-12 encoding vectors. Results described here indicate that VV IL-12-mediated enhancement of the specific CMI response against a model antigen HIV-1 env was time- and dose-dependent and that the antigen and the cytokine could be expresed from two different rVVs modulating the doses of the vectors and allowing for enhancement of a specific CMI response. Moreover, the use of IL-12 during DNA prime/VV boost regimens enhanced the specific anti-HIV-1 env cellular response 20 times compared to that generated after a single rVVenv inoculation. Variables such as: a) dose of the cytokine applied, b) time of its administration and c) routes of inoculation play a critical role in the final outcome of the response. The findings presented here can be extended to other antigens, suggesting that immunomodulatory cytokines can be useful in the development of the future vaccines against numerous infectious diseases and tumors.

Key words: Immune modulation, Cytokines, IL-12, Vaccines, Prime/boost, HIV, Vaccinia virus recombinants

Introduction

In the development of strategies for vaccination the main objective is the induction of the most appropriate type of immune response able to control pathogen replication. The type of immune response induced during an immunization is in part dependent on the types of CD4+ helper T cells that are generated. 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 antibody immune response respectively. Th1 and Th2 cells develop from naive CD4+ T cells, and the differentiation process of these cells is initiated by interaction of the T cell receptor with peptide antigen and directed by cytokines present during the initiation of the cell response. Within the cytokine network, IL-4 promotes Th2 development and IL-12 plays a pivotal role in the development of Th1 cells from naive precursor T cells. Th1 cells secrete a characteristic pattern of cytokines (IFN-γ, TNF-α and ß, IL-2) promoting cell-mediated immune responses, acting against intracellular pathogens. Th2 cells produce cytokines (IL-4, IL-5, IL-6, IL-10, IL-13) that are involved in the development of humoral immunity which protects against extracellular pathogens. The cell-

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mediated immune response (especially that due to cytotoxic T lymphocytes) plays a crucial role in host resistance to intracellular infections caused by bacteria, parasites and viruses. The aim of vaccines for many important infectious diseases is therefore to induce CTL populations that recognize specific pathogen-derived epitopes involved in protection. Current understanding of the immune response induced during several infectious diseases for which effective vaccines are not available (malaria, hepatitis C, AIDS) emphasize the importance of CTLs in combating these infections and controlling the development of the severe pathologies. Particularly, in the case of immunodeficiencies caused by HIV-1 and SIV infection, CD8+ T-cell cytotoxic subsets are essential in controlling viremia and represent an immune parameter that correlates with protection (Gallimore et al., 1995; Rowland-Jones, 1997; Schmitz et al., 1999). To develop an effective vaccine against AIDS, it is essential to design strategies that could enhance the specific immune response to HIV, as well as to steer it towards the desired cell type. The immunoregulatory role of IL-12 is of central importance in the immunity against such pathogens and tumors that require immunity based on cell-mediated mechanisms, supported by Th1 cells (Orange et al., 1994). These properties make IL-12 an excellent candidate adjuvant for vaccination against infectious diseases and cancer. Moreover, the use of vectors delivering cytokines, like IL-12, in conjunction with the appropriate antigen is a promising strategy to induce strong CMI against pathogens of world-wide importance such as human immunodeficiency virus, hepatitis C and Plasmodium falciparum.

IL-12 general properties

IL-12 is a protein hereterodimer of 70 Kda (p70) formed by two covalently linked glycosylated subunits of 40 Kda (p40) and 35 Kda (p35), and is secreted by phagocytic cells, B cells and other types of antigen presenting cells (Trinchieri, 1994). Among the main functional features of IL-12 are: a) it potentiates cytokine production, particularly IFN-γ in T lymphocytes and NK cells, b) it acts as a growth factor for pre-activated T and NK cells, and c) it is involved in the generation and optimal differentiation of CTLs and in the activation of cytotoxicity in both CD8+ T and NK cells (Trinchieri, 1994). Expression of the mature functional form of the IL-12 is a complex process, as expression of two different genes must be triggered. The presence of p40 transcripts correlates with the ability of a cell to express the cytokine whereas the p35 mRNA is ubiquitously expressed in almost all haemopoietic and non-haemopoietic cells (Trinchieri, 1994).

During the first stages of an infection by intracellular pathogens this cytokine is secreted by antigen presenting cells (APCs) in a T cell-independent manner, and acts as a key regulator of the innate resistance (Fig. 1). In these stages IL-12 acts as a potent inducer of IFN-γ secretion

by T and NK cells, thus leading to the activation of the phagocytic cells. In addition to the direct induction of IL-12 observed in response to infectious agents, production of IL-12 by APC, either activated macrophages or dendritic cells (Fig. 1) can be induced by interaction with antigen-specific T cells. This T cell-dependent induction of IL-l2 production is based on the interaction of CD40 ligand (CD40L) on the surface of activated T cells with CD40 on the APC and can be mimicked by crosslinking CD40 on the surface of IL-12-producing cells with anti-CD40 antibodies (Shu et al., 1995) or recombinant CD40L (Kennedy et al, 1996). In vivo, both pathways of IL-12 production are probably activated, either during the inflammatory phase of the innate resistance (T-cell-independent pathway) or during the foregoing adaptive immune response (T-cell-dependent pathway, see Fig. 1). Dendritic cells (DC) are the most potent antigen-presenting cells for Th cells. The interaction between the CD40 molecules on DC and the CD40L present on T cells transmits signals which are
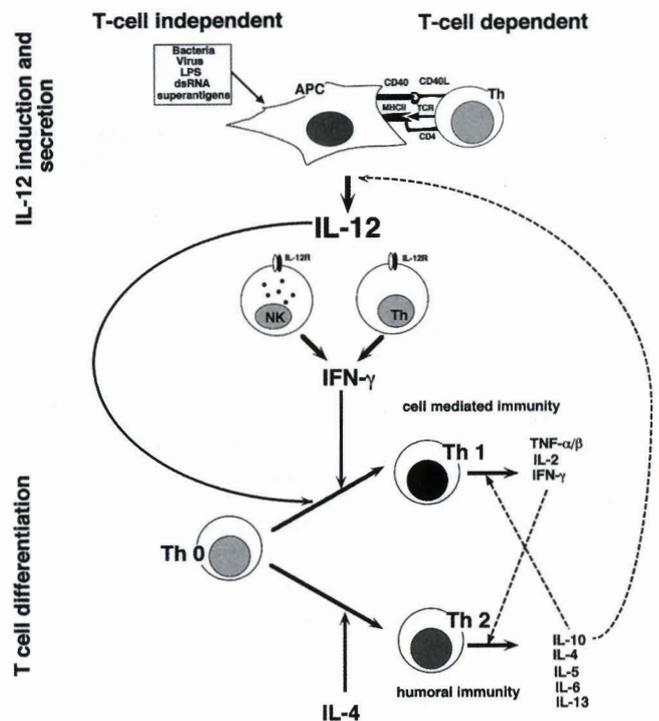

Fig. 1. Schematic representation of the expression of IL-12. Different stimuli, such as bacterial products, virus, LPS, or superantigens, can induce the T-cell-independent secretion of IL-12 by APCs. In these stages IL-12 acts as a potent inducer of IFN-γ secretion by T and NK cells leading to activation of phagocytic cells. APCs can also secrete IL-12 in a T-dependent manner: activated T cells during antigen presentation, can stimulate production of IL-12 by APC, either macrophages or dendritic cells. The mechanism of this T cell-dependent induction of IL-12 production is based on the interaction of CD40 ligand (CD40L) on the surface of activated T cells with CD40 on the APC. Both IL-12 and IFN-γ participate in the T cell differentiation events, favouring the generation of a Th1-type of immune response enhancing the specific cell-mediated immunity against the antigens.

critical for T helper cell development and IL-12 production. When an antigenic stimulus induces a Th2 response different possibilities may explain this fact: the first one is that Th2-inducing DCs belong to a particular lineage, and second, there is a unique lineage of DC that induces Th1 or Th2 differentiation based on the influence of factors on the IL-12 secretion (Sinigaglia et al., 1999). Thus, many pathogens can control the type of immune response generated through the modulation of IL-12 production by dendritic cells. Once secreted, IL-12 activates the Th1-specific transcriptional program on T cells, activating selectively the transcription activator factor STAT4 (Szabo et al., 1995), and the IFN-regulatory factor-1 (IRF1) (Coccia et al., 1999) on Th1 cells, which have been implicated in the development of Th1 cells. The lymphocyte response to IL-12 is dependent on the expression of the high affinity IL-12R, that has two subunits (ß1 and ß2). The IL-12Rß2 expression represents the main checkpoint for the responsiveness to IL-12 in developing T cells (Rogge et al., 1997; Szabo et al., 1997). Thus, in the absence of IL-12, IL-12Rß2 is expressed at low levels but in the presence of IL-12, IL-12Rß2 is upregulated. Thus, cells that develop along the Th1 pathway continue to express both IL-12R subunits, and there is a selective loss of the IL-12Rß2 transcripts during differentiation of T cells along the Th2 pathway. In a recent report (Rogge et al., 1999), a monoclonal antibody to the IL-12 receptor ß2 chain recognized T cells from patients with a Th1-type but not with a Th2-type inflammatory disease, suggesting that expression of IL-12Rß2 chain might be considered as a marker of Th1 cells.

Another property of IL-12 is its role in T cell trafficking, mediated by its ability to upregulate a subset of genes functionally related to leukocyte trafficking, thus confering a selective ability to Th1 cells to penetrate and localize within certain tissues for the immune response (Sallusto et al., 1998; Lim et al., 1999). Overall, the main characteristic of IL-12 is to be a principal immunoregulatory molecule that influences the interaction among pathogens and the host immune system.

The use of IL-12 in therapy with animal and human models of infectious diseases

The ability of IL-12 to skew the immune response into the cellular immune arm forms the basis of its activity as an adjuvant in vaccinations against infectious diseases and cancer. In animal models of infectious diseases, IL-12 was first shown to be effective as a prophilactic agent for *Leishmania major* infection (Afonso et al., 1994). This activity was then extended to other intracellular pathogens such as *Listeria monocytogenes* (Miller et al., 1995) and intracellular parasites such as *Schistosoma mansoni* (Wynn et al., 1995). In a recent study (Kenney et al., 1999), recombinat human IL-12 has been employed in conjuction with alum as adjuvants with killed antigen in

a primate model of cutaneous leishmaniasis. Promising results were obtained providing a basis for further use in clinical human trials. The IL-12 benefits as adjuvant have also been employed in both systemic (Operschall et al., 1999) and intranasal (Arulanandam et al., 1999) administrations during immunization of mice against viral infections such as respiratory syncitial virus (Tang and Ghaham, 1995), pseudorabies virus (Schijns et al., 1995), and influenza virus (Arulanandam et al., 1999). Several studies have evaluated the IL-12 capacity to enhance the specific CMI against HIV-1. Thus, during immunization strategies based on DNA, the coadministration of plasmids encoding IL-12 plus HIV-1 antigens increased both the specific CTL and Th1 proliferative responses (Takashi et al., 1997; Kim et al., 1998). In other studies, IL-12 has been shown to act sinergistically with GM-CSF and TNF-α (Okada et al., 1997; Takashi et al., 1997), in the enhancement of specific CTL against HIV-1 antigens. The cytokine has also been administered from viral vectors in conjunction with desired antigens and is the subject of discussion in the next section.

Finally, a number of studies performed in various animal models addressed the potent antitumor effects of IL-12. Administration of IL-12 showed a therapeutic effect controlling growth of primary and metastatic tumors at non-toxic doses (Rodolfo and Colombo, 1999). The use of IL-12 as a candidate adjuvant for vaccination against cancer is under consideration by a number of laboratories. However, the adverse toxic effects in humans have hampered its further development into clinical applications. The first reported trial in which adverse effects were observed was during Phase II studies conducted by the Genetics Institute of Cambridge (Cohen, 1995). In this study 17 patients with renal cell carcinoma that received the cytokine showed symphtoms of toxicity that affected multiple organs, and two of the treated patients died. In other clinical trials the cytokine was well tolerated when minor doses of IL-12 were subcutaneously administered instead of intravenously (Lamont and Adorini, 1996). Later, it was shown in murine and primate models that pretreatment with a single injection of IL-12 before the administration of a daily IL-12 regime protected the animals from the toxic effects, a fact that was associated with minor IL12-driven IFN-γ levels. However in some studies it was shown that while the predosing approach desensitizes animals to the toxic effects, it can also compromise the IL-12 therapeutic benefits (Coughlin et al., 1997). Gene therapy may be a suitable option to overcome toxicity effects, and this approach was employed in several animal models and in clinical protocols (Rodolfo and Colombo, 1999). In a Phase I clinical study with melanoma metastasic patients, vaccination with autologous IL-12 gene modified tumor cells, and induced immunological changes leading to an increased antitumor immune response (Sun et al., 1998).

Other non-desired side effects associated with IL-12 administration were potential immunnosuppresive

actions. Indeed, transient IL-12 suppression of the immune response was observed in several murine models when administered exogenously as a soluble product (Kurzawa et al., 1998) or when expressed from vectors (Lasarte et al., 1999). This suppressive effect appears to be mediated by the nitric oxide (NO) generated from activated macrophages (Kurzawa et al., 1998).

Another potential use of IL-12 is associated with immune restoration on HIV-1-infected individuals during immuno-therapy treatment. Data generated from different laboratories (Clerici et al., 1993; Chechimi et al., 1994), suggest that a shift from Th1 to Th2 may be associated with a decline in the clinical status of HIV-1-infected patients. Thus, studies with PBMC from HIV-1-infected patients revealed an impairment in the production of IL-12 against different stimuli when compared to healthy donors, suggesting that a primary IL-12 defect may lead to secondary deficiencies, and in turn amplify the immunodeficiency during HIV-1 infection (Marshall et al., 1999). In addition, a direct role

of IL-12 in controlling HIV-1 replication on T cell clones has been reported (Salgame et al., 1998). However, while administration of recombinant human IL-12 to chronically SIVmac-infected rhesus monkeys was well tolerated, it did not cause reduction in virus load of the infected animals (Watanabe et al., 1998). This controversy limited the use of IL-12 as a therapeutic agent to restore immune parameters in HIV-1 immunosuppressed individuals.

IL-12 expressed from rVV as an attenuated model vector for the delivery of cytokines

Studies on live-based vectors represent a promising target area of vaccine research. Probably the best studied vector is vaccinia virus (VV), the prototype member of the Poxvirus family, that was succesfully used as a live vaccine to erradicate smallpox (WHO, 1980). 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
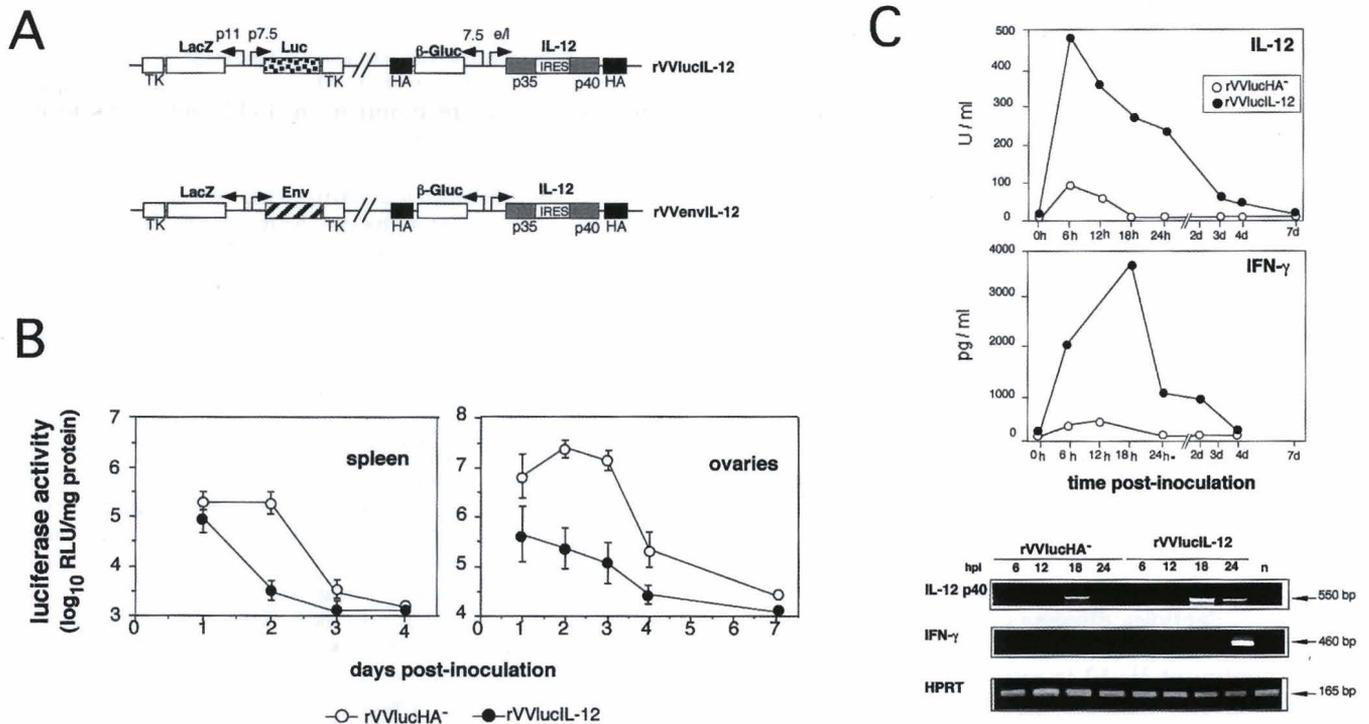

Fig. 2. A. Scheme of the rVV genomes. The genes coding for luciferase (Luc) and HIV -1 Env (Env) proteins were inserted into the thymidine kinase (TK) locus of the VV genome. The DNA cassette containing the genes coding for IL-12 was inserted into the HA locus to generate the double recombinants, rVVluc IL-12 and rVVenvIL-12. P11, p7.5 and e/l represent different VV promoters. **B.** Expression of IL-12 by rVV severely compromises virus replication in mice. BALB/c mice were inoculated i.p. with 5x10^7 PFU/animal of rVVluc HA- or rVVluc IL-12, and ovaries and spleens were collected on the indicated days. Luciferase activity in the different homogenate tissue samples was measured, and values were related to the amount of protein present in tissue extracts (relative luciferase units (RLU) per milligrams of protein). Background levels found in control uninfected tissues were 3 and 4 log10 RLU per milligrams of protein for spleen and ovaries, respectively. Results represent mean values (with the SD) from samples of three animals per day and group. **C.** Kinetics of IL-12 and IFN-γ expression following inoculation of mice with rVVluc IL-12. Groups of mice were inoculated i.p. with 5x10^7 PFU/animal of rVVlucHA- (open symbols or rVVlucIL-12 (filled symbols), and at the indicated time points sera and spleens were obtained from three mice per group. In the upper pannel serum levels of IFN-γ and IL-12 were evaluated (by ELISA and bioassay respectively). In the lower pannel levels of IL-12 and IFN-γ mRNA in spleens were determined by RT-PCR. The expected sizes of the PCR products are indicated. Levels of HPRT mRNA were used as an internal control.

variety of foreign antigens (Moss, 1996). Recombinant VV vectors have also been proven effective in field animal vaccination programs (Brochier et al., 1995). When rVV were tested in different vaccination schedules on simian hosts, specific cellular and humoral immunity, both systemic (Hirsch et al., 1996) or mucosal (Belyakov et al., 1998) were elicited. The above findings suggest that VV-based vaccination approaches using highly attenuated strains might be a promising prophylactic strategy against several infectious agents of world-wide health importance. In our laboratory we are interested in evaluating the effectiveness of the use of rVV as live-based viral vectors that could contribute to the development of safe vaccines against pathogens like Plasmodium, Leishmania and HIV-1 to whose protection relies on CMI responses.

In the case of HIV-1, the scope of our approaches are directed to enhance the specific CMI response against the HIV-1 env gene product, either by the delivery of IL-12 and gp160 (env) from rVV, or by the application of bimodal immunization schedules that exploit the boosting capacity of rVVs on heterologous primed CTL responses (particularly in DNA prime/VV boost regimens) in combination with IL-12 delivery from DNA. We have generated (Fig. 2A) double rVVs expressing the murine IL-12 subunits p35 and p40 in an expression cassette using the EMCV IRES strategy, inserted in the hemagglutinin locus of the Western Reserve (WR) strain of VV and the clade B HIV-1 env gene from the IIIB strain or the luciferase reporter gene inserted in the thymidine kinase viral locus (rVVenvIL-12 and rVVlucIL-12, respectively). Following systemic inoculation of susceptible BALB/c mice with a high dose of rVVluc IL-12, a marked attenuation of the virus occurs, as indicated by a more rapid kinetic of virus clearance from target tissues and lower peak viral titres than in mice infected with control HA⁻ rVV that do not express the cytokine (Fig. 2B). Serum and spleen levels of IL-12 after rVVenvIL-12 inoculation were five times higher than during a control VV infection. These differences were directly correlated with the IFN-γ levels in rVVenvIL-12 mice (Fig. 2C). Importantly, the specific immune response against vector-derived antigens or to the HIV-1 gp160 was skewed to a Th1 type as revealed by the IgG2a/IgG1 ratio, the number of specific CD8+ T-cells and the profile of cytokines secreted during T-cell restimulation (IFN-γ vs. IL4 levels) (Fig. 3). However, the magnitude of these responses was lowered with increased doses of the rVVenvIL-12 used and such effect correlated with higher IFN-γ serum levels at shorter times post-inoculation (12 hpi) in mice immunized with high doses of the virus (Fig. 4). These findings indicate that the IL-12-mediated enhancement of the specific CMI response against the HIV-1 env product was dose-dependent, a result of relevance in further uses of IL-12. Such undesired property exerted at high doses of IL-12 has been adressed in other experimental models following viral infection with LCMV or MCMV upon administration of recombinant mIL-12 and seems to be associated with IFN-γ-driven NO induction (see below). Moreover, our exploration on the optimal conditions of IL-12 delivery in mice using rVVs indicated that a profitable strategy is to express the specific antigen (the HIV-1 env gene) and the cytokine from two different
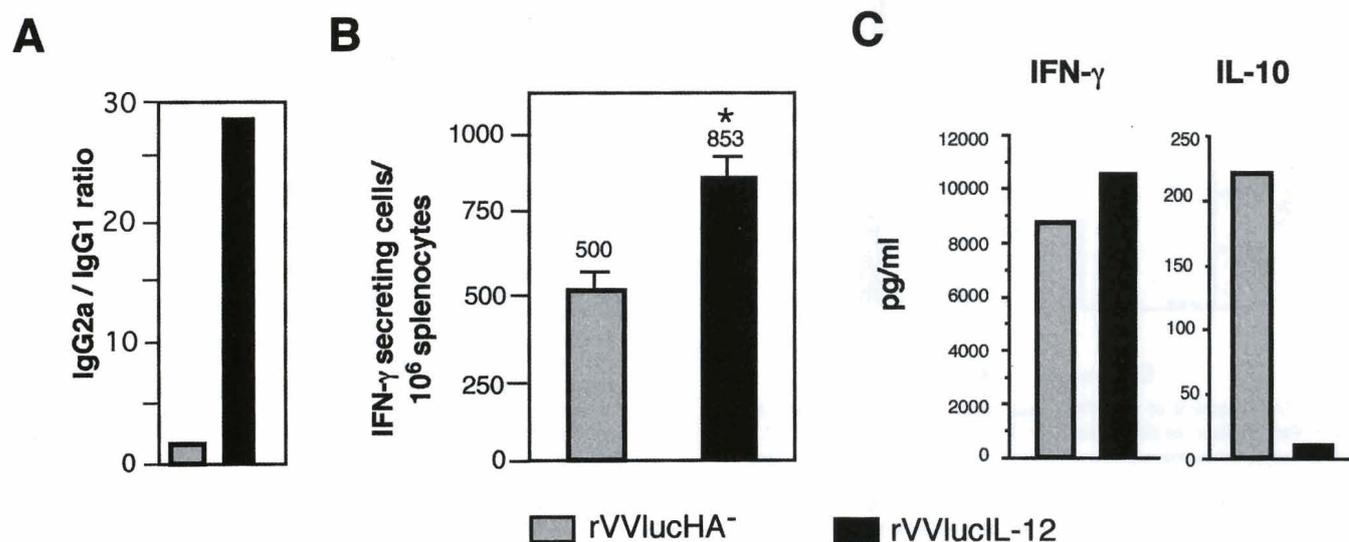

Fig. 3. Immune response induced against VV antigens 14 days after i.p. inoculation with 5 x10⁷ PFU/animal of rVVlucHA⁻ or rVVlucIL-12. **A.** Serum-specific anti-vaccinia IgG2a/IgG1 ratio. Anti-vaccinia IgG antibodies were determined by ELISA and titers calculated as the log2 dilution that gave an absorbance at 492 nm >0.1 O.D. **B.** Determination by the ELISPOT assay of the number of the IFN-γ–secreting CD8+ T cells specific for VV Ags. Spleen cells were employed as responder cells in the ELISPOT assay using P815 cells infected with VV as targets. Data are representative of at least two independent experiments each in triplicate. **C.** Pattern of cytokine secretion in supernatants of spleen cells after Ag restimulation. Splenocytes were cultured with in vitro with VV Ag (UV inactivated) at 1 μg/ml, and supernatants were collected and cytokines determined at 48 hs (IL-10) or 72 hs (IFN-γ) by ELISA. Three different experiments were conducted and the results of one representative experiment are shown.

rVVs. We found that by this approach it is easier to find the combination of viral doses that generate better specific CMI responses. In our particular system it appears that when a low dose (2×10^4 pfu/mice) of rVVIL-12 is combined with a moderate dose (1×10^7 pfu/mice) of rVVenv, nearly a three-fold increase in the number of specific anti-gp160 CD8+ T-cells can be raised in comparison with immunization with higher doses of either rVVenv or rVVenvIL-12. (Fig. 5).

The prime/boost aproach as a new vaccination strategy

Recombinant VV expressing particular antigens has proven to promote an expansion of specific CD8+ T-cells when administered as a boost after a prime given with either recombinant proteins, VLP, non-related viral vectors (influenza virus, Semliki Forest virus, Sindbis virus) or DNA vectors (Kent et al., 1998; Hanke et al., 1999). This prime/boost approach to enhance specific CD8+T cells was first documented in a malaria mouse model (Miyahira et al., 1998) and thereafter it has been shown to be effective in raising protection against malaria and SIV infection in murine (Scheneider et al., 1998) and primate non-human models (Robinson et al., 1999) respectively.

Although underlying immune phenomena of this property of VV is still elusive, it is currently accepted that during the prime/boost approach, a moderate CD8+ T-cell response is primarily induced against a few epitopes after priming, particularly with DNA immunizations, using a single antigen. A boost with an rVV expressing the same antigen allows expansion of the CD8+T cell population, and although the VV boost also primes naive VV-specific immune cells, the already primed antigen-specific memory T-cells can react faster and therefore expand rapidly (Fig. 6)

DNA and VV antigen delivery systems are suitable vaccine vectors due to their ability to elicit both humoral and cell-mediated immune responses; both vectors nonetheless have limitations in practical applications due
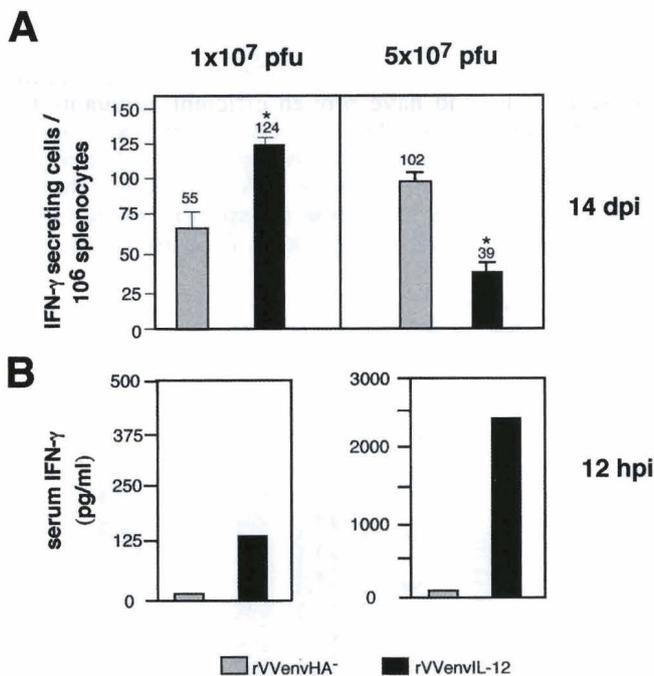

Fig. 4. Enhancement of specific cellular immune response against the HIV-1 Env protein is dependent on the viral dose of rVVenvIL-12. **A.** Data represents the number of IFN-γ- secreting CD8+T cells specific for the V3 loop epitope of the HIV-1 Env protein. Splenocytes from four mice immunized i.p. with the indicated dose of the rVVenvHA⁻ or rVVenvIL-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 aa of the V3 loop of the HIV IIIB Env protein) 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.
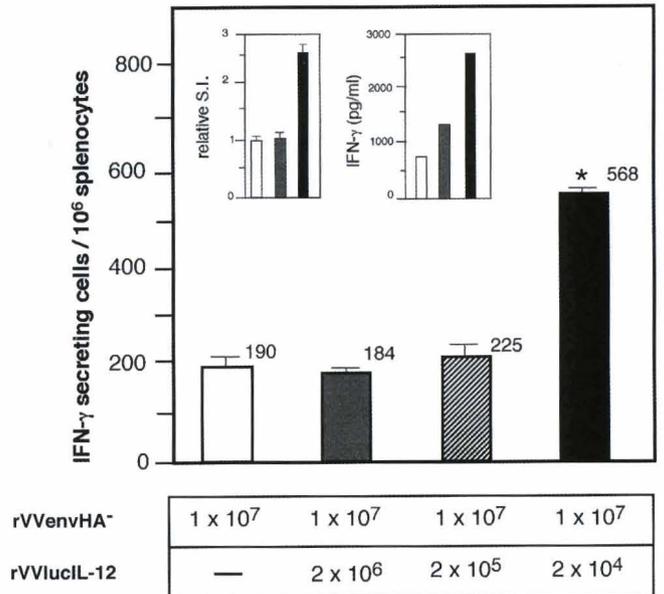

Fig. 5. A combined immunization scheme improved the IL-12-mediated enhancement of the cellular immune response against the HIV-1 Env Ag. Numbers of IFN-γ-secreting CD8+T cells specific for the V3 loop epitope of the HIV-1 Env protein are shown. Splenocytes from four mice immunized with the indicated doses of the rVVenv or rVVIL-12 viruses were pooled 14 days after immunization, and the number of specific IFN-γ-secreting CD8+T cells was determined by ELISPOT assay after coculture with P815 cells coated with the specific peptide (9 Env). Bars represent the mean±SD for triplicate cultures. In the insert the relative stimulation index and levels of IFN-γ in supernatants from cultures of splenocytes from the different immunized groups of mice is shown. Fourteen days after immunization splenocytes from the different indicated groups were tested for T cell proliferation by stimulation with either gp160 protein (1 μg/ml) or medium (negative control). To the left, the graph represents the relative specific T cell proliferative response against gp160, calculated as relative stimulation index [counts per minute in the combined immunization group/counts per minute in the control group (rVVenvHA⁻)]. On the right are the levels of IFN-γ in supernatants

to weak responses in single immunizations with DNA vaccines (Boyer et al., 1997; Donnelly et al, 1997) and strong immune responses against the viral antigens during VV infections, which diminish the efficacy of repeated booster immunizations with the same vector (Kunding et al., 1993). 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, as well as the use of attenuated VV strains, like MVA (Scheneider et al., 1999; Ramírez et al., 2000a,b). In fact, recent approaches (Hanke et al., 1998, 1999; Kent

Fig. 6. Schematic representation of the mechanism operating during the prime-boost vaccination strategy. A. Priming with an rVV vector induces VV-specific cells T1 and T2 (CD4 and CD8 T cells) (white cells) and recombinant antigen-specific T1 and T2 cells (grey cells). Boosting (second immunization) with the same viral vector boosts both Ag-specific and VV-specific responses at the same level. B. Priming with a DNA vector (plasmid) induces a moderate but focused immune response (both T1 and T2 type of cells) against the delivered antigen. During the boosting with an rVV this population of memory cells reacts rapidly expanding the Ag-specific population immune cells. C. If during the priming of the immune response a DNA vector codifying for a T1-type cytokine like IL-12 is applied, the immune response induced can be biased to a type 1 response. After the boosting with an rVV amplification of the primary Ag-specific T1 response occurs, resulting in a significant enhancement of the number of specific T1 cells obtained after the vaccination protocol.

et al., 1998; Sedegah et al., 1998; Caver et al., 1999; Robinson et al., 1999) in HIV-1, SIV and malaria models found that a DNA prime/VV boost regimen is a very efficient procedure to elicit an enhanced CMI response against the specific antigen and protection in murine and primate non-human models.

Improvements of the immunization regimen when combined with the delivery of cytokines

The use of immunomodulators during DNA immunization procedures as a way to further improve the CMI has been established by diffferent approaches. Thus, cytokines (Xiang and Ertl, 1995) and other molecules involved in co-stimulation signaling (Kim et al., 1998) expressed during DNA immunization act as adjuvants modulating the specific immune responses. For example, co-expression of IL-2 or GM-CSF with the antigen in a DNA vector enhances the specific humoral response (Chow et al., 1997; Sin et al., 1997) and DNA immunization strategies against HIV-1 antigens expressed together with GM-CSF and TNF-α improve specific CTL responses (Ahlers et al., 1997; Okada et al., 1997; Takashi et al., 1997). Indeed, other Th1 cytokines, such as IL-15 and IL-18 have also been co-administered with HIV-1 antigens from DNA vectors in mouse models, and have proven efficient adjuvants to modulate the specific CMI (Xin et al., 1999; Kremer et al., 1999).

IL-12 as an adjuvant in prime-booster vaccination strategies

As mentioned previously, IL-12 biological functions that promote the generation of specific CMI have been exploited successfully by both DNA and VV immunization schedules (Ahlers et al., 1997; Okada et al., 1997; Takashi et al., 1997; Kim et al., 1998; Gherardi et al., 1999). Our findings with rVVenvIL-12 prompted us to study the conditions of efficient use of IL-12 delivery in DNA prime/VV boost bimodal immunizations. The application of an immunomodulator like IL-12 during these vaccination approaches, has the advantage of directing the immune response to a Th1 type of response during the generation of the specific immune response that occurs during the priming (Fig. 6).

We adressed the factors that might influence the final outcome of the specific anti-HIV-1 gp160 immune response during this immunization scheme. We focused our investigation to determining both the appropriate moment and dose of administration of IL-12 during bimodal immunizations in comparison with single immunization schedules in which either rVV or DNA vectors were employed. Our findings indicate (Fig. 7A) that expression of IL-12 by DNA vaccine (penv and pIL-12) during the generation of the primary immune response, followed by rVVenv boost increases by 2-fold the number of specific anti-gp160 CD8+ T-cells raised when the cytokine is not present during bimodal
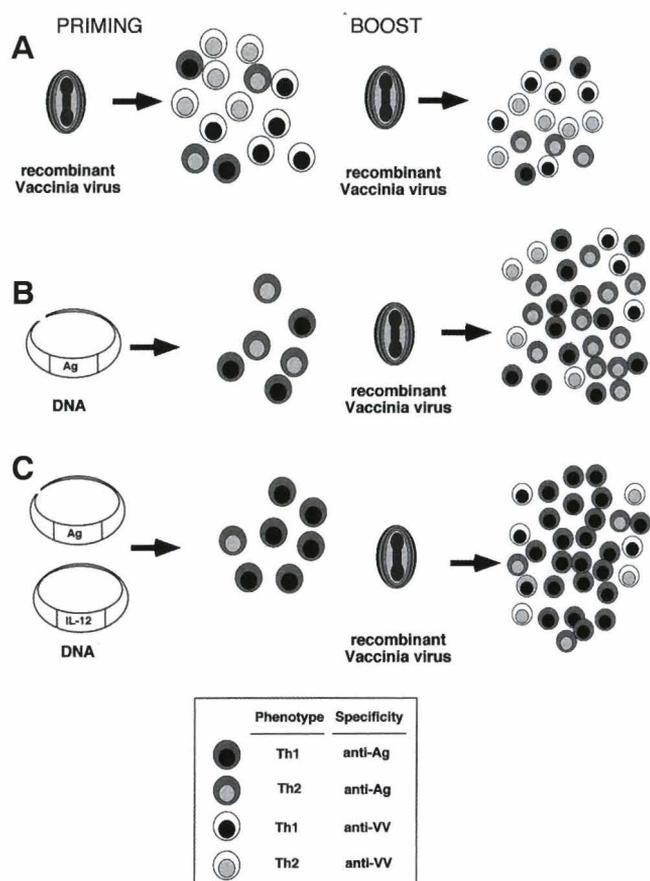
immunization (penv/rVVenv), or were generated only by rVV (rVVenv and rVVIL-12). Previous studies have pointed out the importance of both the route of administration of the vectors and the amount of the immunogen in the extent of the immune response elicited (Parish, 1996). By modifying both parameters we have found that the final response can be increased 3-fold if intravenous (i.v) instead of the intraperitoneal (i.p.) route is used for the rVV boost. This response can be further increased 2-fold if the DNA is applied intramuscularly (i.m.) during priming in comparison with the intradermal route at the optimal dose applied. (Fig. 7B).

Interestingly, we have also found that if the vector encoding the IL-12 is present only at boost or at both prime and boost, the IL-12-mediated enhancement of the specific CMI was not observed (see open bars in Fig. 8). These data suggest that it is not possible to redirect the type of Th response induced at priming by the

expression of the cytokine at boost, and that apparently IL-12 given in the booster promotes a suppressive effect on the cytokine-directed CMI enhancement during the priming of the immune response. Moreover, we observed that the IL-12-driven immunosuppression increased with the rVVIL-12 dose administered (Fig. 8).

As we previously mentioned, a transient IL-12 immunosuppression has been observed in different murine models (Kurzawa et al., 1998a,b; Lasarte et al., 1999), a fact that appears to be mediated by NO. To explain these observations, several interactions between the inducible NO synthetase (iNOS) and IL-12 have been reported, including the inhibition of macrophage IL-12 production by NO, the possible induction of the homodimeric p40 IL-12 antagonist by NO, and (iNOS)-dependent suppression of T-cell responses by IL-12 (Huang et al., 1998; Tarrant et al., 1999). The latter have been demonstrated in studies in mice, and it was shown that the events leading to immunosuppression by high
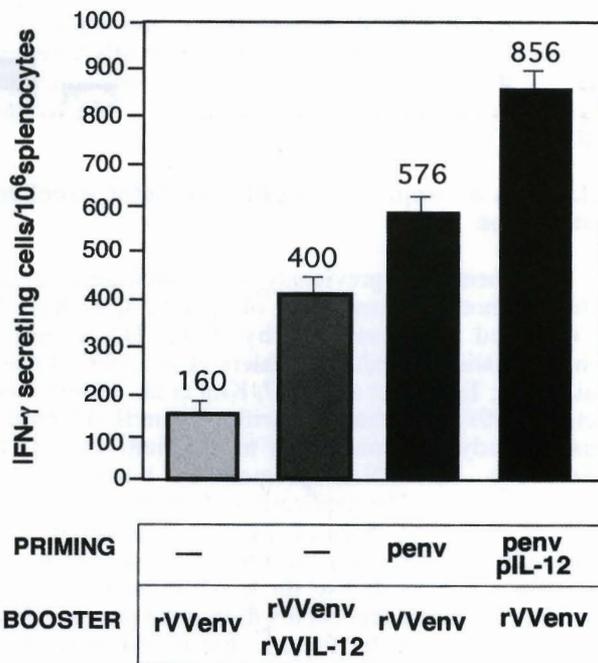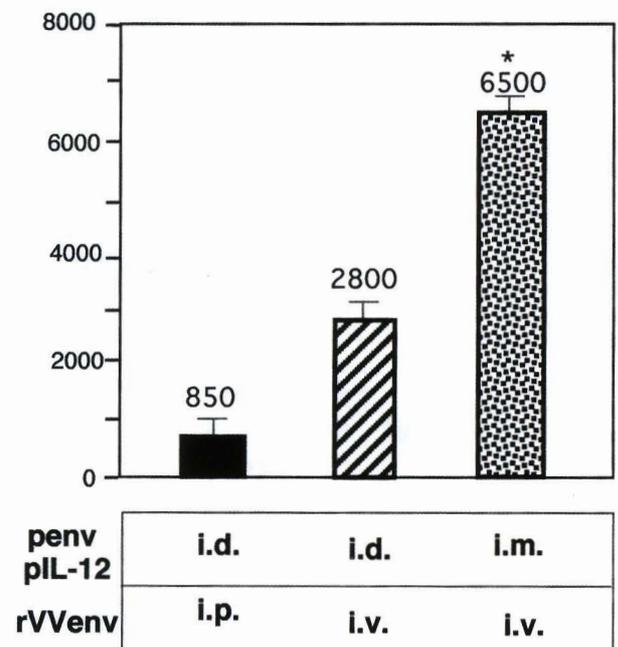
Immunization regimen

Fig. 7. A high CMI response against HIV-1 Env is obtained in a DNA/VV immunization regimen in the presence of IL-12 at priming. **A.** 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/plasmid per mouse. Fourteen days later, animals were boosted i.p. with 1x10^{7} PFU of rVVenv given alone or in combination with 2x10^{4} PFU of rVVIL-12. Two weeks later, spleen cells were used to evaluate the number of gp160-specific IFN-γ- secreting cells by the ELISPOT assay. For accurate comparisons, control animals receiving only one dose of rVV were injected when their counterparts were boosted and the immune response was analyzed at the same time post-inoculation. **B.** Effects of the route of the 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. or i.m. with 50 µg of the indicated plasmids. Fourteen days later, animals were boosted i.p. or i.v. with 1x10^{7} PFU of rVVenv. Two weeks after the second immunization, the number of HIV-1 gp160-specific IFN-γ-secreting cells was determined by ELISPOT. Mean values±SD from triplicate pooled splenocyte cultures are shown. *: significant differences (p<0.01), with respect to all other experimental groups.

IL-12 doses are initiated by the 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 (Bissinger et al., 1998; Duhé et al., 1998). These previous observations led us to propose that the immunosuppressive action of IL-12 when administered via rVV in the booster might be NO-mediated. To address this, we studied the effects on the CMI response against HIV-1 env when the reversible inhibitor of the iNOS, L-NAME, was administered during the booster. We observed that when the drug was inoculated simultaneously with rVVs, an enhancement in the number of anti-HIV-1 env CD8+ T-cells was detected, restoring the response observed in the control (Fig. 8, left). The nature of these effects was transient since at 40 days after the last immunization no main differences were observed with respect to control immunized mice (with no cytokine delivered at boost) (Fig. 8, right). These results concur with previous studies (Kurzawa et al., 1998) showing transient immunosuppression mediated by soluble rIL-12 in a murine model of vaccination against tumour cells.
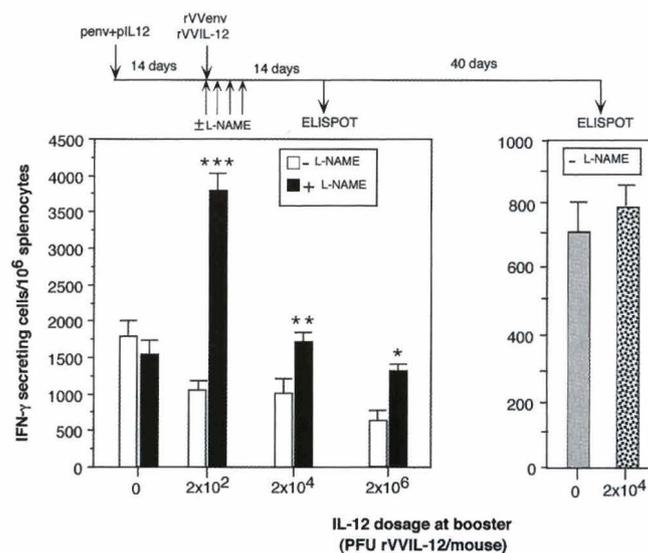
In conclusion, our findings (Gherardi et al., 2000) established that the bimodal DNA prime/VV boost immunization regimen has the capacity to enhance the specific anti-HIV-1 env response almost ten times when compared with a single immunization with a rVVenv and that if the IL-12 expression vector is properly administered, in both the dose and route and more importantly in a precise time window during the immunizing schedule, a 20-fold increase in the number of env-specific CD8+ T-cells can be raised when compared with a rVVenv inoculation (Fig. 9).

Future prospects

While prime/booster combination is gaining acceptance in the vaccine field and several phase I clinical trials for AIDS and malaria are underway, it is likely that as knowledge is obtained in the optimal use of
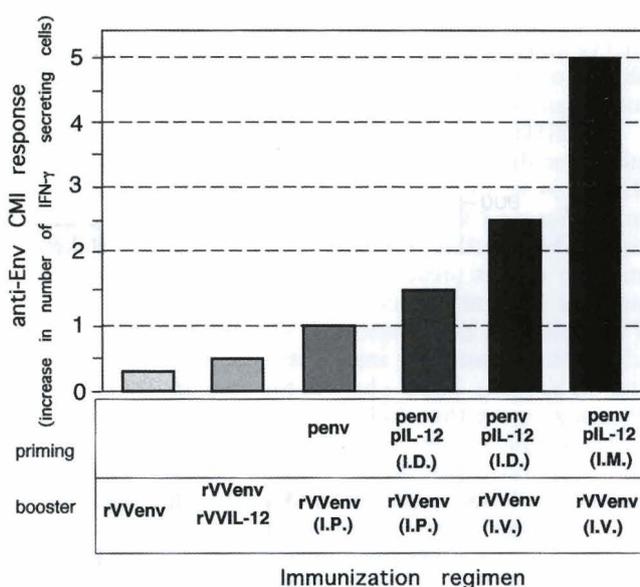

Fig. 8. The inhibitory efffects of IL-12 delivered at booster are reversed by the iNOS inhibitor L-NAME. A scheme summarizing the immunization procedure is shown. Three mice per group were primed i.d. with a mixture of 50 μg/plasmid per mouse of penv + pIL-12 and boosted i.v. 14 days later with 1x10⁷ PFU of rVVenv and the indicated amount of rVVIL-12. At this time and for the next three 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. (filled bars) in 200 μl of PBS or were left untreated (open bars). Fourteen (left panel) or 40 days (right panel) after boost, an ELISPOT assay was performed to determine the number of HIV-1 gp160-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. Bars indicate the mean±SD for triplicate cultures.

Fig. 9. Enhancement of specific HIV-1 env IFN- γ-secreting cells as a function of protocols of immunization. The diagram represents the fold increase in the number of anti-env-specific IFN-γ-secreting CD8+ T-cells generated after the indicated immunization schedules. Values were obtained comparing the data from at least three immunized mice in duplicate experiments. The number of anti-env specific IFN-γ-secreting CD8+ T-cells raised during the bimodal DNA/VV prime/boost is arbitrarily considered as 1 and used to calculate the other values. The figure emphazises the effect of the presence of IL-12 delivered in the priming as well the positive effect in the final outcome of the immune response measured by optimizing the route of both rVV and DNA vaccine inoculation (i.p., intraperitoneal; i.m., intramuscular; i.v., intravenous). The enhancement obtained in our studies by varying the dose of DNA injected and the optimal conditions for rVV delivery of IL-12 are ommited. Immunization procedure was as follows: a priming inoculation with 50 μg of each plasmid/mouse was followed fourteen days later by a boosting inoculation with 1x10⁷ PFU/mouse of rVVenv. Single immunized mice were inoculated with either 1x10⁷ PFU/mouse of rVVenv or with 1x10⁷ PFU of rVVenv and 2x10⁴ PFU of rVVIL-12 per mouse. The measurement of the number of specific IFN-γ-secreting cells was made by an ELISPOT assay two weeks after the last inoculation.

cytokines to enhance specific CMI to antigens with protective efficacies, the next generation of vaccines will include selected cytokines (IL-12, IL-18, GM-CSF, IFN-γ, IL-15) in the vaccination protocols. In view of this likelihood, it will be necessary to expand our current understanding of the co-delivery of cytokines as adjuvants.

This review highlights the notion that immuno-modulators, like IL-12, can be efficiently used during prime/boost immunization regimens based on rVV. For optimal benefit it is extremely important to take into consideration the main parameters that compromise the success of this approach. For many diseases, the number of CTLs during vaccination appears to be an index of the protective status. Our results clearly show that factors involved in such responses can improve quantitatively the specific CMI raised when IL-12 was used. Variables like dose of the cytokine applied, time of its administration and routes of inoculation play a critical role in the final outcome of the response. Thus, these parameters must be considered and assayed in different animal-models during the different developmental phases of the vaccination process applied to particular combinations of antigens and cytokines.

A difficulty in achieving protection against infectious diseases transmitted by the mucosal route, like HIV-1, is to induce an efficient specific response of antibodies and CTLs at the mucosal entry sites of the virus. Thus, further approaches should be undertaken to improve results presented here in terms of induction of mucosal immunity in combination with more attenuated VV strains. In this respect, IL-12 has been used as an adjuvant for mucosal immunity (Boyaka et al., 1999) and MVA has been shown to be a more efficient boosting agent than WR-VV (Ramirez et al., 2000b). Future prime/booster immunizations should explore the mucosal route for induction of CMI and protection against challenge in animal models. Another approach to be considered is the synergistic action of IL-12 and IL-18. In this case, it has been demonstrated that when both cytokines are combined, high levels of IFN-γ are induced (Okamura et al., 1995). IL-18 has also potentiated IL-12-driven Th1 development in BALB/c mice (Robinson et al., 1997). Moreover, a previous study in a mouse model of allergic asthma showed a synergistic action of these cytokines in preventing a Th2 development (Hofstra et al., 1998), and recent work has shown that rIL-18 can potentiate the IL-12 driving induction of an antigen-specific Th1 response against a recombinant antigen (Eberl et al., 2000). Delivering both cytokines from rVV might provide a synergistic action on the protective effects against the virus, which might be associated with a higher specific CTL response against viral antigens. It is tempting to speculate that similar results might be obtained against recombinant antigens expressed from VV.

In summary, here we have discussed some of the multiple applications of the cytokine IL-12, specially its use as an adjuvant. The DNA/VV prime/boost approach has been succesfully applied in different experimental models, proving to be an efficient strategy to enhance induction of specific CD8+T cells. Importantly, our experiments showed that this approach can be improved by applying cytokines that act as immunomodulators during the priming of the immune response. The prime-booster approach might be extended to other cytokines and antigens as part of a vaccination regimen against a wide range of pathogens and tumors. In all cases, critical parameters such as: a) dose of the cytokine, b)time of delivery, and c) route of administration, together with the antigen, should be characterised for each disease.

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