Live attenuated measles vaccine expressing HIV-1 Gag virus like particles covered with gp160ΔV1V2 is strongly immunogenic

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Although a live attenuated HIV vaccine is not currently considered for safety reasons, a strategy inducing both T cells and neutralizing antibodies to native assembled HIV-1 particles expressed by a replicating virus might mimic the advantageous characteristics of live attenuated vaccine. To this aim, we generated a live attenuated recombinant measles vaccine expressing HIV-1 Gag virus-like particles (VLPs) covered with gp160ΔV1V2 Env protein. The measles–HIV virus replicated efficiently in cell culture and induced the intense budding of HIV particles covered with Env. In mice sensitive to MV infection, this recombinant vaccine stimulated high levels of cellular and humoral immunity to both MV and HIV with neutralizing activity. The measles–HIV virus infected human professional antigen-presenting cells, such as dendritic cells and B cells, and induced efficient presentation of HIV-1 epitopes and subsequent activation of human HIV-1 Gag-specific T cell clones. This candidate vaccine will be next tested in non-human primates. As a pediatric vaccine, it might protect children and adolescents simultaneously from measles and HIV.

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Introduction

Despite 25 years of intensive research, the HIV/AIDS pandemic is still expanding over the world, and prevention efforts appear unable to slow the spread of HIV, particularly in Sub-Saharan Africa. The best hope for controlling this devastating disease is the development of an effective preventive vaccine affordable to the most exposed populations living in developing countries. There are currently more than 30 ongoing clinical trials with preventive HIV vaccine candidates. Despite a number of promising Phases I and II studies, the most advanced candidates have been unsuccessful, so far. The halting of Merck’s adenovirus serotype 5 (Ad5)-based HIV vaccine Phase Ib trial was a disappointment (Sekaly, 2008). The vaccine induced HIV-specific T cell responses that were not effective at preventing HIV infection or in reducing virus levels in infected individuals. This result challenges the «T cell based vaccine» strategy, at least using a single non-replicating Ad5 vector. Earlier, the monomeric recombinant gp120-based vaccine AIDSVax, designed to induce neutralizing antibodies, proved to neither elicit detectable antibody titers nor affect viral load in a Phase III trial (Flynn et al., 2005). However, human monoclonal antibodies have been described to effectively and broadly neutralize primary isolates (Mascola et al., 1997), protect macaques from infection (Baba et al., 2000; Mascola et al., 2000) and induce a relative control of viremia in patients (Trkola et al., 2005), thus demonstrating that antibodies contribute to HIV control. Moreover, although a live attenuated HIV vaccine is not currently under consideration for safety reasons, attenuated simian immunodeficiency virus (SIV) is able to protect macaques effectively (Daniel et al., 1992). It appears thus that a strategy inducing both T cells and neutralizing antibodies against native assembled HIV particles expressed in the context of a replicating virus should mimic the characteristics of live attenuated SIV without the risk of reversion observed with live persisting lentiviruses.

Because the rate of HIV infection in adolescents remains high in Africa, adolescents are key targets of prevention efforts, including vaccination (McClure et al., 2004). A pediatric HIV vaccine to immunize children and adolescents in poor countries should induce long-lasting responses after a few administrations, be produced at low cost and scaled up to millions of doses. Live attenuated vaccines are particularly appropriate for mass vaccination in these countries as they are inexpensive to manufacture and induce a strong immunity and long-term memory after a single injection. To evaluate such a pediatric vaccine approach, we previously developed a vector derived from the live attenuated Schwartz strain of measles virus (MV) (Combredet et al., 2003). MV vaccine is a live attenuated negatively-stranded RNA virus proven to be one of the safest and most effective...
human vaccines. Produced on a large scale in many countries and distributed at low cost through the Extended Program on Immunization (EPI) of WHO, this vaccine induces life-long immunity to measles after one or two injections. We previously showed that MV vector stably expressed different HIV proteins and induced strong and long-term specific neutralizing antibodies and cellular immune responses to HIV, even in presence of preexisting immunity to MV (Lorin et al., 2005; Lorin et al., 2004). We also demonstrated that this vector induced protective neutralizing antibodies against flaviviruses (Brandler et al., 2007; Desprès et al., 2005). A clinical development was initiated in collaboration with an industrial vaccine manufacturer to evaluate the safety and immunogenicity of MV–HIV recombinant vector in humans with preexisting immunity to measles. This Phase I trial allows the building of GMP and regulatory logistics for such a vaccine and will evaluate for the first time a replicating vector as an HIV vaccine.

In the present work, we developed a next generation of replicating MV–HIV recombinant vectors that allow the production of fully assembled HIV-1 virus like particles (VLP) containing trimeric envelope glycoproteins. Since the first use of HbS–Ag particles as an effective vaccine against hepatitis B (Blumberg, Millman, and London, 1985), subviral particles have further demonstrated their human vaccine potential with the recent marketing of the papillomavirus VLP vaccine (Ault, 2006). HIV-1 Gag–Env VLPs (HIV-VLP) have a number of advantages for eliciting humoral and cellular immune responses, particularly as they present the envelope glycoproteins in an authentic trimeric form (Hammonds et al., 2007; Ludwig and Wagner, 2007). Moreover, HIV-1 VLPs are captured by antigen presenting cells and efficiently presented to T cells by MHC molecules (Buseyne et al., 2001). Expressed by a live replicating vector, HIV-VLPs should bud from cells infected in vivo, thus increasing their immunogenicity through Toll-like receptor (TLR) induction (Blander and Medzhitov, 2006; Schulz et al., 2005). We produced a series of recombinant MV vectors expressing either HIV-1 Gag only or both Gag and Env, and allowing or not the assembly of HIV-VLPs. We analyzed their growth capacity and the level of HIV-VLP production. We then compared the cellular immune responses and the neutralizing antibodies induced in immunized-transgenic mice susceptible to MV infection. Lastly, we evaluated their capacity to infect human antigen presenting cells (monocyte-derived dendritic cells and B cells) and the resulting presentation of HIV-1 antigens to both CD4 and CD8 HIV-specific T cells.

**Fig. 1.** Recombinant MV–HIV vectors expressing HIV-1 antigens. (A) Schematic representation of HIV-1 Gag and Env constructs and of recombinant MV Schw vector. The G→A mutation in position 1 of p17 protein, the V1V2 deletion in position 130 of gp160 and the peptide signal (PS) are indicated. The HIV-1 Gag and Env sequences indicated were cloned into the ATU-1 and ATU-2 of MV Schw vector using BsiWI/BssHII sites. The MV genes are indicated as follows: nucleoprotein (N), phosphoprotein and V/C accessory proteins (PVC), matrix (M) fusion (F), hemagglutinin (H) and polymerase (L). T7 RNA polymerase promoter (T7), T7 RNA polymerase terminator (T7t), hepatitis delta virus ribozyme (h), hammerhead ribozyme (hh). (B) Immunofluorescence detection of HIV-1 proteins in Vero cells infected for 24 h with MV-p55Gag/Env virus (picture a is Gag labeling and pictures b–c correspond to Env labeling of permeabilized and non-permeabilized cells, respectively). (C) Western blot analysis of HIV-1 proteins expression in cell lysates and supernatants of Vero cells infected by the different MV–HIV viruses (cell lysates are 20 times more concentrated than supernatants).
Results

Generation of rMVs expressing HIV-1 Gag and Env proteins

To construct recombinant MV able to express HIV-1 VLPs, we inserted the sequences corresponding to the HIV-1 gag and env genes into two distinct additional transcription units (ATU) of a single pTM-MVScw plasmid, which contains an infectious MV C DNA corresponding to the antigenome of the Schwarz MV vaccine strain (Combredet et al., 2003) (Fig. 1A). Taking advantage of the gradient of gene expression generated by MV replication (viral mRNAs are produced in decreasing amounts from the 3′ to the 5′ end of MV genomic RNA (Plumet, Duprex, and Gerlier, 2005)), we inserted the gag gene in the ATU-1 (high expression level) and the env gene in the ATU-2 (lower expression level). This choice was based on the observation that mature HIV-1 particles have a low number of Env spikes on their surface (Zhu et al., 2006) and that a high level of Gag expression is necessary for a high amount of VLP production. Full-length (p55gag) and truncated (Δp17p24gag) HIV-1 gag synthetic sequences (consensus clade B) were generated. As a negative control unable to assemble Gag particles, we introduced a Gly→Ala substitution at the first amino-acid of Δp17p24gag (p17p24gagΔ) to abrogate myristylation, which is essential for HIV-particle formation. The different Gag sequences were introduced in ATU-1 of pTM-MVScw vector. A synthetic sequence coding for the anchored form of HIV-1 Env gp160 deleted of the two hypervariable regions V1 and V2 (EnvΔV1V2 consensus clade B) was generated and inserted into ATU-2 of recombinant MV-Gag vectors. We used a delta V1V2 envelope because previous studies showed that deletion of hypervariable loops increased the induction of broader neutralizing antibodies as compared to wild-type form (Kang et al., 2005; Lorin et al., 2005; Srivastava et al., 2003). In our previous work, a triple ΔV1V2V3 mutant induced the broadest and most potent neutralizing antibodies (Lorin et al., 2005). However, other studies indicated that highly conserved subregions within the V3 region are able to elicit strong neutralizing antibody responses (Yang et al., 2004) and that the anti-V3 antibodies can neutralize diverse strains of HIV (Zolla-Pazner et al., 2008). We decided thus to keep the V3 region in the Env construct inserted into MV-Gag vectors. Any other Env mutant might easily be inserted in this vector platform in the future according to the HIV clade targeted by the vaccine composition. These double vectors enable the production of MV–HIV recombinant viruses expressing Gag particles covered with EnvΔV1V2 glycoproteins (Fig. 1A). Six recombinant viruses (MV-p55Gag, MV-Δp17p24Gag, MV-p17p24GagΔ, MV-p55Gag/Env, MV-p17p24Gag/Env, MV-Δp17p24GagΔ/Env) were rescued by transfecting the corresponding plasmids into helper cells and propagation on Vero cells, as previously described (Combredet et al., 2003).

rMV–HIV vectors replicate efficiently

We analyzed the replication of recombinant MV–HIV vectors on Vero cells by single-step growth curves using an MOI of 1 (Fig. 2). The growth of recombinant viruses was similar to that of control MV and their final titers were comparable. Despite the presence of two transgenes in the vector, MV–HIVGag–Env viruses had only a slight delay in their growth and achieved the same titer than MV–HIVGag–Envatically recombinant viruses. MV-p55Gag and MV-p55Gag/Env viruses grew to the same titer than parental MV vector. The efficient growing of MV-p55Gag/Env that produces high amount of HIV-1 VLPs (see below) demonstrates that HIV particles budding does not prevent efficient MV replication. Surprisingly, MV-p17p24Gag and MV-Δp17p24Gag/Env viruses grew slightly less efficiently than others, suggesting that the accumulation of p17p24Gag precursors at the inner side of the cell membrane might disturb the budding of MV particles.

Cells infected with rMV–HIV vectors release HIV-1 Gag–Env VLPs

The expression of HIV-Gag protein is sufficient to drive the process of particle assembly and to generate the production of HIV virus-like particles (VLPs) without the presence of other additional HIV proteins. To analyze the effective release of VLPs from cells infected with the
different MV–HIV-Gag and MV–HIVGag/Env recombinant viruses, we collected cellular supernatants 36 h after infection and concentrated them 100× by ultracentrifugation through a 20% sucrose cushion. As shown in Fig. 1C, Gag proteins were present in the concentrated supernatants of MV-p55Gag, MV-p17p24Gag, MV-p55Gag/Env, and MV-p17p24Gag/Env, but not in the concentrated supernatants of MV-p17p24GagΔ and MV-p17p24GagΔ/Env, which are unable to assemble Gag particles. The Env glycoprotein was not detectable in any concentrated supernatant (data not shown), likely because of gp120 shedding during ultracentrifugation. We also quantified by ELISA the production of p24 antigen in supernatants and lysates of cells infected by MV-p55Gag/Env and MV-p17p24GagΔ/Env viruses (Table 1). 40 h post-infection, the total amount of p24 antigen produced by both viruses was very high and similar (400–500 ng per 10⁶ cells), confirming that mutation of the myristylation site does not affect the total Gag-protein production (Chen et al., 2005). 46% of the total amount of p24 produced in MV-p55Gag/Env infected cells was found in supernatant, compared to only 6% for the particle-incompetent. At earlier times of infection (18 and 24 h), only the supernatant from MV-p55Gag/Env infected cells contained p24 antigen, indicating active secretion. Moreover, as shown in Fig. 1C, the minimal amount of p24 antigen found in supernatant of MV-p17p24GagΔ/Env infected cells did not correspond to secreted VLPs.

The release of assembled HIV-VLPs was then analyzed by equilibrium density centrifugation of concentrated supernatants on 20–60% sucrose gradients and detection of p24 protein by western blot analysis of the different fractions (Fig. 3). The p24 antigen corresponding to HIV-VLPs was found floating in the middle of the gradient, while MV nucleoparticles (N protein) were found at the bottom. This profile corresponds to the expected densities of 1.16 for HIV particles and 1.30 for MV.

To confirm that cells infected by rMV–HIV vectors effectively released HIV-1 Gag–Env VLPs, we performed transmission electron microscopy observation and specific immunogold labeling 30 h after infection (Fig. 4). Vero cells infected with MV-p55Gag and MV-p55Gag/Env were found to intensively produce immature HIV-like particles. These particles were so numerous that they arranged in semicrystalline arrays around cells (Fig. 4A). Their size and morphology were appropriate for HIV-VLPs, with a diameter of 100–130 nm and a characteristic high density Gag layer located underneath the viral membrane (Fig. 4B). Numerous particles were found budding from infected cells (Figs. 4C, D). The enumeration of particles present around cells in 10 large-scale fields allowed us to evaluate at 1500 the number of HIV-VLPs secreted per cell in 70 nm sections. We thus estimated that approximately 50,000 particles were released by a single Vero cell. Considering this number, a T175 cell culture flask containing 20 millions MV-p55Gag or MV-p55Gag/Env infected cells is likely to produce 10¹² HIV-VLPs. The efficient replication of these particle-competent vectors (Fig. 2) demonstrates that the massive budding of HIV particles from infected cells does not prevent MV replication.

When MV-p55Gag/Env infected cells were immunostained with an anti-gp120 antibody before electron microscopy observation, numerous gold particles (10 nm diameter) were found surrounding the Gag particles (Figs. 4E, F). This antibody labels the HIV Env spikes anchored on the surface of particles. The average density of gold nanoparticles was 13±6 per VLP (range 4–28, 30 VLPs were counted). Although gold nanoparticles may also label non-trimeric Env proteins anchored on VLPs, the low density that we observed is close to the trimeric Env spike density previously observed by cryo-electron microscopy tomography on wild-type HIV-1 virions (Zhu et al., 2006).

On the other hand, Vero MV-p17p24Gag and MV-p17p24Gag/Env infected cells did not release HIV-like particles, but were found to produce a high amount of very large empty vesicles together with MV particles (Fig. 4G). Some of these Gag vesicles incorporated HIV Env gp160, as detected by immunogold staining with anti-gp120 antibody (Fig. 4H, white arrow). We did not observe Env gold labeling surrounding MV particles (Fig. 4H, stars), suggesting that HIV Env glycoprotein is not able to incorporate into MV particles membrane. This observation confirms previous studies demonstrating that for incorporating a heterologous glycoprotein on the surface of MV particles, the transmembrane and intracytoplasmic domains need to be swapped with those of MV glycoproteins (Spielhofer et al., 1998). Regarding safety, this observation indicates that recombinant MV–HIV vectors will keep the cell tropism of MV, which is broader than HIVs. Lastly, the empty MV vector induced the production of standard MV particles with an average size of 150–350 nm (Fig. 4f). In these particles, the genomic ribonucleoparticle was clearly noticeable (particle with a star in Fig. 4f).

**Immunization of mice with rMV–HIV vectors**

To select the most immunogenic candidate before evaluating its efficacy in a non-human primate model of HIV infection, we compared the ability of the different rMV–HIV vectors to raise specific anti-MV and anti-HIV antibody and cellular responses in genetically modified mice susceptible to MV infection (Mirkic et al., 1998). These mice express CD46, the human receptor for vaccine MV strains, and lack the INF-α/β receptor (IFNAR). They are commonly used as a preclinical model to evaluate the immunogenicity of recombinant MV before non-human primates experiments (Brandler et al., 2007; Combredet et al., 2003; Després et al., 2005; Liniger et al., 2008; Lorin et al., 2005; Lorin et al., 2004; Singh, Cattaneo, and Biller, 1999; Zuniga et al., 2007). Six-week-old CD46-IFNAR mice (6 mice per group) received 10⁷ TCID₅₀ of the different rMV–HIV vectors by intraperitoneal (ip) injection at days 0 and 28. As a control, CD46-IFNAR mice were immunized similarly with empty MV vector.

![Fig. 3. HIV-1 virus like particles (VLP) release from rMV–HIV infected Vero cells. Western blot analysis of sucrose gradient fractions obtained after ultracentrifugation of infected Vero cell supernatants.](image-url)
rMV–HIV vectors induce high levels of antibodies in mice

We looked for antibodies raised specifically to MV and to HIV Gag and Env in mice sera before immunization, 1 month after the first injection, and also 3 weeks and 11 weeks after the second injection (Fig. 5). Antibodies to MV were induced at similar levels in all immunized mice. The first injection induced MV antibodies with limiting dilution titers of about $10^4$ and the boost increased these titers 10 times. A persisting antibody memory response to MV was observed 15 weeks after the first immunization, with titers stabilized between $10^4$ and $10^5$, as previously described (Lorin et al., 2004) (Fig. 5A). Anti-Gag antibodies were induced at high level in all mice (titers of $10^5$–$10^6$ after boost, remaining above $10^5$ at 15 weeks post prime) (Fig. 5B). Anti-Env antibodies were induced in all mice that received rMV–HIV expressing HIV Env (Fig. 5C). A single injection of both MV-p55Gag/Env and MV-p17p24Gag/Env vectors was sufficient to raise titers up to $10^4$, and the second injection increased only marginally these titers that remained at the same level over time up to 15 weeks post priming. On the other hand, the particle-incompetent MV-p17p24GagΔ/Env vector induced a much lower titer of anti-Env antibodies after the first injection ($10^2$), and boosting was necessary to increase titer to a level that remained below $10^4$ at 15 weeks after priming. This difference could be due to a different level of Env protein expressed by the vectors. However, although MV-p55Gag/Env vector expressed a higher level of Env, as observed in Fig. 1C, both MV-p17p24Gag/Env and MV-p17p24GagΔ/Env vectors expressed a similar level (Fig. 1C). Moreover, the 3 Env-expressing vectors had a similar immunogenicity to MV and to Gag (Figs. 5A and B), and only the particle-incompetent vector had a lower immunogenicity to Env. This result demonstrates that expressing Env onto secreted particles allows achieving a higher titer of anti-Env antibodies after a single injection.

rMV–HIV expressing HIV-1 Gag–Env VLPs induces neutralizing antibodies

As a preliminary test of functionality, we evaluated the capacity of sera from immunized mice to neutralize a heterologous clade B HIV-1 primary isolate (Bx08 virus, NIH) in a single-cycle virus infectivity assay on P455 indicator cells (Barin et al., 2004; Lorin et al., 2004). P455 cells express the CD4, CXCR4 and CCR5 HIV-1 receptors and have been stably transfected with an HIV LTR-LacZ construct. Therefore, they are susceptible to HIV-1 isolates and express β-galactosidase upon infection. The sero-neutralization assay was validated using a combination of anti-HIV immunoglobulin (HIVIG, 2.5 mg/ml) and monoclonal antibodies (2F5 and 2G12, 25 μg/ml each) that were previously shown to synergistically neutralize primary HIV isolates (Mascola et al., 1997). We assessed the neutralizing activity induced in immunized mice on 1/30 dilutions of sera collected 15 weeks after priming. To take into account the non-specific HIV-1 inhibition by mouse serum and the possible effects of Gag antibodies, the 100% level of infectivity was determined in the presence of the same dilution of a mix of sera from mice immunized with vectors expressing Gag only. The neutralizing activity was expressed as the percentage of inhibition of infection observed in the presence of the sample sera compared to
Neutralizing activity, while the highest neutralizing activity (around that obtained with control sera (Fig. 5D). The HIVIG/2F5/2G12 combination neutralized more than 90% of infectivity in this assay, as previously observed (Lorin et al., 2004), and no inhibition of infection was observed with sera from mice immunized with empty MV vector, showing that anti-MV immunity did not neutralize HIV non-specifically. The sera from mice immunized with rMV–HIVGag/Env vectors were able to neutralize the infection with various efficiencies. The particle-incompetent vectors induced only a weak neutralizing activity, while the highest neutralizing activity (around 70%) was observed with sera from mice immunized with MV-p55Gag/Env. Similar neutralizing titers were observed against Bx08 virus in our previous studies with MV vectors expressing HIV Env only (Lorin et al., 2005; Lorin et al., 2004). However, in these previous studies the Env sequence was inserted in ATU-1 of MV vector, which induces a 10 times higher level of expression than the ATU-2 used for Env expression in the present study (Plumet, Duprex, and Gerlier, 2005). This result indicates that, in the context of a replicating measles virus, HIV-1 Env is more immunogenic when expressed onto assembled Gag VLPs, even despite a lower expression level. To express simultaneously Gag and Env from the same vector and to favor the production of high amounts of Gag VLPs, we choose in this study to insert Gag in ATU-1 and Env in ATU-2. In a next construct the reverse situation will be evaluated. This promising result needs to be confirmed with other Env constructs in a non-human primate model, which is more appropriate for neutralizing antibodies.

rMV–HIV vectors induce cellular immune responses to HIV

We then looked for cellular immune responses raised specifically against MV and HIV Gag and Env epitopes both at 1 and 15 weeks after priming. As a quick and simple test, we used enzyme-linked immunospot (ELISPOT) assay to evaluate the capacity of splenocytes from immunized mice to secrete IFN-γ in response to a specific stimulation with either UV-inactivated MVSchw virus or HIV-1 Gag and gp160 peptide pools (Fig. 6). The level of MV-specific T cell response was high after priming (1300–1800 spots/10⁶ cells) and remained still high after 15 weeks (900–1600 spots/10⁶ cells) showing that a single immunization induced a saturated response. On the contrary, the ELISPOT response to HIV-1 Gag and Env increased with time. Again, the MV-p55Gag/Env vector expressing HIV-1 Gag–Env VLPs was the most immunogenic and induced a strong T cell response both to Gag (1200 spots/10⁶ cells) and to Env (400 spots/10⁶ cells). The global level of ELISPOTs raised by this vector against HIV-1 (1800 spots/10⁶ cells) was similar to the level raised against MV. These responses were detected 15 weeks after priming, indicating that memory was established, as for antibodies. The presence of Env gp160 expressed onto Gag p55 VLPs increased significantly the ELISPOT response to Gag, suggesting a cross presentation mechanism due to a better capture of HIV-1 Gag/Env particles than HIV-1 Gag only particles by antigen presenting cells.

The strong capacity to raise T cell responses that persist on long term in lymphoid organs is a hallmark of live attenuated vaccines. MV is particularly efficient at generating live long memory CD4 and CD8 T cells that help to maintain neutralizing antibodies and to prevent from reinfection (Naniche et al., 2004; Ovsyannikova et al., 2003). A number of studies have shown that a strong CD8 T cell response to HIV Gag reduces the chronic as well as the primary viral load in macaques and even protects from a heterologous SIV challenge (Reynolds et al., 2008). As well, the long-term control of SIV replication in vaccinated macaques has been correlated to the preservation of central memory CD4 T cell (Kawada et al., 2007). The induction of strong and long-term CD4 and CD8 T cells to Gag is thus a major component of an HIV vaccine. HIV VLPs have long been shown to stimulate specifically the immune response to HIV. As well, the long-term control of SIV replication in vaccinated macaques has been correlated to the preservation of central memory CD4 T cell (Kawada et al., 2007). The induction of strong and long-term CD4 and CD8 T cells to Gag is thus a major component of an HIV vaccine.
permissive to MV infection (Grosjean et al., 1997) leading to the up-regulation of co-stimulatory molecules (Klagge, ter Meulen, and Schneider-Schaulies, 2000; Schnorr et al., 1997; Servet-Delprat et al., 2000). Upon virus infection, immature DCs undergo maturation and transport the virus to regional lymph nodes, where viral antigens are presented to lymphocytes to initiate immune response (Banchereau and Steinman, 1998). To evaluate the ability of rMV–HIV vectors to induce class-I or class-II presentation of HIV-1 epitopes in a human system, we performed a previously described in vitro antigen presentation assay (Moris et al., 2004; Moris et al., 2006), using HIV-specific CD4 and CD8 human T cell clones and HLA-matched human APCs (Fig. 7). Because of limited HLA-matching possibilities, we used DCs for presentation to CD8 cells and B-cells for presentation to CD4 cells. Monocyte-derived DCs and EBV-transformed human B-cells (B-EBV) were infected with rMV–HIV vectors at different MOIs. The production of p24 antigen in infected cells was quantified by flow cytometry 24 h post-infection. DCs were highly susceptible to rMV–HIV vectors, even at very low MOIs, and the particle-incompetent vectors MV-p17p24GagΔA and MV-p17p24GagΔA/Env produced twice as much p24 in DCs than p55Gag-expressing vectors (Fig. 7A1). Higher MOIs were required for efficient infection of B cells and MV-p17p24GagΔA/Env produced the highest level of p24 (Fig. 7B1). After 24 h of infection, infected DCs and B-EBV were co-cultivated for additional 18 h with autologous HLA-matched CD4+ or CD8+ HIV-1–specific T cell clones: infected DCs were co-cultivated with the HLA-A2-restricted CD8+ T cell clone EM40 specific of HIV-1 Gag–p17 to p24 gagΔ, and infected B-EBV were co-cultivated with the HLA-DRβ1*01-restricted CD4+ T cell clone F12 specific of HIV-1 Gag–p24 to p29 gag2 epitope (Moris et al., 2004; Moris et al., 2006). Then, the number of IFN-γ secreting CD4+ and CD8+ T cells was quantified by ELISPOT.

Fig. 6. T cell responses induced in mice immunized with pMV–HIV viruses. IFN-γ ELISPOT analysis of MV and HIV-1 specific T cell responses stimulated in mice immunized with the different vectors as indicated. Splenocytes were collected 1 and 15 weeks after priming (grey and black bars, respectively) and restimulated with either UV-inactivated MV or HIV-1 specific peptides for 40 h. Results are expressed as number of spots per million cells. Results are presented as the mean values obtained from 6 mice performed in triplicate each ± standard deviation.

We first observed that viral replication was required for efficient specific T cell activation, as UV-inactivated viruses did not induce any ELISPOT (Figs. 7A2 and B2). Mock-infected cells and cells infected with empty MVSChw vector (not shown) did not stimulate Gag-specific T cells. When DCs and B-EBV were exposed to increasing MOI of rMV–HIV viruses, the activation of Gag-specific T cells was dose dependent (Figs. 7A2 and B2), except for MV-p55Gag virus in DCs and MV-p17p24GagΔA/Env virus in B-EBV. In these cases, the maximum activation was already achieved with the lowest MOI, likely reflecting a better presentation of Gag epitopes by infected cells. To estimate the efficiency of epitope presentation relatively to the number of infected presenting cells, we divided the number of IFN-γ positive spots by the percentage of Gag-positive DC or B cells. At the lowest MOI tested (0.01 for DCs and 0.2 for B cells), the result shows that vectors expressing p55Gag VLPs were about 5 times more efficient for Gag-specific CD8+ T cells activation by DCs than particle-incompetent vectors (Fig. 7A3). At higher MOIs, this difference diminished but the tendency remained the same (not shown). In B cells, vectors expressing the Env were twice as efficient for Gag-specific CD4+ T cell activation at the lowest MOI (Fig. 7B3), but this observation was not confirmed at higher MOIs. In conclusion, these results demonstrate that replicating rMV–HIV vectors infect human professional antigen-presenting cells (DCs and B-cells) and induce efficient presentation of HIV-1 epitopes to HLA-matched T cells and subsequent activation of cytokine secretion.

Discussion

Live attenuated vaccines are the most appropriate for global mass immunization. They induce long-term protection because they replicate in vivo after administration. They are easy to multiply because they replicate in vitro in cell culture. Although a live attenuated HIV vaccine is not currently considered for safety reasons, a strategy inducing both T cells and neutralizing antibodies to native assembled HIV particles expressed by a replicating virus might mimic the protective characteristics of live attenuated SIV (Koff et al., 2006). In this work, we generated a live attenuated measles vaccine expressing HIV-1 Gag VLPs covered with gp160ΔV1V2 and demonstrated its capacity to induce at long-term a high level of antibody with neutralizing activity, as well as a strong T cell immune response to both Gag and Env epitopes. This strategy provides a recombinant vaccine that might protect children and adolescents simultaneously from measles and HIV and be affordable to populations through the Expanded Program on Immunization of WHO in the regions affected both by HIV and measles infections.

Live attenuated virus vaccines, such as those against mumps, measles, polio and rubella viruses induce both cell-mediated and humoral immunity. Protection is afforded by the stimulation of polyclonal neutralizing antibodies, and memory is maintained through an efficient cellular immunity. For the chronic and highly variable HIV-1, the ability to induce broadly neutralizing antibodies and long-term protective memory through vaccination has been a discouraging task for several years. The hallmark of HIV-1 is its capacity to establish chronic infection and to generate tremendous viral genetic diversity, mostly in the only proteins present on the virus surface, gp120/gp41 that evolved many mechanisms to evade host neutralizing responses (Burton et al., 2004; Wei et al., 2003).
Considering that there are currently 33 million infected individuals harboring a large set of HIV-1 variants that continue to spread and evolve in the human population, providing protection through vaccination appears an enormous challenge. However, current models predict that if vaccination could substantially lower the level of acute viraemia or reduce the chronic "set point" in an individual, then the transmission rate in the population would be reduced (Davenport et al., 2004). Therefore, vaccine strategies able to stimulate long-term polyclonal neutralizing antibodies to the most conserved parts of the HIV-1 Env, together with strong cellular immunity composed of both CD4⁺ and CD8⁺ T cells directed also to the most conserved viral epitopes, need to be further worked out.

With this aim, we derived a vector from the most safe and efficacious pediatric live attenuated measles vaccine and developed its use as a bivalent recombinant vaccine to immunize simultaneously against measles and other infectious diseases (Combredet et al., 2003). This vector demonstrated a strong capacity to stimulate both cellular and humoral neutralizing immunity against a number of antigens in several animal models (Brandler et al., 2007; Brandler and Tangy, 2008; Desprès et al., 2005; Lorin et al., 2005; Lorin et al., 2004; Fig. 7. Infection of human DCs and B cells by recombinant MV–HIV viruses, and MHC-I and MHC-II-restricted antigen presentation. DCs (A) and B cells (B) were infected at the indicated MOI. 24 h post-infection, a part of the cells were fixed and stained for HIV-p24Gag expression [1]. The remaining cells were co-cultivated with anti-Gag specific HLA-matching CD8⁺ (A) or CD4⁺ (B) T cells for 24 h before IFNγ ELISPOT analysis of T cell responses. The data are shown as the number of IFNγ positive cells for 2500 T cells (mean value from triplicate wells ± standard deviation) [2] and as the ratio of IFNγ positive spots related to the percentage of p24Gag positive DCs or B cells determined at the lowest MOI (3). The negative control of infection (NI) was mock-infected DCs or B cells. Results are presented as the mean values of three independent experiments performed in triplicate ± standard deviation.

Fig. 7. Infection of human DCs and B cells by recombinant MV–HIV viruses, and MHC-I and MHC-II-restricted antigen presentation. DCs (A) and B cells (B) were infected at the indicated MOI. 24 h post-infection, a part of the cells were fixed and stained for HIV-p24Gag expression [1]. The remaining cells were co-cultivated with anti-Gag specific HLA-matching CD8⁺ (A) or CD4⁺ (B) T cells for 24 h before IFNγ ELISPOT analysis of T cell responses. The data are shown as the number of IFNγ positive cells for 2500 T cells (mean value from triplicate wells ± standard deviation) [2] and as the ratio of IFNγ positive spots related to the percentage of p24Gag positive DCs or B cells determined at the lowest MOI (3). The negative control of infection (NI) was mock-infected DCs or B cells. Results are presented as the mean values of three independent experiments performed in triplicate ± standard deviation.
The strong immunogenicity of MV is likely due to its capacity to infect productively human professional antigen-presenting cells, such as macrophages, dendritic cells and B cells. Using MV as a vaccination vector presents a number of advantages: the mandatory global vaccination against measles is still needed for a foreseeable future, vaccine strains are genetically stable, MV does not recombine or integrate genetic material, vaccine does not persist or diffuse, MV-specific CD8 T cells and IgG are detected in vaccinees up to 25–34 years after a single MV vaccination (Griffin, 2001; Naniche et al., 2004; Ovsyannikova et al., 2003) and boosting increases this memory (Ovsyannikova et al., 2003). The remarkable stability of transgenes expression by single-stranded negative RNA viruses results from the absence of geometric constraints on the size of the genome in these pleomorphic viruses with helicoidal nucleocapsids (Schnell et al., 1996 2197) and allows the use of MV vector as a platform to express large amounts of foreign genes.

In this work, we generated replicating MV–HIV recombinant vaccine expressing HIV-1 VLPs covered with envelope glycoproteins, and evaluated its preclinical immunogenicity. VLPs have been explored as HIV-1 vaccine candidates for several years (Doan et al., 2005; Ludwig and Wagner, 2007; Young et al., 2006) and have demonstrated their immunogenicity (Deml et al., 2005). However, most of the evaluated strategies are based on repeated administrations of high doses of VLPs produced in vitro in heterologous systems, as insect cells (Buonaguro et al., 2001; Wagner et al., 1992; Yao et al., 2000) or yeast (Sakuragi et al., 2002). Other delivery strategies used non-replicative canarypox vectors (Fang, Kuli-Zade, and Spearman, 1999; Hammongs et al., 2007) or naked DNA (McBurney et al., 2006; Young, Smith, and Ross, 2004), for which, again, a high number of administrations are necessary. Live replicating MV vector allows massive in vivo production and diffusion through the organism of VLPs at the time of vaccination.

We generated rMV–HIV viruses expressing HIV-1 Gag and Env proteins able to assemble a high amount of VLPs with gp160 spike density very close to that of wild-type HIV-1 virions (Zhu et al., 2006), thanks to the gradient of gene expression generated by MV replication (Plumet, Duprex, and Gerlier, 2005). The versatility of this vector allows generating further constructs in which the gag and env genes can be flipped over to increase the spike density on VLPs, although it would probably lower the yield of VLP production. We produced six rMV–HIV vectors expressing different forms, secreted or not, of p17p24 or p55 Gag antigens with or without gp160AV1V2. The vectors replicated efficiently in cell culture, demonstrating that MV and HIV particles can bud simultaneously without interference from infected cells, and they produced very high amounts of Gag antigen. HIV-1 VLPs were secreted efficiently from cells infected with MV-p55Gag/Env virus. In electron microscopy a huge number of HIV-1 particles were found budding from infected cells or surrounding them. Immunogold labeling demonstrated the presence of Env spikes at the surface of VLPs with an average density of 13±6 per particle. The heterologous HIV Env glycoprotein did not incorporate into MV particles. Regarding the safety of such live recombinant vaccine, this observation indicates that MV cell tropism will be conserved, although MV readily infects all HIV target cells.

In mice sensitive to MV infection, these vectors stimulated a high level of antibodies that persisted up to 15 weeks after immunization. Anti-HIV Gag antibodies were raised at similar titers than anti-MV antibodies. Anti-Env antibodies were also induced at high titer after a single injection with MV-p55Gag/Env virus, demonstrating the better immunogenicity of HIV-1 Env glycoprotein inserted into Gag particles. Although at moderate titer, these antibodies neutralized a heterologous clade B HIV-1 primary isolate. On the contrary, sera from mice immunized with vectors expressing Gag VLPs without Env slightly enhanced HIV infection in vitro. It is tempting to speculate that a vaccine strategy based on the expression of Gag VLPs without Env might induce enhancement of infection. MV is known to stimulate a persisting mucosal immunity that protects from a disease transmitted through mucosa. If such neutralizing antibodies are present on the mucosal sites of HIV-1 infection, they will help to control the first steps of infection (Miller and Abel, 2005; Miller et al., 2005). A humoral response composed of IgG and IgA has been evidenced in exposed non-infected individuals (Buchacz et al., 2001; Devito et al., 2000), and the presence of specific IgA has recently been associated to HIV-1 resistance in Kenyan sex workers (Hirbod et al., 2008).

The rMV–HIV viruses also stimulated a strong cellular immunity that persisted up to 15 weeks. The magnitude of the response against HIV-1 Gag and Env proteins was identical to that against MV, as enumerated by ELISPOT. Again, the MV-p55Gag/Env vector was the most immunogenic, reflecting the high level of Gag/Env VLPs expressed. Lastly, we demonstrated that rMV–HIV vectors infect human professional antigen-presenting cells (DCs and B-cells) and induce efficient presentation of HIV-1 epitopes to HLA-matched T cells. The subsequent activation of cytokine secretion by human HIV-1 Gag-specific T cell clones demonstrates the immunogenic potential of these recombinant vectors in humans. The efficient release and re-uptake of HIV-1 VLPs by DCs infected with MV-p55Gag/Env vector likely induced cross-presentation of captured VLPs, thus increasing the standard class-I presentation of HIV-1 antigens produced in infected DCs and resulting in an efficient CD8+ activation. Replication of the vectors was required to induce activation. MV-infected DCs induce cell apoptosis in neighboring cells (Fugier-Vivier et al., 1997). This activity is mediated by the secretion of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is induced by MV replication (Vidalain et al., 2000). Cross-presentation by dendritic cells to CD8+ T cells of very small amounts of HIV proteins from apoptotic infected CD4+ T lymphocytes is very efficient (Maranon et al., 2004). This suggests a mechanism by which DCs infected by rMV–HIV increase the presentation of HIV antigen to CD8+ T cells.

In conclusion, we demonstrated that a recombinant measles vaccine expressing a high level of fully assembled HIV-1 VLPs covered with gp160AV1V2 can be produced at high titers. This MV-p55Gag/Env vaccine candidate was strongly immunogenic in a mouse preclinical model of infection and deserves to be tested in non-human primates. If better Env immunogens capable of inducing broadly neutralizing antibodies to a large variety of HIV-1 quasi-species are obtained eventually, they will be easily included into this vector, thus providing a human HIV vaccine platform with numerous potential advantages.

Materials and methods

Cell culture

Vero cells (African green monkey kidney cells) were maintained in DMEM-Glutamax (Gibco-BRL) supplemented with 5% heat-inactivated fetal calf serum (FCS, Invitrogen, Frederick, MD). Stable helper 293-T7-NE cells were previously generated by transduction with recombinant lentiviral vectors and cloning (Tangi, Charnau, and Jacob, 2006). They were used for viral rescue and grown in DMEM 10% FCS, P4CS indicator cells (Helix, CD4+, CCR5+, CXCR4+, 50–100 pg/mL HIVLTR–LacZ) (Marechal et al., 1998) were cultured in DMEM 10% FCS supplemented with 0.5 mg/ml G418 (Geniticin; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem). Clinical-grade DCs were prepared as described elsewhere (Goxe et al., 1998; Moris et al., 2006) and maintained in AIMV medium containing 500 U/ml GM-CSF (Genitaur; Gibco) and 50 μg/ml hygromycin (Calbiochem).
Plasmid construction

The plasmid pTM-MVSchw, which contains an infectious MV cDNA corresponding to the anti-genome of the Schwarz MV vaccine strain, has been described elsewhere (Combedret et al., 2003). Consensus codon-optimized HIV-1 clade B sequences coding for the full-length p55Gag or truncated p17p24Gag polyproteins, and for the EnvAV1V2 envelope glycoprotein, were synthesized (GeneArt) and amplified from plasmid with primers containing unique BsiWI and BssHII sites for subsequent cloning in MV vector (Fig. 1). The p17p24Gag sequence was modified to create the p17p24GagΔ protein, incompetent for the formation of Gag-particles. Briefly, directed mutagenesis was performed on the p17p24gag sequence to introduce a glycine-to-alanine mutation in the N-terminal myristylation site of the protein. All the constructs cloned in pCR2.1-TOPO plasmid (Invitrogen) were sequenced. They respect the “rule of six”, which stipulates that the number of nucleotides of MV genome must be a multiple of six (Calain and Roux, 1993). The sequences corresponding to p55Gag, p17p24Gag and p17p24GagΔ were introduced in ATU-1 of pTM-MVSchw vector after BsiWI/BssHII digestion. The resulting plasmids were designated pTM-MV-p55Gag, pTM-MV-p17p24Gag and pTM-MV-p17p24GagΔ. The sequence corresponding to EnvAV1V2 was then inserted in ATU-2 of each MV-Gag vector. The resulting vectors were designated pTM-MV-p55Gag/Env, pTM-MV-p17p24Gag/Env and pTM-MV-p17p24GagΔ/Env. All constructs were verified by complete sequencing.

Rescue of recombinant MV-Gag and MV-Gag/Env viruses

Recombinant MV–HIV viruses were recovered from the pTM-MV-Gag and pTM-MV-Gag/Env plasmids using a helper-cell-based rescue system (Tangy, Charneau, and Jacob, 2006) that was similar to that developed by Radecke et al. (1995) and modified by Parks et al. (1999). The titers of recombinant MV were determined by an endpoint dilution assay on Vero cells. Briefly, Vero cells were seeded into 96-well plates (7500 cells/well) and infected by serial 1:10 dilutions of virus sample in DMEM–5% FCS. After incubation for 7 days, cells were stained with crystal violet and the TCID50 values were determined on Vero cells as described above.

Analysis of VLP production by sucrose gradient purification

Vero cells (3 T–150 flasks) were infected with either empty MV vector or MV–HIV recombinant viruses at MOI 1. Supernatants collected after 36 h of infection were clarified by centrifugation at 3000 rpm for 30 min, layered on 20% sucrose cushion in PBS and centrifuged at 41,000 rpm for 2 h in a SW41 rotor. Pellets were resuspended in PBS 1% BSA and applied on the top of a 20% to 60% sucrose gradient, followed by centrifugation in a SW41 rotor for 16 h at 28,000 rpm. Fractions (1 ml) were collected and the protein content of each fraction was analyzed by SDS-PAGE followed by immunoblotting, as described above.

Electron microscopy and gold immunostaining

Vero cells were seeded on cover glass strips and infected at a MOI of 1 with the different MV–HIV recombinant viruses. Cells were fixed 30 h post-infection with 2% PFA in PBS for 5 min and with 4% PFA for 2 h at room temperature. Non-specific sites were saturated for 10 min with 1% bovine serum albumin and cells were probed for 40 min with anti-HIVgp120 antibody (2G12; NIH-AIDS Research and Reference Reagent Program) in PBS–1% BSA. After washing, cells were incubated for 20 min with 10-nanometer colloidal gold particles coated with protein A (University Medical Center, Utrecht). Stained cells were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4 °C, postfixed with 1% osmic acid, dehydrated in ethanol, and embedded in Epon. Ultrathin sections of 70–80 nm were cut with a diamond knife, stained with uranyl acetate and Reynolds lead citrate, and observed at 80 kV accelerating voltage using JEOL JEM 1010 Electron Microscope and an Eloïse Mega View III camera. Control, uninfected Vero cells were included in this analysis.

Animal immunizations

CD46–IFNAR mice susceptible to MV infection were produced as previously described (Combedret et al., 2003). These mice express the human CD46 gene with human-like tissue specificity, within addition, a target mutation inactivating the interferon receptor type 1 (Mrkic et al., 1998). Mice were housed under specific pathogen-free conditions at the Pasteur Institute animal facility and all experiments were approved and conducted in accordance with the guidelines of the Office of Laboratory Animal Care at Pasteur Institute. Six-week-old CD46–IFNAR mice were inoculated intraperitoneally (ip) with 1 x 10^6 TCID50 of MV–HIV recombinant viruses and boosted 1 month later with the same dose of recombinant viruses. Control mice were immunized with the same dose of empty MVSchw vector. For early cellular immune responses analysis, mice were euthanized at 7 days post-immunization and spleens were collected. For antibody determination, blood samples were collected via the periorbital route 1 month after the first inoculation, then at 3 and 11 weeks after boosting. To analyze long-term cellular responses, mice were euthanized 11 weeks after boosting and spleens were collected.
Characterization of humoral responses

Sera collected from immunized mice were heat-inactivated for 30 min at 56 °C and the presence of anti-MV, anti-HIVGag and anti-HIVenv antibodies was assessed using enzyme-linked immunosorbent assay (ELISA). A commercial ELISA kit (Trinity Biotech) was used for anti-MV antibody detection. For anti-HIV antibodies, 96-well plates were coated with either HIV-1 p24 antigen (Jena Bioscience) or HIVgal gp120 recombinant protein (NIH AIDS Research and Reference Reagent Program) diluted in carbonate buffer pH 9.6 at a concentration of 1 μg/ml. The plates were incubated overnight at +4 °C and washed with PBS–0.05% Tween 20 (PT). Unspecific interactions were blocked with 1% BSA in PBS for 1 h at 37 °C. After washing, plates were incubated with serial dilutions of mouse sera for 90 min at room temperature. Horse-radish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch laboratories) was used as secondary antibody and plates were revealed with TMB substrate (Pierce). The endpoint titers for each individual serum were calculated as the reciprocal of the last dilution giving twice the absorbance (450 nm) of sera from MV inoculated mice that served as negative control.

HIV-1 neutralization assay

Seroneutralization was tested against the clade B HIV-1 primary isolate Bx08 (NIH-AIDS Research and Reference Reagent Program). HIV-1 neutralization assays were performed by using the P4CS indicator cell line (Marechal et al., 1998) as previously described (Barin et al., 2004; Lorin et al., 2004). Briefly, P4CS cells were seeded into 96-well plates (20,000 cells per well) and incubated for 24 h at 37 °C in DMEM–10% FCS supplemented with hygromycin plus G418. The medium was replaced with 30 μl of DMEM–10% FCS–DEAE Dextran (10 μg/ml) and cells were incubated for 30 min at 37 °C. HIV-1 virus Bx08 (0.5 ng of p24 per well) was incubated with 1:30 diluted sera in 30 μl of FCS-free culture medium at 37 °C for 45 min and the virus–serum mixtures were added to the cells in triplicate. 100 μl of DMEM–10% FCS were added to each well 2 h later. After 48 h of incubation, the β-galactosidase activity was measured using a chemiluminescent reporter gene assay (Roche). The mean neutralization for each serum tested was reported to the value recorded in wells containing control serum from non-immunized (NI) mice at the same dilution that served as reference for 100% infection. A mix of neutralizing monoclonal antibodies (Mab) 2F5 and 2G12 (25 μg/ml each; NIH-AIDS Research and Reference Reagent Program) plus anti-HIV IgG (2.5 mg/ml; NIH-AIDS Research and Reference Reagent Program) was used as a positive control of neutralization.

Cellular immune responses

The capacity of splenocytes from immunized mice to secrete IFN-γ upon specific stimulation was tested by enzyme-linked immunospot (ELISPOT) assays. Multiscreen-HA 96-well plates were coated overnight at +4 °C with 5 μg/ml of anti-mouse IFN-γ (BD Pharamingen) in carbonate buffer 50 mM pH 9.6. After washing, unspecific sites were blocked for 2 h at 37 °C with RPMI–10% FCS. The medium was then replaced by 100 μl of cell suspension (1 × 10^6 splenocytes/well in triplicate) and 100 μl of stimulating agent in complete RPMI, and plates were incubated for 40 h at 37 °C. Cells were stimulated with either SEB (Staphylococcal enterotoxin B, Sigma 2 μg/ml) as positive control, complete RPMI (RPMI–5% FCS supplemented with non-essential amino-acids 1%, sodium pyruvate 1% and β-mercaptoethanol) as negative control, a mixture (2 μg/ml each) of HIV-1 Gag (15-mer) or HIV-1 gp160 (20-mer) peptides (NIH-AIDS Research and Reference Reagent Program), or UV-inactivated MVSchw virus (1 TCID50 per cell). After incubation and washing, biotinylated anti-mouse IFN-γ antibody (BD Pharamingen) was added (50 μl; 2.5 μg/ml in PBS–0.05% Tween 20), and plates were incubated for 90 min at room temperature. After extensive washes, streptavidin–alkaline phosphatase conjugate (Roche) was added (50 μl; 1 U/ml in PBS–0.05% Tween 20) and plates were incubated 1 h at room temperature. Spots were developed with BCIP/NBT (Promega) and counted in an ELISpot Reader (Bio-Sys).

Activation of human HIV-specific T cells

Human dendritic cells and B-EBV cells (Moris et al., 2004; Moris et al., 2006) were used to analyze the presentation of HIV-1 Gag epitopes to HIV-1 Gag-specific CD8+ T cells and HIV-1 Gag-specific CD4+ T cells, respectively. DCs and B-EBV were exposed to recombinant MV–HIV viruses at different MOI (0.5, 0.05, 0.01 for DCs and 5, 1, 0.2 for B-EBV) in 0.5 ml RPMI for 2 h at 37 °C, 5% CO2. Following infection cells were washed, resuspended at 1 × 10^6/ml and incubated in 24-well plates (2 ml per well) at 37 °C, 5% CO2. After 24 h, a sample of each condition was removed for ELISPOT assay, and the remainder returned to incubation for additional 24 h. Gag expression in infected DCs or B cells was measured on permeabilized cells using anti-Gaggp24 mAb (KC57, FITC– or RDI-coupled, Coulter) and analyzed by flow cytometry (FACS Calibur and FlowJo software). For IFN-γ ELISPOT assay, 96-well ELISPOT plates (MAHA S45, Millipore) were coated overnight at 4 °C with mouse anti-human IFN-γ antibody (Mabtech) at 10 μg/ml in 50 μl PBS. The plates were washed with PBS and blocked with PBS–5% FCS for 1 h at room temperature. Infected DCs or B-EBV cells were distributed in triplicate at 1 × 10^5 cells per well. HIV-Gag specific CD8+ (clone EM40) or CD4+ (clone F12) T cells were added at a ratio of 1:40 to DCs and B-EBV, respectively. As a positive control, DCs and B-EBV cells previously incubated with HIV-1 peptides (SL9 and gag2) were also incubated with T cell clones at the same ratio. The plates were incubated for 20 h at 37 °C, washed with PBS–0.05% Tween 20 and incubated for 2 h with a biotin-conjugated anti-human IFN-γ antibody (Mabtech). After washing, a streptavidin-horseradish peroxidase complex (Roche) was added for 2 h incubation at room temperature. The peroxidase activity was revealed with BCIP/NBT (Promega France) and counted (ELISpot Reader; Bio-Sys).

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References


