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Nonreplicating vectors are being considered in HIV-1 vaccine design. However, nonreplicating viruses are typically weak immunogens, leading to efforts to target the vaccine to mature dendritic cells (DCs). We have studied a single-cycle form of HIV-1, prepared by pseudotyping envelope-defective HIV-1 plasmids with the envelope from vesicular stomatitis virus (VSV) G protein (VSV-G), to which most humans lack preexisting immunity. The nonreplicating, VSV/HIV-1 efficiently infected the immature stage of DC development, in this case represented by monocytes cultured with GM-CSF and IL-4. A majority of the cells reverse transcribed the HIV-1 RNA, and a minority expressed gag protein. The infected populations were further matured with CD40 ligand, leading to strong stimulation of autologous T cells from HIV-1-infected individuals, but not controls. Enriched CD8+ T cells from 12/12 donors released IFN-γ (50–300 enzyme-linked immunospots/200,000 T cells) and proliferated. Macrophages were much less efficient in expanding HIV-1-responsive T cells, and bulk mononuclear cells responded weakly to VSV/HIV-1. CD4+ T cells from at least half of the donors showed strong responses to VSV/HIV-1-infected DCs. Presentation to CD8+ T cells, but not to CD4+, was primarily through an endogenous pathway, because the responses were markedly reduced if envelope-defective virus particles or reverse transcriptase inhibitors were added. Therefore, nonreplicating vaccines can be targeted to immature DCs, which upon further maturation induce combined and robust CD4+ and CD8+ immunity. The Journal of Immunology, 2000, 165: 6620–6626.

The efficacy of current HIV-1 vaccines needs to be improved. Strong combined CD4+ and CD8+ T cell immunity is considered desirable. Compelling evidence has shown that virus-specific CD8+ T cells resist SIV in macaques (1–3). Activated CD8+ T cells not only kill virus-infected cells (4–6) but also may reduce HIV-1 spread by releasing antiviral chemokines (7). For many viruses, specific CD4+ T cells are additionally valuable, e.g., for maintaining CD8+ memory T cells (reviewed in Ref. 8).

Dendritic cells (DCs) are efficient APCs for initiating immunity (see refs. 9 and 10 for review). Immature DCs take up Ag in the periphery, as illustrated by the capacity of skin DCs to capture and express DNA vaccines (11–13). The DCs then migrate via afferent lymphatics to lymphoid organs, where processed Ags are presented to T cells. Following maturation, DCs prime Ag-specific CD4+ and CD8+ T cells, including T cell memory (14). Given the many specializations that DCs exhibit to initiate T cell immunity, such as the expression of abundant MHC-peptide complexes and T cell costimulators (15, 16), it would seem important for HIV-1 vaccine design to target this physiologic pathway.

It is well documented that envelope-defective HIV-1, when pseudotyped with another viral envelope encoded by a distinct plasmid in the virus-producer cells, undergo only one cycle of replication. These pseudotyped vectors are easy to prepare and provide a safe way to deliver all HIV-1 genes other than env into cells. Page (17) and Landau (18) were the first to describe replication-defective HIV-1 vectors containing deletions in the HIV-1 envelope gene. Several subsequent studies used vesicular stomatitis virus (VSV) G protein (VSV-G)-pseudotyped vectors to transduce dividing and nondividing cells (19–23). Recently, we used VSV-pseudotyped HIV-1 to infect mature DCs and found that a small fraction of the cells undergo a single full round of viral infection and protein synthesis (24). Here, we infected monocyte-derived DCs with VSV-pseudotyped HIV-1 to assess Ag presentation to CD4+ and CD8+ T cells from infected individuals. To mimic the delivery of vaccines in vivo, we delivered the nonreplicating vector to an immature stage of DC development. When the infected DCs were matured to maximize T cell stimulatory activity, the DCs presented HIV-1 Ags as efficiently as pox vectors to CD4+ and CD8+ T cells from HIV-1-infected individuals. The mature DCs greatly expanded CD4+ and CD8+ IFN-γ-secreting cells and induced proliferative responses in enriched CD8+ T cells. Therefore, VSV-pseudotyped virus is a readily available, safe approach for transducing DCs and obtaining strong presentation of HIV-1 to CD4+ and CD8+ T cells.

Materials and Methods

Patients

Patients from two different institutions were all on HAART therapy that was initiated after their CD4+ T cell counts began to fall. Blood from individual patients was studied at least twice, and comparable results were obtained.
Cell preparation

DCs were generated from the monocytes of normal donors and HIV-1-infected individuals. CD14+ monocytes were positively selected from PBMCs using CD14 magnetic beads, as suggested by the vendor (Miltenyi Biotec, Auburn, CA). A total of 1 x 10^7 CD14+ cells were plated in six-well trays in 3 ml consisting of RPMI 1640 supplemented with 5% human hematopoietic growth medium-1 (100 IU/mL, Leukine; Immunix, Seattle, WA) and recombinant human IL-4 (1000 U/mL, Genzyme, Cambridge, MA). The cells were fed on day 2 and 4 with 1 ml fresh medium containing the same concentration of cytokines. At day 6, most of the nonadherent cells were immature DCs, showing little or no expression of the CD83, p55, DC-LAMP, and CD25 markers expressed by mature, more stimulatory DCs. To promote maturation, we replated the nonadherent DCs at 10^6 cells/well for 4 days in 3 ml medium, supplemented with cytokines as above and either LPS (Sigma, St. Louis, MO; 20 ng/ml) or irradiated (3000 rad) CD40 ligand (CD40L)-transfected fibroblasts (a kind gift of Dr. J. Banchereau, Baylor Institute for Immunology Research, Dallas, TX) at a ratio of 1 fibroblast to 7 DCs. In some experiments, soluble CD40L was used. The CD14+ population was the source of T cells and was kept frozen until use. In many experiments, CD14+ cells were separated by nylon wool columns to obtain bulk T cells or by positive and negative selection with CD4 and CD8 magnetic beads (Miltenyi Biotec).

Viruses and infection of cells

We were kindly provided with two envelope plasmids to pseudotype HIV-1: a VSV-G expression construct pHCMV-G from J. Burns (University of Rochester, Troy, NY) and a murine leukemia virus envelope plasmid from D. Landau (Aaron Diamond AIDS Research Center, New York, NY). An enve-defective HIV-1, expressing the green fluorescent protein, was provided by D. Gabuzda (Dana-Farber, Boston, MA). VSV wild-type virus was a gift of J. K. Rose (Yale University, New Haven, CT). Recombinant vaccinia and avipox were obtained from Virogenetics Corporation (Troy, NY). Vaccinia vectors were NYVAC parental vector and vP989; NYVAC-HIV-1 gag/pol (IIIB). Avipox vectors were ALVAC parental and vCP300:ALVAC-HIV-1 gp 160; gag/pro; net/pol CTL. Complementation of enve-defective HIV-1 plasmid with the VSV-G plasmid yielded pseudotyped virus that could only undergo a single round of infection. To produce virus, 3 x 10^6 293T cells were cotransfected with 20 µg/ml of the enve-defective and 5 µg/ml VSV-G plasmids using the calcium phosphate method. Virus particles lacking envelope were produced by transfection of 293T cells with enve-defective HIV-1 plasmid only. Virus stocks were prepared by collecting the culture supernatants after 72 h, filtering through 0.45-µm Millipore filters (Bedford, MA), and storing in aliquots at 80°C. Virus stocks were spun down through a 3-mL 25% sucrose cushion at 25,000 rpm for 2 h in SW41 rotor. Viral titers (infectious units) were measured by infection of 1 x 10^5 293T cells with 20 µg/ml of virus and plaques counted after 48 h of infection.

Detection of HIV-1 p24 protein

For FACS assays, infected DCs were fixed with 4% paraformaldehyde in PBS for 30 min on ice. Cells were permeabilized for 15 min in 1% saponin on ice, stained with anti-p24 clone 183, AIDS Research and Reference Reagent Program) followed by FITC-labeled anti-mouse Ig (Tago Scientific, Burlingame, CA), and double stained with anti-CD68 PE (PharMingen, San Diego, CA). For Western blotting, cells were lysed in RIPA buffer and equivalent cell numbers were loaded in 9% acrylamide gels. After transfer, the nitrocellulose was probed with anti-VSV-G (1.5 µg/ml; Boehringer Mannheim, Indianapolis, IN) or anti-p24 Abs and revealed with enhanced chemiluminescence (Amerham).

Results

Infection of DCs with VSV-pseudotyped HIV-1

We have previously shown that replication-incompetent VSV/HIV-1 undergoes a single cycle of viral protein synthesis in a small fraction of mature DCs (24). To model the immature stage of DC that persists in peripheral tissues, we examined cells that were generated by culturing blood monocytes with GM-CSF and IL-4 (27–29). These cells are competent in Ag capture and support infection with wild-type M-tropic HIV-1 (30), but lack the full costimulatory activities of mature DCs (28, 29).

We used viruses complemented with VSV-G after finding that these were more infectious than those complemented with murine leukemia virus envelope (data not shown). Following infection with VSV/HIV-1, a consistent but small percentage (<10%) of the immature DCs expressed a green fluorescence protein reporter in our HIV-1 plasmid (not shown). HIV-1 infection was monitored by the production of reverse transcripts containing HIV-1 long-terminal repeat and gag sequences; this production was sensitive to pretreatment with AZT or 3TC (Fig. 1A). A vigorous infection with VSV/HIV-1 was evident by the very high number of full-length transcripts, >10^6 for 2 x 10^4 cells (Fig. 1A). When analyzed by Western blot, the infected DCs expressed HIV-1 gag (Fig. 1B). As expected, VSV-G envelope expression was minimal because the pseudotyped virus should lack an envelope gene, whereas DCs infected with wild-type VSV had readily detectable G protein (Fig. 1C). When the infection of DCs was studied with replicating, wild-type VSV, most of the cells were infected as detected by immunofluorescence with anti-VSV-G Ab (not shown), again showing that most DCs were competent for entry via the VSV envelope. These results show that VSV/HIV-1 efficiently infects immature DCs. To monitor the production of viral protein and to assess the effects of the VSV/HIV-1 on DC maturation, we cultured noninfected and infected immature DCs with the CD40L maturation stimulus for 4 days and then used the FACS to measure p24 expression and various DC markers (CD83, CD25, CD86). As illustrated by the high expression of the CD86 costimulator, the presence of VSV/HIV-1 infection did not alter DC maturation (compare the top and bottom rows of Fig. 1D). However, only 2–10% of the most mature DCs expressed p24. Although a higher proportion of DCs might be infected and not be detected with the current methods, it does appear that there is a block to the virus life cycle in DCs, a block that occurs after reverse transcription. We conclude that immature DCs can capture VSV-pseudotyped HIV-1 and then undergo maturation as well as expression of HIV-1 genes.
Mature DCs are more effective in HIV-1 Ag presentation than immature DCs

We first tested the presentation of HIV-1 Ags by DCs that were infected with nonreplicating virus and either kept in an immature state or allowed to mature. To study Ag presentation, DCs were prepared from several HIV-1-infected individuals being followed in two medical centers (Table I). CD14+ blood cells were cultured in GM-CSF and IL-4 for 6 days and then infected with VSV/HIV-1 or left noninfected. Then parallel aliquots were either kept in cytokines (immature) or stimulated 4 days with CD40L to mature and express HIV-1 Ags. The DCs were added to autologous T cells, and the response was measured in an IFN-γ ELISPOT assay. Mature infected DCs were much better stimulators of patient T cells than immature infected DCs (Fig. 2; top, DCp and Tp). Noninfected DCs did not stimulate ELISPOT-producing T cells. Also, the VSV/HIV-1-infected DCs from normal donors did not stimulate IFN-γ release from autologous T cells (Fig. 2, DCn + Tn). When patient DCs were infected with VSV/SIV, they failed to stimulate T cells (not shown), indicating that the T cell response was HIV-1 specific. In all four experiments of this kind, mature DCs were better stimulators for autologous T cells than immature DCs.

Several stimuli are known to induce DC maturation, such as the inflammatory cytokines TNF-α and IL-1β, LPS, monocyte-conditioned medium (MCM), and CD40L. Infected DCs matured with CD40L were best able to present Ags to Ag-specific autologous T cells relative to DCs matured with LPS (Fig. 3) and MCM (not shown). Therefore, in all subsequent experiments, DCs were infected at the immature stage and matured with CD40L.

VSV/HIV-1-infected DCs present Ags to both CD4+ and CD8+ T cells

To identify subsets of T cells that could respond to the infected DCs, CD4+ and CD8+ T cells were enriched either by positive or negative selection using magnetic beads. T cell selections were verified by FACS, and positively selected cells were found to be 98% enriched for the specific subset. The T cell subsets were then stimulated for 36 h with autologous uninfected and VSV/HIV-1-infected DCs in an IFN-γ ELISPOT assay. As shown in Fig. 4A, the CD8+ T cells were more active in the production of IFN-γ, but in some patients the infected DCs could also stimulate IFN-γ secretion from CD4+ T cells (Fig. 4A). The number of T cells that responded to DCs expressing HIV-1 Ags was high, 50–200 cells per 2 × 10⁵ CD8+ cells. Comparable responses were obtained when positively or negatively selected (CD8+ or CD4+) populations were studied, ruling out an involvement of NK cells in the IFN-γ production. Nonetheless, in some cases we also removed the CD56+ NK cells, which represented 1–5% of the CD8+ cells, with CD56 beads, and this did not change the results (data not shown). We conclude that VSV/HIV-1, a vector that is reliably prepared to a high viral titer in a straightforward manner, was HIV-1 specific. In all four experiments of this kind, mature DCs were better stimulators for autologous T cells than immature DCs. In all experiments of this kind, mature DCs were better stimulators for autologous T cells than immature DCs.

Expansion of Ag-specific T cells with DCs infected with VSV-pseudotyped HIV-1

We next tested whether infected DCs could expand Ag-specific T cells in 5-day cultures. As in the case of the ELISPOT assay above (Fig. 2), T cells from uninfected donors did not show proliferative responses to autologous, VSV/HIV-1-infected DCs. In contrast, CD8+ T cells from all patients proliferated in response to VSV/HIV-1-infected DCs and did not show a significant syngeneic MLR to uninfected DCs (Fig. 4B and Table I). At the DC:T ratios that we used (1:10–1:20), uninfected DCs did stimulate CD4+ T cell proliferation, as a result of the syngeneic MLR, but in at least half the patients studied, the infected DCs were further stimulatory for CD4+ T cells (Fig. 4B and Table I). Therefore, VSV/HIV-1-infected DCs are more effective in HIV-1 Ag presentation than immature DCs.
infected DCs can stimulate growth in CD4+ and CD8+ T cells from HIV-1-primed donors.

To assess expansion of cytokine-producing effectors, we cocultured VSV/HIV-1-infected DCs for 7 days with autologous T cells. The ELISPOT assay was then carried out using restimulation with autologous monocytes pulsed with p24 or recombinant HIV-1 vectors. Fig. 5 shows a typical experiment in which T cells from an individual donor were evaluated for IFN-γ secretion in fresh bulk PBMCs (Fig. 5A), in T cells stimulated with autologous DCs infected with various vectors (Fig. 5B), and with T cells expanded for 7 days with DCs (Fig. 5C). It is evident that 1) VSV/HIV-1 is not effective in fresh PBMCs (Fig. 5A), 2) VSV/HIV-1 is comparably effective to recombinant pox viral vectors when DCs are used as APCs with fresh T cells (Fig. 5B), and 3) DCs infected with VSV/HIV-1 clearly expand IFN-γ-secreting T cells, both CD4+ and CD8+ T cells (Fig. 5C).

**Comparison of DCs and macrophages for presentation of VSV/HIV-1 virus**

We then set up dose response curves to compare the efficacy with which DCs and macrophages, infected with pseudotyped HIV-1, expand effector T cells. Mature DCs proved to be markedly more potent. At a DC:T cell ratio of 1:30, the response was much greater than macrophages at a ratio of 1:5, and at 1:30, macrophages had no activity (Fig. 6). Taken together, the data on APC requirements indicate that mature DCs should be targeted with nonreplicating viral vectors to expand HIV-1 Ag-reactive T cells.

**Endogenous and exogenous pathways for HIV-1 presentation by infected DCs**

We have shown above (Fig. 1A) that inhibitors of reverse transcription block the infection of DCs with VSV/HIV-1. To evaluate the relative contributions of the endogenous (AZT-sensitive) and exogenous (AZT-resistant) pathways of HIV-1 Ag presentation, we conducted ELISPOT assays in which CD4+ and CD8+ cells were stimulated with DCs that were treated with AZT. To block endogenous presentation, we also studied envelope-deficient HIV-1 particles produced by 293 cells in the absence of an env plasmid. Fig. 7 shows that pretreatment of DCs with AZT substantially reduced IFN-γ secretion by the CD8+ cells without affecting cytokine release by the CD4+ T cells. Similar results were obtained when DCs were pretreated with 3TC, another reverse transcriptase inhibitor (not shown). DCs infected with virus particles devoid of envelope also did not stimulate CD8+ T cells (Fig.

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* Patients 1–7 were from institution 1 (Rockefeller University) and patients 8–15 were from institution 2 (Mount Sinai School of Medicine).

* Spots scored with stereo microscope. +, 50–100 spots; ++, 100–300 spots.

* Proliferation determined by TdR incorporated in cell culture. +, 10% of counts above background; ++, 10–50% of counts above background.
7). As in the case of DCs infected in the presence of AZT, DCs pulsed with envelope-deficient virus were able to stimulate CD4^+ T cells. Therefore, we conclude that VSV/HIV-1 charges MHC class I molecules in DCs primarily by the endogenous pathway and MHC II by an exogenous route.

**Discussion**

HIV-1 lacking its envelope gene and pseudotyped with other viral envelopes, e.g., the G protein from VSV, has been useful for several studies of HIV-1 biology. Pseudotyped virus bypasses the normal CD4 and chemokine receptor entry mechanism required for intact HIV-1 and also limits the ensuing infection to a single cycle. Here we have studied Ag presentation of VSV-pseudotyped HIV-1, which reverse transcribes to a high efficiency in DCs (about 50%, Fig. 1A). However, VSV/HIV-1 expresses HIV-1 protein (but not VSV-G) in a small fraction of DCs (<10%, Fig. 1B–D). VSV-pseudotyped vectors are easy to prepare and, as long as they are administered via mature DCs, prove to be very effective at stimulating autologous, nylon wool-purified T cells in an ELISPOT assay at different DC:T ratios: 1:10 = 1.5 × 10^9 DCs and 1.5 × 10^9 T cells; 1:20 = 7.5 × 10^9 DCs and 1.5 × 10^9 T cells.

At this time, many nonreplicating forms of HIV-1 are being considered as vaccine vectors, particularly avipox and modified vaccine Ankara (see Ref. 31 for review). Our current studies are an initial exploration of the use of VSV-pseudotyped virus to charge DCs with HIV-1 Ags. We want to assess the hypothesis that targeting of a nonreplicating vaccine to mature DCs will enhance T cell-mediated immunity and thereby provide an opportunity to study the resistance that a well-primed T cell system can provide to challenge with immunodeficiency viruses, HIV-1 and SIV. Strong T cell immunity, comparable to that seen with immunogenic viruses, is not being observed when humans or monkeys are vaccinated with current pox virus vectors.

Because mature DCs are not typically found in the periphery, we assessed whether VSV/HIV-1 can be captured by immature DCs. Whereas pox viruses have toxicity to immature DCs (32, 33) or block their maturation (34), immature DCs allow nontoxic entry of pseudotyped virus through the acid-sensitive VSV envelope. Interestingly, the level of full-length reverse transcripts is very high (at least 10^4 copies in 2 × 10^4 DC in Fig. 1A), yet the percentage of DCs expressing HIV-1 gag is relatively low (Fig. 1D), suggesting a block in the HIV-1 life cycle beyond reverse transcription.

**FIGURE 2.** Mature DCs present HIV-1 Ags more efficiently than immature DCs. Immature DCs were generated from normal individuals (DCn) or from HIV-1 patients (DCp). The cells were infected or not with VSV/HIV-1 and cultured without (immature) or with CD40L (mature) for 4 days. The different populations were used to stimulate autologous, nylon wool-purified T cells in an ELISPOT assay at different DC:T ratios: 1:10 = 1.5 × 10^9 DCs and 1.5 × 10^9 T cells; 1:20 = 7.5 × 10^9 DCs and 1.5 × 10^9 T cells.

**FIGURE 3.** Effect of maturation stimuli on HIV-1 Ag presentation by DCs infected as immature cells. Immature DCs were generated with GM-CSF and IL-4 from the blood of HIV-1 patients and infected at day 6 with VSV/HIV-1. The cells were matured with LPS (20 ng/ml) or irradiated CD40L-transfected fibroblasts (1 CD40L cell for 7 DCs). After 4 days, DCs were added to T cells to stimulate the release of IFN-γ as measured by ELISPOT.

**FIGURE 4.** VSV/HIV-1-infected DCs stimulate both CD4^+ and CD8^+ T cells. A. IFN-γ release in ELISPOT assays by 1.5 × 10^5 positively and negatively selected T cells stimulated with infected DCs derived from an HIV-1 patient at a DC:T cell ratio of 1:10. B. Proliferative responses in identical cultures as A, but assessed after 5 days by pulsing the cultures for 8 h with 1 μCi of [3H]thymidine.
Although our current studies exploit the VSV-G as the entry mechanism, additional uptake receptors are known to be expressed by DCs. These include the macrophage mannose receptor (35) and DEC-205 (36), both multilectin receptors for adsorptive endocytosis. Other newly recognized C-type lectins, Langerin (37) and DC-SIGN (38), are not yet known to lead to Ag presentation.

Following uptake into immature DCs, the cells could be induced to mature and became more potent in presenting HIV-1 Ags (Figs. 2 and 3). We tested several maturation stimuli and found CD40L to be superior to LPS and MCM. Together, our data indicate that a nonreplicating vaccine can be captured by immature DCs, and then maturation harnesses the T cell stimulatory potential of these APCs.

There are interesting advantages to the use of pseudotyped virus for delivering HIV-1 Ags to the immune system in vivo. The first is safety. The virus is not pathogenic, because it is genetically unable to undergo more than a single cycle of replication. We have verified this previously by showing that pseudotyped virus is not transmitted from DCs to T cells, as occurs with wild-type HIV-1 (24), nor does the virus replicate in DCs cultured for up to 7 days. A second advantage is the ease of preparation. High titer virus (10^6–10^7 infectious units/ml in the Magi assay) is produced by simple cotransfection of the 293 T producer line with the VSV-G and env-deficient HIV-1 plasmids. Third, as discussed above, VSV-pseudotyped virus gains access to immature DCs, which are rich in acidic, endocytic compartments and represent the most logical sites for capture of vaccines in vivo (9, 10). Fourth, anti-envelope Abs should not be induced when pseudotyped viruses are targeted to DCs. VSV-G is poorly expressed in the infected DCs, so B cells should not be selected to produce anti-envelope Abs. Moreover, most humans do not have preexisting neutralizing Abs to VSV. A fifth advantage, and the emphasis of this paper, is that...
VSV/HIV-1 is efficiently presented to both CD4+ and CD8+ T cells by DCs. Both CD4+ and CD8+ subsets are probably required for strong T cell memory and protection against HIV-1 (39–41). In fact, DCs charged with VSV/HIV-1 induce robust T cell responses from primed T cells, both in terms of the absolute number of IFN-γ-secreting cells and the high levels of DNA synthesis, especially in CD8+ cells.

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