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Baculovirus-Derived Human Immunodeficiency Virus Type 1 Virus-Like Particles Activate Dendritic Cells and Induce Ex Vivo T-Cell Responses

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We have recently developed a candidate human immunodeficiency virus type 1 (HIV-1) vaccine model based on HIV-1 Pr55^{gag} virus-like particles (HIV-VLPs), produced in a baculovirus expression system and presenting a gp120 molecule from a Ugandan HIV-1 isolate of clade A (HIV-VLP$_A$s). The HIV-VLP$_A$s show the induction in BALB/c mice of systemic and mucosal neutralizing antibodies as well as cytotoxic T lymphocytes, by intraperitoneal as well as intranasal administration. In the present article, the effects of the baculovirus-expressed HIV-VLPs on human immature monocyte-derived dendritic cells (MDDCs) have been evaluated. The HIV-VLPs efficiently induce maturation and activation of MDDCs and are incorporated into MDDCs preferentially via an actin-dependent macropinocytosis and endocytosis. The HIV-VLP-activated MDDCs show enhanced Th1- and Th2-specific cytokine production, and the effects of HIV-VLPs on MDDCs are not mediated through Toll-like receptors 2 and 4 (TLR2 and -4) signaling. Finally, HIV-VLP-loaded MDDCs are able to induce a primary and secondary response in autologous human CD4$^+$ T cells in an ex vivo immunization assay. Our results on the interaction and processing of baculovirus HIV-VLPs by MDDCs give an insight into the mechanisms underlying the immune response induced by HIV-VLP$_A$s in vivo.

Virus-like particles (VLPs) represent a novel form of subunit vaccine based on viral capsid proteins which show the ability to self-assemble into highly organized particulate structures (21, 35). VLPs closely resemble immature virus particles but are both replication and infection incompetent, lacking regulatory proteins as well as infectious genetic material. VLPs can be employed to deliver additional antigenic structures, such as whole proteins or specific individual epitopes, and have been shown to generally induce more effective humoral and cellular immune response than their soluble counterparts. In fact, while endogenous antigens (Ags) efficiently fuel the major histocompatibility complex (MHC) class I pathway, exogenous antigens, including inactivated virus, cannot reach the MHC class I pathway and fail to induce cytotoxic T-lymphocyte (CTL) responses (3, 5). On the contrary, VLPs are highly complex exogenous antigens and efficiently reach the MHC class I pathway in the absence of infection or intracellular replication (4, 41, 42).

Considering these properties, VLPs represent a highly attractive vaccine approach and have been produced from a broad spectrum of enveloped and nonenveloped viruses, regardless of whether the particle structure is based on single or multiple capsid proteins (37).

The VLPs developed in our laboratory are based on the human immunodeficiency virus type 1 (HIV-1) Pr55^{gag} precursor protein (HIV-VLPs) and display an entire gp120 molecule, anchored through the transmembrane (TM) portion of the Epstein-Barr virus gp220/350. The heterologous TM increases the expression and stability of the glycoprotein on the HIV-VLP surface, without affecting its oligomerization (7). The gp120 glycoprotein selected for these HIV-VLPs (HIV-VLP$_A$s) is derived from a Ugandan HIV-1 isolate of the A clade (6, 8), which represents the second most prevalent HIV-1 subtype worldwide (approximately 25%) and is predominant in many developing countries.

The HIV-VLP$_A$s show strong immunogenicity in BALB/c mice, without adjuvants, and HIV-1-specific CD4$^+$ and CD8$^+$ T-cell responses as well as cross-clade neutralizing antibodies have been detected in immunized animals (9). Moreover, the intraperitoneal and intranasal administration of HIV-VLP$_A$s in mice induces antibody responses at systemic as well as mucosal (vaginal and intestinal) levels (10).

Immature monocyte-derived dendritic cells (MDDCs) are professional antigen-presenting cells (APCs) that specialize in the uptake of antigen in the periphery and play a crucial role in the initiation of immune responses. Different pathways have been described for the Ag uptake by APCs, such as phagocytosis, clathrin-mediated uptake, caveolae-mediated uptake, macropinocytosis, and non-clathrin, non-caveola-mediated uptake (16, 45).

During the maturation induced by the antigen, DCs upregulate costimulatory molecules such as CD40, CD80, CD83, and CD86, necessary for efficient T-cell activation. This effect is elicited by the recognition and binding of pathogen-associated molecular patterns (PAMPs) to pathogen-recognition recep-

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tors (PRRs) expressed on the DCs, including Toll-like receptors (TLRs) and C-type lectins (47, 50, 51).

The priming of T-cell responses depends on the presentation of Ag-derived peptides by MHC molecules. In particular, MHC class I-restricted $CD8^+$ cytotoxic T-cell responses have been generally thought to be triggered only by Ags synthesized by the APC (endogenous pathway), either when infected by a live pathogen or when targeted by a DNA vaccine. However, it is now established that some exogenous Ags, besides the "classical" MHC class II pathway, may "cross over" to the endogenous pathway to gain access to MHC class I, a phenomenon defined as "cross-presentation" or "cross-priming," inducing $CD8^+$ cytotoxic T-cell responses (23, 36).

Most VLP models, developed in the last few years, have been shown to be highly effective at stimulating CD4 proliferative responses and CTL responses in addition to humoral immunity (37), and ours is no exception. Nonenveloped human papillomavirus-based VLPs have been reported to induce activation and maturation of DCs through the TLR4 (28, 29, 52). Similarly, yeast-derived HIV-VLPs have been shown to activate DCs partially through the TLR2 signaling pathway (49). Our enveloped HIV-VLPs are produced in a baculovirus expression system, presenting a whole gp120 molecule on the surface within a cell membrane environment which may influence the mechanisms of interaction between VLPs and APCs and the pattern of secreted Th-polarizing cytokines.

The results showed that, indeed, baculovirus-expressed HIV-VLPs are able to induce maturation and activation of MDDCs and that this effect is not mediated by the surface TLRs 2 and 4. The HIV-VLP-activated MDDCs produce a pattern of cytokines indicative of both Th1 and Th2 pathways and induced primary and secondary responses in autologous human $CD4^+$ T cells in an ex vivo immunization assay. Finally, the uptake of HIV-VLPs by DCs appears to be mainly mediated by a cytochalasin D-sensitive pathway.

MATERIALS AND METHODS

Cell culture medium. DC culture medium consisted of RPMI 1640 medium (Life Technologies, Carlsbad, Calif.) supplemented with 2 mM L-glutamine (Sigma), 1% nonessential amino acids (Life Technologies), 1% sodium pyruvate (Life Technologies), 50 μM 2-mercaptoethanol (Sigma), 50 μg of gentamicin (Life Technologies) per ml, and 10% fetal calf serum (Life Technologies).

MDDC preparations. All human specimens were obtained under informed consent, as approved by the University of Maryland—Baltimore Institutional Review Board. MDDCs were generated as described previously (43), with minor modifications. Briefly, human peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and were enriched for $CD14^+$ monocytes by negative selection with a cocktail of monoclonal antibodies (MAbs) from StemCell Technologies (Vancouver, British Columbia, Canada), according to the instructions of the manufacturer. Typically, greater than 80% of the cells were $CD14^+$ after enrichment, as verified by flow cytometry. The isolated monocytes were allowed to adhere to plastic by plating in six-well plates at 1×10^6 cells per ml in RPMI 1640 medium for 2 h. Adherent monocytes were washed with RPMI 1640 medium and were then cultured for 6 days in DC culture medium supplemented with 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; R&D Systems, Minneapolis, Minn.) per ml and 1,000 U of recombinant interleukin-4 (rIL-4; R&D Systems, Minneapolis, Minn.) per ml.

Cell treatments. Agonists and inhibitors were added directly to MDDC cultures in individual wells. MDDCs were pulsed with either 10 μg/ml of HIV-VLPs or 1 μg/ml of lipopolysaccharide (LPS). As negative controls, MDDCs were treated with several regimens. In particular, during the development/optimization of the experimental assay, the absence of MDDC activation following treatment with phosphate-buffered saline (PBS), supernatant of SF9 cell culture transfected with baculovirus expression vector (mock baculovirus supernatants), and heat-denatured VLP suspension in PBS (100°C for 10 min) has been repeatedly observed. Thus, the complete set of experiments was subsequently performed using only the PBS treatment as negative control. The HIV-VLPs presenting on their surface a CCR5-specific, clade A Ugandan gp120 molecule were prepared as previously described (7, 10), and the residual endotoxin activity possibly present in the HIV-VLP preparation was inhibited by preincubation with polymyxin B sulfate (Sigma) at a concentration of 10 μg/ml. The absence of interference due to the polymyxin B sulfate in the activation results was verified in parallel on MDDCs treated only with polymyxin. For inhibition experiments, MDDCs were preincubated with purified anti-TLR2 (TL2.1, IgG2a), anti-TLR4 (HT125, IgG2a), and an unrelated control IgG2a (15 μg/ml) (eBioscience, San Diego, CA). After 16 h, the cells were harvested, washed, and stained for phenotypic analysis by flow cytometry. The cellular supernatants were collected for quantification of cytokine production by enzyme-linked immunosorbent assay (ELISA) (University of Maryland Cytokine Core Lab, Baltimore, MD).

Isolation of naïve $CD4^+$ T cells. Naïve $CD4^+$ T cells were isolated from peripheral blood mononuclear cells by negative selection with a mixture of MAbs from StemCell Technologies (Vancouver, British Columbia, Canada), according to the manufacturer's instructions. The mixture included MAbs to CD8, CD14, CD16, CD19, CD45R0, CD56, and glycophorin A. An anti-human HLA-DR tetrameric MAb (StemCell Technologies) was also included to increase the purity of the naïve $CD4^+$ T cells. The isolated cells were >95% $CD3^+$ $CD4^+$ and were completely depleted of monocytes, NK cells, B cells, and $CD8^+$ T cells. Generally, 92 to 99% of the isolated $CD4^+$ T cells were naïve as judged by surface expression of CD62L ($CD62L^+$), low expression of CD11a, and the lack of expression of CD25, CD45R0, and HLA-DR (data not shown).

Antigen-specific stimulation of autologous naïve $CD4^+$ T cells ex vivo. Carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) labeling of naïve $CD4^+$ T cells was carried out as described previously (32). MDDCs were harvested after 6 days of culture in MDDC culture medium (supplemented with IL-4 and GM-CSF), and 2×10^3 cells/per well were used for priming autologous naïve $CD4^+$ T cells (2×10^5 cells/per well) in 200 μl of total culture volume in 96-well U-bottom plates. MDDCs used for priming were either untreated or were loaded with 100 ng/ml of *Staphylococcus* enterotoxin B (SEB; Sigma) or with 10 μg/ml of HIV-VLPs. The cocultures were incubated in a humidified incubator in the presence of 5% CO_2 at 37°C for 7 or 15 days. At the two time points, T-cell proliferation was estimated by both cell count and CFSE dilution using flow cytometry. At 7 days, the primary T-cell response was evaluated by the surface expression of CD62L and CD45R0 markers. At 15 days, memory T-cell response was evaluated reexposing T cells for 5 h to freshly loaded autologous MDDCs, and the response was measured as intracellular gamma interferon (IFN-γ) production and surface CD69 expression. Background responses were determined by omitting antigen from the cultures. For flow cytometry, cultured cells were harvested, washed in wash buffer, and incubated for 30 min at 4°C with MAbs, washed, and fixed for flow cytometric analysis.

Flow cytometry. Cells were incubated for 30 min at 4°C with murine monoclonal antibodies specific for the indicated MAbs (BD Pharmingen, San Diego, CA), washed, and then fixed with 2% paraformaldehyde for analysis with a FACScalibur flow cytometer (BD Pharmingen). Data analysis was carried out with FlowJo software (Tree Star Inc., San Carlos, CA). The fraction of MDDCs that responded by upregulation of activation markers on the cell surface was calculated by overlaying the histograms of treated and untreated MDDCs and Overton subtraction of the curves.

HIV-VLP uptake experiments. HIV-VLPs were labeled with 10 μM carboxyfluorescein diacetate (CFDA) (Vybrant CFDA Cell Tracer kit; Molecular Probes, Eugene, OR) for 1 h at room temperature. After this incubation, the preparation was centrifuged through a separation membrane to remove the excess unbound dye marker. MDDCs were incubated with 10 μg of HIV-VLP-CFDA for 90 min at 37°C. At indicated time points, cells were both observed under fluorescence microscope and collected for evaluation by flow cytometry. For inhibition of uptake, cytochalasin D (1 μM) or a DC-SIGN ligand polyacrylamide-based polymer, Man9 (200 nM), was added to the cells 1 h before the HIV-VLP loading.

The percentage of inhibition of HIV-VLP-CFDA uptake in the presence of inhibitors was determined as follows: % inhibition = [(MFI of cells stained without inhibitors) − (MFI of cells stained with inhibitors)] × 100/(MFI of cells stained without inhibitors), where MFI is mean fluorescent intensity.

Statistical analyses. Intergroup comparisons were performed with the Mann-Whitney U test (for univariate nonparametric group analysis). All *P* values were two tailed and were considered significant if <0.05.

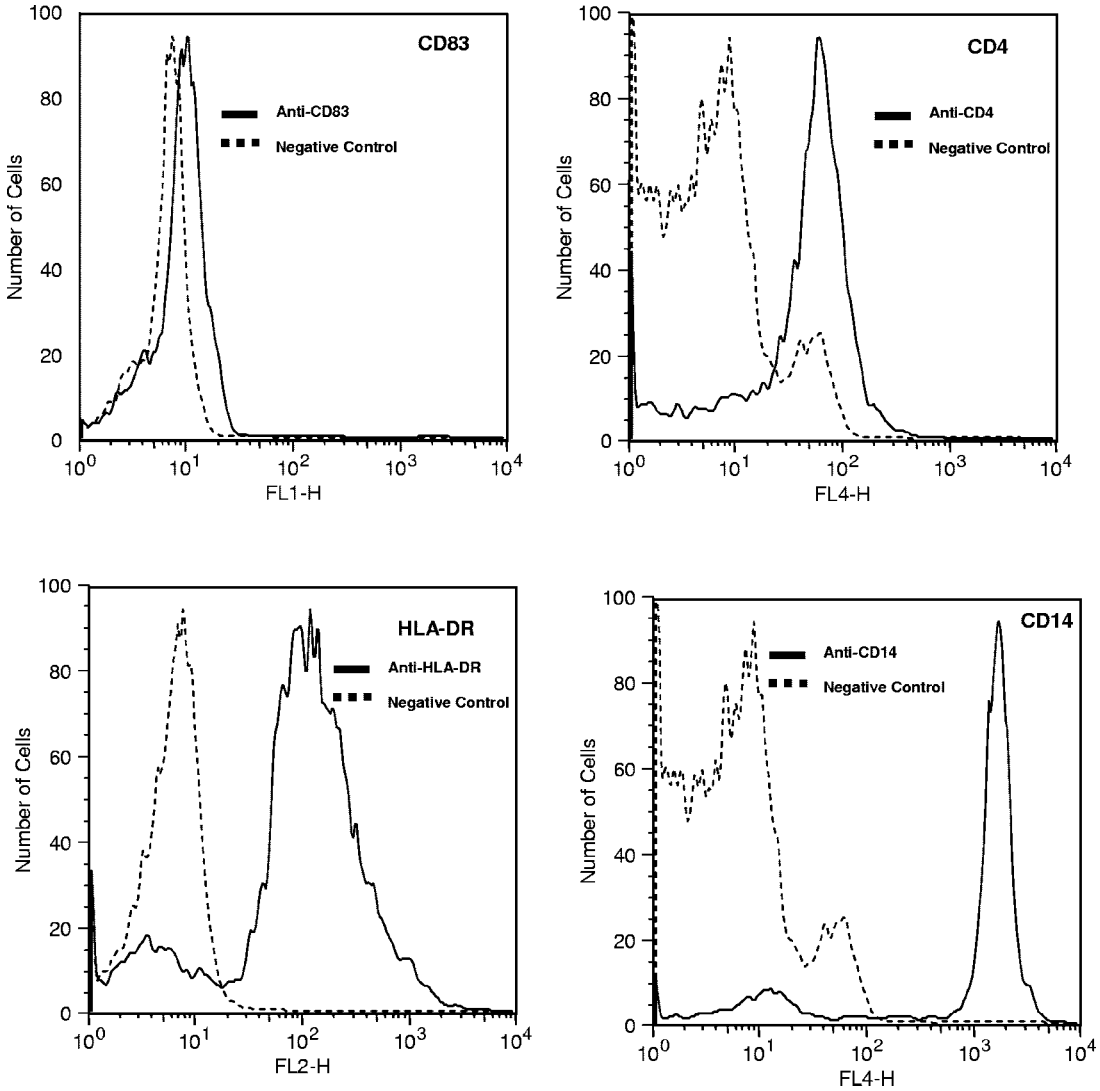


FIG. 1. Evaluation of monocyte purity. Human peripheral blood mononuclear cells, isolated by Ficoll-Hypaque density gradient centrifugation, were enriched for $CD14^+$ monocytes by negative selection. The purity of the obtained monocyte population was evaluated by flow cytometry using MAbs specific for the indicated cellular markers.

RESULTS

Baculovirus HIV-VLPs induce a maturation phenotype of MDDCs. Human peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and were enriched for $CD14^+$ monocytes by negative selection. The purity of the obtained monocyte population was evaluated using a set of MAbs in flow cytometry. The very high intensity of CD14 expression in >80% of enriched cells, coupled to HLA-DR and CD4 expression as well as the absence of CD83 expression, indicates the purity of the starting monocyte population for the in vitro generation of immature MDDCs (Fig. 1).

After 6 days of IL-4 and GM-CSF induction, immature MDDCs were incubated for 16 h with 10 μg/ml of HIV-VLPs and the expression of surface maturation markers, including CD80, CD83, CD86, and HLA-DR, was examined. In parallel, MDDCs were incubated with LPS (1 μg/ml), a well-known activator of MDDCs' maturation, or with PBS, as negative control. As shown in Fig. 2A, the expression of all of the four maturation markers was upregulated by treatment with HIV-VLPs, at levels equivalent to those with the LPS treatment. Given that MDDC maturation is characterized by the production of cytokines that are important for the activation of T cells, the level of IFN-γ, tumor necrosis factor α (TNF-α), IL-6, IL-12 p70, and IL-10 was assessed in the supernatant of MDDCs loaded with HIV-VLPs or LPS. With the exception of IFN-γ, which was not induced in these cultures, HIV-VLP-loaded MDDCs produced high levels of the other cytokines. In particular, compared to LPS-loaded MDDCs, the levels of TNF-α, IL-12 p70, and IL-10 were on average higher (2.5×, 8.7×, and 2.7×, respectively) and those of IL-6 were 2.89× lower ($P < 0.001$). The standard deviation of the cytokine levels produced in five independent experiments was <10% of the mean value (Fig. 2B). The expression of additional Th1-

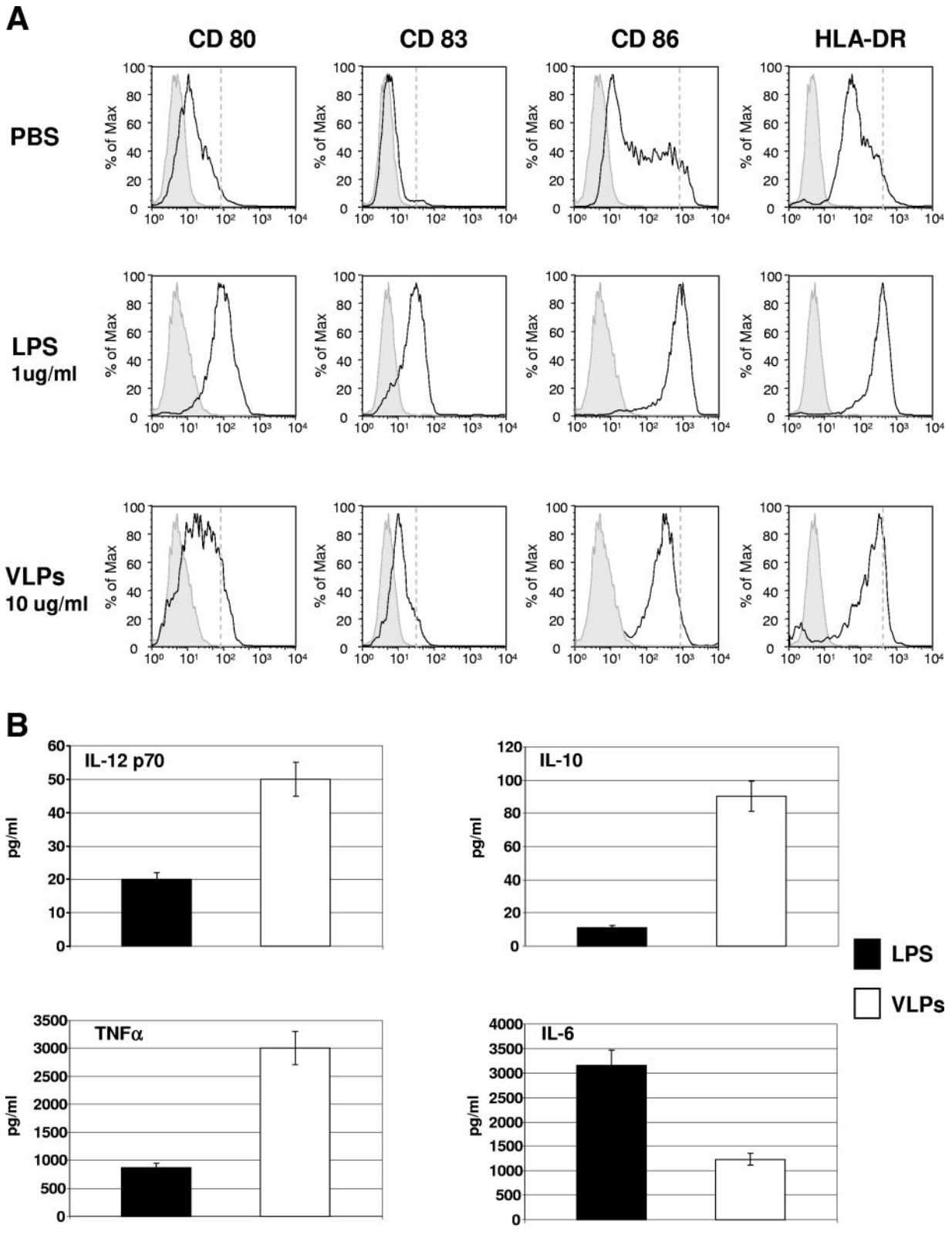


FIG. 2. Maturation of DCs by baculovirus-expressed HIV-VLPs. Immature MDDCs were incubated in the presence of the indicated stimulus for 16 h. (A) The expression of CD80, CD83, CD86, and HLA-DR was analyzed on fixed cells by FACScalibur flow cytometer, and data analysis was carried out with FlowJo software. The results of one representative experiment out of five are shown; the shaded curve represents the background fluorescence, and the dotted line indicates the peak LPS response. (B) The level of cytokines in the culture supernatant was measured by ELISA. The results represent the average of five experiments.

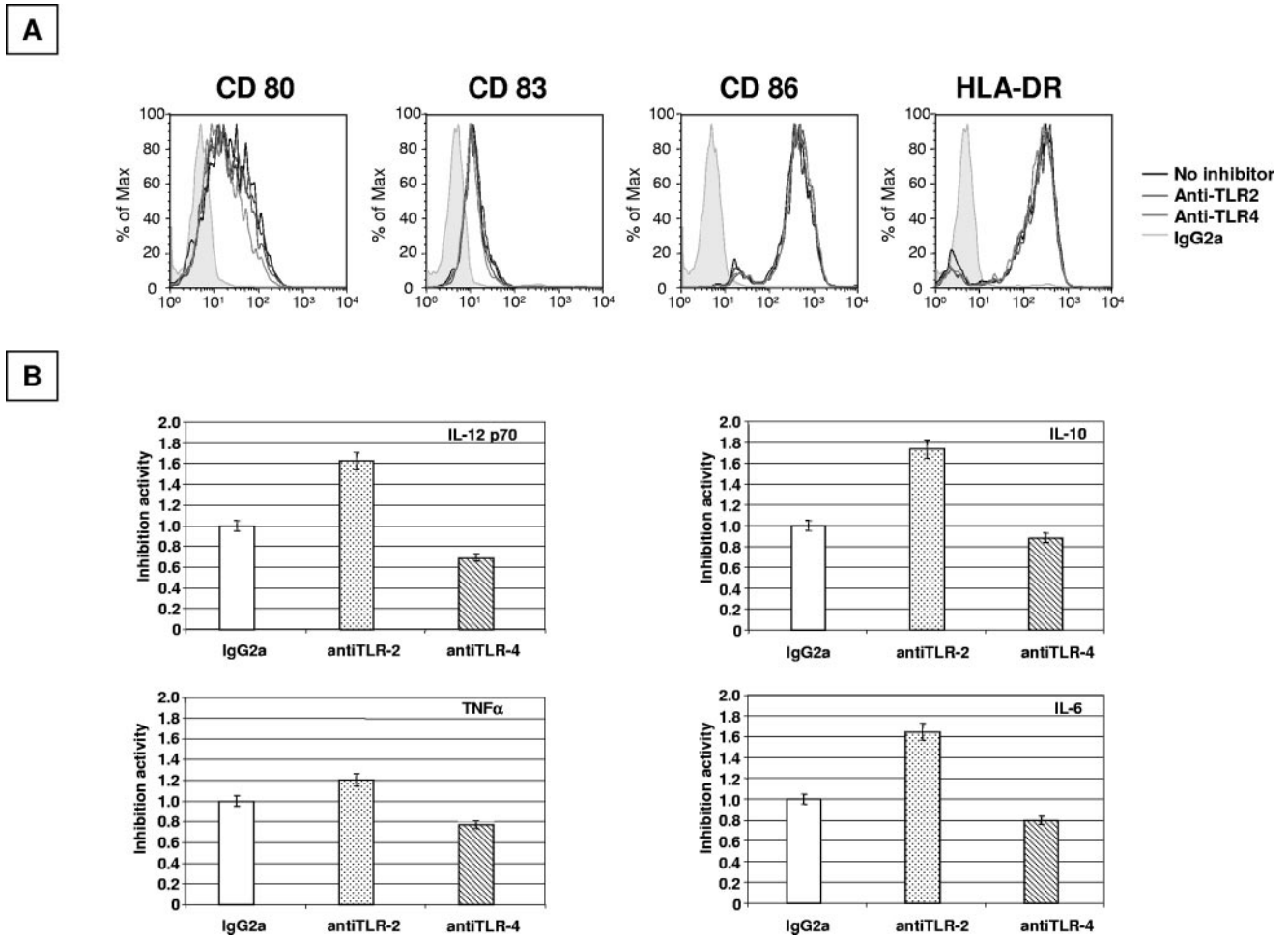


FIG. 3. Surface TLRs do not mediate the maturation of DCs by baculovirus-expressed HIV-VLPs. Immature MDDCs were preincubated for 1 h with purified anti-TLR2 (TL2.1, IgG2a) and anti-TLR4 (HT125, IgG2a) or an unrelated control IgG2a (15 μg/ml) and then with HIV-VLPs (10 μg/ml). (A) After 16 h, the expression of CD80, CD83, CD86, and HLA-DR was analyzed on fixed cells by FACScalibur flow cytometer and data analysis was carried out with FlowJo software. The results of one representative experiment out of three are shown. The shaded curve represents the untreated cells; the gray curves represent the cells treated in the presence of the MAbs. (B) The level of cytokines in the culture supernatant was measured by ELISA and is expressed as percentage of production in the presence of the anti-TLR2 and -4 antibodies, compared to cells treated with the unrelated control IgG2a. The results represent the average of three experiments.

related markers (IL-2, IL-18R, and IL-23A) as well as Th2-related markers (IL-13 and IL-15) has been found increased in the same HIV-VLP-loaded MDDCs, evaluated in parallel by a gene array analysis (2). The PBS treatment did not induce the expression of the surface maturation markers nor the production of cytokines in the cell culture supernatant (levels of sensitivity of the ELISA, <2 pg/ml). These results indicate that baculovirus HIV-VLPs induce an MDDC maturation that is coupled with the production of cytokines involved in the Th1 and Th2 pathways.

Maturation of MDDCs by HIV-VLPs is not mediated by signaling through surface TLR2 and -4. TLRs represent a family of surface and intracellular pattern recognition receptors that signal the presence of a pathogen to the host. They play an important role in MDDC maturation, inducing a cascade of events leading to the generation of effector responses such as Th1/Th2 polarization and CTL effector responses (38, 44). Among TLRs, the surface TLR4 recognizes LPS of gram-negative bacteria and TLR2 recognizes bacterial lipoproteins and lipoteichoic acids, whereas intracellular TLRs recognize double-stranded or single-stranded viral RNA nucleic acid as well as unmethylated bacterial and virus CpG DNA (13, 24, 25, 34, 46). The role of the surface TLRs in the response of MDDCs to HIV-VLPs was investigated by evaluating the expression of surface maturation markers and the levels of cytokines produced by DCs stimulated with HIV-VLPs in the presence of blocking specific antibodies. As shown in Fig. 3A, the stimulation of immature MDDCs with HIV-VLPs in the presence of MAb specific for TLR2 and -4 did not induce any alteration in the expression of surface maturation markers, resulting in an effective maturation profile. Moreover, while the blocking with the anti-TLR2 MAb did not decrease cytokine production, the anti-TLR4 MAb induced only a modest (10 to 30%) inhibition of cytokine production in HIV-VLP-stimulated MDDCs. This result, however was not supported by statistical significance ($P < 0.087$). As a control, DCs were

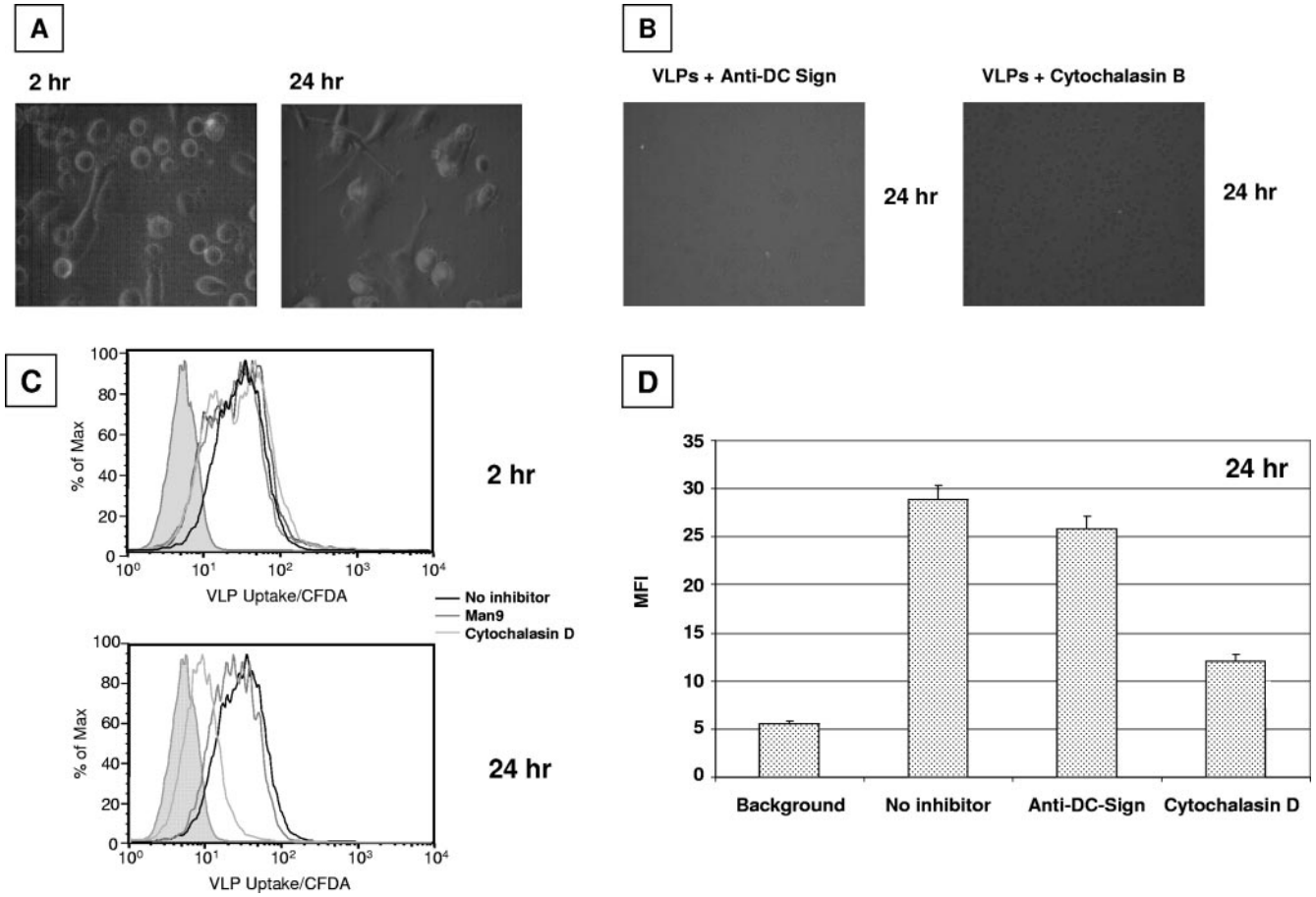


FIG. 4. Uptake of baculovirus-expressed HIV-VLPs by MDDCs. CFDA-labeled HIV-VLPs were loaded on immature MDDCs, untreated or preincubated for 1 h with cytochalasin D (1 μM) or DC-SIGN ligand Man9 (200 nM). DCs were collected after 2 and 24 h and observed under a fluorescence microscope (panels A and B); in parallel, aliquots of cell culture were collected and fixed with paraformaldehyde for measurements by flow cytometry (C). The shaded curve represents the background, the black curve represents the cells loaded with CFSE-labeled HIV-VLPs without inhibitors, and gray curves represent the cells preincubated with inhibitors and then loaded with CFSE-labeled HIV-VLPs (dark gray, Man9; light gray, cytochalasin D). The MFIs of cells with different treatments are shown in arbitrary units in panel D.

loaded in the presence of an unrelated control IgG2a MAb without effects (Fig. 3B). The standard deviation of the cytokine levels produced by each treatment in three independent experiments was <10% of the mean value. Taken together, these results suggest that MDDC maturation induced by baculovirus HIV-VLPs is not significantly mediated by surface TLR2 and -4.

Uptake of baculovirus HIV-VLPs by MDDCs. Pathogens and particulate molecules can enter APCs via endocytosis (14, 44). DC-SIGN is a C-type lectin expressed on the DCs' surface and has been shown to function as receptor for the endocytosis of viral envelope glycoproteins, including HIV-1 gp120 (17, 20, 26). CFDA-labeled HIV-VLPs were used to determine the mechanism of HIV-VLP uptake used by DCs in the presence and absence of a DC-SIGN ligand or cytochalasin D. Cytochalasin D is an inhibitor of the actin polymerization and cellular processes such as phagocytosis that are actin dependent. Considering that cytochalasin D inhibits the uptake of HPV-VLPs by DCs (16), we evaluated its ability to inhibit the uptake of our HIV-VLPs by MDDCs.

The results show that CFDA-labeled HIV-VLPs, in the absence of inhibitors, are effectively taken up by DCs localizing after 2 h along the cellular membrane and, after 24 h, inside of intracellular organelles (Fig. 4A and B).

In the inhibition studies, the cytochalasin D showed an efficient (>60%), although not complete, inhibition of the HIV-VLP uptake by MDDCs after 24 h of incubation. The MDDCs treated with cytochalasin D, in fact, showed on average 40% of the mean fluorescence intensity observed in the untreated cells (Fig. 4C and D).

The synthetic polyvalent Man9 is a polyacrylamide-based polymer containing 15% high-mannose type N-glycan (Man9), which has been shown to bind the DC-SIGN molecule, inhibiting the binding of the HIV-1 (BAL) gp120 and blocking the capture of HIV-1 (BAL) by DC-SIGN-expressing BTPH cells at nanomolar concentrations (L. X. Wang and T. Fouts, unpublished data). The pretreatment of MDDCs with the Man9 molecule induced only a modest (<10%) inhibition of the HIV-VLP cellular uptake after 24 h of incubation, and cells showed on average 90% of the MFI observed in the untreated cells (Fig. 4C and D). These results indicate that, under the experimental conditions used for this study, HIV-VLPs are preferentially taken up by MDDCs in an actin-dependent manner.

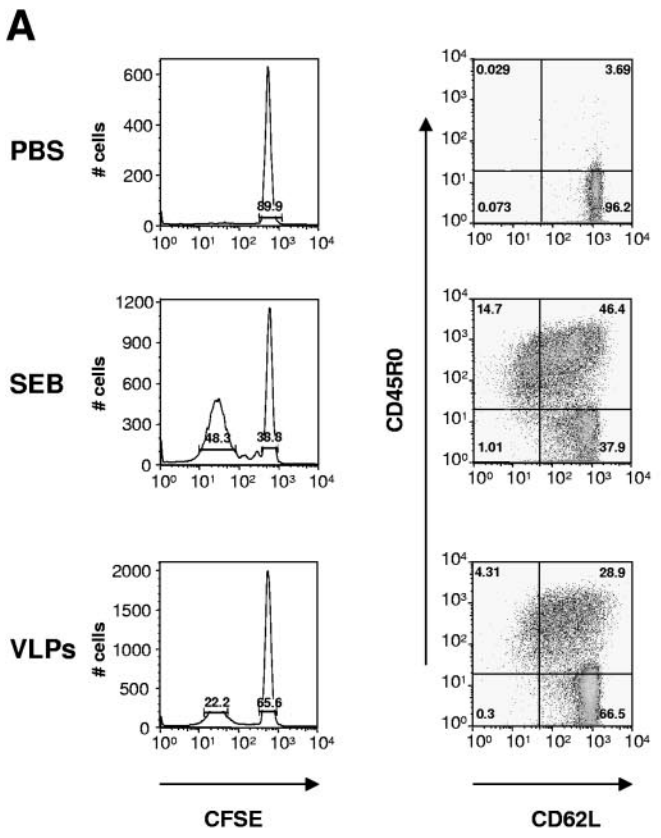
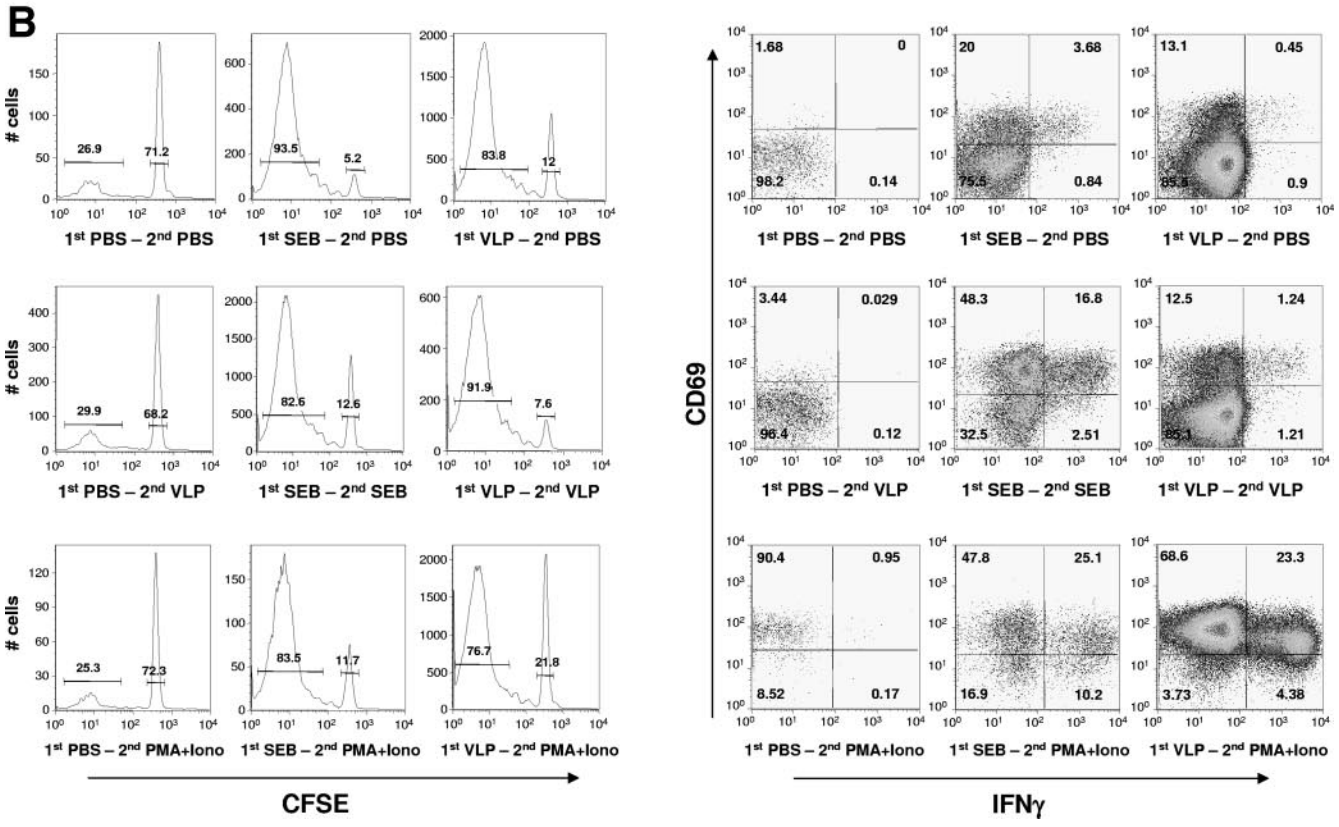


FIG. 5. Baculovirus-expressed HIV-VLPs are cross-presented by MDDCs. Immature MDDCs were pulsed for 16 h with the indicated reagents and cocultured with CFSE-labeled autologous naïve $CD4^+$ T cells. (A) After 7 days of cocultivation, T-cell activation was evaluated as decreased CFSE intensity and expression of CD45R0 marker. (B) T cells were cocultivated, in parallel wells, for 15 days with autologous DCs pulsed with the indicated reagents and restimulated for 5 h with fresh autologous DCs pulsed with the indicated reagents. T-cell activation was evaluated as decreased CFSE intensity and increased CD69 expression; the Th1 polarization was evaluated as intracellular production of IFN-γ. Cellular viability in all three sets of cocultivation was verified by PMA and ionomycin (Iono).



Generation of primary and secondary $CD4^+$ T-cell responses ex vivo by HIV-VLP-treated MDDCs. MDDCs loaded with HIV-VLPs were used to prime and boost autologous $CD4^+$ T cells in an ex vivo system. Highly purified naïve $CD4^+$ T cells, isolated from the same donor used for the MDDC preparation, were labeled with CFSE and mixed with HIV-VLP-loaded MDDCs in a 100:1 T-cell/MDDC ratio. After 7 days of cocultivation, >25% of T cells appeared activated, as measured by CFSE dilution, and the duplicating T cells showed a robust positivity for the conventional activation/memory CD45R0 marker (Fig. 5A). On the contrary, naïve $CD4^+$ T cells cocultivated with unloaded autologous MDDCs did not show any activation, as measured by CFSE dilution, nor induction of the CD45R0 memory marker. In this experimental system, the positive control was represented by naïve $CD4^+$ T cells cocultivated with autologous MDDCs loaded with the superantigen SEB. These results indicate that HIV-VLP-loaded MDDCs are fully activated and able to prime autologous $CD4^+$ T cells, inducing their activation.

To demonstrate a boosting effect, after 15 days of cocultivation, $CD4^+$ T cells were restimulated with Ag-loaded autologous MDDCs to evaluate the induction of a secondary response. The results showed that a population of $CD4^+$ T cells, after a 15-day "primary" stimulation with HIV-VLP-loaded DCs, were still viable. When restimulated with fresh HIV-VLP-loaded autologous MDDCs, the resting cells showed IFN-γ production, suggesting the presentation of HIV-VLPs by MDDCs in an MHC-restricted context.

In parallel, the $CD4^+$ T cells primed for 15 days with unloaded autologous MDDCs, did not synthesize IFN-γ upon restimulation. As a positive control, the $CD4^+$ T cells primed for 15 days with SEB-loaded autologous MDDCs, induced a strong IFN-γ response (Fig. 5B). The cellular viability in all three sets of cocultivation was verified by phorbol myristate acetate (PMA) and ionomycin treatment which induced a maximal activation of the CD69 marker and IFN-γ production.

DISCUSSION

We have previously reported that baculovirus-expressed HIV-VLP_As, which display a gp120 derived from a Ugandan HIV-1 isolate of subtype A, are strongly immunogenic in BALB/c mice, inducing HIV-1-specific $CD4^+$ and $CD8^+$ T-cell response as well as cross-clade neutralizing antibodies at systemic and mucosal sites (9, 10).

Here we have shown that baculovirus-expressed HIV-VLPs are able to induce maturation of immature human MDDCs, resulting in the expression of surface maturation markers (CD80, CD83, CD86, and HLA-DR) as well as the increased production of cytokines, such as TNF-α, IL-6, IL-12 p70, and IL-10. Moreover, in the same HIV-VLP-loaded MDDCs the expression of additional Th1-related markers (IL-2, IL-18R, and IL-23A) as well as Th2-related markers (IL-13 and IL-15) has been found increased by a gene array analysis (2). These results indicate that baculovirus HIV-VLPs induce the production of cytokines involved in the Th1 and Th2 pathways; in particular, the production of the IL-12 p70, which represents the critical Th1 polarizing cytokine (19, 39, 48), indicates the ability of the baculovirus HIV-VLPs to induce a cell-mediated immunity and are consistent with the results obtained repeatedly in vivo (9–11).

Different kinetics of cytokine production have been previously described in activated DCs, showing early production (3 to 4 h postactivation) for TNF-α and IL-6 and delayed production (6 to 10 h postactivation) for IL-10 and IL-12 p70. This has been explained by the possible different roles played by the individual cytokines. In particular, TNF-α would be released by DCs still in peripheral tissues to further recruit DC precursors and sustain the antigen capture and presentation. IL-12, on the contrary, would be released by DCs in lymph nodes to polarize Th cells toward a Th1 phenotype (27). The production of these cytokines is increased in HIV-VLP-loaded DCs, indicating that both functions should be induced by this vaccine model in peripheral tissues. However, all of our data have been generated on DC supernatants at a single time point (16 h postactivation), and, therefore, such a sequential cytokine production cannot be ruled out. Furthermore, the significant production of TNF-α and IL-10 by HIV-VLP-activated DCs, which are known to inhibit the IL-12 p70 production, are a common finding in Ag-activated DCs and might be explained as a feedback control mechanism, intrinsic to the immune system (12, 33). In the present study, MDDCs activated by baculovirus-expressed enveloped VLPs do not release measurable IFN-γ in the cell culture supernatant, although upregulation of the IFN-γ transcription in these cells has been detected by gene expression profile analysis (2). The apparent discordance of these results could be due to a different optimal timing for the sample collection and needs to be evaluated by more extensive observations. Nevertheless, the production of IFN-γ by DCs induced with enveloped or nonenveloped VLP produced in different expression systems (prokaryotic, yeast, and baculovirus) is still a debated issue (18, 28, 30, 49, 53, 54). In particular, Yang et al. (53), reported that the $CD8\alpha^+$ $CD11C^+$ subpopulation of DCs and not the $CD4^+$ $CD11C^+$ subpopulation releases IFN-γ in response to baculovirus-expressed HPV16-L1 VLPs, although the contribution of $CD8\alpha^+$ $CD11C^+$ splenic DCs to Th1 response in vivo remains to be validated. On the other hand, all of these studies, including the present one, show the induction of IL-12 p70 production in VLP-activated MDDCs, which represents the critical Th1 polarizing cytokine.

Blocking experiments performed with MAb specific for the surface TLR2 and -4 suggested that the ability of HIV-VLPs to induce maturation of MDDCs is not mediated by these receptors. The expression of surface maturation markers was not affected at all by blocking with either of the two MAbs, and the moderate inhibition of cytokine production induced by the anti-TLR4 MAb was not statistically significant. Additional studies will be necessary to evaluate the role of other TLRs in the baculovirus-expressed HIV-VLP-induced activation of MDDCs. Yeast-derived HIV-VLPs have been previously shown to activate MDDCs through the TLR2 (49), suggesting that the expression system used for the VLP production may significantly influence the pathway involved in the biological effects induced on APCs.

Baculovirus-expressed HIV-VLPs appear to be preferentially taken up by DCs via macropinocytosis in an actin-dependent manner. The preincubation of MDDCs with cytochalasin D, however, induces a partial block of the HIV-VLP uptake, suggesting the potential involvement of additional pathways

which need to be further investigated. Moreover, the data also showed that, to a much smaller extent, the HIV-VLP uptake is mediated by the binding to the DC-SIGN receptor. The latter, in particular, belongs to the C-type lectins present on the DCs' surface to recognize carbohydrate structures within the pathogens' cell wall and to drive the pathogens' internalization for their lysosomal degradation, antigen processing, and presentation to T cells (15, 17). DC-SIGN recognizes the viral envelope glycoproteins expressed by a broad range of viruses that contain a large number of N-linked carbohydrates, including HIV-1 gp120 (1, 20, 22, 31, 40). The HIV-VLPs, which express a gp120 molecule on the surface, can take advantage of different pathways to be efficiently internalized, processed, and presented by DCs to the immune system. Finally, our data show that HIV-VLP-loaded DCs are able, in an ex vivo system, to activate autologous naïve $CD4^+$ T cells and to drive them toward a Th1 polarization, measured as IFN-γ production.

The VLP-based vaccine approach, therefore, has been confirmed as a good platform to deliver promising HIV antigens for generating efficient humoral and cellular immune responses. Additional studies on the VLP interactions and processing by different subsets of DCs, and on possible adjuvanting molecules, will lead to a better understanding of how the VLP-induced immunity might be enhanced and/or guided toward an optimal preventive and/or therapeutic immune response.

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REFERENCES

1. **Alvarez, C. P., F. Lasala, J. Carrillo, O. Muñiz, A. L. Corbi, and R. Delgado.** 2002. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in *cis* and in *trans*. J. Virol. **76:**6841–6848.
2. **Aricò, E., E. Wang, M. L. Tornesello, M. Tagliamonte, G. K. Lewis, F. M. Marincola, F. M. Buonaguro, and L. Buonaguro.** 2005. Immature monocyte derived dendritic cells gene expression profile in response to virus-like particles stimulation. J. Transl. Med. **3:**45.
3. **Bachmann, M. F., T. M. Kundig, C. P. Kalberer, H. Hengartner, and R. M. Zinkernagel.** 1993. Formalin inactivation of vesicular stomatitis virus impairs T-cell- but not T-help-independent B-cell responses. J. Virol. **67:**3917–3922.
4. **Bachmann, M. F., M. B. Lutz, G. T. Layton, S. J. Harris, T. Fehr, M. Rescigno, and P. Ricciardi-Castagnoli.** 1996. Dendritic cells process exogenous viral proteins and virus-like particles for class I presentation to CD8+ cytotoxic T lymphocytes. Eur. J. Immunol. **26:**2595–2600.
5. **Braciale, T. J., L. A. Morrison, M. T. Sweetser, J. Sambrook, M. J. Gething, and V. L. Braciale.** 1987. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. Immunol. Rev. **98:**95–114.
6. **Buonaguro, L., F. M. Buonaguro, F. Russo, M. L. Tornesello, E. Beth-Giraldo, R. Wagner, H. Wolf, and G. Giraldo.** 1998. A novel gp120 sequence from an HIV-1 isolate of the A clade identified in north Uganda. AIDS Res. Hum. Retrovir. **14:**1287–1289.
7. **Buonaguro, L., F. M. Buonaguro, M. L. Tornesello, D. Mantas, E. Beth-Giraldo, R. Wagner, S. Michelson, M.-C. Prevost, H. Wolf, and G. Giraldo.** 2001. High efficient production of Pr55gag virus-like particles expressing multiple HIV-1 epitopes, including a gp120 protein derived from an Ugandan HIV-1 isolate of subtype A. Antivir. Res. **49:**35–47.
8. **Buonaguro, L., E. Del Gaudio, M. Monaco, D. Greco, P. Corti, E. Beth-Giraldo, F. M. Buonaguro, G. Giraldo, and the Italian-Ugandan Cooperation AIDS Program.** 1995. Heteroduplex mobility assay and phylogenetic analysis of V3 region sequences of human immunodeficiency virus type 1 isolates from Gulu, northern Uganda. J. Virol. **69:**7971–7981.
9. **Buonaguro, L., L. Racioppi, M. L. Tornesello, C. Arra, M. L. Visciano, B. Biryahwaho, S. D. K. Sempala, G. Giraldo, and F. M. Buonaguro.** 2002. Induction of neutralizing antibodies and CTLs in Balb/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A (HIV-VLPAs). Antivir. Res. **54:**189–201.
10. **Buonaguro, L., M. L. Visciano, M. L. Tornesello, M. Tagliamonte, B. Biryahwaho, and F. M. Buonaguro.** 2005. Induction of systemic and mucosal cross-clade neutralizing antibodies in BALB/c mice immunized with human immunodeficiency virus type 1 clade A virus-like particles administered by different routes of inoculation. J. Virol. **79:**7059–7067.
11. **Deml, L., R. Schirmbeck, J. Reimann, H. Wolf, and R. Wagner.** 1997. Recombinant human immunodeficiency $Pr55^{gag}$ virus-like particles presenting chimeric envelope glycoproteins induce cytotoxic T-cells and neutralizing antibodies. Virology **235:**26–39.
12. **De Smedt, T., M. Van Mechelen, G. De Becker, J. Urbain, O. Leo, and M. Moser.** 1997. Effect of interleukin-10 on dendritic cell maturation and function. Eur. J. Immunol. **27:**1229–1235.
13. **Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa.** 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science **303:**1529–1531.
14. **Doxsey, S. J., F. M. Brodsky, G. S. Blank, and A. Helenius.** 1987. Inhibition of endocytosis by anti-clathrin antibodies. Cell **50:**453–463.
15. **Engering, A., T. B. Geijtenbeek, S. J. van Vliet, M. Wijers, E. van Liempt, N. Demaurex, A. Lanzavecchia, J. Fransen, C. G. Figdor, V. Piguet, and Y. van Kooyk.** 2002. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. J. Immunol. **168:**2118–2126.
16. **Fausch, S. C., D. M. Da Silva, and W. M. Kast.** 2003. Differential uptake and cross-presentation of human papillomavirus virus-like particles by dendritic cells and Langerhans cells. Cancer Res. **63:**3478–3482.
17. **Figdor, C. G., Y. van Kooyk, and G. J. Adema.** 2002. C-type lectin receptors on dendritic cells and Langerhans cells. Nat. Rev. Immunol. **2:**77–84.
18. **Garcia-Pineres, A. J., A. Hildesheim, M. Trivett, M. Williams, L. Wu, V. N. Kewalramani, and L. A. Pinto.** 2006. Role of DC-SIGN in the activation of dendritic cells by HPV-16 L1 virus-like particle vaccine. Eur. J. Immunol. **36:**437–445.
19. **Gately, M. K., L. M. Renzetti, J. Magram, A. S. Stern, L. Adorini, U. Gubler, and D. H. Presky.** 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. **16:**495–521.
20. **Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. Kewalramani, D. R. Littman, C. G. Figdor, and Y. van Kooyk.** 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell **100:**587–597.
21. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde.** 1989. Assembly and release of HIV-1 precursor $Pr55^{gag}$ virus-like particles from recombinant baculovirus-infected insect cells. Cell **59:**103–112.
22. **Halary, F., A. Amara, H. Lortat-Jacob, M. Messerle, T. Delaunay, C. Houles, F. Fieschi, F. Arenzana-Seisdedos, J. F. Moreau, and J. Dechanet-Merville.** 2002. Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. Immunity **17:**653–664.
23. **Heath, W. R., and F. R. Carbone.** 2001. Cross-presentation in viral immunity and self-tolerance. Nat. Rev. Immunol. **1:**126–134.
24. **Heil, F., P. Ahmad-Nejad, H. Hemmi, H. Hochrein, F. Ampenberger, T. Gellert, H. Dietrich, G. Lipford, K. Takeda, S. Akira, H. Wagner, and S. Bauer.** 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur. J. Immunol. **33:**2987–2997.
25. **Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer.** 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science **303:**1526–1529.
26. **Kwon, D. S., G. Gregorio, N. Bitton, W. A. Hendrickson, and D. R. Littman.** 2002. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. Immunity **16:**135–144.
27. **Langenkamp, A., M. Messi, A. Lanzavecchia, and F. Sallusto.** 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat. Immunol. **1:**311–316.
28. **Lenz, P., P. M. Day, Y. Y. Pang, S. A. Frye, P. N. Jensen, D. R. Lowy, and J. T. Schiller.** 2001. Papillomavirus-like particles induce acute activation of dendritic cells. J. Immunol. **166:**5346–5355.
29. **Lenz, P., C. D. Thompson, P. M. Day, S. M. Bacot, D. R. Lowy, and J. T. Schiller.** 2003. Interaction of papillomavirus virus-like particles with human myeloid antigen-presenting cells. Clin. Immunol. **106:**231–237.
30. **Lenz, P., D. R. Lowy, and J. T. Schiller.** 2005. Papillomavirus virus-like particles induce cytokines characteristic of innate immune responses in plasmacytoid dendritic cells. Eur. J. Immunol. **35:**1548–1556.
31. **Lin, G., G. Simmons, S. Pöhlmann, F. Baribaud, H. Ni, G. J. Leslie, B. S. Haggarty, P. Bates, D. Weissman, J. A. Hoxie, and R. W. Doms.** 2003. Differential N-linked glycosylation of human immunodeficiency virus and Ebola virus envelope glycoproteins modulates interactions with DC-SIGN and DC-SIGNR. J. Virol. **77:**1337–1346.
32. **Lyons, A. B., and C. R. Parish.** 1994. Determination of lymphocyte division by flow cytometry. J. Immunol. Methods **171:**131–137.
33. **Ma, X., J. Sun, E. Papasavvas, H. Riemann, S. Robertson, J. Marshall, R. T. Bailer, A. Moore, R. P. Donnelly, G. Trinchieri, and L. J. Montaner.** 2000.

Inhibition of IL-12 production in human monocyte-derived macrophages by TNF. J. Immunol. **164:**1722–1729.
34. **Matsumoto, M., K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, A. Yamamoto, and T. Seya.** 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J. Immunol. **171:**3154–3162.
35. **Miyanohara, A., T. Imamura, M. Araki, K. Sugawara, N. Ohtomo, and K. Matsubara.** 1986. Expression of hepatitis B virus core antigen gene in *Saccharomyces cerevisiae*: synthesis of two polypeptides translated from different initiation codons. J. Virol. **59:**176–180.
36. **Moron, V. G., P. Rueda, C. Sedlik, and C. Leclerc.** 2003. In vivo, dendritic cells can cross-present virus-like particles using an endosome-to-cytosol pathway. J. Immunol. **171:**2242–2250.
37. **Noad, R., and P. Roy.** 2003. Virus-like particles as immunogens. Trends Microbiol. **11:**438–444.
38. **Palliser, D., Q. Huang, N. Hacohen, S. P. Lamontagne, E. Guillen, R. A. Young, and H. N. Eisen.** 2004. A role for Toll-like receptor 4 in dendritic cell activation and cytolytic CD8+ T cell differentiation in response to a recombinant heat shock fusion protein. J. Immunol. **172:**2885–2893.
39. **Park, A. Y., B. D. Hondowicz, and P. Scott.** 2000. IL-12 is required to maintain a Th1 response during Leishmania major infection. J. Immunol. **165:**896–902.
40. **Pöhlmann, S., J. Zhang, F. Baribaud, Z. Chen, G. J. Leslie, G. Lin, A. Granelli-Piperno, R. W. Doms, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. **77:**4070–4080.
41. **Ruedl, C., T. Storni, F. Lechner, T. Bachi, and M. F. Bachmann.** 2002. Cross-presentation of virus-like particles by skin-derived CD8$^-$ dendritic cells: a dispensable role for TAP. Eur. J. Immunol. **32:**818–825.
42. **Ruedl, C., K. Schwarz, A. Jegerlehner, T. Storni, V. Manolova, and M. F. Bachmann.** 2005. Virus-like particles as carriers for T-cell epitopes: limited inhibition of T-cell priming by carrier-specific antibodies. J. Virol. **79:**717–724.
43. **Sallusto, F., and A. Lanzavecchia.** 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. **179:**1109–1118.
44. **Schnare, M., G. M. Barton, A. C. Holt, K. Takeda, S. Akira, and R. Medzhitov.** 2001. Toll-like receptors control activation of adaptive immune responses. Nat. Immunol. **2:**947–950.
45. **Sieczkarski, S. B., and G. R. Whittaker.** 2002. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. J. Virol. **76:**10455–10464.
46. **Takeda, K., T. Kaisho, and S. Akira.** 2003. Toll-like receptors. Annu. Rev. Immunol. **21:**335–376.
47. **Thoma-Uszynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Rollinghoff, P. L. Bolcskei, M. Wagner, S. Akira, M. V. Norgard, J. T. Belisle, P. J. Godowski, B. R. Bloom, and R. L. Modlin.** 2001. Induction of direct antimicrobial activity through mammalian toll-like receptors. Science **291:**1544–1547.
48. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **13:**251–276.
49. **Tsunetsugu-Yokota, Y., Y. Morikawa, M. Isogai, A. Kawana-Tachikawa, T. Odawara, T. Nakamura, F. Grassi, B. Autran, and A. Iwamoto.** 2003. Yeast-derived human immunodeficiency virus type 1 p55^{gag} virus-like particles activate dendritic cells (DCs) and induce perforin expression in Gag-specific CD8$^+$ T cells by cross-presentation of DCs. J. Virol. **77:**10250–10259.
50. **Underhill, D. M., and A. Ozinsky.** 2002. Toll-like receptors: key mediators of microbe detection. Curr. Opin. Immunol. **14:**103–110.
51. **Weis, W. I., M. E. Taylor, and K. Drickamer.** 1998. The C-type lectin superfamily in the immune system. Immunol. Rev. **163:**19–34.
52. **Yan, M., J. Peng, I. A. Jabbar, X. Liu, L. Filgueira, I. H. Frazer, and R. Thomas.** 2005. Activation of dendritic cells by human papillomavirus-like particles through TLR4 and NF-kappaB-mediated signalling, moderated by TGF-beta. Immunol. Cell Biol. **83:**83–91.
53. **Yang, R., F. Martinez Murillo, K.-Y. Lin, W. H. Yutzy, I. V., S. Uematsu, K. Takeda, S. Akira, R. P. Viscidi, and R. B. S. Roden.** 2004. Human papillomavirus type-16 virus-like particles activate complementary defense responses in key dendritic cell subpopulations. J. Immunol. **173:**2624–2631.
54. **Zhang, R., M. Li, C. Chen, and Q. Yao.** 2004. SHIV virus-like particles bind and activate human dendritic cells. Vaccine **23:**139–147.