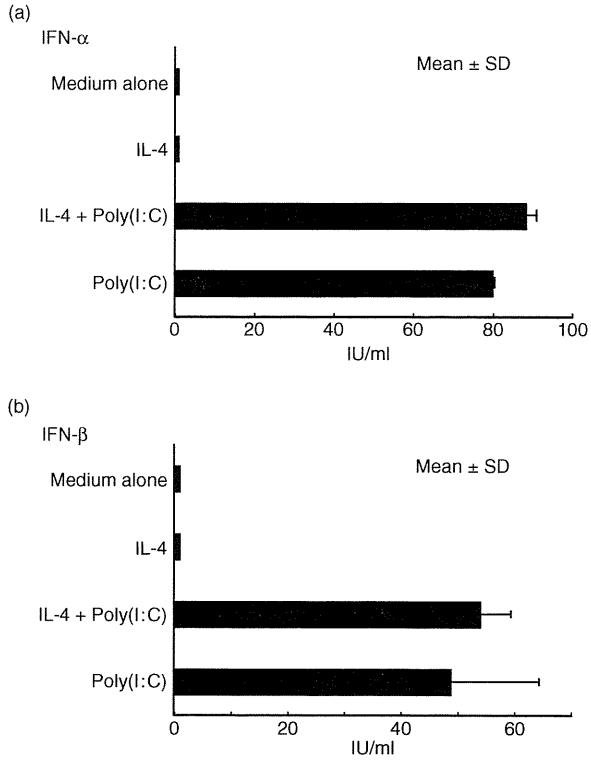


Figure 3. Measurement of type I interferon (IFN) secretion in the culture supernatant of breast milk macrophages (BrMMø) stimulated with poly(I:C). Both freshly isolated and interleukin (IL)-4-treated BrMMø produced and secreted both IFN-α and IFN-β after incubation with 50 μg/ml poly(I:C) for 24 hr, as measured by enzyme-linked immunosorbent assay (ELISA). The data shown are representative of at least four different experiments and results are expressed as the mean ± standard deviation.

external factors, such as IL-4, suggests that such treatment may inhibit DC-SIGN-mediated MTCT of HIV-1. As also shown in the dot-plot analyses in the two right-hand panels of Fig. 4b, co-culture of BrMMø with IFNs and IL-4 usually generated two subpopulations, DC-SIGN positive and negative. We found that the percentage of CD4 expression in the DC-SIGN-negative population was always higher than that in the DC-SIGN-positive population, indicating that the HIV-1-susceptible population may persist in the presence of IFNs, in particular IFN-β, when BrMMø are co-cultured with IL-4 (data not shown).

Moreover, we previously demonstrated that a dramatic reduction in chemokine receptor expression for both CXCR4 and CCR5 on BrMMø led to weak susceptibility to HIV-1 when cells were co-cultured with IL-4.[3] The reduced expression of CXCR4 and CCR5 as well as CD4 on IL-4-treated BrMMø was not augmented by treatment with IFNs (data not shown).

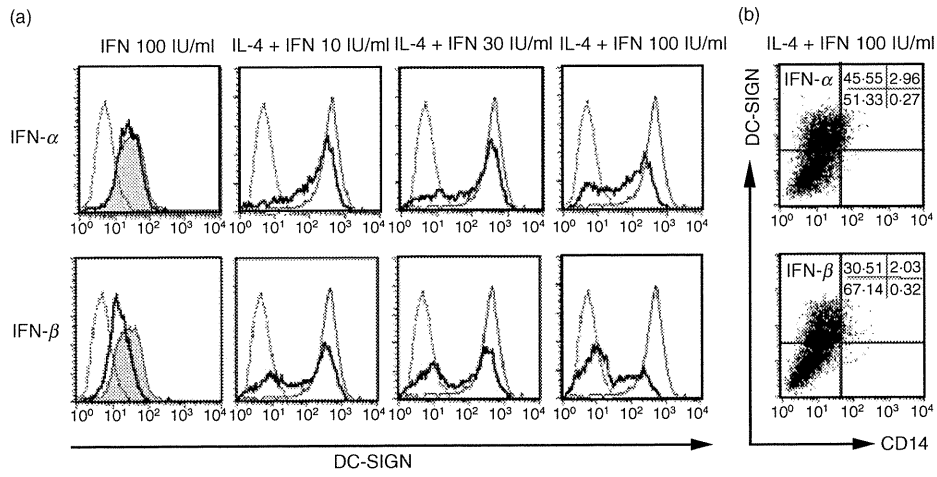
Taken together, the results suggest that, although co-culture of BrMMø with IFNs and IL-4 may reduce their capacity to transmit HIV-1 via DC-SIGN, IFN treatment of IL-4-stimulated BrMMø will not affect their HIV-1 transmissibility and susceptibility. It is also important to note that pretreatment of BrMMø with anti-IFN-α/β-receptor-specific antibodies produced a remarkable abrogation of poly(I:C)-mediated inhibition of DC-SIGN expression on BrMMø (data not shown). The findings indicate that IFNs secreted by stimulated BrMMø may down-modulate DC-SIGN expression via their IFN-α/β receptors.

DC-SIGN-mediated transmission of HIV-1 via BrMMø was significantly inhibited by treatment not only with poly(I:C) but also with IFNs

Collectively, the above findings indicate that not only IFN inducers, such as poly(I:C), but also IFNs themselves have the capacity to inhibit mother-to-child HIV-1 transmission via breast milk through down-modulation of DC-SIGN. Thus, we first co-cultured BrMMø for 24 hr with 50 μg/ml poly(I:C) and further incubated them with IL-4 for an additional 4 days after extensive washing to remove free poly(I:C). Poly(I:C)-treated, IL-4-co-cultured BrMMø were infected with a 0·2 MOI dose of the R5-type macrophage-tropic HIV-1 isolate NL(AD8) for 2 hr at 37°. After washing three times to remove free NL(AD8) virions, 1×10^4 Ghost X4/R5 cells expressing GFP were added to NL(AD8)-pulsed BrMMø and incubated for an additional 16 hr. After incubation, the loaded BrMMø were removed by gentle washing with warmed medium (D-10) and NL(AD8)-infected Ghost X4/R5 cells expressing GFP were analysed by flow cytometry to estimate transmissibility. As expected, in comparison with IL-4-treated BrMMø, poly(I:C)-pretreated BrMMø had a markedly reduced capacity to transmit HIV-1 virions to susceptible cells (Fig. 5a). Also, IL-4-mediated enhancement of HIV-1 transmission was slightly inhibited by post-treatment with poly(I:C) (Fig. 5a). It should be noted that blocking with anti-DC-SIGN-specific antibody produced a 60–70% reduction of IL-4-mediated HIV-1 transmission of BrMMø (Fig. 5a). Similarly high transmissibility of IL-4-treated BrMMø was strongly inhibited by pretreatment with type I IFNs (Fig. 5b). As also shown in Fig. 5b, it should be noted that the magnitude of inhibition was always much greater with IFN-β than with IFN-α treatment. These results indicate that, although there may be DC-SIGN-independent pathways for HIV-1 transmission of BrMMø, down-modulation of DC-SIGN expression on BrMMø through TLR3 signalling mediated by poly(I:C) or through their product IFNs may reduce HIV-1 transmission via breast-feeding.

Discussion

In the present study, although TLR3 was not detected in PBMo, TLR3 expression was clearly observed in freshly



Cell treatment	DC-SIGN expression MFI (mean ± SD)	Percentage of positive cells (%) (mean ± SD)	Viability of cells at 5 d culture (%) (mean ± SD)
Medium alone	2·9 ± 1·4	15·2 ± 4·2	87·5 ± 4·1
IL-4	47·4 ± 3·9	93·7 ± 4·7	85·5 ± 2·3
IFN-α 100 IU/ml	2·9 ± 1·4	16·0 ± 2·8	93·4 ± 2·7
IL-4 + IFN-α 10 IU/ml	20·1 ± 1·8	79·2 ± 4·9	86·9 ± 8·6
IL-4 + IFN-α 30 IU/ml	15·9 ± 3·6	69·5 ± 4·9	85·7 ± 5·3
IL-4 + IFN-α 100 IU/ml	8·8 ± 2·1	48·8 ± 5·3	87·9 ± 8·8
IFN-β 100 IU/ml	1·9 ± 1·1	6·0 ± 5·3	90·9 ± 3·4
IL-4 + IFN-β 10 IU/ml	14·6 ± 4·7	70·7 ± 3·6	84·0 ± 7·6
IL-4 + IFN-β 30 IU/ml	6·2 ± 1·2	52·9 ± 1·2	85·5 ± 0·9
IL-4 + IFN-β 100 IU/ml	2·9 ± 1·4	29·7 ± 2·8	83·3 ± 7·8

Figure 4. Effect of type I interferons (IFNs) on dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN) expression on breast milk macrophages (BrMMø). BrMMø co-cultured with IFN for 5 days are shown as thick lines and isotype controls as dotted lines (a). Both type I IFNs, particularly IFN-β (lower panels), showed a strong capacity to suppress the enhancement of DC-SIGN expression in BrMMø cultured with IFN for 5 days in the presence of 20 ng/ml IL-4 (indicated as thin lines), and this suppression was dose-dependent. As controls (the two left-hand panels), DC-SIGN expression in BrMMø cultured for 5 days in medium alone is shown as shaded lines, that for isotype controls as dotted lines, and that for IFN-treated BrMMø as thick lines. (b) Dot-plot analyses for BrMMø co-cultured with IFNs and IL-4. The effect of IFNs on DC-SIGN expression on BrMMø co-cultured with IL-4 is shown in the table, which gives the percentage of positive cells and their mean fluorescence intensity (MFI) and viability (mean ± standard deviation). Data shown are representative of at least three separate experiments.

isolated BrMMø producing GM-CSF intracellularly. As expected, TLR3 was detected in PBMo when the cells were incubated with GM-CSF. In contrast, freshly isolated neutrophils that expressed all TLRs except TLR3 did not express TLR3 even when co-cultured with GM-CSF.[16] It has also been reported that GM-CSF enhances the expression of TLR2 or TLR4 in neutrophils and TLR2 in monocytes.[17] These findings indicate that there may be at least two distinct subpopulations of PBMo, one that provides protection against extracellular bacteria, such as neutrophils expressing TLR2 and TLR4, and one that provides protection against intracellular viruses, such as macrophages expressing TLR3, and that GM-CSF seems to have the capacity to reinforce the innate alert system

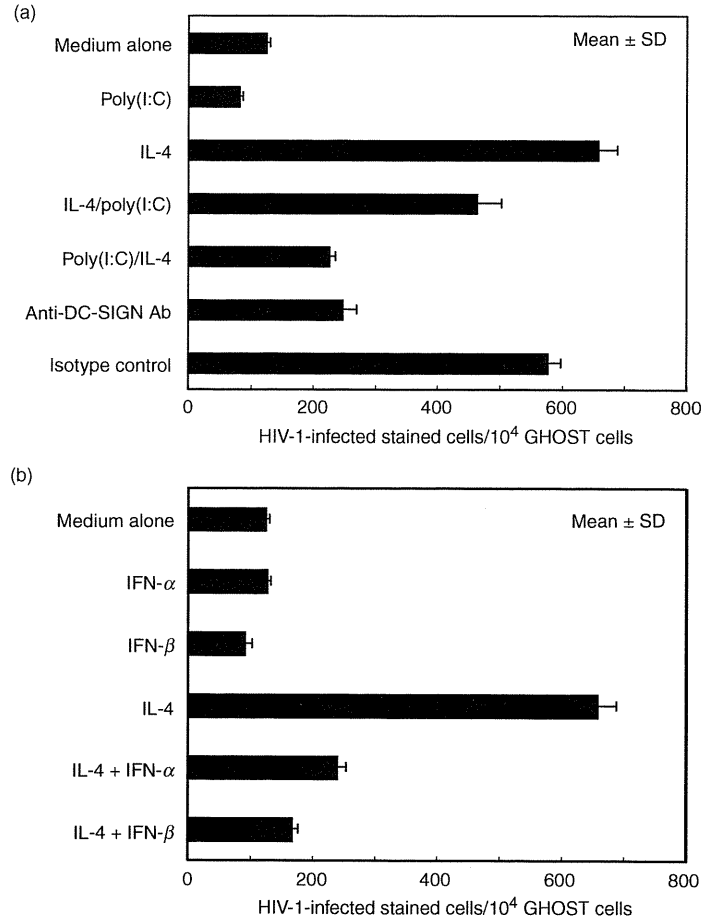


Figure 5. Dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN)-**mediated transmission of human immunodeficiency virus type 1 (HIV-1) via** breast milk macrophages (BrMMø) **was inhibited by treatment not only with poly(I:C) (a) but also with interferons (IFNs) (b). (a) To examine the effect of poly(I:C) treatment on DC-SIGN-mediated HIV-1 transmission, BrMMø incubated for 24 hr with 50 μg/ml poly(I:C) were further incubated with either culture medium or interleukin (IL)-4 for an additional 4 days after extensive washing to remove free poly(I:C). Also, BrMMø co-cultured with IL-4 for 4 days were further incubated with 50 μg/ml poly(I:C) for the final 24 hr. These poly(I:C)-treated BrMMø were infected with** a 0·2 **multiplicity of infection** (MOI) dose of the R5-type **macrophage-tropic HIV-1 isolate NL(AD8) for 2 hr at 37°. After extensive washing to remove free NL(AD8) virions, 1×10^4 Ghost X4/R5 cells were added to NL(AD8)-pulsed BrMMø and incubated for an additional 16 hr. After incubation, the HIV-1-loaded BrMMø were gently removed with warmed D-10, and NL(AD8)-infected Ghost X4/R5 cells expressing green fluorescent protein (GFP) were analysed by flow cytometry to estimate transmissibility. Also, to examine the possibility of a DC-SIGN-independent pathway for HIV-1 transmission by BrMMø, IL-4-stimulated BrMMø pretreated with 20 μg of anti-DC-SIGN monoclonal antibody (mAb) for 30 min on ice were infected with NL(AD8) for 2 hr at 37° and added to Ghost X4/R5 cells. (b) Similarly, to investigate the effect of IFNs on DC-SIGN-mediated HIV-1 transmission, BrMMø co-cultured with IFNs and IL-4 for 5 days were infected with a 0·2 MOI dose of NL(AD8) for 2 hr at 37°, washed to remove free virions, and incubated with 1×10^4 Ghost X4/R5 cells for an additional 16 hr before analysis by flow cytometry. Data are representative of at least four experiments and the results are expressed as the mean ± standard deviation.**

in PBMo providing protection against various invasive pathogens through enhancement of the TLR network.

As we reported previously, GM-CSF production was detected in freshly isolated BrMMø, and PBMo gained the ability to produce GM-CSF upon exposure to breast milk.[4] The findings strongly suggest that breast milk has the potential to stimulate PBMo to become TLR3-positive cells. Moreover, freshly isolated TLR3-positive BrMMø also expressed DC-SIGN, which captures various virions, such as HIV-1 and West Nile virus (WNV), and transmits them to susceptible cells. It has recently been reported that binding of the glycosylated WNV envelope protein to DC-SIGN leads to a reduction in the expression of TLR3 in macrophages from young donors via the signal transducer and activator of transcription 1 (STAT1)-mediated pathway.[18] Thus, binding signals of DC-SIGN mediated by target virions may down-modulate the internal expression of TLR3, which would act as an alert signalling the invasion of foreign viruses. Indeed, we have observed a similar reduction of TLR3 expression in IL-4-treated

BrMMø, which have up-regulated expression of DC-SIGN and down-regulated expression of chemokine receptors,[3] when incubated with HIV-1 (Y.Y., E.W., and H.T., unpublished observations). The findings suggest that the direct attachment of HIV-1 to DC-SIGN on BrMMø may cause a negative signal to down-regulate TLR3 expression.

Conversely, TLR3-mediated alert signalling seems to inhibit both intracellular virus replication and intercellular virus transmission via DC-SIGN. Here we found a reduction in DC-SIGN expression on both freshly isolated BrMMø and IL-4-treated BrMMø when they were stimulated with a synthetic TLR3 ligand, poly(I:C). These findings indicate that TLR3 in BrMMø appears to act as a 'button' that can be pressed to cancel DC-SIGN-mediated virus transmission, and this cancelling signal is sent via the products of internally replicated virions. Collectively, DC-lineage BrMMø seem to be unique HIV-1 carriers equipped with receptors allowing entrance of the virus (CD4 and chemokine receptors), a mechanism for virus attachment (DC-SIGN), and a 'cancel button' (TLR3, the cellular expression of which seems be regulated by GM-CSF). The reason why BrMMø have such equipment for use against viruses remains to be determined.

Breast milk from an HIV-infected women may contain both cell-free HIV-1 and cell-associated virus.[19] However, as we reported previously, high transmissibility is mediated through HIV-1 virions captured by DC-SIGN but not through cell-free virus particles released from HIV-1-infected cells,[3] although the actual impact of human breast milk on HIV infection in CD4 cells remains poorly understood. Nevertheless, it has recently been reported that breast milk itself may have a protective function against cell-free HIV-1 but may be less effective at blocking infection by cell-associated virus.[20] Thus, to prevent vertical transmission of HIV-1 through breast-feeding, it may be important to inhibit the acquisition of free HIV-1 virions via specific receptors such as DC-SIGN on the BrMMø of HIV-infected women.

When TLR3 in BrMMø was stimulated by the dsRNA poly(I:C), a considerable amount of type I IFNs, such as IFN-α and IFN-β, was detected in the culture supernatant. In addition, significant down-modulation of DC-SIGN on BrMMø was observed when they were stimulated with IL-4 together with IFN-α or IFN-β. As expected, in comparison with IL-4-treated BrMMø, poly(I:C)-pretreated and IL-4-expanded BrMMø had markedly reduced capacities to transmit HIV-1 virions to susceptible cells. Similarly, the high transmissibility of IL-4-treated BrMMø was also markedly inhibited by pretreatment with either IFN-α or IFN-β. Also, DC-SIGN expression on IL-4-treated BrMMø was slightly down-modulated by 24 hr of poly(I:C) exposure, which also inhibited HIV-1 transmission to some extent (Fig. 5a). Moreover, as shown in Fig. 5a, anti-DC-SIGN antibody treatment produced a 60–70% reduction of HIV-1 transmission by IL-4-treated mature BrMMø. Therefore, blocking of DC-SIGN-mediated transmission of HIV-1 may have great potential to prevent MTCT HIV transmission, although the possible existence of a DC-SIGN-independent transmission pathway should also be considered.[21]

These results indicate that decreased DC-SIGN expression may be the cause of decreased transmission of HIV-1 virus, and that BrMMø have the ability to reduce their external DC-SIGN expression by stimulating their internal TLR3, and thus the TLR3 in BrMMø seems to be a critical molecule to determine the MCTC of HIV-1. The findings presented here may be of use in developing strategies for preventing MTCT of HIV-1 via breast-feeding.

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Disclosures

The authors have no competing financial interests or conflicts of interests to disclose.

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Research Article

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HIV-1 Gag-Virus-Like Particles Induce Natural Killer Cell Immune Responses via Activation and Maturation of Dendritic Cells

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Key Words
Gag-virus-like particles · Dendritic cells · Natural killer cells · HIV · Innate immunity

Abstract
Despite the extensive efforts that have been made to combat acquired immune deficiency syndrome (AIDS), the number of people infected each year with human immunodeficiency virus type 1 (HIV-1) is still increasing worldwide, and a safe and effective vaccine to control HIV infection is urgently needed. Recently, the natural killer (NK) cell-mediated innate immune response, which represents the first line of defense against infections, has attracted attention for its role in combating HIV infection and disease progression. In the present study, we investigated the immunogenic ability of HIV-1 Gag-virus-like particles (Gag-VLPs) to induce NK cell immune responses in vitro and in vivo. Gag-VLPs efficiently activated human monocyte-derived dendritic cells (MDDCs), eliciting MDDC maturation with an associated increase in the surface expression of CD80, CD86 and MHC classes I and II, MDDC proliferation and proinflammatory cytokine production. Gag-VLP-treated MDDCs subsequently activated autologous NK cells, leading to their proliferation and production of interferon-γ and to the upregulation of NK cell cytotoxicity against YAC-1 cells and HIV-1-infected $CD4^+$ T cells. In addition, we introduced a 2-phase immunization strategy in BALB/c mice to assess the role of DCs in the induction of NK cell immune responses by Gag-VLPs in vivo. Our findings reveal that Gag-VLPs efficiently activate DCs, which in turn induce innate and Gag-specific immune responses in NK cells.

Introduction

Control of human immunodeficiency virus (HIV) infection by highly active antiretroviral therapy has greatly decreased morbidity and mortality due to acquired immune deficiency syndrome (AIDS). However, despite enormous efforts, the number of HIV-1-infected individuals increases on a daily basis, and we still lack prophylactic and therapeutic vaccines against HIV-1. Several vaccine components such as live attenuated viruses, recombinant viral vectors and DNA vaccines have been proven to be effective in inducing cellular and humoral immune responses; however, serious safety concerns and the limited efficacy of some of these vaccine components prevent their clinical application as anti-HIV vaccines. The possible risks of live attenuated viruses and recombinant viral vectors, including (1) potential reversion to a virulent pathogenic form of the attenuated virus, especially in immunodeficient recipients, (2) possible recombination of the vaccine strain with wild-type pathogenic

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virus in an infected individual, (3) dysregulation of the immune system by viral proteins and (4) ability of the proviral genome to integrate into the host genome, have prevented their approval for use in humans [1, 2]. Furthermore, several potential risks of DNA vaccines, including tumor induction due to chromosomal integration, autoimmune reactions as a result of the induction of anti-DNA antibodies or adverse reactions due to the biological activity of the vector or the expressed antigens have not yet been ruled out [3]. Because of these difficulties, a safe and effective HIV vaccine suitable for prophylactic and therapeutic use in humans is urgently needed.

Virus-like particles (VLPs) are stable, self-assembling, nonreplicating and noninfectious particles capable of inducing immune responses without containing any viral genome or other potentially toxic viral gene products [4–6]. Recent research in HIV vaccine strategies has focused on the use of VLPs as highly attractive HIV-1 vaccine candidates because of their ability to elicit humoral and cellular immunity more effectively than soluble proteins [7, 8].

VLPs are incorporated into dendritic cells (DCs) via actin-dependent macropinocytosis and endocytosis. VLP uptake is also induced by receptor-mediated endocytosis. Mannose-recognizing receptors, including the DC-SIGN receptor, which belongs to the C-type lectin family, are reportedly involved in this process. The DC-SIGN receptor, present on the DC surface, recognizes VLPs and enhances internalization for lysosomal degradation, antigen processing and cross-presentation on both MHC class I (MHC I) and MHC class II (MHC II) molecules. VLPs induce maturation and activation of monocyte-derived DCs (MDDCs), and this effect is partially mediated through Toll-like receptors [9–11]. Previous reports have shown that Gag-VLPs induce $CD4^+$ T cell activation and $CD8^+$ cytotoxic T lymphocyte (CTL) responses as well as B cell-mediated humoral immunity [9, 12–17]. Despite these findings, little is known about the ability of Gag-VLPs to elicit natural killer (NK) cell immune responses.

DCs are the most potent antigen-presenting cells of the immune system and play a pivotal role in the initiation and regulation of immune responses to various antigens. Antigen-loaded DCs acquire a mature phenotype associated with the production of proinflammatory cytokines and stimulate $CD4^+$ T cells and CTL responses [18–20]. The mature DCs migrate towards the lymphoid organs, where they interact with NK cells, inducing the activation and proliferation of these cells. The interaction between DCs and NK cells initiates innate signals that orchestrate subsequent adaptive immune responses [21–25].

NK cells are the primary effector cells of the innate immune system and are essential for the immune control of infections and tumors through production of cytokines such as interferon (IFN)-γ and the potent lysis of infected cells or tumor cells, without the need for prestimulation. Notably, other than activated T cells, NK cells are the only known source of IFN-γ which shapes the pattern of innate and adaptive immune responses [26–28]. The innate immune response of NK cells against HIV is crucial both for the subsequent course of the infection and for the induction of an efficient adaptive response. Both NK cell cytotoxicity and cytokine production are impaired in HIV-1 viremic patients. Preservation of NK cell activity and cell number correlates with lower plasma viral load and slower progression to AIDS [29, 30]. Moreover, because NK cell responses following vaccination correlate with clinical outcome more closely than do T cell responses, the effectiveness of an HIV vaccine should be evaluated by monitoring the potentially important NK cell responses after immunization [31].

In this article, we demonstrate that Gag-VLP-treated MDDCs (VLP-DCs) underwent activation and maturation characterized by the increased surface expression of costimulatory molecules, including CD80, CD86, MHC I and MHC II, and the production of proinflammatory cytokines, including IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ. MDDCs subsequently activated NK cells, inducing their proliferation, release of IFN-γ and enhanced cytotoxicity. Next, we immunized BALB/c mice with Gag-VLPs in a phase I immunization study to examine the activation of DCs and NK cells in vivo. These experiments were followed by a phase II immunization study in which mice were inoculated with splenic DCs of phase I-immunized mice to assess the contribution of DCs to the induction of NK cell immune responses.

In conclusion, we show that Gag-VLPs induce activation and maturation of DCs, which then mediate NK cell innate and Gag-specific immune responses. Our findings reveal that Gag-VLPs are an effective HIV-1 vaccine candidate for prophylactic and therapeutic purposes in humans.

Materials and Methods

Mice and Cell Culture

Six- to 8-week-old female BALB/c mice were purchased from Nippon SLC (Hamamatsu, Japan). All mice were maintained under specific-pathogen-free conditions. All animal experiments were carried out in compliance with institutional guidelines and approved by the animal experimentation committee of the Chiba

Institute of Technology. *Spodoptera frugiperda* (Sf9) insect cells were grown at 27°C in BD Gold serum-free medium containing 100 μg/ml kanamycin sulfate. HeLa cells and HEK-293T cells were maintained in DMEM culture medium (Sigma), and YAC-1 cells were maintained in RPMI-1640 culture medium (Sigma). Both DMEM and RPMI-1640 were supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma).

Generation of Recombinant Baculovirus and Wild-Type HIV-1

The baculovirus transfer vector pAcCAG-gag containing the HIV-1 gag gene was generated by cloning of cDNA encoding the HIV-1 (NL4-3) gag gene into the multiple cloning site of the baculovirus transfer vector pAcCAG-MCS2, under control of the mammalian CAG promoter. HIV-1 gag was first cloned into the *Dra*III and *Nru*I sites of pcDNA3.1 and then excised with *Kpn*I and *Not*I endonucleases and ligated into the *Kpn*I and *Not*I sites of the pAcCAG-MCS2 vector. Recombinant baculovirus (AcCAG-gag) was generated by cotransfection of Sf9 cells with the baculovirus transfer vector pAcCAG-gag and AcMNPV DNA using a BD Gold Baculovirus transfection kit (BD Biosciences) according to the manufacturer's protocol. AcCAG-gag was expanded in Sf9 cells, and the titer was determined by plaque assay. Wild-type HIV-1 NL4-3 (X4-tropic) was generated by transfection of HEK-293T cells with HIV-1 plasmid (pNL4-3) using FuGENE 6, and culture supernatant was collected on day 2. Viral p24 content was measured by ELISA using Lumipulse.

Production of Gag-VLPs in HeLa Cells

HeLa cells were infected with recombinant baculovirus AcCAG-gag at a multiplicity of infection of 100 for 1 h at 37°C, washed twice with PBS to thoroughly remove the baculovirus and then replenished with fresh culture medium. After incubation for 3 days, the culture supernatant was clarified by centrifugation at 2,500 rpm for 20 min and filtered through a 0.45-μm filter. VLPs were then pelleted at 19,000 rpm for 2 h at 4°C with a Beckman NVT-100 rotor, resuspended in PBS, purified by 20–60% sucrose gradient centrifugation at 25,000 rpm for 90 min and again resuspended in PBS. Protein concentrations of purified Gag-VLP samples were determined using a BCA™ protein assay kit (Pierce). Gag-VLP preparations were determined to be free of endotoxin (<0.01 endotoxin units/ml) using a Pyrodick endotoxin kit (Seikagaku Co., Tokyo, Japan).

Preparation of Human MDDCs

Human peripheral blood mononuclear cells were separated from buffy coats using Ficoll-Paque density gradient centrifugation. Monocytes were allowed to adhere to plastic plates at 37°C for 2 h, and nonadherent cells (peripheral blood lymphocytes, PBLs) were removed. Monocytes were washed with medium and cultured for 6 days in DC culture medium consisting of RPMI-1640 (Sigma) supplemented with 2 mM L-glutamine (Sigma), 50 μM 2-mercaptoethanol (Sigma), 1% nonessential amino acids (Gibco) and 10% fetal calf serum in the presence of 20 ng/ml GM-CSF and IL-4 (PeproTech, London, UK). On days 3 and 5, one half of the volume of medium was replaced with fresh medium supplemented with GM-CSF and IL-4. On day 6, MDDCs were purified by positive selection using a $CD1c^+$ (BDCA-1) DC isolation kit (Miltenyi Biotec Inc., Auburn, Calif., USA).

MDDC Activation and Analysis

Human MDDCs (1×10^6) were incubated with Gag-VLPs (10 μg/ml), lipopolysaccharide (LPS; 1 μg/ml; Sigma) or medium alone (control) for 24 h. For phenotypic analysis, MDDCs were washed and stained with FITC-conjugated anti-human CD1a and PE-conjugated anti-human CD80, CD86, HLA-ABC or HLA-DR (eBioscience) and analyzed using a FACSCalibur flow cytometer and CellQuest software. Culture supernatants were collected to quantify IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ levels using ELISA kits (BD Biosciences, San Diego, Calif., USA). MDDC proliferation was evaluated using a CellTiter 96 proliferation assay kit (Promega) and carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) labeling. For the proliferation assay, MDDCs treated with Gag-VLPs, LPS or medium alone were incubated in a 96-well plate (5×10^3 cells/well in 100 μl) for 5 days in triplicate wells. CellTiter 96 reagent containing MTS (20 μl) was added to each well, and samples were incubated for 4 h at 37°C. The absorbance of light released from formazin was recorded with a plate reader. For CFSE labeling, MDDCs (5×10^5) were loaded with 5 μM CFSE and cultured for 5 days. Proliferation was analyzed by measuring the decrease in CFSE fluorescence intensity by flow cytometry after staining cells with anti-CD1a-PE.

NK/DC Coculture and Analysis of NK Cell Activation

MDDCs were treated with Gag-VLPs, LPS or medium for 24 h and washed thoroughly with PBS before coculturing with NK cells. Autologous human NK cells were isolated from PBLs using a $CD56^+/CD16^+$ NK cell isolation kit (Miltenyi Biotec) and cultured for 12 h in the presence of IL-2. NK cells (5×10^5) were cocultured with MDDCs (1×10^5) at a ratio of 5:1 for 18 h. NK cell activation was analyzed by flow cytometry after staining cells with anti-CD56-PE and anti-CD69-FITC.

Intracellular Cytokine Staining

Intracellular cytokine staining for IFN-γ-producing NK cells from NK/DC cocultures was performed after 18 h of coculture. The transport inhibitor brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, Mo., USA) was added to cocultures for the last 4 h. NK cells were surface stained with anti-CD56-PE and permeabilized with Cytofix/Cytoperm reagent (BD Pharmingen) according to the manufacturer's instructions. Cells were then stained with anti-IFN-γ-FITC and examined by FACS analysis.

NK Cell Proliferation

NK cell proliferation was analyzed using the CellTiter 96 proliferation assay and CFSE labeling. NK cells were cocultured with MDDCs treated with Gag-VLP or LPS or with untreated control MDDCs at a DC to NK ratio of 1:5 for 5 days. NK cell proliferation was analyzed using the CellTiter 96 proliferation assay kit (Promega) as described above. For CFSE labeling, NK cells were labeled with 5 μM CFSE and cocultured with MDDCs at a DC to NK ratio of 1:5 for 5 days. To assess the degree of proliferation, CFSE-labeled NK cells were then stained with anti-CD56-PE, and the decrease in CFSE fluorescence intensity was measured by flow cytometry.

NK Cell Cytotoxicity Assays

Autologous $CD4^+$ T cells were separated from PBLs using a human $CD4^+$ T cell isolation kit (Miltenyi Biotec) and cultured for 12 h in the presence of IL-2. $CD4^+$ T cells were infected with

HIV-1 (NL4-3; 100 ng of p24 for 1 × 10^6 cells) for 2 h. $CD4^+$ T cells or YAC-1 cells were seeded into a 96-well round-bottomed tissue culture plate in triplicate (1 × 10^4 cells/well) and used as target cells. Effector NK cells were cocultured with Gag-VLP or LPS-treated MDDCs for 24 h and added to target cells at effector to target ratios of 10:1, 5:1, 2.5:1 and 1.25:1, in a total volume of 100 μl, and incubated for 4 h. Unstimulated NK cells were added as a control. Lysis of target cells was determined by measuring lactate dehydrogenase (LDH) release using the Cytotox 96 nonradioactive cytotoxicity assay kit (Promega Corp., Madison, Wisc., USA). Spontaneous release of LDH from target cells was less than 15% of the maximum release.

Immunization of Mice and Analysis of Immune Responses

Six-week-old female BALB/c mice were immunized using a 2-phase immunization procedure. In the phase I immunization, 4 groups of BALB/c mice, each consisting of 3 subgroups with 4 mice each, were injected intramuscularly with 50 μg of Gag-VLPs or 1 μg of LPS resuspended in 100 μl of sterile PBS without addition of adjuvants. Control mice were left untreated. Twenty-four hours after priming, mice from the first group were sacrificed, spleens were removed and activation of splenic DC and NK cells was assessed by flow cytometry. Seven days after priming, the first booster immunization was administered to the remaining 3 groups of mice using the same doses. Twenty-four hours after the first booster injection, mice from the second group were sacrificed and spleens were removed for flow cytometry. On day 14, the remaining 2 groups of mice were given a second booster injection using the same doses. After 24 h, mice from the third group (donor mice) were sacrificed, and blood samples were collected for ELISA quantification of serum IFN-γ. Spleens were removed for flow cytometry. At the same time, splenic DCs were separated from the remaining spleen cells using a mouse $CD11c^+$ DC isolation kit (Miltenyi Biotec) and subsequently used in phase II immunizations. Mice from the fourth group were sacrificed 3 weeks after the second booster injection, and spleens were collected. A portion of the spleen cells was used for intracellular staining of IFN-γ-producing NK cells without re-stimulation. The remaining spleen cells were re-stimulated overnight with 5 μg/ml Gag-VLPs and used for ex vivo cytotoxicity assays and intracellular staining of IFN-γ-producing NK cells. For intracellular staining, brefeldin A (10 μg/ml) was added, and samples were incubated for an additional 4 h and processed as described above.

In the phase II immunization, 4 groups of (recipient) BALB/c mice, each consisting of 5 animals, were intraperitoneally injected with purified splenic DCs (1 × 10^6 cells/mouse) from each group of mice from the phase I immunization. All mice were sacrificed after 24 h, serum samples were collected for the quantification of IFN-γ and spleens were removed for flow cytometry analysis. All immunizations were performed in 2 independent experiments in accordance with institutional animal experiment guidelines.

Ex vivo Cytotoxicity Assay

NK cells were separated from the spleens of the fourth group of phase I-immunized or control mice using a mouse NK cell isolation kit (Miltenyi Biotec) after restimulation with Gag-VLPs and used as effector cells. Spleen cells isolated from non-immunized BALB/c mice were used as target cells. Target spleen cells were pulsed with Gag-VLPs (5 μg/ml) for 2 h and seeded in a 96-well round-bottomed tissue culture plate (1 × 10^4 cells/well) in triplicate. Effector NK cells were then added at effector to target ratios of 10:1, 5:1, 2.5:1 and 1.25:1 and incubated for 4 h. Lysis of target cells was determined by measuring LDH release using the Cytotox 96 nonradioactive cytotoxicity assay kit as mentioned above.

Statistical Analysis

All data are presented as means ± SD. Statistical analysis was performed using Student's t test. A p value of <0.05 was considered a significant difference.

Results

Gag-VLPs Induce Activation and Proliferation of DCs

Given the crucial role of DCs in antiviral innate immunity, we sought to investigate whether HIV-1 Gag-VLPs could activate human DCs in vitro. Treatment of MDDCs with Gag-VLPs resulted in increased expression of the activation markers CD80, CD86, MHC I and MHC II. MHC II expression was especially elevated and reached levels higher than those observed upon treatment of MDDCs with LPS (fig. 1a). We next examined the secretion of IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ by VLP-DCs in the culture supernatant. The levels of cytokines induced by VLPs were comparable to those induced by LPS (fig. 1b). We then investigated the proliferation of MDDCs using a proliferation assay kit and CFSE labeling. The results of both experiments showed that VLP-DCs and LPS-stimulated DCs (LPS-DCs) proliferated to a similar extent (fig. 1c). These results indicate that Gag-VLPs induce activation and maturation of DCs, which are associated with the production of specific cytokines and proliferation in vitro.

Activation of NK Cells by VLP-DCs

To determine whether human NK cells were functionally modified by VLP-DCs in vitro, MDDCs were cocultured with autologous NK cells at a 1:5 ratio for 18 h, and NK cell activation was evaluated by measuring the expression of the NK cell activation marker CD69. NK cells cocultured with VLP-DCs exhibited a statistically significant 6-fold increase in CD69 expression over NK cells cocultured with control DCs (fig. 2a). We next assessed the amount of IFN-γ in cocultured supernatants by ELISA. NK cells cocultured with VLP-DCs produced higher levels of IFN-γ than did NK cells cocultured with control DCs (fig. 2b, left panel). This finding was further substantiated by intracellular staining of NK cells, which showed an increase in the frequency of IFN-γ-producing NK cells following coculture with VLP-DCs (fig. 2b, right panel).

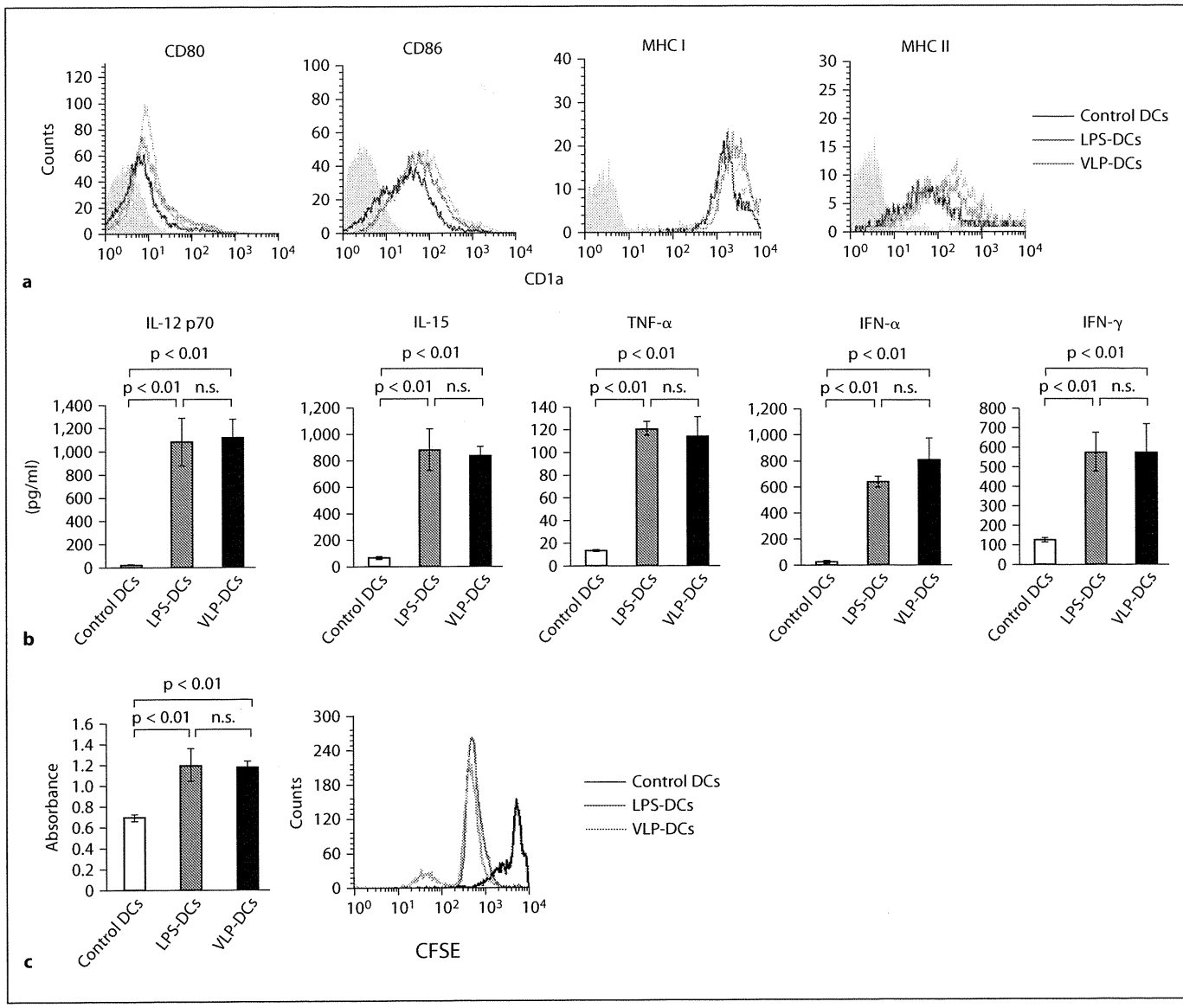


Fig. 1. Gag-VLPs induce activation and proliferation of MDDCs. **a** Expression of the costimulatory molecules CD80, CD86, MHC I and MHC II on human MDDCs treated with Gag-VLPs (10 μg/ml), LPS (1 μg/ml) or medium alone for 24 h was analyzed by flow cytometry. Gray curves represent isotype controls. Results are representative of 3 independent experiments using cells from 3 different donors. **b** Levels of the cytokines IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ in culture supernatants were measured by ELISA. All results are expressed as means ± SD of triplicate wells. Statistical analysis was performed using Student's t test. **c** MDDCs were treated with Gag-VLPs, LPS or medium and cultured for 5 days. MDDC proliferation was evaluated using a proliferation assay kit. The results are expressed as means ± SD of triplicate wells. For CFSE labeling, MDDCs were labeled with 5 μM CFSE and treated with Gag-VLPs, LPS or medium and cultured for 5 days. MDDC proliferation was evaluated by measuring the decrease in CFSE fluorescence intensity by flow cytometry. The results are representative of 3 independent experiments from 3 different donors.

We next examined NK cell proliferation after coculture with VLP-DCs. The results of both proliferation assays (fig. 2c, left panel) and CFSE labeling of NK cells (fig. 2c, right panel) demonstrated that NK cells cocultured with VLP-DCs proliferated at a rate comparable to that of NK cells cocultured with LPS-DCs after 5 days, while NK cells cocultured with control DCs showed only a basal level of proliferation. To investigate NK cell cytotoxicity, we used NK-sensitive YAC-1 cells (fig. 2d, left panel) and HIV-infected autologous $CD4^+$ T cells (fig. 2d,

right panel) as target cells and NK cells cocultured with VLP-DCs, LPS-DCs or control DCs as effector cells in cytotoxicity assays. Both the non-specific and the Gag-specific cytolytic activity of NK cells were shown to be strongest in NK cells cocultured with VLP-DCs (fig. 2d).

In vivo Activation of DC and NK Cells by Gag-VLPs

To investigate whether Gag-VLPs can induce immune responses in vivo, we introduced a 2-phase immunization strategy in BALB/c mice as illustrated in figure 3a. In the phase I immunization, we assessed the efficacy of Gag-VLPs in activating murine DCs and NK cells in vivo. To examine whether booster immunizations of mice with Gag-VLPs may be required to induce stronger NK cell activation and Gag-specific memory responses in vivo, we immunized 4 groups of mice and evaluated NK cell responses after each immunization.

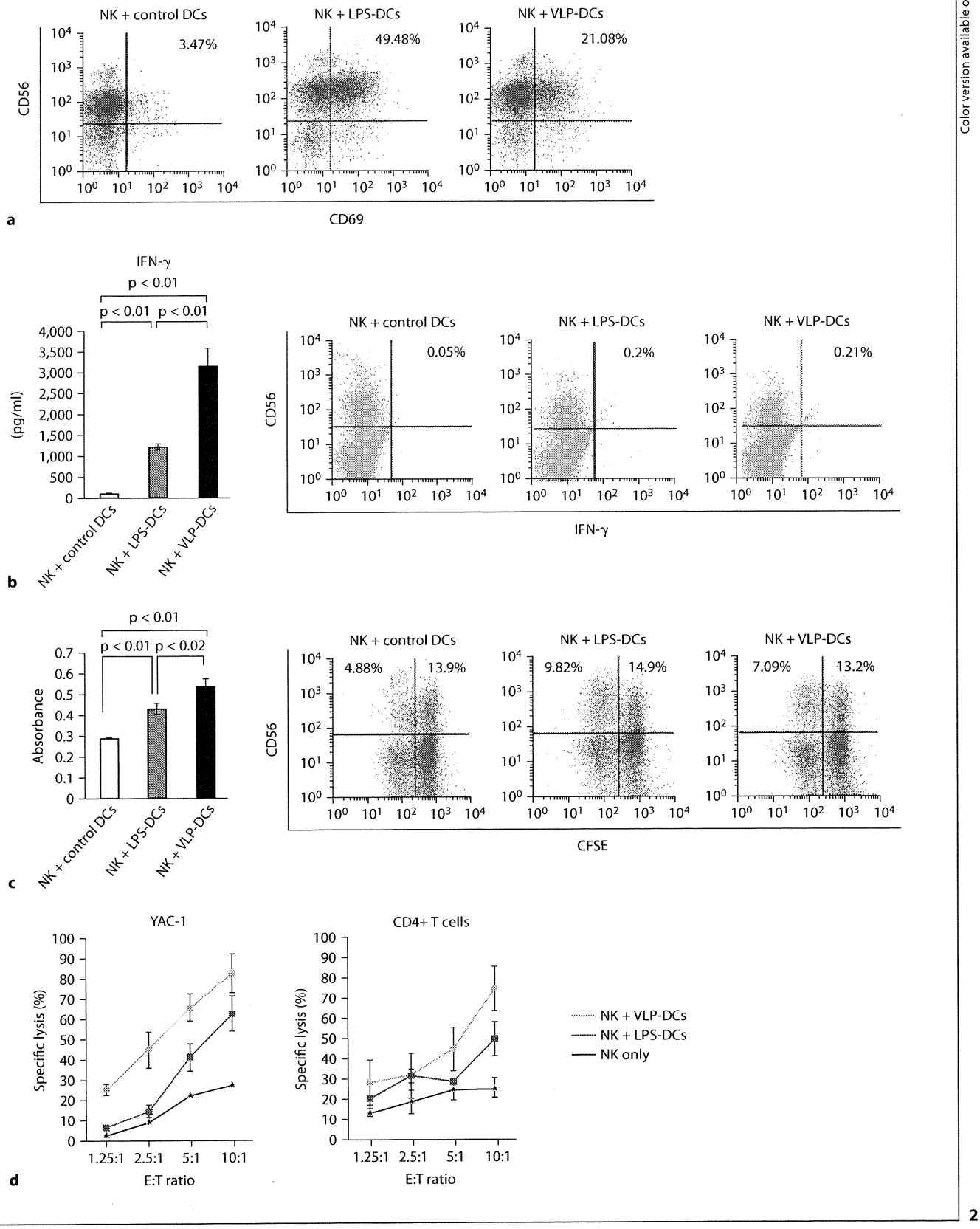
Splenic DCs of Gag-VLP-immunized mice expressed higher levels of MHC I, MHC II and the DC activation markers CD80 and CD86 than splenic DCs of control mice, and the degree to which these markers were up-regulated was similar to that observed in splenic DCs of LPS-immunized mice (fig. 3b). In vivo activation of NK cells was examined by analyzing CD69 expression on splenic NK cells after each immunization. NK cells from Gag-VLP-immunized mice showed a significant increase in the expression of CD69 after the second booster injection (fig. 3c). We next investigated serum IFN-γ levels 24 h after the second booster injection (fig. 3d, left panel) and the frequency of IFN-γ-secreting splenic NK cells after restimulation with Gag-VLPs, 3 weeks after the second booster injection. Only immunization with VLPs resulted in a Gag-specific memory response, as evidenced by an increased frequency of IFN-γ-producing splenic NK cells only in VLP-immunized mice (fig. 3d, right panel).

Next, to investigate whether Gag-VLPs could induce Gag-specific NK cell cytolytic responses, splenic NK cells (effector cells) of the fourth group of phase I-immunized mice or control mice were restimulated and cocultured with Gag-VLP-loaded splenocytes (target cells) at various effector to target ratios, and cytotoxicity was analyzed by measuring the lysis of target cells. NK cells from Gag-VLP-immunized mice displayed a dose-dependent increase in Gag-specific cytolytic activity higher than that observed in control mice and LPS-immunized mice (fig. 3e). Together, these data demonstrate that HIV-1 Gag-VLPs induce not only NK cell innate immune responses but also Gag-specific memory responses in vivo.

DCs from Gag-VLP-Immunized Mice Trigger NK Cell Activation and IFN-γ Production in vivo

One of the major aims of this study was to investigate whether activation of DCs and NK cell immune responses could be induced by Gag-VLPs. Remarkably, immunization of mice with splenic DCs isolated from phase I, Gag-VLP-immunized mice resulted in NK cell activation in vivo to an extent nearly as great as that observed in mice immunized with DCs isolated from LPS-immunized mice. In contrast, NK cells of mice immunized with DCs isolated from control mice showed no increase in expression of CD69 (fig. 4a). Furthermore, ELISAs (fig. 4b, upper panel) and intracellular staining (fig. 4b, lower panel) demonstrated that inoculation of mice with DCs isolated from VLP- or LPS-immunized mice resulted in increased IFN-γ secretion in vivo.

Fig. 2. Activation of human NK cells cocultured with VLP-DCs. MDDCs were treated with Gag-VLPs, LPS or medium for 24 h. Purified autologous NK cells were added at a DC to NK ratio of 1:5 and incubated for 18 h. **a** NK cell activation was analyzed by flow cytometry after staining with anti-CD56-PE and anti-CD69-FITC. **b** Production of IFN-γ was detected by ELISA (left panel) and intracellular cytokine staining (right panel). IFN-γ in culture supernatants was measured using an ELISA kit. All results are expressed as means ± SD of triplicate wells. Statistical analysis was performed using Student's t test. For intracellular cytokine staining, NK cells were stained with anti-CD56-PE, permeabilized and stained with anti-IFN-γ-FITC. IFN-γ-producing NK cells were examined by FACS analysis. The results are representative of 3 independent experiments. **c** NK cell proliferation was analyzed using a lymphocyte proliferation assay kit (left panel) and CFSE labeling (right panel). NK cells were cocultured with MDDCs for 5 days, and NK cell proliferation was evaluated using a proliferation assay kit. The results are expressed as means ± SD of triplicate cultures from 3 independent experiments. For CFSE labeling, NK cells were loaded with 5 μM CFSE and cocultured with MDDCs for 5 days. Proliferation was assessed by measuring the decrease in the intensity of CFSE fluorescence by flow cytometry after staining cells with anti-CD56-PE. The results are representative of 3 independent experiments using cells from 3 different donors. **d** To examine nonspecific and Gag-specific cytotoxicity, NK cells were cocultured with MDDCs for 24 h. YAC-1 cells (left panel) and HIV-1 NL4-3-infected $CD4^+$ T cells (right panel) were then added to each well (1×10^4 cells/well), and samples were incubated for another 4 h. As a control, unstimulated NK cells were incubated with target cells. Lysis of target cells was determined by measuring LDH release using the Cytotox 96 non-radioactive cytotoxicity assay kit. The results are expressed as means ± SD of triplicate cultures. Comparable results were obtained in 2 independent experiments. E:T = Effector to target.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a
NK + control DCs
NK + LPS-DCs
NK + VLP-DCs
3.47%
49.48%
21.08%
CD56
CD69
b
IFN-γ
p < 0.01
p < 0.01 p < 0.01
(pg/ml)
NK + control DCs
NK + LPS-DCs
NK + VLP-DCs
0.05%
0.2%
0.21%
IFN-γ
c
p < 0.01
p < 0.01 p < 0.02
Absorbance
4.88%
13.9%
9.82%
14.9%
7.09%
13.2%
CFSE
d
YAC-1
CD4+ T cells
Specific lysis (%)
E:T ratio
1.25:1 2.5:1 5:1 10:1
NK + VLP-DCs
NK + LPS-DCs
NK only

2

In conclusion, we show that Gag-VLPs efficiently activate human MDDCs in vitro and murine DCs in vivo. Furthermore, VLP-activated DCs subsequently induce innate immune responses as well as HIV-1-Gag-specific immune responses in NK cells.

Discussion

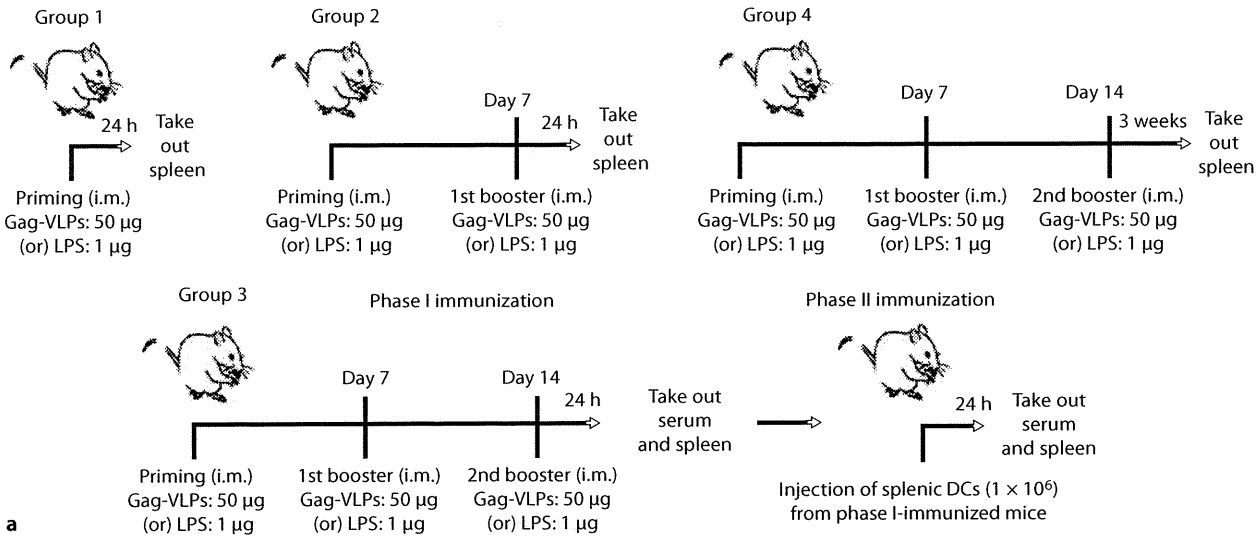
Despite extensive efforts to combat AIDS, the global HIV-infected population continues to increase on a daily basis. Highly active antiretroviral therapy results in significant suppression of HIV viral load but is unable to eradicate the virus from the body because of the appearance of resistant strains. One of the greatest hopes for preventing HIV infection and progression to AIDS is the development of an effective prophylactic or therapeutic vaccine.

In recent years, Gag-VLPs have been shown to be highly attractive HIV vaccine candidates because of their ability to activate multiple cell types such as DCs, T cells and B cells [9, 15, 16]. In this report, we used an HIV-1 Gag-VLP as an immunogen in a vaccine against HIV to induce DC-mediated NK cell immune responses. Previous reports showed that HIV envelope glycoprotein 120 (Env-gp120)-containing VLPs mainly induced Th2-polarizing cytokines (IL-6, IL-10 and TNF-α) and failed to induce significant levels of Th1-polarizing cytokines (IL-12 p70 and IFN-γ). The high levels of IL-10 and TNF-α induced by Env-VLPs could explain the aberrant function of immature DCs and impairment of T cell proliferation [32]. Moreover, gp120 induces production of IL-4, which drives synthesis of IgE and inhibits synthesis of antiviral IgG by B cells, inactivation of Th1 cells and inhibition of CTL responses [33]. The results from phase III trials showed that purified recombinant Env-gp120 failed to protect against HIV-1 infection and also failed to induce production of neutralizing antibodies to diverse primary isolates. The presence of HIV-1 Env-gp120 in Gag-VLPs switches the Th2 polarization in peripheral blood mononuclear cells from HIV-infected subjects [34–36]. For these reasons, HIV-1 Gag-VLPs are preferred as an HIV-1 vaccine candidate.

Internalization of VLPs into DCs occurs through actin-dependent macropinocytosis and endocytosis. VLP uptake is also induced by DC-SIGN-mediated endocytosis. The ability of Gag-VLPs to target DCs is an important advantage over other HIV vaccines because activation of DCs is essential for the induction of subsequent innate and adaptive immune responses. Although numerous reports have demonstrated Gag-VLP-mediated induction of $CD4^+$ T cell activation, CTL responses and B-cell-mediated humoral immunity, reports on the induction of NK cell immune responses by Gag-VLPs are virtually absent.

Recent reports indicate that long-term non-progressing HIV-1 infection does not necessarily require the presence of broadly cross-reactive neutralizing antibodies; however, a strong antiviral cytotoxic activity has been shown to correlate temporally with the clearance of viremia during primary infection [37]. In contrast, reports showing a negative correlation between the number of Gag-specific $CD8^+$ T cells and HIV viral load or progression to AIDS made it necessary to reevaluate the efficiency of HIV vaccines [38]. Recently, it became apparent that a $CD8^+$ T cell-inducing vaccine failed in a phase IIB clinical trial. The trial was suspended after an interim analysis showed that the vaccine did not protect the trial participants against HIV infection and indicated that the presence of high numbers of cytokine-producing HIV-specific $CD8^+$ T cells does not guarantee a better clinical outcome [39]. Taken together,

Fig. 3. Immunization of mice with Gag-VLPs and assessment of DC and NK cell activation. **a** Schematic representation of the phase I and phase II immunization regimens. In phase I immunizations, 4 groups of female BALB/c mice, each consisting of 3 subgroups with 4 animals, were immunized by intramuscular (i.m.) injection of 50 μg of Gag-VLPs or 1 μg of LPS or were left untreated. Twenty-four hours after priming, mice from the first group were sacrificed and their spleens were collected for analysis of DC and NK cell activation. The remaining groups of mice were given first booster injections using the same doses 7 days after priming. Mice from the second group were sacrificed after 24 h and their spleens were collected. Mice from the third and fourth groups were given a second booster injection on day 14. After 24 h, mice from the third group (donor mice) were sacrificed, and blood samples were collected for the quantification of serum IFN-γ by ELISA. Spleens were removed for flow cytometry analysis. At the same time, splenic DCs were purified using a mouse $CD11c^+$ DC isolation kit (Miltenyi Biotec) and subsequently used in phase II immunizations. Mice from the fourth group were sacrificed 3 weeks after the second booster injection, and spleens were collected for flow cytometry. A portion of splenocytes was restimulated overnight with 5 μg/ml Gag-VLPs and used for intracellular staining of IFN-γ-producing NK cells and ex vivo cytotoxicity assays. In phase II immunizations, 4 groups of (recipient) BALB/c mice, each consisting of 5 animals, were adoptively transferred with purified splenic DCs (1×10^6 cells/mouse) from phase I-immunized (donor) mice from the third group. After 24 h, all mice were sacrificed and sera and spleens were collected for immunological assays. **b** Expression of mouse CD80, CD86 and MHC I and MHC II molecules on splenic DCs from the first group of phase I-immunized mice was analyzed by flow cytometry.



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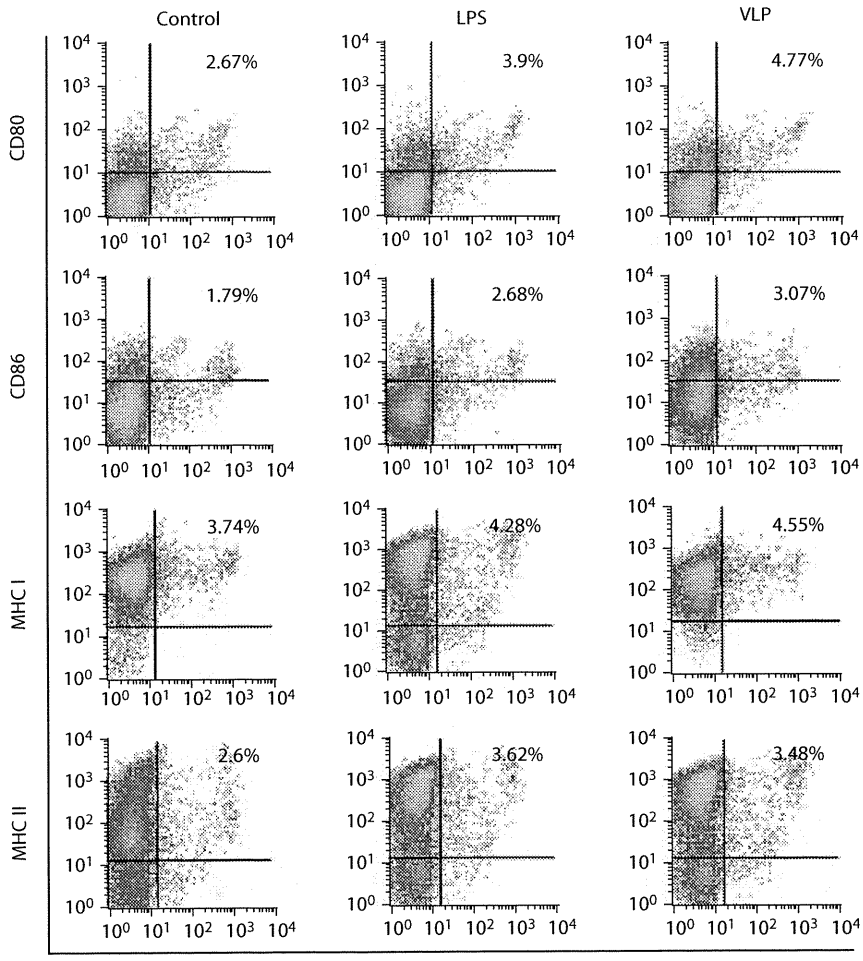

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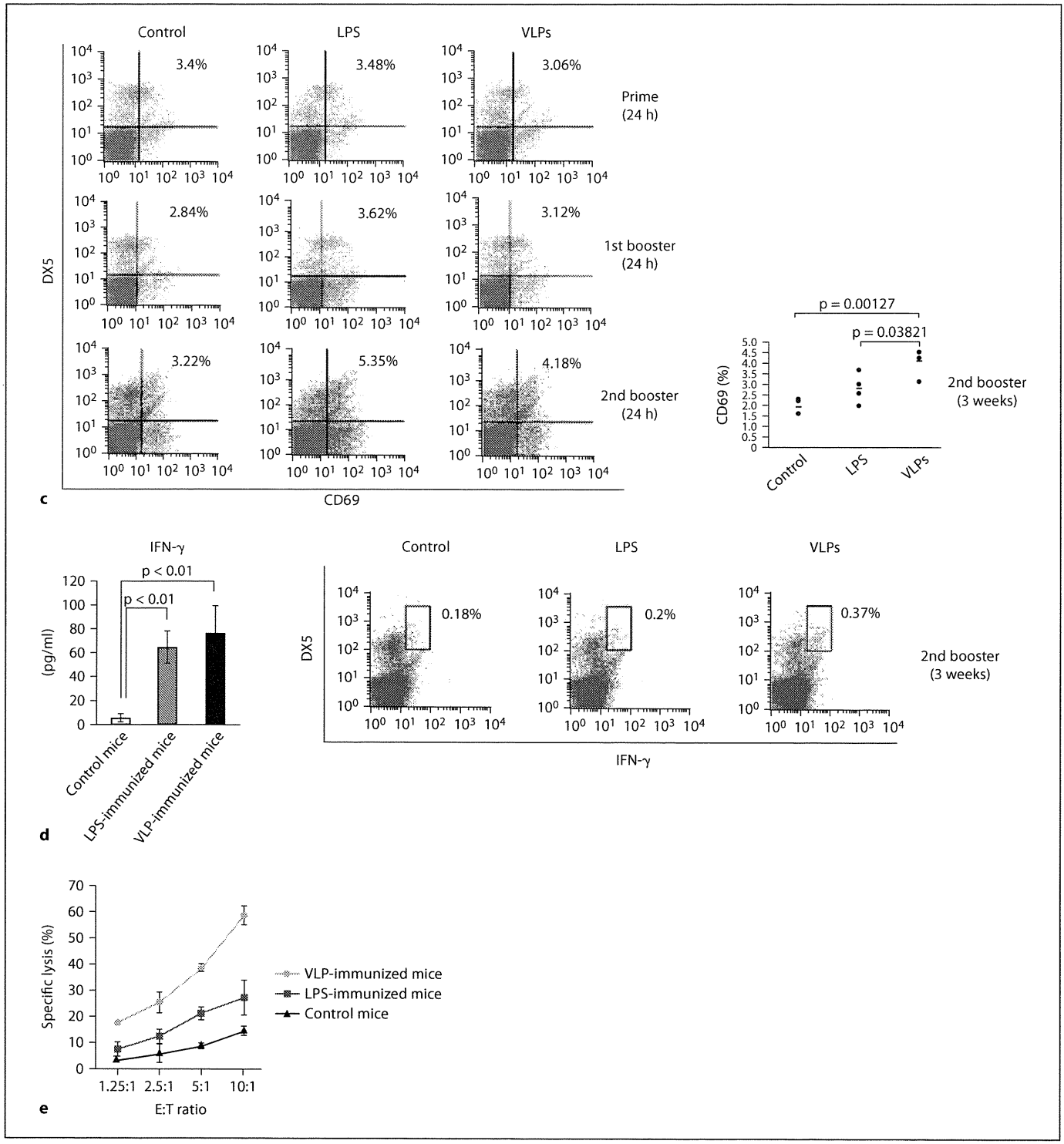

Fig. 3. Immunization of mice with Gag-VLPs and assessment of DC and NK cell activation. **c** CD69 expression on NK cells from each group of phase I-immunized mice was analyzed by flow cytometry. **d** Serum IFN-γ levels from the third group of phase I-immunized mice were determined by ELISA. IFN-γ-producing splenic NK cells from the fourth group of phase I-immunized mice after restimulation with Gag-VLPs were examined by intracellular cytokine staining. The results are representative of 3 independent experiments. **e** Gag-specific NK cell cytolysis of Gag-VLP-loaded spleen cells was assessed by measuring LDH release using the Cytotox 96 non-radioactive cytotoxicity assay kit. The results are expressed as means ± SD of triplicate cultures. Comparable results were obtained in 2 independent experiments. E:T = Effector to target.

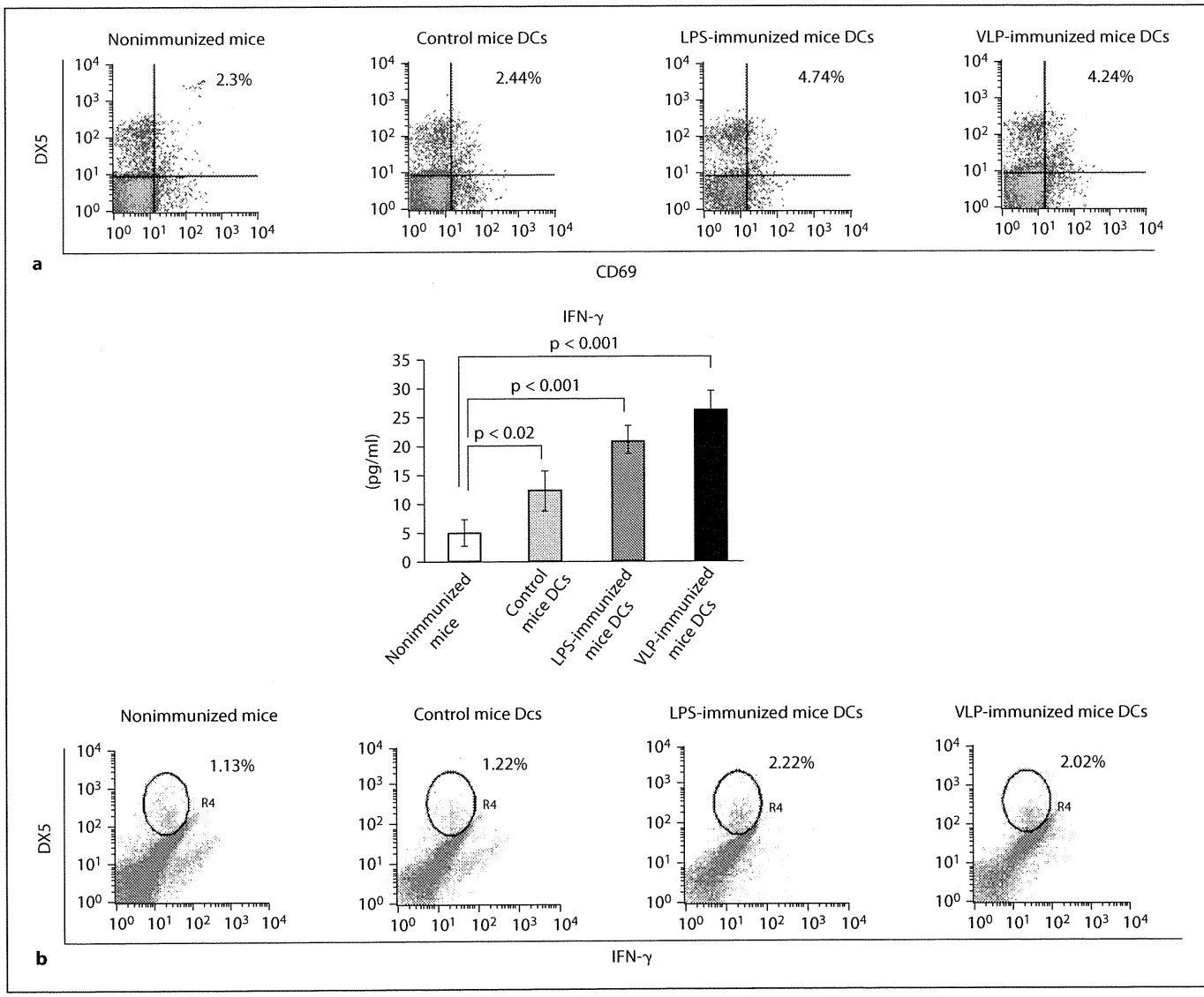

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Fig. 4. DCs from phase I-immunized donor mice induce in vivo activation of NK cells. **a** NK cell activation in phase II, DC-immunized recipient mice was analyzed by flow cytometry after staining the spleen cells with antibodies against DX5-PE and anti-CD69-FITC. **b** Serum IFN-γ levels of phase II-immunized or control mice were determined using a mouse IFN-γ ELISA assay kit. All results are expressed as means ± SD of triplicate wells. Statistical analysis was performed using Student's t test. IFN-γ-producing murine NK cells were examined by intracellular cytokine staining and FACS analysis. The results are representative of 3 independent experiments.

these results suggest that in order to achieve an effective potential HIV vaccine, a strategy that results in induction of both nonspecific innate immune responses and specific immune responses against HIV is necessary.

In this study, we first demonstrated the activation of DCs and NK cells by Gag-VLPs both in vitro and in vivo by immunization with Gag-VLPs or splenic DCs isolated from VLP-immunized mice in a 2-phase immunization experiment.

We generated HIV-1 Gag-VLPs in mammalian HeLa cells to exclude the possible baculovirus contamination of VLP preparations, which may have affected the results of experiments intended to analyze the immunogenicity of VLPs. Our studies using these VLP preparations showed that incubation of human MDDCs with Gag-VLPs resulted in upregulation of the DC maturation markers MHC I and MHC II and of costimulatory molecules such as CD80 and CD86 (fig. 1a). DC activation is

also characterized by the production of cytokines that are important for the priming of innate and adaptive immune cells such as NK cells, $CD4^+$ and $CD8^+$ T cells. Thus, to determine the type of responses elicited by DCs stimulated with VLPs, we examined the secretion of IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ in culture supernatants and demonstrated that Gag-VLP-treated DCs produced increased levels of cytokines (fig. 1b). The quality and the quantity of DCs are important factors when attempting to induce effective immune responses in T cells and NK cells. To be effective, a vaccine must restore both the function and the number of DCs in HIV-infected persons. In vivo expansion of DCs after immunization has been shown to be necessary for the activation of other immune cells [16]. Moreover, since DC immunotherapy requires a sufficient quantity of functional autologous DCs, it is important that stimulation with Gag-VLPs should induce the activation and maturation of DCs as well as proliferation of functional DCs. We next used 2 methods to investigate the ability of Gag-VLPs to induce proliferation of human DCs in vitro. The results of CFSE labeling experiments and experiments using a proliferation assay kit both demonstrated the proliferation of VLP-treated MDDCs (fig. 1c). To further prove the capability of VLP-treated DCs to stimulate NK cells, coculture experiments were performed to analyze NK cell immune responses. These experiments demonstrated that NK cells cocultured with VLP-treated DCs showed upregulation of CD69 expression, IFN-γ production, proliferation and cytotoxicity against YAC-1 cells and HIV-infected CD4+ T cells (fig. 2). Furthermore, MDDCs produced IFN-γ after stimulation with Gag-VLPs or LPS, but the presence of NK cells in cocultures significantly increased IFN-γ levels (about 8-fold). Because the number of DCs in cocultures was 10 times less than that in DC-only cultures, we hypothesized that the IFN-γ in NK/DC cocultures was produced mainly by NK cells.

Recent reports have demonstrated NK cell memory for specific antigens and NK cell involvement in both innate and adaptive immunity. Rapid NK cell responses during innate immune activation are also associated with long-lasting antigen-specific NK cell memory responses [40–44]. NK cells can develop memory-like properties based on prior activation. These amplified NK cell responses are important for the early response to pathogens, and it may be possible to boost the NK cell response to subsequent infection by stimuli that result in the memory-like NK cell phenotype. NK cells can orchestrate specific immune responses to infection by recognizing pathogens through germ-line-encoded receptors such as toll-like receptors [45].

In our studies, immunization of mice with Gag-VLPs resulted in activation of murine DCs and NK cells in vivo, supporting the results of in vitro experiments (fig. 3b–e). We evaluated NK cell activation 24 h after each immunization with Gag-VLPs (prime, first and second booster injections) to verify the immediate NK cell response to each immunization and again 3 weeks after the last immunization to verify Gag-specific NK cell memory as shown in figure 3d (right panel) and e. We found that the prime/boost regimen using Gag-VLPs significantly enhanced NK cell activation and Gag-specific NK cell immunity. In both LPS-immunized mice and Gag-VLP-immunized mice, nonspecific activation of NK cells was evidenced by the increase in CD69 expression shortly after (24 h) each immunization (fig. 3c). In contrast, long-lasting Gag-specific NK cell immune responses of NK cells were only seen in Gag-VLP-immunized mice (fig. 3d, right panel, e). These results demonstrate that Gag-VLPs are able to induce NK cell innate immune responses as well as HIV-1 Gag-specific memory responses.

Sailaja et al. [16] injected mice intraperitoneally with Gag-VLPs once and assessed the activation of splenic DCs, T cells and B cells on day 3. Our data showed that 24 h after priming, NK cell activation was not detected in the first group of mice. This is likely due to the early time point at which the mice were examined, a short time (24 h) after injection with Gag-VLPs, which is likely too short a time period to induce DC-mediated NK cell activation in vivo. NK cell activation was found to increase after each booster injection due to the increased activation of DCs induced by booster injections (fig. 3c).

Adoptive transfer of DCs from VLP-immunized mice in the phase II immunization experiments revealed that Gag-VLP-immunized DCs could induce NK cell innate and Gag-specific immune responses in vivo (fig. 4a, b).

In most previous adoptive transfer studies, mice were injected intraperitoneally with 1×10^6 DCs [46]. Transferred DCs reportedly localized in the spleen as early as 3 h after intravenous injection and persisted for 24 h. DCs entering the popliteal lymph nodes from the footpad were also located 3 h after subcutaneous transfer [47]. Eggert et al. [48] injected mice subcutaneously, intraperitoneally and intravenously with 1×10^6 DCs/mouse. In intravenously injected mice, DCs localized in the lung 5 min after injection and in the spleen as early as 2 h after injection. DCs were detected in the draining lymph nodes 8 h

after intraperitoneal injection [48]. DCs adhere to endothelium within the splenic marginal zone through endothelium-specific homing receptors and chemokine receptor expression [49]. The required number of DCs, DC trafficking/migration patterns and subsequent immune responses differ greatly according to cell type, route of inoculation and the immune competency of recipient mice.

In this study, we evaluated the ability of Gag-VLPs to induce NK cell innate responses and HIV-1 Gag-specific NK cell adaptive immunity, which is essential for the immunological control of HIV-1 infection. These studies highlighted the role of DCs in VLP-based vaccine strategies. Our findings have significant implications for the design of a highly effective vaccine model for prophylactic and therapeutic purposes in HIV infection.

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Disclosure Statement

The authors declare that they have no competing interests.

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RESEARCH ARTICLE

Induction of antitumor immunity against mouse carcinoma by baculovirus-infected dendritic cells

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A dendritic cell (DC) vaccine strategy has been developed as a new cancer immunotherapy, but the goal of complete tumor eradication has not yet been achieved. We have previously shown that baculoviruses potently infect DCs and induce antitumor immunity against hepatomas in a mouse model. Baculovirus-infected, bone marrow-derived DCs (BMDCs) display increased surface expression of costimulatory molecules, such as CD80, CD86 and major histocompatibility complex (MHC) classes I and II, and secrete interferons and other proinflammatory cytokines. In this study, we evaluated the induction of antitumor immunity in mice by baculovirus-infected BMDCs against lung cancer and melanoma. After treatment with baculovirus-infected BMDCs, murine lung tumors caused by Lewis lung carcinoma (LLC) cells were significantly reduced in size, and the survival of the mice was improved. In addition, experiments using a melanoma mouse model showed that baculovirus-infected BMDCs inhibited tumor growth and improved survival compared with controls. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine levels remained normal in baculovirus-infected BMDC-treated mice. Our findings show that baculovirus-infected DCs induce antitumor immunity and pave the way for the use of this technique as an effective tool for DC immunotherapy against malignancies.
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Keywords: dendritic cells; natural killer cells; T cells; tumor Immunity

INTRODUCTION

Dendritic cells (DCs) play a major role as professional antigen-presenting cells in the activation of natural killer (NK) cells, T cells and B cells. In particular, DCs are important in the initiation of innate and adaptive immune responses against tumors and invading pathogens. Recently, DC vaccination has been reported as a new immunotherapeutic strategy for the treatment of cancers.[1] However, very few clinical studies using DC immunotherapy have been reported, and those studies demonstrated insufficient efficacy.

The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus, is an enveloped insect virus that has a 130-kb double-stranded circular DNA genome.[2] Baculoviruses have long been used as biopesticides[3,4] and recombinant protein expression systems.[5,6] Recent research has focused on the use of baculoviruses as vectors in gene therapy because of (1) their ability to infect, but not replicate in, mammalian cells; (2) their low cytotoxicity; and (3) their ability to carry large foreign genes into their genome.[7–10] In several reports, baculoviruses were developed as vaccines against pathogens.[11–15] Abe *et al.* reported that baculovirus-infected host cells produced type I interferons and proinflammatory cytokines through Toll-like receptor 9 and interferon regulatory factor 7 signaling.[16,17] Schütz *et al.* reported that baculovirus-infected, human monocyte-derived DCs expressed cell-surface activation markers and produced tumor-necrosis factor alpha (TNF-α).[18] In addition, Hervas-Stubbs *et al.* reported that baculoviruses strongly induced the secretion of type I interferon and proinflammatory cytokines in mice.[19] Conventional splenic DCs and plasmacytoid DCs displayed upregulation of surface major histocompatibility complexes (MHCs) and costimulatory molecules in response to baculovirus infection *in vivo*. Jordan *et al.* demonstrated that vaccination with insect cells infected with recombinant, baculovirus-encoding peptide–MHC complexes generated peptide-specific cytotoxic T-cell responses and antitumor immunity.[20] We have previously described the induction of antitumor effects using wild-type baculovirus in a mouse model of hepatoma.[21] In particular, DCs were more potently infected by baculovirus than were other immune cells, and the DCs underwent activation and maturation. Baculovirus-infected bone marrow-derived dendritic cells (BMDCs) (BV-DCs) showed upregulation of costimulatory molecules, such as CD80, CD86 and MHC classes I and II, and increased secretion of interferons and other proinflammatory cytokines.[22]

In this study, we showed that the inoculation of BV-DCs induced efficient $CD8^+$ T cell- and NK cell-dependent, $CD4^+$ T cell-independent antitumor immunity. Furthermore, BV-DC administration did not cause any functional damage to the mouse liver or kidneys. Our results demonstrate that BV-DC-induced antitumor immunity *in vivo* has strong potential as a future DC immunotherapy against various malignancies.

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