CD1d-restricted Human Natural Killer T Cells Are Highly Susceptible to Human Immunodeficiency Virus 1 Infection

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Abstract

Human natural killer (NK) T cells are unique T lymphocytes that express an invariant T cell receptor (TCR) Vα24-Vβ11 and have been implicated to play a role in various diseases. A subset of NKT cells express CD4 and hence are potential targets for human immunodeficiency virus (HIV)-1 infection. We demonstrate that both resting and activated human Vα24^{+} T cells express high levels of the HIV-1 coreceptors CCR5 and Bonzo (CXCR6), but low levels of CCR7, as compared with conventional T cells. Remarkably NKT cells activated with α-galactosylceramide (α-GalCer)-pulsed dendritic cells were profoundly more susceptible to infection with R5-tropic, but not X4-tropic, strains of HIV-1, compared with conventional CD4^{+} T cells. Furthermore, resting CD4^{+} NKT cells were also more susceptible to infection. After initial infection, HIV-1 rapidly replicated and depleted the CD4^{+} subset of NKT cells. In addition, peripheral blood NKT cells were markedly and selectively depleted in HIV-1 infected individuals. Although the mechanisms of this decline are not clear, low numbers or absence of NKT cells may affect the course of HIV-1 infection. Taken together, our findings indicate that CD4^{+} NKT cells are directly targeted by HIV-1 and may have a potential role during viral transmission and spread in vivo.

Key words: HIV • NKT cell • chemokine receptors • CD1d tetramer • cytokines

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Introduction

NKT cells are a distinct subset of T lymphocytes with highly conserved TCR in both humans and mice (1–3). Human NKT cells express a monoclonal TCR consisting of an invariant Vα24-Jα18 chain preferentially paired with a Vβ11 chain (4–7). NKT cells also share some phenotypic similarities with classical NK cells, such as the coexpression of C-type lectin NKRP-1A (CD161) (7). However, a subset of conventional T cells also expresses CD161 and therefore, the most reliable marker of NKT cells in humans is their invariant TCR (8). NKT cells are either CD4^{+} (∼30–60%) or CD4^{-}CD8^{-} with respect to their coreceptor expression (9, 10). Phenotypically, NKT cells are also defined by constitutive expression of the memory marker CD45RO, both in adults and in neonates (11–13). This effector/memory phenotype of NKT cells suggests chronic stimulation by activating self-antigens.

The NKT TCR is specific for glycolipid antigens presented by the nonpolymorphic MHC class I–like molecule CD1d (1); however, the natural antigens that activate NKT cells in the context of CD1d remain unknown. The glycosphingolipid α-galactosylceramide (α-GalCer), which is derived from a marine sponge, is the only known antigen that can bind to CD1d and activate all NKT cells expressing the invariant TCR (14–16). Activation of NKT cells in this manner results in the rapid secretion of large amounts of cytokines such as IFN-γ and IL-4 (17–20).

In mice NKT cells have been implicated in protective immune responses against the pathogens *Borrelia burgdorferi* (21), *Leishmania major* (22), and diabetogenic encephalomyocarditis virus (23). However, it is not clear how NKT cells mediate their protective function during infections and whether they play a role against a wider range of pathogens, which may include HIV-1 (24, 25).

HIV-1 entry into target cells requires cellular expression of chemokine receptors, in conjunction with CD4 (26). CCR5 is the major coreceptor for R5 strains (R5-tropic) of HIV-1 and most SIV strains, while CXCR4 allows entry of X4 strains (X4-tropic) (26). Naive and memory subsets

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of conventional CD4$^+$ T cells differ in their expression of chemokine receptors (27). CXCR4 is predominantly expressed on the resting naive subset of T cells, whereas CCR5 is almost exclusively expressed on the activated or memory subset of human T cells (28). Therefore, only CD4$^+$ memory T cells are susceptible to infection with R5-tropic viruses, whereas X4-tropic viruses can enter into both naive and memory subsets (29–31). During viral transmission and early in the course of disease R5-tropic viruses predominate. Thus, memory CD4$^+$ T cells are selectively infected and lost in HIV-1 infected individuals during the early stages of the infection (32).

Expression of the HIV-1 receptor CD4 on a subset of human NKT cells as well as their effector/memory phenotype under physiological conditions prompted us to investigate whether these cells are targets for HIV-1 infection. Herein, we report that human NKT cells express very high levels of the HIV-1 or SIV coreceptors CCR5 and CXCR6, and that CD4$^+$ NKT cells are highly susceptible to infection with R5-tropic HIV-1 strains. We further demonstrate profoundly reduced numbers of NKT cells and a selective depletion of the CD4$^+$ NKT cell subset in HIV-infected adults. These findings suggest a possible role for NKT cells during the establishment of HIV-1 infection and as a reservoir for rapid viral spread.

Materials and Methods

Preparation of Primary Human T Cells and Dendritic Cells. PBMC were separated from buffy coats of healthy donors through Ficoll-Hypaque (Amersham Pharmacia Biotech). Resting CD4$^+$ T cells were purified as described previously (33). Briefly, PBMCs were incubated with anti-CD4 mAbs conjugated with Dynabeads (Dynal) for 30 min and the bead-bound cells were recovered using a magnet (Dynal). The bead-bound cells were then washed and the CD4$^+$ T cells were detached from the beads using Detachabead, according to the manufacturer's instructions (Dynal). These cells were then incubated with anti–HLA-DR and CD14 antibodies (BD Biosciences) followed by Dynabeads conjugated with goat anti–mouse IgG (Dynal) and magnetic removal of bead-bound preactivated cells and residual monocytes or dendritic cells (DCs)*. This purification protocol typically resulted in 99.5% purity of positively selected cells, as determined by postpurification FACS® analysis. In some experiments purified CD4$^+$ T cells were further negatively sorted into CD45RO$^+$ and CD45RA$^+$ subsets by staining the cells with anti-CD45RA and anti-CD45RO antibodies (BD Biosciences), respectively and magnetically removing the stained cells using goat-anti–mouse IgG beads. Vα24$^+$ T cells were purified by incubating PBMCs with Vα24 antibody (Coulter), followed by staining with goat-anti–mouse IgG antibody conjugated with MACS® beads (Miltenyi Biotec). Bead-conjugated cells were then positively sorted using a MACS® sorter. The culture media used in all experiments was RPMI 1640 (Life Technologies) supplemented with 10% FCS (Hyclone), penicillin (50 U/ml; Life Technologies), streptomycin (50 ug/ml), sodium pyruvate (1 mM; Life Technologies) and glutamine (2 mM; Life Technologies) (10% RPMI 1640). All cytokines were purchased from R&D Systems. DCs were generated as described previously (34). Briefly, CD14$^+$ monocytes were purified using the MACS® system (Miltenyi Biotec) and cultured in the presence of IL-4 (100 ng/ml) and GM-CSF (50 ng/ml) (both from R&D Systems) for 4–6 d.

Study Subjects. Healthy donors were adults negative for HIV-1 and with no history of chronic viral infections such as Hepatitis B or C. Mean age of healthy donors was 34, 64% were male and 36% were female. Whole blood samples from adults with HIV-1 infection were obtained during routine primary care visits at the Comprehensive Care Center, Vanderbilt University Medical Center, Nashville, TN. Mean age of the HIV-1 infected donors was 40, 74% were male and 26% were female. There were no selection criteria based on race or sex. All subjects provided written informed consent, and the study was approved by the Vanderbilt Institutional Review Board.

Virus Production and Infections. Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped replication incompetent HIV-1 particles were generated as described previously (33). Briefly, HEK-293T cells were transfected with 20 μg of proviral HIV-1 vector and 12 μg of pL-VSV-G plasmid per 3×10^6 cells seeded on 10-cm plates. Supernatants were collected at 48 h after transfection, centrifuged, and passed through 0.4-μm filters to remove fine debris and stored at -80°C. The viral titers were determined by infection of the human T cell line Hut78 with serially diluted virus supernatant. R5- or X4-tropic replication-competent viruses were prepared similarly by transfecting 293T cells with HIV-1 that encodes R5- or X4-tropic (BAL and NL4–3 respectively) envelope and EGFP (CLONTECH) in place of the *nef* gene as described previously (33). Typically viral titers ranged from $0.5–2 \times 10^6$ ifu/ml for replication competent viruses and $5–10 \times 10^6$ for VSV-G pseudotyped HIV-1. Wild-type virus (NL4–3) with X4-tropic or with R5-tropic envelope (BAL) and virus (R8) encoding heat stable antigen (HSA) in place of *vpr* (35) with intact *nef* gene were gifts of Drs. Vineet KewalRamani, National Cancer Institute, Frederick, MD, and Chris Aiken, Vanderbilt University School of Medicine, Nashville, TN, respectively. The titers for the wild-type viruses were determined using the sensitive GHOST assay as described previously (36). Viral replication in T cell cultures was determined by either FACS® analysis using GHOST assay or by measuring p24 levels within supernatants by an ELISA.

Detection of NKT Cells by CD1d-Tetramers. Tetramers of mouse CD1d were produced by streptavidin-mediated tetramerization of folded CD1d1-β2m heterodimers which will be described in detail elsewhere (unpublished data). Briefly, mouse CD1d and human β2m were expressed in Drosophila cells (S2; Invitrogen). CD1d1-β2m complexes were purified by metal affinity chromatography and biotinylated using biotin protein ligase (Avidity). Free biotin was removed by dialysis, and biotinylation efficiency was ascertained with a colorimetric assay using avidin peroxidase (Sigma-Aldrich). CD1d1 was loaded with ligand by incubating monomers with a threefold molar excess of α-GalCer (obtained from Kirin Brewery Co., Japan) for 12–16 h at room temperature. Tetramers for flow cytometry were prepared by mixing monomers with streptavidin-allophycocyanin (Molecular Probes) at a molar ratio of 4:1. Staining was performed by incubating cells on ice for 30 min at a concentration of 10 μg/ml.

Flow Cytometry. Cells were stained with the relevant antibody on ice for 30 min in PBS buffer with 2% FCS and 0.1% Sodium azide, washed twice, fixed with 1% paraformaldehyde, and

**Abbreviations used in this paper:* α-GalCer, α-galactosylceramide; DC, dendritic cell; GFP, green fluorescent protein; HSA, heat stable antigen; MOI, multiplicity of infection; VSV-G, vesicular stomatitis virus glycoprotein.

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analyzed with a FACSCalibur™ four-color cytometer, using the CELLQuest™ program. Live cells were gated based on forward and side scatter properties. The following anti–human antibodies were used in stainings: CD3, CD4, CD8, CD11b, CD25, CD45RO, CD45RA, HLA-DR, CD161, (all from BD Biosciences), and Vα24, Vβ11 (Coulter), antibodies to chemokine receptors CCR1, CCR3, CCR5, CXCR4, CXCR6 (R&D Systems), CCR7 and CXCR3 (BD Biosciences). Anti–mouse HSA (CD24) was purchased from BD PharMingen. Cytokines in the culture supernatant were measured using Cytometric Bead assay (CBA) by flow cytometry, according to the manufacturer's (BD Biosciences) instructions. Cell death was assessed by staining the cells with propidium iodide.

Statistical Analyses. Continuous variables were compared by Mann-Whitney U test, or by determining Pearson correlation coefficients. Linear regression analysis used an entry method, with entry and removal criteria of 0.05 and 0.01, respectively. All significance levels were based on Student's two-tailed tests. Statistical analyses were performed using SPSS version 9.0.

Results

Isolation, Expansion, and Phenotypic Characterization of Vα24⁺ NKT cells. To expand NKT cells ex vivo in short-term cultures, $V\alpha24^+$ T cells were first enriched by magnetic cell sorting from PBMCs of healthy individuals. We found that 20–80% of purified $V\alpha24^+$ T cells also expressed Vβ11, suggesting that these cells were of the NKT cell lineage (data not shown). Purified $V\alpha24^+$ T cells were then stimulated with autologous DCs) pulsed with α-GalCer and expanded in IL-2 for 1–2 wk. In parallel, conventional $CD4^+$ T cells were purified from the same individual and activated with Staphylococcus enterotoxin B (SEB)-pulsed DCs, which were expanded and maintained in IL-2. After 10 d of culture, all of the α-GalCer–stimulated Vα24 T cells also expressed Vβ11, whereas $V\alpha24^+V\beta11^+$ T cells were virtually undetectable among the SEB stimulated T cell cultures (Fig. 1 A). To determine whether $V\alpha24^+$ $V\beta11^+$ T cells belong to the NKT cell lineage, ex vivo expanded T cell lines were stained with CD1d α-GalCer tetramers (henceforth, CD1d-tetramers) which selectively bind with Vα24-Jα18 NKT cells. All of the $V\beta11^+$ T cells in α-GalCer–stimulated cultures also stained with the CD1d-tetramer, demonstrating that these cells express NKT cell–specific receptors (Fig. 1 A). Further, a portion of the NKT cell lines coexpressed CD161 characteristic of NK cells, whereas SEB-stimulated $CD4^+$ T cell lines were negative for these markers (Fig. 1 B). A sizeable portion of in vitro derived NKT cells also expressed CD4 (Fig. 1 B), which varied between 20–60% of total cells (of 22 healthy donors analyzed, data not shown), and a smaller subset of the NKT cells were $CD8^+$ or $CD4^+CD8^+$ (Fig. 1 B).

Chemokine Receptor Expression and Cytokine Production by Activated NKT Cells. HIV-1 entry into cells requires, in addition to CD4, one of the coreceptors CCR5 or CXCR4 (26). Little is known about the chemokine receptor/HIV coreceptor expression patterns of NKT cells. Therefore, activated NKT and $CD4^+$ T cells were stained with mAbs directed against various chemokine receptors. We found that almost all of the NKT cells expressed very high levels of CCR5 and most were also positive for CXCR6 and CXCR3 (Fig. 2 A). Remarkably, the expression levels of these chemokine receptors on NKT cells were much higher than those on SEB stimulated $CD4^+$ T cells (Fig. 2 A and B). In contrast, expression of the T-tropic HIV-1 coreceptor CXCR4 was lower on NKT cells than on $CD4^+$ T cells. Of interest, chemokine receptor CCR7 was expressed by very few NKT cells (Fig. 2 A). CCR3 was not expressed by either type of T cells (data not shown). This chemokine receptor expression profile of NKT cells is consistent with that of conventional effector/memory T cells (37). Similar expression patterns were observed in three other independently derived NKT cell cultures from three different healthy individuals (data not shown).

To assess the effector function of NKT cells isolated in this manner, we determined their cytokine secretion patterns. NKT cells were restimulated with α-GalCer–pulsed DCs and compared with a conventional T cell line stimulated with SEB-pulsed DCs. Cytokine levels in supernatants were quantitated using CBA assay (38). Activated NKT cells secreted distinctive and copious amounts of IL-4, IL-5, IFN-γ, and TNF-α (data not shown) as has been reported (15, 39).

Expression of Chemokine Receptors on Resting NKT Cells. Our in vitro–derived NKT cell lines express high levels of CCR5 and CXCR6 (Fig. 2). To exclude the pos-

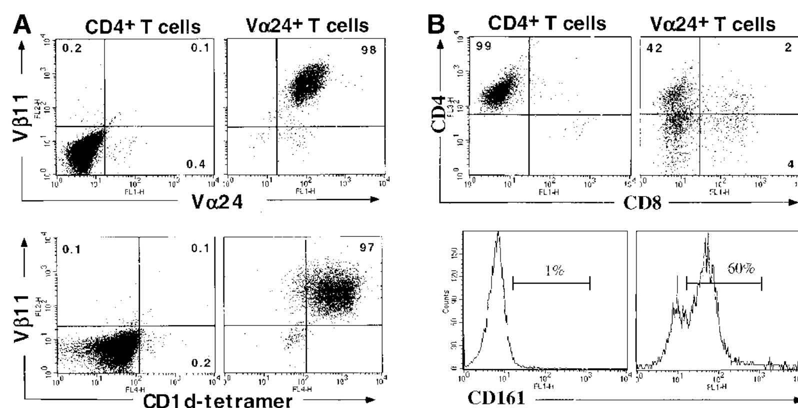

Figure 1. Phenotype of in vitro–expanded NKT cells. Purified $CD4^+$ T cells were activated by SEB (100 ng/ml) and purified $V\alpha24^+$ T cells were stimulated with α-GalCer (100 ng/ml) in the presence of autologous DCs. Cells were expanded in IL-2 for 14 d. (A) To determine expansion of NKT cells in vitro–expanded T cell lines were stained with anti-Vβ11-PE, anti-Vα24-FITC, and CD1d-tetramer-APC. (B) Expression of CD4, CD8, and NK markers on SEB-stimulated $CD4^+$ or α-GalCer-stimulated NKT cell lines. Cells were costained with anti-CD4-PercP and anti-CD8-FITC, and individually stained with purified anti-CD161 followed by FITC-conjugated anti–mouse IgG.

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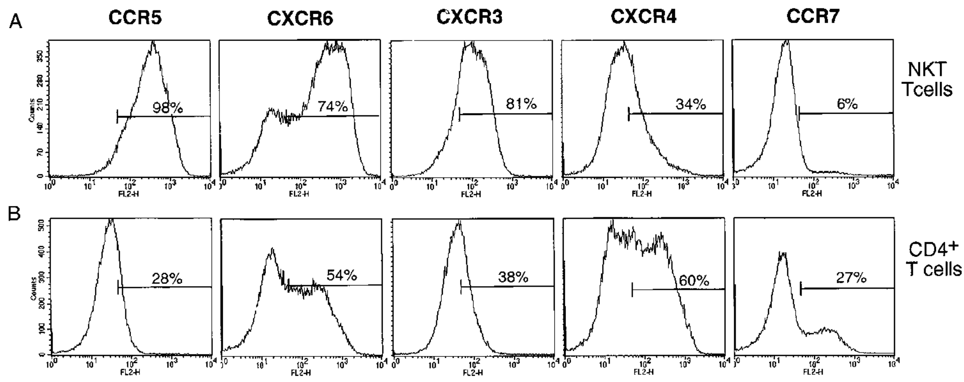

Figure 2. Expression of chemokine receptors on in vitro–activated NKT cells. T cell lines were expanded as described in the legend of Fig. 1 and stained with chemokine receptor antibodies followed by PE-conjugated anti–mouse IgG. (A) α-GalCer–stimulated NKT cells. (B) SEB-stimulated CD4$^+$ T cells.

sibility that the expression of these chemokine receptors resulted from in vitro activation or cell culture in the presence of IL-2, the expression levels of CCR5 and CXCR6 were examined on resting NKT cells. For these studies we employed a four-color FACS® protocol. Freshly derived PBMCs were stained with NKT cell–specific TCR (anti-Vα24 and anti-Vβ11 or CD1d-tetramer) and anti-CD3 antibodies in combination with various cell surface markers (Fig. 3). As shown in Fig. 3, ~50% of NKT cells express CD4 and a small subset expresses CD8, but at lower levels than conventional CD8$^+$ T cells (Fig. 3). In addition all NKT cells express the memory T cell marker CD45RO and the majority of these cells also express CD161 (Fig. 3). Importantly, like in vitro–expanded NKT cells, resting NKT cells express high levels of CCR5 and CXCR6 but lower levels of CCR7 (Fig. 3). NKT cells also expressed CXCR4 at lower levels than conventional T cells (Fig. 3), and some donors also expressed high levels of CCR1 and CXCR3 (data not shown). These data demonstrate that our in vitro expanded NKT cells recapitulate the phenotype of resting NKT cells freshly derived from PBMCs.

HIV-1 Infects CD4$^+$ NKT Cells. High expression levels of HIV-1 coreceptors CCR5 and CD4 on a subset of NKT cells prompted us to determine whether these cells are susceptible to HIV-1 infection. Because NKT cell cultures contain both CD4$^+$ and CD4$^-$ cells, in some experiments NKT cells were purified into CD4$^+$ and CD4$^-$ subsets. Both NKT and conventional T cell lines were infected, at varying multiplicity of infection (MOI), with green fluorescent protein (GFP) encoding replication-competent HIV-1 strains, which use CCR5 (R5-tropic) or CXCR4 (X4-tropic) as coreceptors for entry. As a control, cells were infected with replication-defective HIV-1 pseudotyped with VSV-G envelope that bypasses receptor

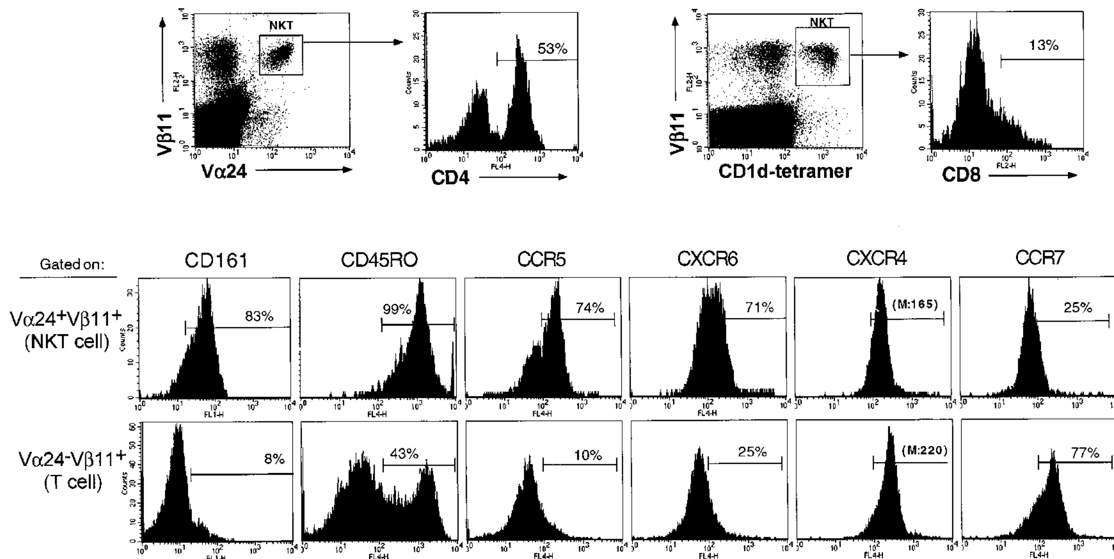

Figure 3. Phenotype of resting NKT cells in PBMCs. PBMCs from healthy donors were first stained with anti-Vβ11-PE, anti-CD3-PercP.Cy5.5, and either anti-Vα24-FITC or CD1d-tetramer-APC in combination with anti-CD4-APC, and anti-CD8-PE. To stain with mAbs directed against CD161, CD45RO, and chemokine receptors, CCR5, CXCR6, CCR7, CXCR4, cells were first incubated with purified antibodies, followed by second step staining with anti–mouse IgG-APC. After extensive washing cells were preincubated with mouse Ig to neutralize any free anti–mouse Ig. Cells were then stained with anti-Vβ11-PE, Vα24-FITC, and CD3-PercP-Cy5.5. Electronic gates were set on CD3$^+$ Vα24$^+$Vβ11$^+$ or CD3$^+$Vβ11$^+$CD1d-tetramer$^+$ T cells or Vα24$^-$Vβ11$^+$ cells to analyze expression of molecules, listed above, on NKT and conventional T cells, respectively. For CXCR4 expression, mean intensity of fluorescence is shown.

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requirements for viral entry. We found that CD4+ NKT cells were highly susceptible to HIV-1 infection with R5-tropic strains (Fig. 4 A). After 3 d of infection, with R5-tropic viruses at MOI of 1, ~50% of the CD4+ NKT cells and 4–5% of conventional CD4+ T cells were infected (Fig. 4 A). In contrast, conventional CD4+ T cells were approximately twice as susceptible than CD4+ NKT cells to infection with an X4-tropic virus (Fig. 4 A). CD4− NKT cells were resistant to infection by both R5- and X4-tropic viruses (Fig. 4 A). Although, both viruses lack *nef* gene, a significant fraction of the infected T cells downregulated CD4 (Fig. 4 A), possibly due to interaction with HIV-1 envelope glycoprotein and presence of the accessory protein Vpu (40, 41). CD4+ and CD4− NKT cells as well as conventional CD4+ T cells were equally susceptible to infection by VSV-G pseudotyped HIV-1 (Fig. 4 A), suggesting that dramatic differences in infectivity of

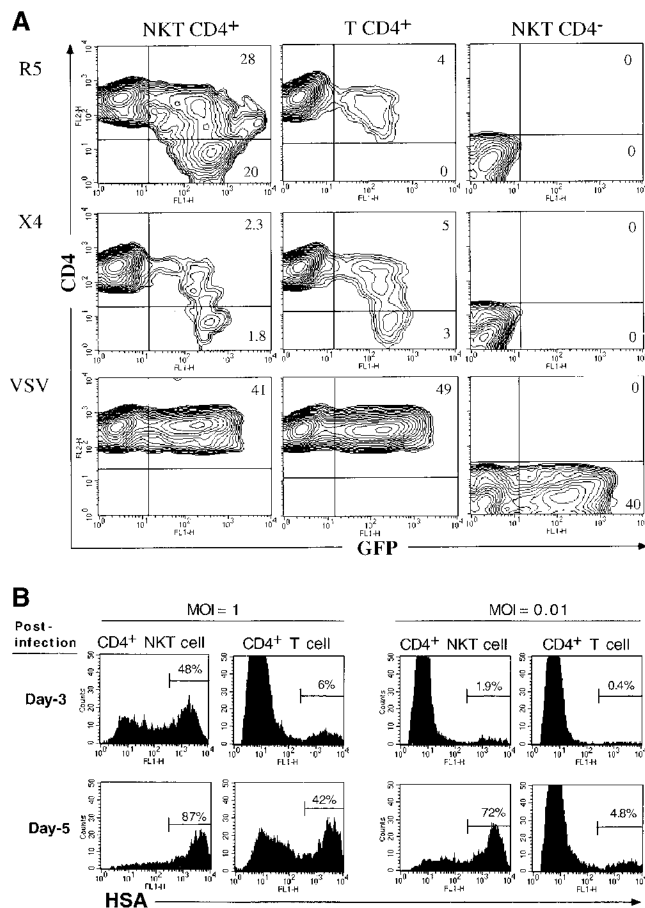

Figure 4. Infection of NKT cells by HIV-1. SEB-stimulated CD4+ T cells and α-GalCer stimulated NKT cell cultures were described in Fig. 1 legend. Activated NKT cells were further sorted into CD4+ and CD4− subsets. At days 7–10 after activation cells were infected with: (A) GFP-encoding R5-tropic, X4-tropic viruses, or VSV-G pseudotyped HIV vector at MOI of 1. After 3 d, cells were stained with anti-CD4-PE, fixed with 2% paraformaldehyde, and GFP expression was analyzed by FACS®. (B) Cells were also infected with HSA-encoding *nef*+ R5-tropic viruses at MOI 1 and 0.01. After 3 and 5 d after infection, cells were stained with FITC-conjugated anti–mouse HSA, fixed with 2% paraformaldehyde, and HSA expression was analyzed by FACS®.

NKT cells and conventional T cells by R5-tropic viruses are caused by differential receptor-mediated viral entry. Because the viruses used in these experiments lacked a functional *nef* gene due to replacement with GFP, we sought to determine whether presence of Nef effects the infectivity of NKT cells. NKT cells and conventional SEB-stimulated CD4+ T cells were infected with virus strains engineered to express the murine HSA antigen surface marker in place of *vpr* gene (35, 42). These viruses express Nef and were shown to fully replicate in an in vivo model of HIV-1 infection (42). Similar to *nef*-deleted GFP expressing viruses, *nef*+ strains infected NKT cells and spread in these cultures at a rate higher than SEB-stimulated CD4+ T cells (Fig. 4 B).

To determine whether resting NKT cells can also be infected, PBMCs were directly infected with GFP- or HSA-expressing viruses. To assess the role of cytokines in the infection of resting NKT cells, PBMCs were also stimulated with either IL-2 or IL-15 for 1 d and infected for 3 more d. As expected infection of resting T cells was much less robust than TCR-stimulated cells (Fig. 5 A). However, the proportion of resting CD4+ NKT cells that were GFP positive was consistently higher (~3–5-fold) than conventional resting CD4+ T cells (Fig. 5 B). Short-term stimulation of the cultures with IL-15 or IL-2 showed a slight increase in the percentage of infected CD4+ NKT and conventional CD4+ T cells (Fig. 5 B). We next sought to compare the infection of freshly isolated NKT cells vis a vis with CD4+ memory or CD4+ naive T cells, activated through the TCR. For this experiment we first purified CD4+ T cells which were further sorted into Vα24+ T cells, CD45RO+ memory, or CD45RA+ naive T cells. The Vα24+CD4+ T cells were then stimulated with α-GalCer plus DCs and CD4+RA+ or RO+ conventional T cells were stimulated with SEB plus DCs. All three sets of T cells were also infected with GFP expressing R5-tropic viruses. At third day of infection T cells were stained with CD1d-tetramers and Vβ11, to identify NKT cells, with CD25 to gate on TCR-activated T cells, (since SEB only activates a portion of T cells) and CD11b to exclude any remaining DCs. As shown in Fig. 5 C, TCR-stimulated resting NKT cells were infected substantially more than both TCR-activated memory or naive CD4+ T cells. These results reinforce our finding that CD4+ NKT cells are infectable with R5-tropic viruses at a higher rate than conventional CD4+ T cells and may be targeted by HIV-1 in vivo. Further, low level infection of resting NKT cells suggest, similar to conventional T cells, antigen-stimulation of NKT cells greatly enhances their susceptibility to HIV-1 infection (Fig. 5).

Replication of HIV-1 in NKT Cells. We next monitored the replication of HIV-1 within the NKT and conventional T cell cultures. NKT cells and conventional T cells were infected at a low MOI of 0.01, and GFP expression by infected cells was assessed at different time points after infection. The R5-tropic virus very rapidly spread in NKT cell cultures (Fig. 6 A). Indeed, infected CD4+ NKT cells, as assessed by GFP expression, increased by

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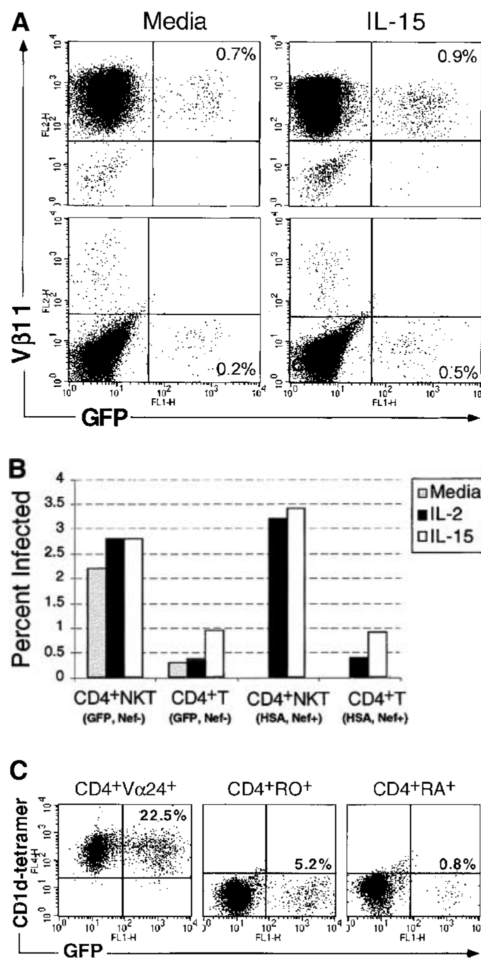

Figure 5. HIV-1 infection of resting or cytokine stimulated NKT cells in PBMCs. PBMCs were isolated and incubated for one day either in media alone or in the presence of IL-2 (50 U/ml) or IL-15 (10 ng/ml). Cells were then infected with R5-tropic virus expressing GFP or HSA. After 3 d of infection PBMCs were stained with CD3-PercP.Cy5.5, Vβ11-PE, and Vα24-biotin followed by strepavidin-APC. Cells that were infected with HSA-expressing viruses were also stained with HSA-FITC. (A) Approximately 10 million events were acquired for each sample to obtain statistically significant number of infected NKT cells. NKT cells were identified by gating on $CD3^+V\alpha24^+$ cells and staining for Vβ11 (top), conventional T cells were gated on $CD3^+V\alpha24^-$ cells (bottom). To facilitate visual comparison, only 1% of events acquired is shown for conventional T cells (bottom). (B) Cells were also stained with CD4-Percp, Vβ11-PE, and Vα24-biotin followed by strepavidin-APC to determine the percent of $CD4^+$ NKT and conventional T cells that are infected with HIV-1. (C) Purified $CD4^+V\alpha24^+$ (NKT), $CD4^+CD45RO^+$ (memory T), $CD4^+CD45RA^+$ (naive T), were stimulated through TCR and infected with R5-tropic HIV-1 expressing GFP. At 3 d after infection cells were stained with CD1d-tetramer-APC, CD25-PE (RA^+ or RO^+ T cells), or with Vβ11-PE ($V\alpha24^+$ T cells) and CD11b-Cy. Electronic gates were set on $CD25^+CD11b^-$ (for RO^+ and RA^+) or $V\beta11^+CD11b^-$ (for $V\alpha24^+$) T cells and on large cell size.

>15-fold from 4 to 60% of the cells between days 3 and 6 (Fig. 6 A), similar to infection with *nef*$^+$ HSA expressing viruses (Fig. 4 B). In contrast, X4-infected NKT cell cultures contained a constant number of GFP^+ cells (between 1 and 2%) up to 12 d after infection (Fig. 6 A, and data not shown). As expected, R5- and X4-tropic viruses replicated in conventional $CD4^+$ T cell cultures, albeit at slower kinetics compared with NKT cells (Figs. 4 B and 6 A). The production of virus by infected cultures were also determined, at different time points, by quantitating HIV p24 antigen and infectious virus in the culture supernatants using ELISA and GHOST assays, respectively. Both wild-type *nef*$^+$ and *nef*$^-$ viruses rapidly replicated in NKT cells, as assessed by p24 levels in the supernatants (Fig. 6 B). At days four and six after infection, NKT cultures contained 10–20-fold more viral particles compared with SEB-stimulated $CD4^+$ T cells (Fig. 6 B). To determine whether the virus produced from NKT cells was infectious, we used highly sensitive GHOST assay, which turns on GFP expression (through Tat protein) when infected with HIV-1 (36). At each time point after infection, NKT cells produced ~10-fold more infectious virus than SEB-stimulated T cells and with replication kinetics that paralleled p24 levels (data not shown).

To assess whether HIV-1 induces cell death, we infected a NKT cell culture, containing ~50% $CD4^+$ cells, either with R5-tropic or X4-tropic viruses. The percentage of $CD4^+$ and dead cells within the postinfection cultures were determined by FACS® analysis. 12 d after infection no $CD4^+$ NKT cells were detectable, even in cultures infected at an MOI as low as 0.01. At high MOI (0.3–1) most $CD4^+$ NKT cells were depleted by day 6 after infection (Fig. 6 C). In stark contrast, in cultures infected with X4-tropic viruses, the proportion of $CD4^+$ NKT cells remained similar to uninfected or VSV-G virus infected cultures (Fig. 6 C, and data not shown). Although, significant cell death was also observed in conventional T cell cultures following infection with both R5- and X4-tropic viruses, at least 50% of these cells were viable after 6 d after infection even at high MOI levels (data not shown). Thus, the R5-tropic virus rapidly replicates and depletes the $CD4^+$ NKT cell subset with faster kinetics compared with conventional $CD4^+$ T cells.

Analysis of NKT Cells in HIV-1 Infected Individuals. Because NKT cell lines are efficient hosts for HIV-1 in vitro and because resting NKT cells express similar levels of CCR5, we concluded that HIV-1 can infect NKT cells in vivo. To gain insight into the role of NKT cells during natural HIV-1 infection, we quantified NKT cell numbers both in HIV-1 infected and healthy individuals. PBMCs were isolated from infected or healthy donors and cells were subjected to four-color FACS® analysis using anti-Vβ11, CD3, and CD4 mAbs in conjunction with either CD1d-tetramer or Vα24-specific antibodies. $CD4^+$ or $CD4^-$ NKT cells were identified in 48 HIV-1 infected adults. Ages ranged from 34 to 59 y, 33% were African-American, 25% were female, 83% were receiving antiretroviral therapy, and the mean $CD4^+$ T cell counts was 402 cells/mm^3 (range 8 to 1,080 cells/mm^3). 10 (21%) had serologic evidence of either hepatitis B or C virus infection. None had active cytomegalovirus disease. As controls 22 uninfected healthy individuals were studied. The HIV-infected patients were slightly older than uninfected patients (mean age 40 versus 34 y; $P = 0.011$). The groups did not

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differ with regard to gender ($P > 0.05$). Approximately 44% of HIV-infected donors had undetectable levels of NKT cells (our detection limit was 0.003%), whereas all of the healthy donors had at least 0.01% NKT cells within their PBMCs ($P < 0.001$) (Fig. 7 A). In fact, 81% of healthy donors contained >0.01% NKT cells and 19% of these donors had >0.1% NKT cells (Fig. 7 A). Only ~30% of HIV-1–infected individuals had >0.01% NKT cells and none had >0.1% (Fig. 7 A).

Characterization of samples from HIV-infected subjects by univariate analysis showed that plasma HIV-1 RNA concentration was inversely correlated with $CD4^+$ T cell count ($r = -0.561$, $P < 0.001$) and percentage ($r = -0.631$, $P < 0.001$), $CD4^+$ NKT cell percentage ($r = -0.548$, $P = 0.006$) (Fig. 7 C), but not with total NKT cell percentage ($r = 0.194$, $P > 0.05$) (Fig. 7 B). Although six subjects were not receiving antiretroviral therapy, neither $CD4^+$ NKT cell nor total NKT cell percentages differed depending on whether antiretroviral therapy was being prescribed ($P > 0.05$). Mean NKT cell percentage of

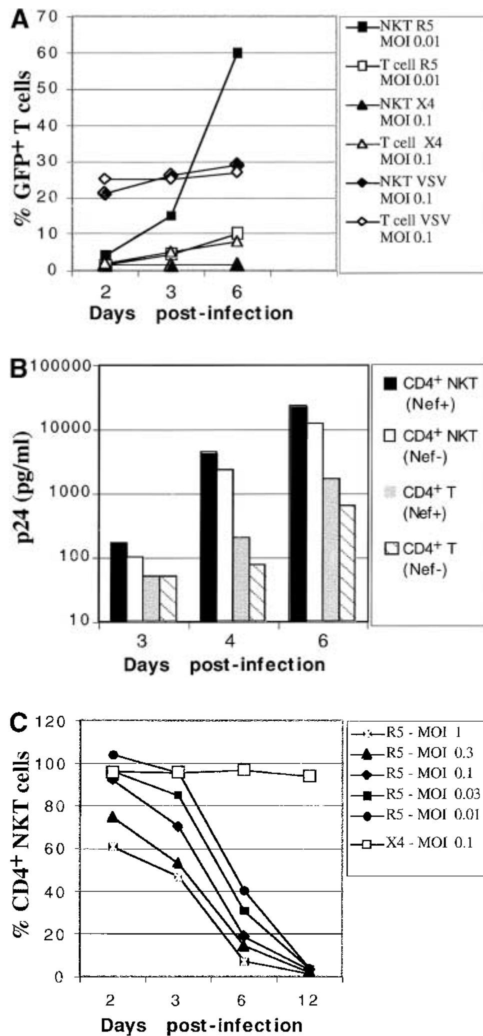

Figure 6. HIV-1 replication in NKT cell lines. (A) $CD4^+$ and NKT cell lines were infected with R5-tropic, X4-tropic or VSV-G pseudotyped HIV-1 at varying MOI (0.01–1). After 2, 3, and 6 d of infection cells were stained with anti-CD4-PE, fixed and analyzed by FACS®. The percentage of GFP^+ cells in the cultures is shown. (B) Supernatants from NKT cell cultures, infected with wild-type *nef*$^+$, or *nef*$^-$ virus MOI of 0.002 (p24: ~200pg/ml), were collected at different time points and HIV p24 levels were measured by ELISA. (C) Numbers of $CD4^+$ NKT cells were monitored in unsorted NKT cell lines at 2, 3, 6, and 12 d after infection by staining with anti-CD4-PE.

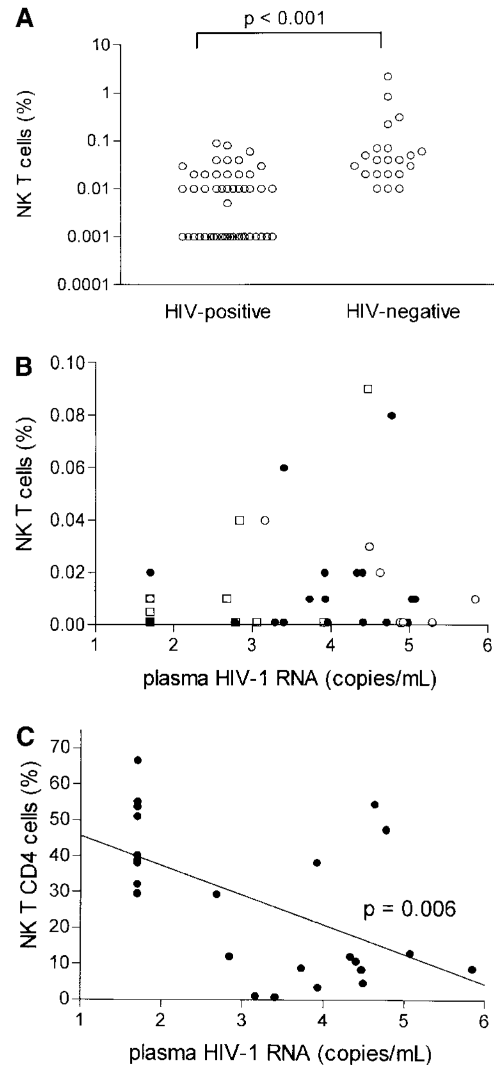

Figure 7. Analysis of NKT cells in HIV-1 infected and healthy donors. PBMCs from healthy and HIV-infected donors were isolated and stained with anti-Vβ11-FITC, CD3-PercP.Cy5.5, CD4-PE, and CD1d-tetramer-APC. Alternatively, cells were stained with anti-Vβ11-PE, Vα24-FITC, CD3-PercP.Cy5.5, and CD4-APC. Electronic gates were set on $CD3^+V\beta11^+V\alpha24^+$ or $CD3^+V\beta11^+CD1d\text{-tetramer}^+$ cells to identify the NKT cell subset. Between 3 and 5 million events were collected for each sample. Analysis was performed on CD3-gated T cells. The sensitivity for detecting NKT cells was at least 0.003%. (A) Portion of NKT cells among $CD3^+$ T cells of 48 HIV-infected (left) and 22 HIV-negative subjects (right). (B) Relationships between NKT cell percentage, plasma HIV-1 RNA concentration, and $CD4^+$ T cell counts in HIV-1–infected individuals. $CD4^+$ T cells were <200 cells/mm^3 (white circles), 200–500 cells/mm^3 (black circles), or >500 cells/mm^3 (white squares). (C) Relationship between $CD4^+$ NKT cell percentage and plasma HIV-1 RNA concentration in HIV-1–infected subjects. The regression line is shown.

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HIV-infected untreated individuals was about sixfold lower (0.038%) compared with healthy donors (0.26%) but this difference was not statistically significant, possibly due to the relatively few patients with untreated HIV infection ($P > 0.05$). To exclude any effects of possible chronic viral infections on NKT cell percentages, we compared the 10 hepatitis seropositive and 38 hepatitis seronegative HIV-infected patients with healthy donors. NKT cell percentages were greatly reduced in both groups which was highly statistically significant ($P < 0.001$ for each comparison). This analysis indicates that differences in NKT cell percentage are not due to hepatitis virus coinfection. When data from HIV-infected subjects was analyzed by linear regression, only $CD4^+$ NKT cell percentage was significantly correlated with plasma HIV-1 RNA concentration ($P = 0.011$). These results demonstrate both a severe reduction in NKT cells and selective loss of the $CD4^+$ NKT subset during HIV-1 infection.

Discussion

We have shown that human NKT cells, resting or activated, express high levels of chemokine receptors, including HIV-1/SIV coreceptors CCR5 and CXCR6, and that they are highly susceptible to infection with R5-tropic strains of HIV-1. The chemokine receptor family is differentially expressed on naive and memory subsets of human T cells. CC family chemokine receptors, including CCR5, CCR6, CXCR3, and CXCR6 are expressed primarily on the $CD45RO^+$ or $CD26^+$ memory/activated subset of T cells (43–46), whereas CXCR4 and CCR7 are expressed at higher levels on naive T cells. The memory/effector phenotype of NKT cells, in vitro and in vivo, is consistent with this expression pattern. Recently, human memory T cells were further subdivided, based on CCR7 expression, into two functionally distinct subsets (47). $CCR7^-$ memory cells lack the lymph node homing receptor, CD62L, express receptors for migration to inflamed tissues and display immediate effector function (47, 48). In contrast, $CCR7^+$ memory cells express lymph node homing receptors and lack immediate effector function. Of interest, the $CCR7^-$ cells are also enriched for expression of CCR5 (47, 49) and $CCR5^+CXCR6^+$ cells predominate in extralymphoid tissues (50). The patterns of chemokine receptor expression on resting or activated NKT cells described here, $CCR7^-CCR5^+CXCR6^+$, are consistent with a phenotype of memory T cells that would be compartmentalized to the organs and/or mucosal tissues where these cells are likely to be exposed to pathogens including HIV-1.

High expression levels of CCR5 and CD4 on NKT cells prompted us to determine their susceptibility to HIV-1 infection. Remarkably, we found that both activated and resting $CD4^+$ NKT cells are more susceptible to infection with R5-tropic, but not X4-tropic, viruses relative to conventional $CD4^+$ T cells. These findings suggest that NKT cells play a key role in establishing infection given their perpetually activated state. Cell surface expression of CCR5 is critical in transmission of HIV-1 infection. Individuals bearing a homozygous 32-base pair deletion in the *CCR5* gene that prevents CCR5 expression are largely resistant to HIV-1 infection (51–53). This compelling observation argues strongly that entry of R5-tropic strains into susceptible target cells is requisite for the establishment of HIV-1 infection. Why the absence of one HIV-1 coreceptor confers resistance remains unclear since these individuals have intact CXCR4 receptors and most are resistant to infection, despite exposure to both R5- and X4-tropic viruses (54, 55). Based on our results we speculate that NKT cells may be targeted at the initial phases of infection because of their high susceptibility to R5-tropic viruses and effector/memory status at sites where HIV-1 gains entry. Possibly the interaction between HIV-1 infected DCs and NKT cells facilitates infection through signaling NKT cells with endogenous ligand(s) and cytokines.

Our findings also show that NKT cells in PBMCs of HIV-1–infected individuals are dramatically reduced as compared with healthy donors. Although disappearance of the $CD4^+$ NKT cell subset is consistent with rapid replication and cell death upon infection with R5-tropic viruses (Fig. 6), it is not clear why $CD4^-$ NKT cells also disappear in HIV-1–infected individuals. It has been reported that NKT cells are reduced in numbers in several pathologic conditions such as autoimmune diseases (56–60) and during bacterial (61) and viral (62) infections. Current evidence suggests that in vivo activation of NKT cells through TCR ligation, results in rapid loss of these cells by apoptosis. This activation induced cell death is dependent on DC-derived IL-12 (63). Therefore, it is possible that during HIV-1 infection, NKT cells are activated by direct (antigen) or indirect mechanisms (cytokines), which result in activation induced cell death. However, after their initial disappearance, NKT cells usually rapidly reappear in vivo whereas during HIV-1 infection there may be either chronic stimulation or loss of NKT precursors, leading to a more permanent defect. It remains to be determined whether NKT cells reappear and fluctuate during the course of HIV-1 infection. As an alternative scenario, it is also possible that reduced numbers of $CD4^-$ NKT cells in HIV^+ individuals are due to expression of CD4 on these cells at some point during their differentiation and thus rendering them susceptible to infection. It will be important to understand whether any of these mechanisms play a role in the reduction of NKT cells during HIV-1 infection.

It will also be important to determine whether loss of NKT cells during HIV-1 infection affects the course of the disease. Recently, NKT cells have been implicated in the regulation of autoimmune diseases through suppression of immune responses against autoantigens (64) and antigens exposed in immune privileged sites (65). Indeed, in murine models of diabetes, stimulation of NKT cells can protect susceptible animals from the disease (66, 67). It has been proposed that NKT cells may also facilitate responses during bacterial (25) and viral infections (24, 68). Therefore, it is possible that NKT cells have a beneficial role in protecting against HIV-1 infection through secretion of chemo-

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kines or other effector functions. It is also possible that NKT cells affect the course of disease through secretion of large amounts of cytokines. Indeed, NKT cells activated by α-GalCer induce extensive bystander proliferation of memory T cells in the spleen and liver through cytokine production (20, 69, 70). During HIV-1 infection, cytokine production from activated NKT cells may recruit bystander resting T cells and render them susceptible to HIV-1 infection (33). Further examination of both in vitro and animal models for HIV-1 infection, particularly SIV infection of macaques, may yield insights toward the roles these cells play in viral replication and pathogenesis.

In conclusion, our findings implicate an important role for NKT cells in HIV-1 infection defining them as a second subset of T cells that undergoes depletion as a result of viral infection. It will be critical to determine at what level NKT cells affect the transmission and propagation of HIV-1 in vivo and how they contribute to the complex pathogenesis underlying AIDS.

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