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Ⅳ. 刊行物別刷（抜粋）

Hematopoietic stem cell–engrafted NOD/SCID/IL2Rγ^{null} mice develop human lymphoid systems and induce long-lasting HIV-1 infection with specific humoral immune responses

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Critical to the development of an effective HIV/AIDS model is the production of an animal model that reproduces long-lasting active replication of HIV-1 followed by elicitation of virus-specific immune responses. In this study, we constructed humanized nonobese diabetic/severe combined immunodeficiency (NOD/SCID)/interleukin-2 receptor γ-chain knockout (IL2Rγ^{null}) (hNOG) mice by transplanting human cord blood–derived hematopoietic stem cells that eventually developed into human B cells, T cells, and other monocytes/macrophages and dendritic cells associated with the generation of lymphoid follicle–like structures in lymphoid tissues. Expressions of CXCR4 and CCR5 antigens were recognized on CD4$^+$ cells in peripheral blood, the spleen, and bone marrow, while CCR5 was not detected on thymic CD4$^+$ T cells. The hNOG mice showed marked, long-lasting viremia after infection with both CCR5- and CXCR4-tropic HIV-1 isolates for more than the 40 days examined, with R5 virus–infected animals showing high levels of HIV-DNA copies in the spleen and bone marrow, and X4 virus–infected animals showing high levels of HIV-DNA copies in the thymus and spleen. Furthermore, we detected both anti–HIV-1 Env gp120– and Gag p24–specific antibodies in animals showing a high rate of viral infection. Thus, the hNOG mice mirror human systemic HIV infection by developing specific antibodies, suggesting that they may have potential as an HIV/AIDS animal model for the study of HIV pathogenesis and immune responses. (Blood. 2007; 109:212-218)

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Introduction

Current animal models for either human immunodeficiency virus type 1 (HIV-1) or simian immunodeficiency virus (SIV) suffer from the lack of a system precisely mirroring human HIV infection and the progression to disease state.[1] In current animal models with HIV infection, such as chimpanzees, animals do not develop AIDS.[1] Past animal models for HIV infection have relied on humanized severe combined immunodeficiency (hSCID) mice models to study prospective anti-HIV drugs and vaccines. SCID-hu (Thy/Liv) mice, engrafted with human fetal thymus and liver tissue in the renal subcapsular region, were first reported as the small-animal model.[2] Because human T cells are generated within the engrafted thymus, this model has been used for the study of thymopoiesis[3-6] and hematopoiesis[7,8] under the burden of HIV-1 infection. However, this model allows for a limited systemic HIV-1 infection, which is restricted mainly to the engrafted thymus. Another HIV mouse model, hu-PBL–SCID mice engrafted with human peripheral blood mononuclear cells (PBMCs),[9] has been actively used as a tool in developing antiretroviral therapy.[9-11] However, the infection persists for only a short time in association with rapid loss of CD4$^+$ T cells because there is no active hematopoiesis or thymopoiesis.[9,12,13] Furthermore, these mouse models fail to mirror certain key aspects of the human immune response, lacking normal lymphoid tissue and functional human antigen-presenting cells such as dendritic cells (DCs).[14] Thus, although these mouse models are valuable as animal models for HIV infection, the development of a mouse model more analogous to human HIV infection is needed if we are to better understand HIV pathogenesis and develop successful anti-HIV therapies and preventive vaccines.

To solve the difficult issue about the development of an ideal HIV mouse model, we initially selected a humanized nonobese diabetic (NOD)/SCID interleukin-2 receptor (IL-2R) γ-chain knockout (NOG) mouse[15] as a model animal because it has been suggested that multilineage cells, including human T, B, and natural killer (NK) cells, differentiate in these mice when given transplants of human CD34$^+$ hematopoietic stem cells.[16-18] In the current study, we further reveal the kinetics of differentiation of human B and T cells, monocytes/macrophages, and DCs in the mice that received transplants, and we characterize the animals by infection with both CCR5 (R5)– and CXCR4 (X4)-tropic HIV strains. Since our hNOG mice show stable and systemic infection of both R5- and X4-tropic HIV for more than

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the 40 days studied, and HIV-specific antibodies are detectable in the animals with high plasma viral loads and HIV-DNA copy numbers, we also discuss the suitability of HIV-hNOG mice as an animal model for HIV-1 infection.

Materials and methods

Transplantation of human CB-derived hematopoietic stem cells in NOG mice

Human cord blood (CB) was obtained from Saiseikai Central hospital (Minato-ku, Tokyo, Japan) and Tokyo Cord Blood Bank (Katsushika-ku, Tokyo, Japan) after obtaining informed consent. All research on human subjects was approved by the Institutional Review Board of each institution participating in the project. CB mononuclear cells were separated by Ficoll-Hypaque density gradient. $CD34^+$ hematopoietic stem cells were isolated using a magnetic-activated cell sorting (MACS) Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. More than 95% of $CD34^+$ cells were positively selected after 2 time-enrichment manipulations. Cells were either immediately used for the transplantation or frozen until use. NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and maintained under specific pathogen-free (SPF) conditions in the animal facility of the National Institute of Infectious Diseases (NIID; Tokyo, Japan). Mice used in these studies were free of known pathogenic viruses, herpes viruses, bacteria, and parasites. They were housed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science (1987) under the Japanese Law Concerning the Protection and Management of Animals, and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of NIID, Japan. Once approved by the Institutional Committee for Biosafety Level 3 experiments, these studies were conducted at the Animal Center, NIID, Japan, in accordance with the requirements specifically stated in the laboratory biosafety manual of the World Health Organization. Female mice (6 to 10 weeks old) were irradiated (300 cGy) and 1×10^4 to 1.2×10^5 $CD34^+$ cells were intravenously injected within 12 hours.

Flow cytometry

The purity of CB-derived $CD34^+$ cells after separation was evaluated by double staining with FITC-conjugated anti–human CD45 (J.33) and PE-conjugated anti–human CD34 (Class III 581) (all from Beckman Coulter, Fullerton, CA). After transplantation (1-7 months), peripheral blood, spleens, bone marrow (BM), and thymi were collected for flow cytometric analysis following staining with the following monoclonal antibodies (mAbs): FITC-conjugated anti–human CD45 (J.33), CD3 (UCHT1), CD4 (13B8.2), CD19 (J4.119), CD45RO (UCHL1) (all from Beckman Coulter), and CCR5 (2D7; BD Pharmingen, San Diego, CA); PE-conjugated anti–human CD4 (13B8.2), CD8 (B9.11), CD19 (J4.119), CD45RA (ALB11) (all from Beckman Coulter), and CXCR4 (44717; R&D Systems, Minneapolis, MN); anti–mouse CD45 (YW62.3; Beckman Coulter); ECD-conjugated anti–human CD45 (J.33; Beckman Coulter); and PC5-conjugated anti–human CD8 (T8) and CD14 (Rm052) (all from Beckman Coulter). Flow cytometric analysis was conducted by 2- or 4-color staining using an EpicsXL (Beckman Coulter).

Immunohistochemistry

Organs were snap-frozen following embedding in OCT compound (Sakura Finetechnical, Tokyo, Japan). Frozen sections were air-dried and fixed in acetone. HIV-1–infected organs were fixed in 4% paraformaldehyde and embedded in OCT compound following immersion in gradient sucrose (5%-30%). Fixed samples were stained with the following mAbs: anti–human CD45 (1.22/4014; Nichirei, Tokyo, Japan), CD3 (UCHT1; DAKO, Glostrup, Denmark), CD20 (L26; DAKO), CD68 (KP1; DAKO), CD205 (MG38; eBioscience, San Diego, CA), and DRC-1 (R4/23; DAKO) for follicular dendritic cells (FDCs); anti–mouse FDC-M1 (BD Pharmingen) for murine FDCs; and HIV-1 Gag p24 (DAKO) for detection of infected cells. Biotin-labeled goat $F(ab')_2$ anti–mouse immunoglobulin (Ig; ICN Biomedicals, Aurora, OH)– or biotin-labeled mouse $F(ab')_2$ anti–rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody. Samples were treated with alkaline phosphatase (AP) or horseradish peroxidase (HRP)–streptavidin conjugate (ZYMED Laboratories Inc, San Francisco, CA). BCIP/NBT, DAB, or AEC (all from DAKO) was used for the visualization. Photographs were taken by light microscopy (Leica DMRA; Leica Microsystems Wetzlar, Wetzlar, Germany) using Leica HC PLAN APO lenses (10×/0.40 NA PH1). Leica Q550 was used for image processing.

Measurement of human Igs in mice plasma

Plasma concentrations of human IgM, IgG, and IgA in NOG mice that received transplants of human stem cells were determined by conventional human Ig quantitation assay at BML Inc (Tokyo, Japan).

Cells and viruses

Human embryonic kidney 293T cells and monkey kidney COS7 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. The 293T cells and COS7 cells were used for transfection of DNA plasmids containing HIV-1_{JRCSF} and simian/human immunodeficiency virus (SHIV)–C2/1, respectively. The SHIV-C2/1 strain contains the *env* gene of pathogenic HIV-1 strain 89.6.[19] Cell-free supernatant was collected and stored at −80°C before use. A primary clinical isolate, HIV-1_{MNp}, was kindly provided by Dr J. Sullivan of the University of Massachusetts Medical School (Worcester, MA). PBMCs isolated from HIV-1–seronegative individuals were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics with 5 μg of phytohemagglutinin (PHA)/mL for 3 or 7 days (PHA-PBMCs). HIV-1_{MNp} was propagated in PHA-PBMCs, and cell-free virus stocks were stored at −80°C.

The 50% tissue-culture infectious dose ($TCID_{50}$) was determined using PHA-PBMCs and the endpoint dilution method. A 4-fold series of dilution was prepared from the virus stock, and then cells were mixed and cultured for 7 days for X4–HIV-1 and 14 days for R5–HIV-1 in RPMI 1640 supplemented with 20% FBS and antibiotics. The endpoints were determined by screening for the p24 antigen using Lumipulse (Fujirevio, Tokyo, Japan).

HIV-1 infection

All procedures for the infection and maintenance of NOG mice were performed in Biosafety Level 3 facilities at NIID under standard caging conditions. On days 102 to 132 after stem cell transplantation, 16 mice were inoculated intravenously with R5-tropic HIV-1_{JRCSF} (65 000 $TCID_{50}$) or X4-tropic SHIV-C2/1 (50 000 $TCID_{50}$). On days 18 to 43 after inoculation, plasma was collected to determine HIV-RNA copy numbers, and spleen cells were prepared as single-cell suspensions to analyze the CD4/CD8 ratio using flow cytometry. A number (14) of other mice were inoculated intravenously with R5-tropic HIV-1_{JRCSF} (200 or 65 000 $TCID_{50}$) or X4-tropic HIV-1_{MNp} (180 or 20 000 $TCID_{50}$) on days 126 to 146 after transplantation. On days 18 to 40 after inoculation, plasma was collected for the determination of HIV-RNA copy numbers, and single-cell suspensions of the spleen, BM, and thymus were prepared for HIV-DNA measurement. The CD4/CD8 ratio in the spleen and percentages of human $CD45^+$ cells in organs were analyzed using flow cytometry.

Virologic analysis

Plasma viral RNA copy numbers were measured using a real-time quantification assay based on the TaqMan system (Applied Biosystems, Foster City, CA). Plasma viral RNA was extracted and purified using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). The RNA was subjected to reverse transcription (RT) and amplification using a TaqMan One-Step RT–polymerase chain reaction (PCR) Master Mix Reagents Kit (PE Biosystems, Foster City, CA) with HIV-1 gag consensus primers

(forward, 5′-GGACATCAAGCAGCCATGCAA-3′; and reverse, 5′-TGCTATGTCACTTCCCCTTGG-3′) and an HIV-1 gag consensus TaqMan probe (FAM-5′-ACCATCAATGAGGAAGCTGCAGAA-3′-TAMRA). For SHIV-C2/1 analysis, primers (forward, 5′-AATGCAGAGCCCCAAGAAGAC-3′; and reverse, 5′-GGACCAAGGCCTAAAAAACCC-3′) and a TaqMan probe (FAM-5′-ACCATGTTATGGCCAAATGCCCAGAC-3′-TAMRA) were designed for targeting the SIVmac239 gag region.[20] Probed products were quantitatively monitored by their fluorescence intensity with the ABI7300 Real-Time PCR system (PE Biosystems). To obtain control RNA for quantification, HIV-1 gag RNA and SIVmac239 gag RNA were synthesized using T7 RNA polymerase and pKS460. Viral DNA was extracted and purified using a QIAamp DNA Mini Kit (Qiagen). Determination of HIV-1 DNA copy numbers was performed by real-time PCR assay with TaqMan Master mixture (PE Biosystems). Primers (forward, 5′-GGCTAACTAGGGAACCCACTG-3′; and reverse, 5′-CTGCTAGAGATTTTCCACACT-3′) and probes (FAM-5′-TAGTGTGTGCCCGTCTGTTGTGTGAC-3′-TAMRA) were designed for targeting the HIV-1 long terminal repeat region, R/U5. The viral DNA was quantified using LightCycler (Roche Diagnostics, Almere, The Netherlands). Viral RNA and DNA were calculated based on the standard curve of control RNA and DNA. All assays were carried out in duplicate.

HIV-antigen ELISA

Levels of anti–HIV-1 Igs against recombinant HIV-1_{IIIB} Env gp120, recombinant HIV-1_{MN} Env gp120, and recombinant HIV-1_{IIIB} Gag p24 (all from ImmunoDiagnostics Inc, Woburn, MA) in plasma from HIV-1–infected and –uninfected control mice were determined using a standard enzyme-linked immunosorbent assay (ELISA). Microplates (96-well) were coated overnight with 200 ng/well antigens, and plasma diluted 1:20, 1:60, and 1:180 with PBS were incubated for 1 hour. AP-labeled anti–human Igs (γ, α, and μ; Sigma-Aldrich, St Louis, MO) were used as secondary antibodies. P-nitrophenylphosphate (pNPP) Solution (WAKO Chemical USA, Richmond, VA) was used for the visualization. The enzyme reaction was stopped by addition of 0.1 M NaOH and read at 405 nm. All assays were carried out in triplicate.

Statistical analysis

Data were expressed as the mean value ± standard deviation (SD). Significant differences between data groups were determined by 2-sample Student *t* test analysis. A *P* value less than .05 was considered significant.

Results

Reconstitution of human lymphoid systems in hNOG mice

The initial studies describing the construction of humanized SCID mice used the human PBMC for infection of immunodeficiency viruses.[9,12,21] However, these hu-PBL-SCID mice showed a partial infection to the R5 virus and a relatively limited period of viral replication. To construct a more suitable mouse model mimicking HIV-1 infection in humans, we selected human CB stem cells as a transplant for NOG mice. NOG mice were inoculated intravenously with human CD34$^+$ hematopoietic stem cells, and their development of human lymphoid systems were then monitored. After transplantation (2 months), human CD45$^+$ leukocytes were recognized in both PB and the spleen, but most of the cells were human B cells (Figure 1A). Human T cells began to be recognized clearly in PB and the spleen 4 months after transplantation (Figure 1B) and gradually increased in level, as did human B cells (Figure 1C).

In Figure 1D, we summarized percentages of human CD3$^+$ T cells in human CD45$^+$ cells from 38 mice from 39 to 213 days after transplantation. Human CD3$^+$ T cells clearly increased 100 days after transplantation in both PB and the spleen. After transplantation (4 months), human CD3$^+$ T cells in the spleen preferably expressed CD45RA rather than CD45RO (70.8% ± 13.4% and 27.3% ± 38.8% in CD3$^+$ T cells, respectively; n = 5; Figure 1E), demonstrating that most of the T cells were in a naive state. In addition, plasma taken from 5 mice 113 to 143 days after transplantation showed that all mice produced human IgM, with concentrations ranging from 0.025 to 0.5 g/L, and that human IgG and IgA was also detected in some of the mice (ranges, 0.015-0.18 g/L and 0.003-0.012 g/L, respectively) (data not shown).

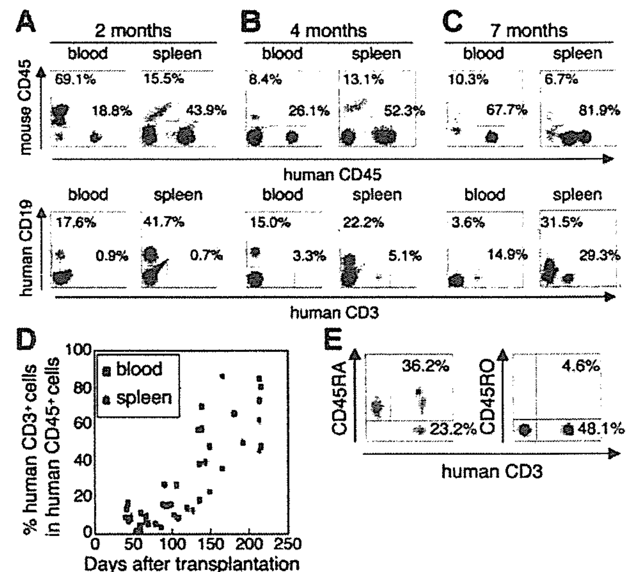

Figure 1. Flow cytometric analysis of human T cells in the peripheral blood and spleen in NOG mice given intravenous transplants of human CB-derived CD34$^+$ cells. (A-C) Representative profiles of the mice 2 months (A), 4 months (B), and 7 months (C) after transplantation. The ratio of human to murine CD45$^+$ cells and that of human CD3$^+$ to CD19$^+$ cells show an incremental increase in human CD45$^+$ cells and human CD3$^+$ cells from 2 to 7 months. (D) Change of net percentages of human CD3$^+$ T cells among human CD45$^+$ cells in peripheral blood and the spleen from 38 mice 39 to 213 days after transplantation. (E) CD45RA is more efficiently expressed than CD45RO on human CD3$^+$ T cells in spleen. A gate was set on the human CD45$^+$ population. The fluorescence-activated cell sorting (FACS) profile is representative of 1 in a group of 5 mice.

By 7 months after transplantation, human CD45$^+$ leukocytes comprised more than 80% to 90% of mononuclear cells in the spleen (Figure 1C), and most of the mice showed symptoms of a wasting condition and a hunched back. Based upon these results, we determined that the suitable period for HIV inoculation would be 4 to 5 months after transplantation.

Formation of lymphoid structures, including monocytes/macrophages, DCs, and FDCs

Next, using the hNOG mice at 4 months after transplantation, we investigated lymphoid structure formation and the development of human monocytes, macrophages, DCs, and FDCs, which are very important factors not only for elicitation of immune responses against foreign antigens, but also for the spread of HIV-1 infection in a body.[22-24] Human CD14$^+$ monocytes were detected in PB, the spleen, and BM using flow cytometry (Figure 2A). During immunohistochemical analysis, human CD45$^+$ leukocytes gathered in a form of follicle-like structures (FLSs) at the end of the central artery in the spleen (Figure 2B). From a serial section of the same region (Figure 2B-G), these structures consisted mainly of human CD20$^+$ B cells (Figure 2C) admixed with a small number of human CD3$^+$ T cells (Figure 2D). Hardly any human FDCs positive for DRC-1 were detected (data not shown), whereas a

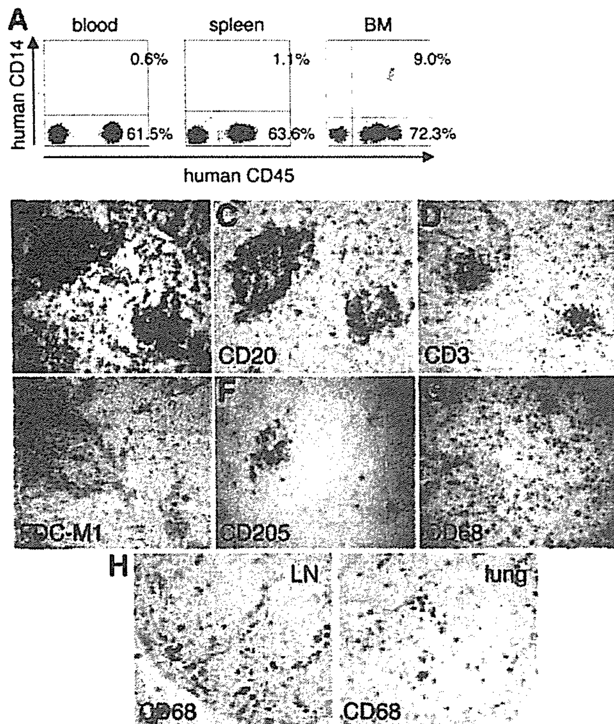

Figure 2. Flow cytometric analysis and immunohistochemical analysis of the expression of myelomonocytic markers in hNOG mice 4 months after transplantation. (A) Human $CD14^+$ monocytes/macrophages are recognized in peripheral blood, the spleen, and BM. (B-G) Immunohistochemical findings from serially sectioned spleen for the expressions of human CD45 (B), human CD20 (C), human CD3 (D), murine FDC (E), human CD205 (F), and human CD68 (G). (H) Human $CD68^+$ macrophages are also detected in the medulla of the LN and lung. Visualization was performed with BCIP (B-D, F-G), DAB (E), and AEC (H). Original magnification, ×100.

loose network of murine FDCs positive for FDC-M1 was recognized in the distal portion of the FLSs (Figure 2E). Human $CD205^+$ DCs were predominantly detected in a cluster form within the FLSs (Figure 2F), while human $CD68^+$ macrophages were scattered throughout the spleen (Figure 2G). Many human $CD68^+$ macrophages were also observed in various other organs, including the lymph nodes (LNs) and the lungs (Figure 2H).

Expression of HIV-1 coreceptors on $CD4^+$ cells in various tissues

Since the development of lymphoid tissues was recognized in hNOG mice, we focused on the expressions of HIV-1 coreceptors CXCR4 and CCR5 on human $CD4^+$ cells in these tissues. CXCR4 antigen was expressed in 36.5% ± 4.2% (n = 4) of the $CD4^+$ cells in PB (Figure 3A) and 78.1% ± 17.1% (n = 5) in the spleen (Figure 3B). $CCR5^+$ cells were detected in 15.5% ± 1.8% (n = 4) of $CD4^+$ cells in PB and 28.6% ± 12.6% (n = 5) in the spleen (Figure 3A-B). In the thymus, $CD4^+CD8^+$ thymocytes existed in 82.9% ± 4.4% (n = 5) as well as small numbers of $CD4^+CD8^-$ cells (6.4% ± 2.4%; n = 5) and $CD4^-CD8^+$ cells (7.7% ± 3.0%; n = 5), with the CXCR4 antigen expressed in 50.1% ± 4.5% (n = 5) of $CD4^+$ cells, while, as with normal human thymocytes,[25] $CCR5^+$ cells were almost undetectable, with less than 1% (0.6% ± 0.1%; n = 5) (Figure 3C). Human $CD3^+$ T cells and $CD14^+$ monocytes in BM were detected only in 3.2% ± 2.1% and 5.8% ± 3.8%, respectively, while $CD4^+$ cells were recognized in 18.1% ± 6.5%, with many expressing both CXCR4 (75.0% ± 23.1%) and CCR5 (81.3% ± 6.6%; n = 5; Figure 3D). Thus, distributions of HIV-1 coreceptor–positive cells in these lymphoid tissues suggest that the hNOG mice allow for sufficient development of human cells to make the study of HIV-1 pathogenesis possible.

Both R5- and X4-tropic HIVs efficiently infect and replicate in hNOG mice

In our preliminary study, using low and high doses of challenge virus, no viral infection was detected in any of the virus-inoculated hNOG mice at 7 days after infection, while some showed detectable plasma viral loads at 14 days (data not shown). Then, we prepared 16 hNOG mice that received transplants of stem cells and inoculated them with a high dose of R5-tropic HIV-1_{JRCSF} (65 000 $TCID_{50}$) and X4-tropic SHIV-C2/1 (50 000 $TCID_{50}$) intravenously through the tail vein at 102 to 132 days after transplantation. Upon HIV-1_{JRCSF} infection, viral copy numbers in plasma rose to a level of 1.6×10^5 to 5.8×10^5 copies/mL (n = 4) on day 33 and 2.0×10^5 to 4.7×10^5 copies/mL on day 43 (n = 4) (Figure 4A). Moreover, for SHIV-C2/1 infection, viral copy numbers in plasma were 1.6×10^3 to 3.2×10^5 copies/mL on day 18 (n = 4) and reached 5.4×10^4 to 1.1×10^5 copies/mL on day 42 (n = 4; Figure 4B). In these mice, no significant decline in the CD4/CD8 ratio was observed throughout entire period of follow-up for the R5-tropic virus infection, while $CD4^+$ cell decline was detected for the X4-tropic virus infection on day 42 after infection ($P = .044$) but not on day 18 after infection (Figure 4C). Four mice that did not receive transplants of human stem cells showed no detectable levels of plasma viral load (less than 500 copies/mL) following HIV/SHIV inoculation (data not shown).

To confirm HIV infection, we used immunohistochemistry to detect the presence of the p24 antigen of the HIV-1 Gag protein in various tissues of mice showing viremia. $p24^+$ cells were clearly identified in the spleen, LN, and lungs (Figure 4D), which include macrophage-like cells.

Different distributions of R5- and X4-tropic viruses in lymphoid tissues

A number of mice (14) were further analyzed for HIV-1 infection on days 126 to 146 after transplantation with a low dose (200 $TCID_{50}$) or a high dose (65 000 $TCID_{50}$) of R5-tropic HIV-1_{JRCSF} and a low dose (180 $TCID_{50}$) or a high dose (20 000 $TCID_{50}$) of X4-tropic HIV-1_{MNp}. Consequently, 2 of the 4 mice given a low

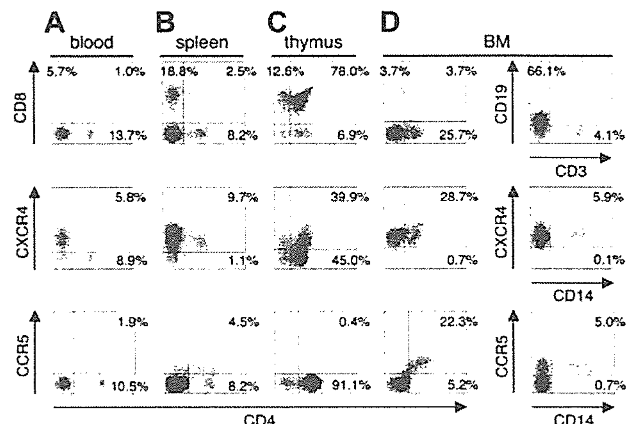

Figure 3. Surface expression of HIV-1 coreceptors on $CD4^+$ cells in various organs of mice 4 months after transplantation. A representative FACS profile of human CXCR4 and CCR5 on $CD4^+$ cells shows the existence of $CXCR4^+CD4^+$ and $CCR5^+CD4^+$ cells in blood (A), spleen (B), and BM (D), but no $CCR5^+CD4^+$ cells in the thymus (C). BM results show that many $CD4^+$ cells are neither $CD3^+$ T cells nor $CD14^+$ monocytes. A gate was set on the human $CD45^+$ population.

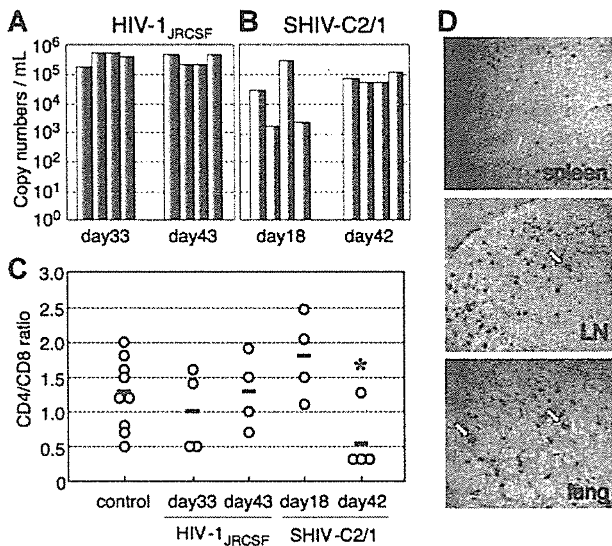

Figure 4. The numbers of RNA viral copies in plasma, $CD4^+/CD8^+$ T-cell ratios in the spleen, and p24 detection in the immunohistochemistry of HIV/SHIV-infected mice. (A) Viral copy numbers of 8 mice inoculated with a high infectious dose of HIV-1_{JRCSF} (65 000 $TCID_{50}$) and killed on days 33 and 43 after inoculation. (B) Viral copy numbers of 8 mice inoculated with a high infectious dose of SHIV-C2/1 (50 000 $TCID_{50}$) and killed on days 18 and 42 after inoculation. Note that all the mice showed high levels of viremia that lasted more than 40 days after inoculation. (C) CD4/CD8 cell ratios in the spleens of 16 infected mice and 9 uninfected control mice. Control mice were not inoculated with HIV/SHIV and were killed on days 105 to 166 after stem cell transplantation. There was no significant rapid loss of $CD4^+$ cells in HIV-1_{JRCSF}–infected mice, while a decline of the CD4/CD8 ratio was detected in SHIV-C2/1–infected mice on day 42 after infection compared with uninfected control mice (*$P < .05$). The short bars indicate the means of each group. (D) $P24^+$ cells are clearly observed in the spleen, LNs, and lungs. Arrow indicates p24 positive for macrophage-like cells. Original magnification, ×100.

dose of HIV-1_{JRCSF} and 2 of the 3 mice given a low dose of HIV-1_{MNp} were successfully infected (Table 1), suggesting that each dose represents an approximately 50% infectious dose of HIV for hNOG mice. High HIV-DNA copy numbers were mainly detected in the spleen and BM of the HIV-1_{JRCSF} –infected mice, and in the thymus and spleen of the HIV-1_{MNp}–infected mice, while their BM showed lower copy numbers (Table 1).

Generation of HIV-specific antibodies in hNOG mice at a high multiplicity of infection

We then tested for generation of human antibodies against HIV-1 from these 14 mice by HIV antigen-specific ELISA. The sera of mice no. 136-3 and no. 157-3 infected with HIV-1_{JRCSF} and HIV-1_{MNp}, respectively, showed significant levels of human antibodies specific for HIV-1_{IIIB}-Env gp120 (Figure 5A), HIV-1_{MN}-Env gp120 (Figure 5B), and HIV-1_{IIIB}-Gag p24 (Figure 5C). In addition, no. 157-4 sera from an HIV-1_{MNp}–infected animal was also weakly positive for their Env and Gag antigens. These animals showed intense plasma viral loads and enhanced proviral DNA copies in the spleen, BM, and thymus (Table 1), suggesting that hNOG mice inoculated with high doses of HIV and showing high rates of viral infection develop HIV-1–specific humoral immune responses that are analogous to those seen in human anti-HIV B-cell responses.

Discussion

Current small-animal models fall short of accurately mirroring human HIV-1 infection and thus have limited usefulness in analyzing the natural course of its progression to the disease state and in developing antiviral countermeasures. Although successful HIV-1 infections in immunodeficiency mice humanized with PBMCs have been reported,[12,13,21] transplanted human cells are soon depleted and do not elicit virus-specific immune responses, shedding little light on pathogenesis and vaccine development. By using NOG mice that received hematopoietic stem cell transplants showing high rates of viral infection, we demonstrated HIV-specific antibody responses and viral infection parameters, including the following: (1) similar levels of susceptibility to both R5- and X4-tropic HIV-1; (2) high levels of viremia stably observed over 40 days; (3) immunohistochemical detection of infected cells in various organs; and (4) a distinct tissue distribution for R5- versus X4-tropic HIV-1s.

Among $CD4^+$ T cells, CXCR4 antigen is primarily expressed on naive and CCR5 on activated or memory cells.[26] hu-PBL-SCID mice become susceptible to R5-tropic HIV-1 strains,[27] since T cells

Table 1. Comparison of viral RNA copies in plasma and HIV-DNA copies in the spleen, BM, and thymus from hNOG mice receiving low- and high-dose viral inoculations

Mouse ID no.	HIV strain	$TCID_{50}$	Time after inoculation, d	RNA viral copies/mL	CD4/CD8 ratio	HIV-DNA copies/10^6 human cells: Spleen	BM	Thymus
Low-dose viral inoculation group								
113-1	HIV-1_{JRCSF}	200	18	6 240	1.8	34 177	11 785	3 495
112-2	HIV-1_{JRCSF}	200	18	<500	1.2	< 100	< 100	< 100
113-2	HIV-1_{JRCSF}	200	40	6 177	1.6	25 855	27 920	3 473
112-3	HIV-1_{JRCSF}	200	40	<500	0.9	< 100	< 100	<100
112-4	HIV-1_{MNp}	180	18	72 477	1.3	18 873	100	ND
113-4	HIV-1_{MNp}	180	40	70 667	0.3	4 947	653	32 163
112-1	HIV-1_{MNp}	180	40	<500	0.9	< 100	< 100	< 100
High-dose viral inoculation group								
136-3	HIV-1_{JRCSF}	65 000	25	252 381	0.8	958 871	1 797 600	232 155
136-2	HIV-1_{JRCSF}	65 000	29	50 167	0.7	41 172	54 521	8 600
141-1	HIV-1_{JRCSF}	65 000	30	67 667	2.2	27 735	52 430	429
161-3	HIV-1_{JRCSF}	65 000	30	13 847	0.9	104 466	14 653	111 080
157-3	HIV-1_{MNp}	20 000	31	1 253 925	0.5	41 053	56 802	976 556
157-4	HIV-1_{MNp}	20 000	31	147 973	0.6	3 634	262	40 796
161-6	HIV-1_{MNp}	20 000	31	108 073	1.7	4 991	< 100	3 673

Seven mice inoculated with a low infectious dose of HIV-1_{JRCSF} (200 $TCID_{50}$) or HIV-1_{MNp} (180 $TCID_{50}$), and 7 mice receiving a high infectious dose of HIV-1_{JRCSF} (65 000 $TCID_{50}$) or HIV-1_{MNp} (20 000 $TCID_{50}$) were listed.

ND indicates not done.

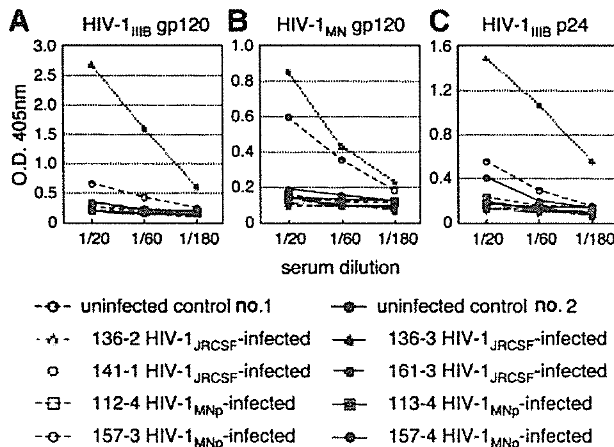

Figure 5. Detection of anti–HIV-1 antibodies from the plasma of HIV-1–infected mice. An ELISA assay was conducted by using plasma from 14 mice inoculated with either HIV-1_{JRCSF} or HIV-1_{MNp}, and from 2 uninfected control mice. Representatives (n = 8) of the 14 HIV-1–inoculated mice, and the 2 uninfected mice, are shown in the panels. Measurements of specific human antibodies for HIV-1_{IIIB} gp120 (A), HIV-1_{MN} gp120 (B), and HIV-1_{IIIB} p24 antigens (C) were shown. Results are expressed as the means from triplicate assays in 3 different experiments.

are initially activated in the xenogenic environment and then become anergic.[14] In contrast, SCID-hu (Thy/Liv) mice are more susceptible to X4 than to R5 strains[6] because HIV-1 infection is restricted mainly to the engrafted thymus that is primarily comprised of immature T cells, suggesting that this model may not be practical overt HIV infection. Our study represents the first attempt to infect NOG mice that received transplants of human hematopoietic stem cells with HIV-1. Very similar infection rates were seen for both R5 and X4 strains in the mouse model. Flow cytometry revealed both $CXCR4^+CD4^+$ and $CCR5^+CD4^+$ cells in PB, the spleen, and BM, but only CXCR4 on thymic $CD4^+$ T cells. It also showed the scattering of human macrophages, known to be susceptible to R5-tropic HIV-1 strains[28,29] and the source of HIV-1,[23,30-32] throughout various organs. $p24^+$ macrophage-like cells were detected in these organs after R5-tropic HIV-1_{JRCSF} infection. These data may help explain the susceptibility of hNOG mice to both R5- and X4-tropic HIV strains and also shed light on the active replenishment of these target cells in mice.

SCID mouse systems have been actively used in the evaluation of anti–HIV-1 drugs.[9,11,21] In most cases, HIV-1 detection levels reach a peak within a month after inoculation and level off, accompanied by $CD4^+$ T-cell depletion.[3,12,13] Although suitable for short-term experiments, it is also true that these models require large numbers of mice because of large variations in infection efficiency. In contrast, very stable infections were noted in our hNOG mice that were inoculated with a high dose of HIVs. They did not show rapid CD4/CD8 decrease in spite of high levels of viremia persisting for more than 40 days. Efficient hematopoiesis and thymopoiesis of human cells probably compensated for the loss of $CD4^+$ T cells, allowing for persistent infection. This capacity of the hNOG mouse system makes it attractive as a model for the long-term evaluation of anti–HIV-1 drugs. In addition to destroying mature blood cells, altered hematopoiesis in BM and the thymus has also been reported to be responsible for immunodeficiency in patients with AIDS.[33,34] To study hematopoietic abnormalities in HIV-1 infection, both SCID-hu (Thy/Liv) mice[8,35,36] and SIV- or SHIV-infected macaque models[20,37-39] have been used. The current hNOG mouse system, in which human cells are efficiently reproduced from stem cells and then settled into hematopoietic organs, offers a promising model for the study of events that occur after infection not only with R5-tropic HIV-1 but also with X4-tropic HIV-1. Indeed, the BM of hNOG mice infected with R5-tropic HIV-1 exhibited exceptionally elevated levels of HIV-DNA copies. On the other hand, the thymus of X4-tropic HIV-1_{MNp}–infected hNOG mice yielded large numbers of HIV-DNA copies, which seemed to correlate with the predominant expression of CXCR4 on the thymocytes. Thus, further observation is essential to address whether AIDS symptoms such as considerable $CD4^+$ T-cell depletion and hematopoietic abnormalities eventually occur in these mice.

It is noteworthy that human antibodies against both HIV-1 Env gp120 and Gag p24 antigens were detected in mice no. 136-3, no. 157-3, and no. 157-4 after exposure to high titers of HIV-1, suggesting that hNOG mice have the ability to respond to HIV-1 antigens. This encourages us to develop antibody-based HIV vaccine candidates, although additional modifications are required for the stable induction of immune responses. Importantly, since the seroconverted mice showed high viremia and high numbers of proviral DNA copies in the spleen, BM, and thymus, abundant viral production may stimulate human B-cell responses against HIV-1 and generate specific antibodies. These mice showed little or no detectable human IgG against HIV-1, as determined by Western blot analysis (data not shown), suggesting that very low levels of class-switching occurred in these mice, though further study is required.

In addition to the humoral immune responses, the induction of primary T-cell responses is critical for the study of HIV-specific immune responses and pathogenesis, as well as for vaccine development. Although we did not demonstrate the T-cell ability to respond to virus antigens, human T cells from the spleen proliferated when stimulated with anti–human CD3 antibodies (data not shown), indicating that the human T cells in the NOG mice that received transplants of hematopoietic stem cells are capable of responding to T-cell receptor–mediated signals and are expected to be able to elicit primary antigen-specific immune responses against foreign antigens. To address whether the specific T-cell responses may be induced will be one of the important studies.

In conclusion, the NOG mice that received transplants of human hematopoietic stem cells successfully achieved systemic and persistent infection with both R5-tropic and X4-tropic HIV-1, and generated humoral immune responses against HIV-1. These capacities of the hNOG mouse model may be very attractive for the study of HIV pathogenesis and humoral immune responses induced by HIV vaccine candidates.

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Authorship

Contributions: S.W., K.T., N.S., M.H., and N.Y. designed the study; S.W., K.T., S.O., S.H., M.Y., Y.S., M.Z.D., and Z.Y. carried out the research; M.I. contributed live mice; S.W., K.T., and T.M. analyzed the data; N.S., M.H., and N.Y. controlled the data; S.W. wrote the paper; and all authors checked the final version of the manuscript.

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Anti-V3 Humanized Antibody KD-247 Effectively Suppresses Ex Vivo Generation of Human Immunodeficiency Virus Type 1 and Affords Sterile Protection of Monkeys against a Heterologous Simian/Human Immunodeficiency Virus Infection

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In an accompanying report (Y. Eda, M. Takizawa, T. Murakami, H. Maeda, K. Kimachi, H. Yonemura, S. Koyanagi, K. Shiosaki, H. Higuchi, K. Makizumi, T. Nakashima, K. Osatomi, S. Tokiyoshi, S. Matsushita, N. Yamamoto, and M. Honda, J. Virol. 80:5552-5562, 2006), we discuss our production of a high-affinity humanized monoclonal antibody, KD-247, by sequential immunization with V3 peptides derived from human immunodeficiency virus type 1 (HIV-1) clade B primary isolates. Epitope mapping revealed that KD-247 recognized the Pro-Gly-Arg V3 tip sequence conserved in HIV-1 clade B isolates. In this study, we further demonstrate that in vitro, KD-247 efficiently neutralizes CXCR4- and CCR5-tropic primary HIV-1 clade B and clade B′ with matching neutralization sequence motifs but does not neutralize sequence-mismatched clade B and clade E isolates. Monkeys were provided sterile protection against heterologous simian/human immunodeficiency virus challenge by the passive transfer of a single high dose (45 mg per kg of body weight) of KD-247 and afforded partial protection by lower antibody doses (30 and 15 mg per kg). Protective neutralization endpoint titers in plasma at the time of virus challenge were 1:160 in animals passively transferred with a high dose of the antibody. The antiviral efficacy of the antibody was further confirmed by its suppression of the ex vivo generation of primary HIV-1 quasispecies in peripheral blood mononuclear cell cultures from HIV-infected individuals. Therefore, KD-247 promises to be a valuable tool not only as a passive immunization antibody for the prevention of HIV infection but also as an immunotherapy for the suppression of HIV in phenotype-matched HIV-infected individuals.

Because most primary strains of human immunodeficiency virus type 1 (HIV-1) are relatively resistant to neutralization, the specificities of antibodies that confer protective immunity against it are still not understood (22). Previously, we and others (9, 31) have reported that chimpanzees can be protected against infection with the T-cell-line-adapted strain HIV-1_{IIIB} by passive transfer of either HIV immunoglobulin (Ig) (HIVIG) or anti-HIV-1_{IIIB} V3 monoclonal antibodies (MAbs). Passive administration of the anti-HIV-1 gp41 human MAb 2F5 (24) to two chimpanzees prior to challenge with primary HIV-1_{5016} resulted in a delay in plasma viremia and reduced viral load. Since the chimpanzee model is limited by the failure of HIV-1 to induce disease in these animals, a pathogenic model was developed in monkeys using a simian/human immunodeficiency virus (SHIV) strain that is capable of inducing high plasma viremia, $CD4^+$-T-cell loss, and simian AIDS (11, 14, 15, 37). Following pathogenic SHIV 89.6P challenge, Mascola and colleagues (20) previously noted a synergistic effect with the passively transferred antibody HIVIG, a MAb against membrane-proximal external region 2F5 (27), and 2G12, a glycan-dependent MAb (41). Monkeys were afforded protective immunity against pathogenic SHIV DH12 by chimpanzee HIVIG and provided sterile protection against the challenge virus when given high-dose inoculations (27, 36). However, sterile protection was strain specific, and the antiserum did not bind a V3 loop peptide or block the interaction of gp120 with CD4. In several passive immunization studies using MAbs, the antibodies 2G12 and 2F5 as well as 4410, a MAb against membrane-proximal external region 4E10 (4), have been shown to inhibit SHIV in monkeys (2, 20, 21). Furthermore, human MAb b12, targeting the CD4-binding domain of gp120, has been reported to elicit complete protection against viral challenge (29) and partial protection against MAb 2G12 (22) in monkeys. Recently, passively transferred antibodies with 2G12, 2F5, and 4E10 were shown to delay the rebound of HIV-1 after the cessation of antiretroviral therapy, with that delay especially pronounced in acutely infected individuals.

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The in vivo effect of the neutralizing antibody cocktail was found to depend on 2G12 activity by escape mutant analysis (42).

It has been established that anti-V3 antibodies, induced by brief immunization protocols in animals, are capable of neutralizing HIV-1 in cell cultures and in animal challenge studies (13, 16, 27, 28). However, that capability has not been fully exploited because the V3 sequence is extremely diverse, and so the anti-V3 antibodies are extremely type specific and displayed little cross-reactivity. In the accompanying paper (8a), we describe how we sequentially immunized mice with V3 peptides derived from several different HIV-1 clade B field isolates. The antibody response could be traced to a tip sequence of the HIV-1 gp120 V3 domain, a relatively conserved motif (11, 18, 45). We reshaped anti-V3 MAb C25 into KD-247, a humanized MAb directed against the V3 tip motif Pro-Gly-Arg of the V3 domain. KD-247 cross-neutralized primary isolates with a matching neutralization sequence motif, suggesting that it could be used to overcome the previous limitations surrounding anti-V3 neutralizing antibody production by active immunization strategies.

In this study, we show that the humanized MAb KD-247 is suitable not only for use as a passive immunization antibody for the prevention of immunodeficiency virus infection but also to passively transfer antibodies for immunotherapy. Using 18 primary HIV-1 isolates, we evaluate the neutralizing capacity of KD-247. We also assess its efficacy against ex vivo generation of HIV from the peripheral blood mononuclear cells (PBMCs) of four HIV-infected individuals. Finally, we examine whether KD-247 can suppress HIV-1 replication in monkeys.

MATERIALS AND METHODS

Passive transfer of KD-247 to monkeys followed by pathogenic virus challenge. All animals used in this study were mature, cycling, male cynomolgus monkeys (*Macaca fascicularis*) from the Tsukuba Primate Center, National Institute of Infectious Diseases (NIID), Japan. They were free of known simian retroviruses, herpesviruses, bacteria, and parasites. They were housed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science under the Japanese Law Concerning the Protection and Management of Animals (1, 38) and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of NIID, Japan. Once approved by an institutional committee for biosafety level 3 experiments, these studies were conducted at the Tsukuba Primate Center, NIID, Japan, in accordance with the requirements specifically stated in the laboratory biosafety manual of the World Health Organization (44a).

The pathogenic SHIV strain C2/1 is an SHIV strain 89.6 variant isolated by in vivo passage in cynomolgus monkeys (37). The original SHIV 89.6 strain was kindly provided by Y. Lu at the Harvard AIDS Institute (Boston, MA) (19, 32). Virus stocks of SHIV C2/1 were stored at −125°C and thawed just prior to use. The challenge stock was provided by K. Shinohara of the National Institute of Infectious Diseases, Tokyo, Japan. Cynomolgus monkeys injected intravenously with SHIV C2/1 showed high levels of viremia and marked $CD4^+$-T-cell depletion within 2 weeks after inoculation (1, 34, 35, 37). Naïve monkeys were intravenously administered 0, 15, 30, or 45 mg/kg of KD-247 along with either 45 mg/kg of purified normal human immunoglobulin (Nihon Pharmaceutical Co., Tokyo, Japan) or saline. Twenty-four hours after antibody transfer, the animals were intravenously challenged with 20 50% tissue culture infective doses ($TCID_{50}$s) of SHIV C2/1.

In vitro virus neutralization assays. The primary clinical isolate HIV-1_{MNp} was kindly provided by J. Sullivan of the University of Massachusetts Medical School, Worcester, MA. The virus was confirmed to be neutralization resistant (5). Laboratory-adapted HIV-$1_{89.6}$ and HIV-1_{MN} were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD. GHOST cell neutralization assays were performed as described previously (5, 38). Briefly, GHOST cells expressing either CXCR4 or CCR5 coreceptors were used as targets of HIV-1 infection. The cells were then analyzed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA). The same concentration of either purified normal human immunoglobulin consisting primarily of the IgG1 subclass (Nihon Pharmaceutical Co.) or saline was used as control.

Neutralization activities in monkey plasma were assayed by detecting the neutralizing titers in the assay measuring 100% neutralization against the challenge virus as described previously by Nishimura et al. (26). In brief, plasma samples were serially diluted and incubated with 100 $TCID_{50}$s of challenge virus, and M8166 cells were then incubated as previously described (26). The neutralization was expressed as the percent inhibition of simian immunodeficiency virus p27 antigen production in the culture supernatants (38, 39). Normal monkey plasma was used as a control.

PBMC-based virus neutralization assay. HIV-1_{MN} (H9/HTLV-III MN) was kindly provided by the AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD (45). The WHO primary isolates 92TH002, 92TH022, 92TH023 (all clade E), and 92TH014 (clade B′) were used as virus stocks (12). The primary isolates HIV-$1_{JR\text{-}CSF}$ and the CS and JCI series of HIV-1 isolates were provided by Y. Koyanagi (40) and Y. Okamoto (27). In vitro virus neutralization assays were performed as previously described (7, 12). Neutralization titers are expressed as either the concentration of serum IgG antibody or the reciprocal of the serum dilution that yielded a 50% (50% inhibitory concentration [IC_{50}]) or 90% (IC_{90}) reduction in HIV-1 p24 production over that seen in controls using purified serum IgG from healthy individuals or preimmune mouse sera.

Ex vivo virus neutralization assays. The PBMCs of patients infected with HIV-1 were depleted of $CD8^+$ cells by magnetic separation using polystyrene beads coated with anti-CD8 MAb (Dynabeads M-450 CD8; Dynal, Oslo, Norway). The negatively selected cells were stimulated with OKT3 antibody (1 μg/ml; Jannsen-Kyowa, Tokyo, Japan) and subsequently cultured in the presence of interleukin-2 (20 U/ml; Boehringer, Mannheim, Germany) together with KD-247 (60 and 240 μg/ml). The amount of HIV-1 p24 antigen in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA) (Dainabot, Tokyo, Japan). Approval by the ethical committee and written informed consent from all the human subjects were obtained according to the guidelines of the Ministry of Health, Labor, and Welfare, Japan, and to those of the Kumamoto University Medical School, Kumamoto, Japan.

Competitive PCR quantitation of SHIV RNA in plasma. Quantitative competitive reverse transcription-PCR was performed as described previously by Piatak et al. (30), with both the substitution of a different competitor RNA and a different DNA template (35). The detection limit of this assay was 500 RNA copies/ml in monkey plasma.

Flow cytometric evaluation of cell surface antigen expression and absolute cell count. Mouse MAbs conjugated with either fluorescein isothiocyanate, phycoerythrin (PE), PE-Cy5, or peridinin chlorophyll protein were used in flow cytometric analyses to detect cellular expression of monkey CD3 (NF-18; BioSource International Inc., Camarillo, CA), human CD4 (Nu-TH/I; Nichirei Co., Tokyo, Japan), CD8 (SK-1; Becton Dickinson & Co., San Jose, CA), and CD95 (CH11 and 7C11; Becton Dickinson) (30). To determine absolute cell counts, samples of whole blood were analyzed following the addition of fluorescein isothiocyanate-conjugated anti-CD3 (BioSource), PE-conjugated anti-CD4 (Becton Dickinson), and peridinin chlorophyll protein-conjugated anti-CD8 (Becton Dickinson) MAbs as previously described (35).

Plasma concentration of KD-247. HIV-1 V3 peptide-based ELISA was used for quantification of KD-247 antibody. In brief, 96-well ELISA plates (Maxisorp; Nunc A/S, Roskilde, Denmark) were coated with 100 μl of a KD-247 antigen peptide (SP1 [YNKRKRIHIGPGRAFYTTKNC]) per well in 50 mM carbonate buffer (pH 9.3) at 1 μg/ml overnight at 4°C. KD-247 was diluted to concentrations ranging from 2.5 to 40 ng/ml as a reference. Bound KD-247 was detected with a peroxidase-conjugated anti-human IgG MAb (in-house preparation; The Chemo-Sero-Therapeutic Research Institute). The concentrations of KD-247 in the plasma of monkeys were determined using a calibration curve (SOFTmax; Molecular Devices Co., Menlo Park, CA).

Statistical analysis. The plasma concentrations at various data points postdose were applied to a two-compartment model using an automatic pharmacokinetic analysis program (nonlinear least-squares method), and pharmacokinetic parameters were calculated.

RESULTS

Neutralization ability of the humanized antibody KD-247 against a panel of primary isolates as determined by a PBMC-based study. In the initial series of the study, we showed that

TABLE 1. PBMC-based neutralization of primary and laboratory isolates by KD-247[a]

Isolate	Env V3 sequence[b]	GHOST cell	KD-247		447-52D IC_{50}[c]
			IC_{90}	IC_{50}	
Laboratory isolates, clade B					
HIV-1_{MN}	CTRPNYNKRKRIHI GPGRAFYTTKNIIGTIRQAHC	X4	1	0.1	0.1
HIV-1_{SF2}	-----N-T--G--- -------A-EK-V-D------	X4	5	1.0	1.0
HIV-$1_{89.6}$	-----N-T-R-LS- -------ARR----D------	R5/X4	2.5	0.2	>10
Primary isolates, clade B					
HIV-1_{JR-CSF}	----SN-K--S--- ---------GE---D------	R5	5	0.4	>10
HIV-1_{CS2-2}	-----N-T--S--M ---K-----GD---N----Y-	R5	>50	>50	ND
HIV-1_{CS3-5}	---I-N-T--S--- -------A-GE---N-K----	R5	10	1.4	ND
HIV-1_{CS4-4}	-I---N-T--G--- -L-- WK--A-G--N------	R5/X4	>50	>50	ND
HIV-1_{CS6-6}	--G--N-T--S-R-QR------V-IGK --NM-----	R5	>50	>50	ND
HIV-1_{CS6-8}	-I---N-T--G--- -------A-D----N------	R5	8	1.2	ND
HIV-1_{JCI-1}	----HKTI------ ----------Q-E-N-----	X4	5	0.4	ND
HIV-1_{JCI-2}	----SN-T-R---- ---------RQ-R-D-----	X4	4	0.2	ND
HIV-1_{JCI-3}	-----N-I--H--- ---------RG -RD--K---	R5	10	0.6	ND
HIV-1_{JCI-5}	-------T--G--- ------V---G -RD--K---	X4	4	0.2	ND
HIV-1_{JCI-6}	----SN-T-R---- -----S--A-Q-RGD------	X4	6	0.7	ND
HIV-1_{JCI-9}	-------T--G--- ------V---G -RD--K---	R5	21	1.6	ND
HIV-1_{JCI-11}	-------TS-G-R- -------ASER -RD--K---	R5	34	3.2	ND
HIV-1_{JCI-22}	-----N-I--H--- ---------RG -RD--K---	R5	12	1.2	ND
Primary isolates, clade B′					
HIV-$1_{92TH014}$	-----N-T--S-PL -----W---GQ---D------	R5	8	0.9	>1.5
Primary isolates, clade E					
HIV-$1_{92TH002}$	----SN-T-TS-T- ---QV--R-GD---D--K-Y-	R5	>50	>50	ND
HIV-$1_{92TH022}$	----SN-T-TS-T- ---QV--R-GD---D--K-Y-	R5	>50	>50	>10
HIV-$1_{92TH023}$	----SN-T-TS-N- ---QV--R-GD---D--K-Y-	R5	>50	>50	ND
SHIV-B					
SHIV 89.6PD	-----N-T-R-LS- -------ARR----D------	R5/X4	5	0.5	ND
SHIV C2/1	-----N-T-E-LS- -------ARR----D------	R5/X4	5	0.5	ND

[a] The HIV-1 sequences were confirmed by proviral DNA sequencing of virus-infected cells.
[b] Dashes indicate sequence homology to HIV-1_{MN}, and spaces represent the presence of a deletion.
[c] ND, not done.

sequential immunization with synthetic V3 peptides from representatives of primary HIV-1 clade B isolates generated cross-reactive antisera and produced a high-affinity humanized MAb, KD-247, directed against the tip of the HIV-1 V3 domain, PGR. Furthermore, the humanized antibody more effectively neutralized several primary isolates of HIV-1 clade B than did previously reported neutralization antibodies (8a, 10, 23, 27). To further analyze the divergence of the cross-neutralization ability of the antibody by a PBMC-based HIV-1 neutralization assay, we used a panel of a total of 23 immunodeficiency viruses: 18 primary isolates of HIV-1 clade B, clade B′, and clade E viruses; 3 laboratory HIV-1 clade B viruses; and 2 highly pathogenic SHIVs (Table 1). The KD-247 antibody effectively neutralized HIV-1_{MN}, HIV-1_{SF2}, and HIV-$1_{89.6}$, containing the consensus V3 sequence of HIV-1 clade B, IGPGR AFY, with an IC_{90} and IC_{50} from 1 to 5 and from 0.1 to 1.0 μg/ml, respectively (Table 1, laboratory isolates, clade B). We next sought to assess whether the neutralization of primary isolates by KD-247 required a matching neutralization sequence motif. As expected, KD-247 effectively neutralized primary CCR5-tropic clade B and B′ isolates (IC_{90} and IC_{50} from 5 to 34 and from 0.4 to 3.2 μg/ml, respectively) and all four of the CXCR4-tropic clade B isolates (IC_{90} and IC_{50} from 4 to 6 and from 0.2 to 0.7 μg/ml, respectively) with matching IGPGR or V3 tip sequences. Thus, CCR5-tropic isolates with an IC_{90} of a mean concentration of neutralization antibody of 13.5 μg/ml were more than 2.8 times less sensitive to the neutralization by KD-247 than primary CXCR4-tropic isolates with a mean IC_{90} of 4.8 μg/ml. In contrast, the neutralization-resistant virus CS2-2 did not match the neutralization sequence motif, and the CS6-6 virus showed a QR insertion in the V3 tip sequence. The HIV-1 isolates containing a glutamine (Q) residue at position 20 in the V3 region, such as those of subtype E, were also resistant to neutralization by KD-247. Therefore, KD-247 effectively neutralizes both the CCR5- and CXCR4-tropic primary isolates with matching neutralization motifs.

Ex vivo suppressive effects of KD-247 on the generation of HIV-1 quasispecies from PBMCs of HIV-infected individuals. To fully assess the antiviral efficacy of KD-247, we next sought to determine whether it would suppress the generation of HIV-1 from PBMCs of HIV-infected individuals and whether it would do so as efficiently as an established anti-V3 humanized antibody, Cβ1 (23). As shown in Table 2, we investigated the effect of KD-247 at concentrations of 60 and 240 μg/ml on the ex vivo generation of HIV-1 using $CD8^+$-T-cell-depleted PBMC cultures from four Japanese individuals infected with HIV-1 clade B (Env V3 sequence in Table 2). In the presence of KD-247 at concentrations of 60 and 240 μg/ml, the gener-

TABLE 2. Ex vivo neutralizing activity of KD-247 against HIV-1 present in PBMC cultures established using cells from HIV-infected individuals[a]

Patient	HIV-1 Env V3 sequence (no. of clones)	PBMCs, (no. of cells/well)	KD-247 (μg/ml)	p24 (log_{10} pg/ml)
KU008	CTRPHNNTRKSIHIGPGRAFYATGDIIGNIRQAHC (3)	6.5×10^5	0	3.93
	-------------------------E---D--R-- (2)		60	0.37
	-------------------------E---D----- (1)		240	0.08
	-----------------------------D----- (1)			
KU045	CTRPNNNTRKGIHIGPGRAFYGTDIVGDIRQAHC (5)	7.3×10^5	0	3.70
	-----------------------E-T-N----Y- (2)		60	0.88
	---------------------------N------ (1)		240	0.56
KU037	CTRPNNNTRKSIPIGPGRAFYATGDIIGDIRKAHC (3)	1.3×10^6	0	3.81
	-------I--------------------------- (1)		60	3.86
	-I------G-------------------------- (1)		240	0.25
KU040	CTRPNNNTRKSVHIGPGRAWYATGEIIGNIRQAHC (2)	8.0×10^5	0	4.12
	---------------A----F-------------- (1)		60	2.34
	------------I--------H------------- (1)		240	2.62
	---H-------I-L---G--H---D--------- (1)			

[a] Ex vivo neutralization activity was directly detected by using $CD8^+$ cell-depleted PBMCs from HIV-infected individuals as described in Materials and Methods.
[b] The number of analyzed DNA clones from each patient is indicated in parentheses. Dashes indicate sequences identical to those of the upper major clone from each patient.

ation of viruses from PBMCs of KU008 was reduced in a dose-dependent manner, with 3.56- and 3.85-log reductions in the culture supernatants, respectively; reductions of 2.82 and 3.14 logs of virus generation from PBMCs of KU045 were also detected in the presence of 60 and 240 μg/ml of KD-247, respectively, KU037 showed a reduction of 3.56 logs at only 240 μg/ml. However, KU040 showed no dose-dependent suppressive effects of virus generation by KD-247. When the irrelevant antibodies of Cβ1 and normal serum IgG were added to cell cultures, they showed no suppressive effects on virus generation (data not shown). These results demonstrate that KD-247 effectively neutralizes nonpassage viruses generated in the primary culture of PBMCs from individuals infected with HIV-1 clade B with neutralization sequence motifs matching that of the quasispecies, IGPGR.

Induction of complete protection of monkeys against a highly pathogenic SHIV strain by a single passive transfer of a high dose of KD-247. PBMCs from 12 juvenile male cynomolgus monkeys were first evaluated in vitro to establish their susceptibility to infection with the SHIV C2/1 challenge stock in standard viral infectivity assays (35, 37) (data not shown). Challenge virus SHIV C2/1 originated from SHIV 89.6 but did share an identical envelope sequence with the parental strain, HIV-$1_{89.6}$, and showed 17 nucleotide mutations with amino acid changes (1, 34). The neutralization sensitivity of SHIV C2/1 to KD-247 was found to be similar to that of HIV-$1_{89.6}$, with an IC_{90} and IC_{50} of 5 and 0.5 μg/ml in human PBMC-based neutralization assays, respectively (Table 1, laboratory isolates, clade B and SHIV-B), suggesting that the neutralization potency of KD-247 in vitro might be sufficient to warrant passive transfer experiments.

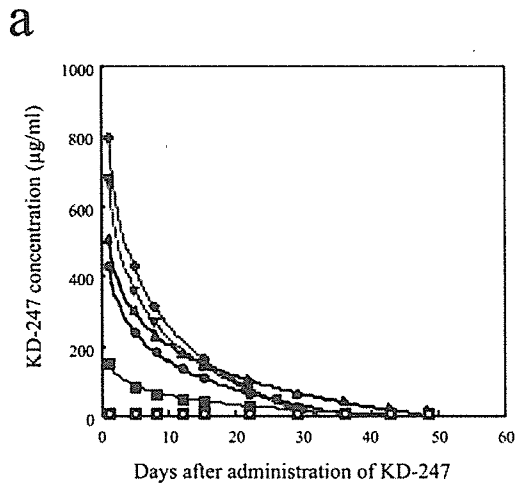
Of the 12 monkeys, 5 were inoculated with KD-247, 2 were inoculated with control normal human IgG (NHIgG) (45 mg/kg), and the remaining 5 were given saline alone. Of the five animals receiving KD-247, two were given a dose of 45 mg/kg, two received 30 mg/kg, and one received 15 mg/kg. Twenty-four hours after antibody transfer, all 12 monkeys were given an intravenous challenge of 20 $TCID_{50}$s/ml SHIV (Fig. 1). At the time of viral challenge, the plasma concentrations of KD-247 were 151, 443, 496, 866, and 678 μg/ml of the antibody in immune sera from monkeys 3968, 3969, 3972, 4092, and 4099, respectively (Fig. 1a). The area under the plasma concentration time curve (AUC) values for monkeys 3968, 3969, 3972, 4092, and 4099 were calculated from the antibody concentration data to be 1.8, 3.5, 5.0, 6.5, and 5.6 mg · day/ml, respectively.

The percentage of $CD4^+$ T cells and the levels of plasma viremia were also monitored after SHIV challenge (Fig. 1b and c). All monkeys that were intravenously inoculated with normal human IgG or saline showed a loss of $CD4^+$ T cells within 7 days of viral challenge, accompanied by plasma viremia reaching 10^7 to 10^8 viral RNA copies/ml (data from the five control monkeys that received saline alone are not shown). Of the two control monkeys that received 45 mg/kg of NHIgG, both seroconverted against SHIV p27 antigen (monkeys 3967 and 3974) (Fig. 1d). At autopsy, all control monkeys showed $CD4^+$-T-cell depletion in lymphoid organs, a finding consistent with our previous observations using this model (35, 37).

Both monkeys that received a single high dose of 45 mg of KD-247 per kg of body weight prior to SHIV challenge were completely protected from viral challenge, maintaining stable $CD4^+$-T-cell counts and not seroconverting or exhibiting plasma viremia (Fig. 1b to e, monkeys 4092 and 4099, indicated by red lines and red characters). When evaluated at autopsy using PCR for SHIV *gag* proviral DNA, their tissues showed no sign of infection (data not shown). The titers in plasma resulting from 100% in vitro neutralization against 100 $TCID_{50}$s of the challenge virus at the time of virus challenge were 1:160 in both monkeys 4092 and 4099. The titers in partially protected monkeys 3969 and 3972 were 1:40 and 1:80, respectively. No neutralization activity of less than 1:10 was measured in the animals receiving 45 mg/kg of NHIgG (monkeys 3967 and 3974). Thus, although the highest titers of neutralization activities were detected in plasma from protected animals, the neutralization activity was high even in animals with only partial protection.

Administration of lower doses of KD-247, 30 mg/kg to two monkeys (monkeys 3969 and 3972, indicated by blue lines and

a



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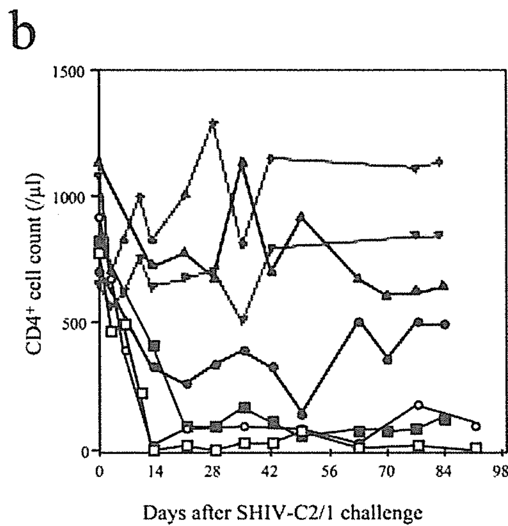

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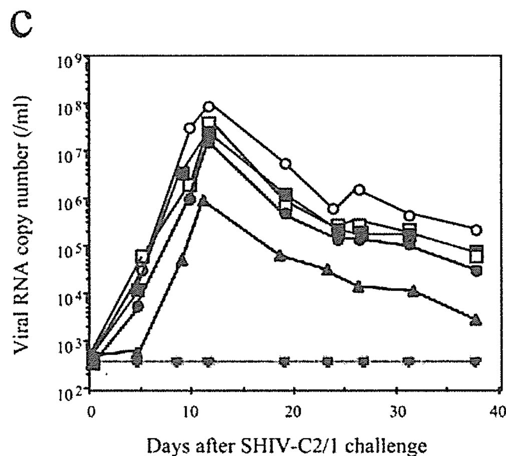

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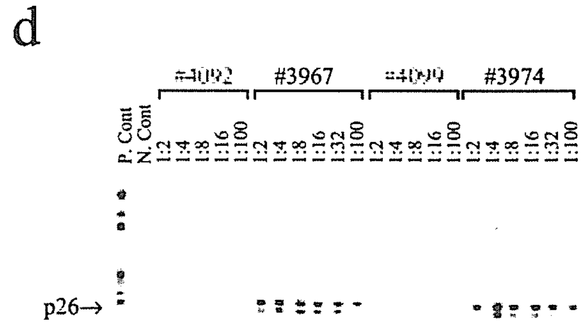
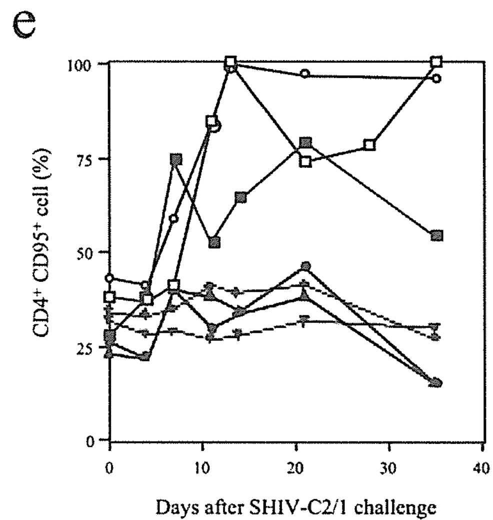


e



#3968 KD-247 15mg/kg followed by SHIV-C2/1
#3969, #3972 KD-247 30mg/kg followed by SHIV-C2/1
#4092, #4099 KD-247 45mg/kg followed by SHIV-C2/1
#3967, #3974 NHIgG 45mg/kg followed by SHIV-C2/1

FIG. 1. KD-247 efficiently protects monkeys from pathogenic virus challenge. A total of 12 cynomolgus monkeys were used for virus challenge studies with SHIV C2/1. In the first group, five monkeys were intravenously inoculated with various doses of KD-247, followed by 20 $TCID_{50}$s of SHIV C2/1 challenge 24 h after antibody transfer. Monkeys in the second and third groups were injected prior to virus challenge with either 45 mg/kg of normal human immunoglobulin (two monkeys) or saline alone (five monkeys). The following parameters were measured in monkeys given KD-247: (a) concentration of KD-247 in plasma following passive transfer, (b) $CD4^{+}$-T-cell counts, (c) plasma viremia, (d) Western blot analysis using an HIV-2 Western blot kit (Diagnostics Pasteur, Marnes-La-Coquette, France) (6) of serum samples obtained at autopsy from monkeys given a single high dose (45 mg/kg) of KD-247 (monkeys 4092 and 4099) or NHIgG controls (monkeys 3967 and 3974), and (e) CD95 antigen expression on PBMCs from monkeys challenged with SHIV.

blue characters in Fig. 1) and 15 mg/kg to one monkey (monkey 3968, indicated by green lines and green characters in Fig. 1), afforded partial protection from SHIV infection. Monkey 3972 (Fig. 1, closed triangle with blue line) showed better partial protection than monkey 3969, which received 30 mg/kg of antibody. That superior degree of partial protection may be related to better blood concentration of the antibody and to better AUC values. All three monkeys described above seroconverted against SHIV p27 antigen (data not shown), but their loss of $CD4^+$ T cells seemed to be inversely proportional to the plasma concentration of KD-247 (Fig. 1a and b). Although the $CD4^+$-T-cell decline indicated minimal protection in the monkey given 15 mg/kg of KD-247 (monkey 3968) (Fig. 1b), CD95 antigen expression, a marker for cell stimulation, was significantly lowered in this animal and completely inhibited in the other four monkeys receiving KD-247 (Fig. 1e), suggesting that KD-247 significantly suppressed PBMC stimulation by the virus challenge in these animals (monkeys 3969, 3972, 4092, and 4099).

These results therefore demonstrate that KD-247 efficiently neutralizes primary HIV isolates regardless of cell tropism. Furthermore, passive immunization with a single dose of 45 mg of antibodies per kg of body weight 24 h prior to viral challenge completely protected animals from viral challenge, showing that at high concentrations, KD-247 lowers the viral load and induces sterilizing immunity in the monkey model.

DISCUSSION

In this study, KD-247 proved an effective antiviral agent for the targeting of phenotype-matched viruses, one capable of both in vitro neutralization of primary isolates and in vivo passive transfer of the antibody as well as of suppressive effects against ex vivo generation of HIV from HIV-infected individuals. Although it has already been established that brief immunizations with a V3 peptide can elicit neutralizing antibodies to homologues of the CXCR4-tropic virus, the limitations of anti-V3 antibodies have been known for over a decade (8, 13, 16, 28). Also, at reasonable IC_{50}s, the anti-V3 antibodies did not neutralize CCR5-tropic strains. In the accompanying paper (8a), we described the derivation of a humanized MAb, KD-247, that was produced by sequential immunization using six different HIV-1 Env V3 peptides derived from HIV-1 clade B field isolates. We suggested that KD-247 could potentially overcome the previous limitations to immunologically exploiting the anti-V3 antibody induced by brief immunization protocols, i.e., its extraordinary sequence variability and the associated isolate specificity of anti-V3 antibodies (27, 38). The findings of our current study suggest that KD-247 may curb the spread of viral infection and reduce viral loads in HIV-infected individuals who have been determined to share the V3 tip sequence of the virus by virus neutralization phenotype-matching analysis.

In vitro, KD-247 has potent neutralizing activity against a variety of primary HIV-1 clade B isolates, including CCR5-tropic viruses, at low concentrations. We found that KD-247 neutralized a variety of clade B primary viruses containing IGPGR V3 sequences, although its neutralization ability was affected by some of the surrounding amino acids of the V3 tip region, as discussed in the accompanying paper (8a). Based upon these results, we should be able to predict the neutralization ability of KD-247 by prior sequencing of the HIV-1 Env V3 region of the target virus. Using the previously published sequences found in the Los Alamos HIV-1 sequence database, we determined that the IGPGRA sequence is present in the majority of HIV-1 clade B isolates (45) to which KD-247 would be expected to have cross-neutralization activity. Moreover, KD-247 significantly curbed the generation of primary HIV-1 quasispecies in ex vivo cultures of $CD8^+$-T-cell-depleted PBMCs from seropositive individuals. However, as described above, the major limitation of KD-247 as an antiviral agent is its inability to neutralize variants expressing amino acid alterations in the binding site PGR motif and additional amino acids.

What are the properties that make KD-247 an effective neutralizer of CCR5-tropic viruses? First, the site-specific binding of KD-247 to epitopes on the virus envelope glycoprotein seems to be key to its virus neutralization ability. Indeed, the results of the Pepscan analysis reported in the accompanying paper suggest that KD-247 can react with core V3 sequences from various HIV-1 clade B isolates (8a). The shortest peptide that was reactive with KD-247 was IGPGR, but that epitope was stabilized by the addition of one or more amino acids. Furthermore, IGPGRA and GPGRAF sequences occur in the majority of HIV-1 isolates from donors in the United States (17). The results of Pepscan with replacement peptides also suggest that KD-247 has broad binding activity to HIV-1. While the number of amino acid substitutions tolerated in the central PGR sequence of the V3 tip peptide was small, replacement of amino acids in the flanking region was relatively permissible. Second, ex vivo neutralization assays using patient-derived isolates containing APGR and GPGG sequences in the V3 tip showed incomplete neutralization (Table 2, KU040). Thus, KD-247 would be expected to bind with HIV-1 quasispecies having a recognition sequence similar to the neutralization phenotype. Third, as the accompanying paper demonstrates, high-affinity antibody binding is apparently required for neutralization, because the kinetic parameters of KD-247 were identified to be fast on and slow off rates, similar to those of a type-specific MAb, Rμ5.5, although the equilibrium dissociation constant value of KD-247 for binding to a control SP1 peptide was higher than that of Rμ5.5 (8a). This is a reasonable assumption, since the epitope of KD-247 (IGPGR) is shorter than that of Rμ5.5 (IHIGPGRAFYT). The high association rate of KD-247 might be responsible for exerting the observed cross-neutralization activity against various primary isolates. These results are consistent with the hypothesis that virus neutralization can be explained by the kinetic parameters of antibody binding.

Most recent passive transfer studies with monoclonal antibodies used common combinations of broadly cross-reactive human MAbs capable of neutralizing primary HIV-1 isolates. In monkeys, human MAbs b12 (29) and 2G12 (20) were shown to induce complete and partial protection, respectively, against viral challenges. In contrast, the MAb chosen for this study, KD-247, is a humanized antibody induced by sequential immunization with a set of V3 peptides from primary isolates. Because the KD-247 IC_{90} value from an in vitro neutralization assay in our study, 5.0 μg/ml of the antibody, approximates that obtained by a single antibody, b12 (3), and a combination of

the two MAbs 2F5 and 2G12 or a triple combination of HIVIG, 2F5, and 2G12, as previously reported (41, 43), we postulated that KD-247 was sufficiently potent to achieve protection of monkeys against a pathogenic SHIV challenge. Since our previous experience (9) has taught us to expect approximately 500 to 1,000 μg/ml in sera from monkeys passively immunized with 30 to 45 mg of antibody per kg of body weight, the potency of KD-247 should prove sufficient for passive transfer experiments of effective antibodies in animals in vivo. We also expected that a single passive transfer of KD-247 via inoculation with 15 and 30 mg of antibody would result in approximately 150 to 500 μg/ml of plasma concentration at the time of viral challenge. As expected, we found an AUC value of 1.8 to 5.0 mg · day/ml. Consequently, we found that animals passively immunized with 45 mg/kg of KD-247 showed 678 and 866 μg/ml of KD-247 in plasma at the time of viral challenge and an AUC value of 5.6 and 6.5 mg · day/ml. Those animals were provided sterile protection against intravenous challenge with the pathogenic virus SHIV C2/1. The protective endpoint titers of neutralization antibodies in plasma at the time of virus inoculation were 1:160 in both animals that elicited sterile immunity, and a high titer of neutralization activity in plasma was similarly detected in completely protected monkeys, as described previously by Nishimura et al. (26) and Parren et al. (29). Thus, the high titers of neutralization activity in plasma confer sterile protection against viral challenge in the passively immunized animals with neutralizing antibodies. Furthermore, the pharmacokinetic information consisting of the plasma concentration of the neutralizing antibodies at the time of viral challenge and the AUC value may be closely related to the ability of the antibody to provide sterile protection against viral challenge. Since those protected macaques demonstrated the inhibition of $CD4^+$ cell loss, the pharmacokinetic properties of KD-247 may also be closely associated with the inhibition of $CD4^+$ cell decline in the peripheral circulation of the challenged monkeys.

In this study, we also detected lower viremia with lesser $CD4^+$ cell decline in animals that were inoculated with intermediate doses of antibody. However, we noted that the lesser doses of the antibody provided complete protection against enhanced rates of the $CD4^+$ $CD95^+$ cell subpopulation in the peripheral circulation of the challenged animals, suggesting that the reshaping MAb might be able to control the activation of peripheral $CD4^+$ T cells in animals by its passive transfer. Although the number of monkeys enrolled in this study was limited, it remains noteworthy that a single inoculation with KD-247, even at a suboptimal dose for viral protection, appeared to be effective for maintaining $CD4^+$ T cells in monkeys inoculated with virus. Since it has been previously reported that the limited effect of neutralizing antibody may be related to the rapid appearance of an escape mutant in infected individuals, high titers of neutralization activity should be generated in the passively immunized animals (25, 33, 44). In our preliminary study, we isolated the escape mutant from the neutralization resistance virus HIV-1_{JR-FL} in the presence of KD-247: at passage 8 of the culture in the presence of 1,000 μg/ml KD-247, one amino acid substitution, GPGR to GPER, was identified in the V3 tip (K. Yoshimura et al., unpublished results). Collectively, these results suggest that KD-247 shows clinical promise both for passive immunization and as a strategy for preventing viral spread in phenotype-matched HIV-infected individuals.

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Induction of Positive Cellular and Humoral Immune Responses by a Prime-Boost Vaccine Encoded with Simian Immunodeficiency Virus *gag/pol*[1]

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It is believed likely that immune responses are responsible for controlling viral load and infection. In this study, when macaques were primed with plasmid DNA encoding SIV *gag* and *pol* genes (SIV*gag/pol* DNA) and then boosted with replication-deficient vaccinia virus DIs recombinant expressing the same genes (rDIsSIV*gag/pol*), this prime-boost regimen generated higher levels of Gag-specific CD4$^+$ and CD8$^+$ T cell responses than did either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone. When the macaques were i.v. challenged with pathogenic simian/HIV, the prime-boost group maintained high CD4$^+$ T cell counts and reduced plasma viral loads up to 30 wk after viral challenge, whereas the rDIsSIV*gag/pol* group showed only a partial attenuation of the viral infection, and the group immunized with SIV*gag/pol* DNA alone showed none at all. The protection levels were better correlated with the levels of virus-specific T cell responses than the levels of neutralization Ab responses. These results demonstrate that a vaccine regimen that primes with DNA and then boosts with a replication-defective vaccinia virus DIs generates anti-SIV immunity, suggesting that it will be a promising vaccine regimen for HIV-1 vaccine development. *The Journal of Immunology,* **2006, 176: 1784–1795.**

The primary goals of any prophylactic HIV vaccine are to induce HIV-specific immune responses capable of preventing the malfunctioning of immune systems and to limit viral transmission due to replication. Clinical studies have demonstrated that CTL immune responses are associated with the reduction of plasma viral load (1, 2) and can control disease progression (3, 4). Replication of pathogenic SIV in vivo has also been shown to be controlled in the macaque model by CD8$^+$ T cell responses (5). Because amino acid sequences of Gag and Pol of HIV-1 proteins are relatively conserved, cross-clade and broad CTL responses targeting those proteins have been observed in both HIV-infected and HIV-exposed individuals, even if the latter group had not become infected (6–8). Thus, one recent focus of HIV vaccine research has been to elicit more protective antiviral immune responses by enhancing the expression levels of HIV-1 Ags of Gag and Pol using a safe vaccine vector.

Recently, several prime-boost regimens consisting of a DNA prime and a recombinant poxvirus boost targeting the immunodeficiency virus have been reported to generate higher levels of HIV-specific T cell immune responses than regimens relying on DNA or recombinant poxvirus vaccine alone (9, 10). In efficacy trials of such heterologous prime-boost vaccines, an SIV Ag encoding DNA prime and a boost of recombinant modified vaccinia virus Ankara (MVA)[3] elicited effective anti-SIV immunity and controlled infection of the nonpathogenic simian-HIV (SHIV) strain as well as of the pathogenic strain SHIV-89.6P in macaques (11–13) by effectively inducing CD8$^+$ CTL immunities. Various poxvirus vectors, i.e., an avipox virus, a canarypox virus, a fowlpox virus, a substrain of vaccinia Copenhagen (NYVAC), and MVA, have been evaluated for their usefulness, either alone or in combination with other vaccine modalities (14–18). To be useful, these vaccine vectors must, of course, be safe. The currently widely used MVA, which was developed toward the end of the campaign to eradicate small pox, has been effectively and safely used in >100,000 people as a small pox vaccine (19). MVA-based recombinant vector has also been reported to be safe in animals (20, 21). Lately, we have developed a replication-defective vaccinia virus DIs strain as a vaccine vector (22, 23). The DIs strain, generated by a 1-day-old egg passage of the DIE strain (24), has been proven safe (25, 26). We also suggested that a new prime-boost vaccine regimen consisting of SIV*gag/pol* DNA and rDIsSIV*gag/pol* might be useful for the development of an HIV-1 candidate vaccine that could induce strong cellular protective responses in mice (23). Lately, similar DNA/MVA vaccine combinations support the idea that the vaccine induced strong Ag-specific T and B cell responses (27). The prime-boost-vaccinated mice generated higher levels of both Gag-specific CD4$^+$ and CD8$^+$ T cell immune responses than those vaccinated with either DNA or rDIs alone. When such mice were challenged with SIV *gag/pol* expressing

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[3] Abbreviations used in this paper: MVA, modified vaccinia virus Ankara; rDIsSIV-*gag/pol*, recombinant DIs expressing SIV*gag* and *pol*; SFC, spot-forming cell; SHIV, simian-human immunodeficiency virus; SIV*gag/pol* DNA, plasmid DNA encoding SIV *gag* and *pol* genes; $TCID_{50}$, 50% tissue culture infectious doses.

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