Highly Active Antiretroviral Therapy Potently Suppresses HIV Infection in Humanized Rag2^{-/-}γ_c^{-/-} Mice

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Abstract

Humanized Rag2^{-/-}γ_c^{-/-} mice (Hu-DKO mice) become populated with functional human T cells, B cells, and dendritic cells following transplantation with human hematopoietic stem cells (HSC) and represent an improved model for studying HIV infection *in vivo*. In the current study we demonstrated that intrasplenic inoculation of hu-DKO mice with HIV-1 initiated a higher level of HIV infection than intravenous or intraperitoneal inoculation, associated with a reciprocal decrease in peripheral CD4⁺ T cells and increase in peripheral CD8⁺ T cells. HIV infection by intrasplenic injection increased serum levels of human IgG and IgM including human IgM and IgG specific for HIV-1 gp120. There was a significant inverse correlation between the level of HIV-1 infection and the extent of CD4⁺ T cell depletion. Highly active antiretroviral therapy (HAART) initiated 1 week after HIV-1 inoculation markedly suppressed HIV-1 infection and prevented CD4⁺ T cell depletion. Taken together, these findings demonstrate that intrasplenic injection of hu-DKO mice with HIV is a more efficient route of HIV infection than intravenous or intraperitoneal injection and generates increased infection associated with an increased anti-HIV humoral response. This animal model can serve as a valuable *in vivo* model to study the efficacy of anti-HIV therapies.

Introduction

IN VIVO ANIMAL MODELS GREATLY FACILITATE investigation of the virology, immunology, and pathogenesis of HIV infection. Studies in nonhuman primates, while invaluable,¹ do not directly recapitulate the pathogenesis and disease course of human HIV infection, and are further complicated by the availability and cost of the animals and biological differences between SIV and HIV.²⁻⁴ As an alternative, various models using humanized severe combined immunodeficiency (hu-SCID) mice have been developed and widely utilized for studying HIV infection,^{5,6} including the hu-PBL-SCID model and the SCID-hu thy/liv model.^{7,8} However, the limitations of these models have been well documented, including restricted T cell and B cell repertoires and minimal development of primary immune responses.^{7,9-13}

Deletion of the Rag2 recombinase and interleukin (IL)-2 receptor gamma chain [gamma common (γ_c) chain] genes severely compromises the immune system of Rag2^{-/-}γ_c^{-/-} mice (DKO mice) by preventing the maturation of functional T cells, B cells, and natural killer (NK) cells.^{14,15} As a conse-

quence of this severe immunodeficiency, DKO mice are highly tolerant for human cell transplantation and support the subsequent differentiation of human hematopoietic stem cells (HSC) into functional lymphoid and myeloid cells^{16,17} after transplantation into sublethally irradiated newborn mice.¹⁸ These humanized DKO mice, hu-DKO mice, form primary and secondary human lymphoid tissues, develop functional immune responses,^{16,17} and are susceptible to HIV infection.¹⁹⁻²⁴ However, studies are still limited regarding their capacity to develop immune responses against HIV after infection and their use to evaluate the efficacy of antiretroviral therapy.

In the present study, we demonstrate that after intrasplenic injection of HIV-1, hu-DKO mice develop productive HIV infection that is associated with reciprocal depletion of the human CD4⁺ T cell population and increase in the human CD8⁺ T cell population that correlates with the degree of HIV infection. The plasma of the HIV-infected hu-DKO mice contained human IgG and IgM directed to gp120. Provision of highly active antiretroviral therapy (HAART) soon after infection potently suppressed productive HIV infection and prevented CD4 depletion. Taken together, these results

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further validate the applicability of hu-DKO mice as a model for the development of HIV-specific therapies.

Materials and Methods

Isolation of hematopoietic stem cells from fetal liver

Human fetal liver was obtained from the Albert Einstein College of Medicine Human Fetal Tissue Repository in accordance with institutional review board regulations. Cells were isolated from the disrupted fetal liver by density gradient centrifugation over Ficoll-Paque Plus (GE Health Care, Piscataway, NJ). Some of the harvested mononuclear cells were enriched for CD34⁺ cells by positive selection after incubation with immunomagnetic beads (CD34 Progenitor Cell Isolation Kit, Miltenyi Biotec Inc., Auburn, CA) and passage through an autoMACS Separator (Miltenyi Biotec Inc., Auburn, CA). The cells were either immediately used for transplantation or frozen until newborn mice were available for injection.

Generation of hu-DKO mice

Rag2^{-/-}γc^{-/-} mice on a C57BL/10 background (DKO mice) were bred and maintained under biocontainment conditions. Newborn mice (within 1–7 days of birth) were sublethally irradiated (3.50 Gy) and within 6 h intrahepatically injected with fetal liver-derived human hematopoietic progenitor cells, either with CD34 enriched cells (0.1–2.5 × 10⁶ cells) or unfractionated mononuclear cells (0.5–70 × 10⁶ cells) as described.¹⁶ All the studies were performed under a protocol approved by the Animal Institute for Animal Studies at Albert Einstein College of Medicine.

Analysis of cell populations by flow cytometry

Human leukocyte engraftment was quantified in the peripheral blood at indicated time points after transplantation to determine the level of human cell engraftment. Selected mice were sacrificed at different time points after infection, and the spleens, bone marrow, thymus, and lymph nodes were harvested. The population of human cells was quantified by staining with antihuman monoclonal antibodies (mAb) directed to CD34, CD45, CD8, CD4, CD19, CD11b, CD14, and CD69 (BD Biosciences, San Jose, CA) and measuring expression of these markers on an LSRII (BD Biosciences) using the Flowjo Analysis Program (Tree Star, Ashland, OR).

HIV-1 infection of hu-DKO mice and analysis

Rag2^{-/-}γc^{-/-} mice reconstituted with human HSC were infected with 100 μl of HIV-1_{JR-CSF} viruses (800, 3600, or 8000 TCID₅₀) at 9 weeks and older by intrasplenic injection as described.²⁵ A control mouse group was injected with heat-inactivated (30 min at 56°C) HIV-1_{JR-CSF} virus by intrasplenic injection.²⁶ To compare the effects of infection by different injection routes, other groups of hu-DKO mice were injected with the same dose of HIV-1_{JR-CSF} virus by either the intraperitoneal or intravenous route. Plasma HIV-1 RNA was quantified using VERSANT HIV-1 bDNA 3.0 (Bayer, Tarrytown, NY) according to the manufacturer's instructions. Frozen plasma from mice in the same experimental group was pooled and diluted in phosphate-buffered saline (PBS) to achieve the 1 ml volume required by the assay. The number of

HIV-1-infected cells in the mouse spleens was quantified by limiting dilution coculture of mouse splenocytes with HIV-1-naïve donor PHA/IL-2-activated human peripheral blood mononuclear cells (PBMCs) (1 × 10⁶/well). HIV-1 p24 antigen in the supernatant was measured 7 days later by a modified enzyme-linked immunosorbent assay (ELISA) as previously described.²⁵

Identification of HIV-infected cells in the spleens of the HIV-challenged mice by intracellular staining for p24 antigen and flow cytometry

Spleen cells harvested from the mouse spleens were pooled in each experimental group and suspended in FACS buffer (PBS with 1% sodium azide). The cells were stained with antihuman CD45 and CD4 mAbs prior to cell fixation with 4% paraformaldehyde. Cells were then incubated for 4°C for 1 h, followed by washing with FACS buffer. Phycoerythrin (PE)-conjugated anti-HIV p24 antibody (clone KC57-RD1, Beckman Coulter, Inc., CA) was added at 1:1000 dilution in 200 μl per sample of staining/washing buffer (PBS containing 1% saponin and 20% FCS), and the cells were incubated for 20 min at room temperature. After incubation, the cells were washed twice with staining/washing buffer, followed by washing with FACS buffer. Cells were then evaluated on an LSRII and analyzed with the Flowjo Program.

Measurement of HIV-1-specific antibodies

HIV-1-specific human IgM and human IgG in the mouse plasma were measured by ELISA. Briefly, a Maxisorp ELISA plate (NUNC, Rochester, NY) was coated with 5 μg/ml of HIV-1 proteins, recombinant HIV-1 IIIB p55Gag (NIH AIDS Research and Reference Reagent Program, Catalog number 3276), or HIV-1 CN54 gp120 (NIH AIDS Research and Reference Reagent Program, Catalog number 7749)^{27,28} in 0.1 M NaHCO₃ buffer. After washing and blocking the plates, diluted plasma samples (1:5) were added and incubated for 1 h at 37°C. Either horseradish peroxidase-conjugated antihuman IgG or IgM monoclonal antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were added and incubated for 1 h at 37°C. Substrate (*o*-phenylenediamine dihydrochloride, Sigma-Aldrich, St. Louis, MO) was added and incubated at room temperature for 30 min. The reaction was stopped with 4 N sulfuric acid and specific absorbance at 490 nm was determined using a microplate reader. Plasma from an HIV-infected human patient was used as a positive control and plasma from hu-DKO mice infected with inactivated HIV, HIV-uninfected hu-DKO mice, and HIV-inoculated DKO mice were used as negative controls.

Administration of HAART to the mice

Mice were treated with a well-described clinical triple combination regimen of two reverse transcriptase inhibitors, AZT and lamivudine, and a protease inhibitor, indinavir, by administering the drugs (45 mg/kg/day for each drug) dissolved in the sterile drinking water at a concentration that provides therapeutic levels of the drugs.²⁹ Antiretroviral therapy was initiated 1 week after infection with HIV. Seven weeks later the mice were sacrificed and analyzed for the level of HIV infection, human CD4⁺ and CD8⁺ T cell subsets, and viral load as described above.

Statistical analysis of data

SPSS version 13 for Windows XP statistical software package was used for the statistical analysis with Pearson correlation and Student's *t*-test. Differences were considered statistically significant for *p*-values < 0.05.

Results

Reconstitution of human lymphoid systems in hu-DKO mice transplanted with enriched CD34⁺ cells and unfractionated human fetal liver cells

DKO mice were sublethally irradiated and injected intrahepatically with human hematopoietic stem cells within 7 days of birth. By 8 weeks after transplantation, human leukocytes were detected in the peripheral blood of almost all of the transplanted mice. Representative FACS staining profiles of mouse bone marrow, spleens, and thymus at 8 weeks after

transplantation, stained with antihuman mAbs, are shown in Fig. 1. The bone marrow of the mice contained a large population of human CD19⁺ B cells as well as a defined population of human CD34⁺ cells (Fig. 1A). The mice exhibited active human thymopoiesis in the mouse thymus as evidenced by the presence of human double-positive CD4⁺CD8⁺ and single-positive CD4 and CD8 human T cells (Fig. 1B). A discrete population of human cells stained by CD45 antihuman mAb was also observed in the spleen of the transplanted mice (Fig. 1C). Of 68 mice transplanted with human fetal liver cells intrahepatically within 7 days of birth, 97% of the animals achieved reconstitution with human CD45⁺ cells (>1%) at 7 weeks or later after transplantation (mean $\pm$ standard error = 14.21% $\pm$ 1.56%). There was no significant difference in the level of engraftment of the mice transplanted with highly purified CD34⁺ cells isolated from fetal liver or with unfractionated fetal liver mononuclear cells (Table 1).

FIG. 1. Population of hu-DKO mice with human immune cells. Newborn DKO mice (1–7 days old) were irradiated (3.50 Gy) and injected intrahepatically with enriched CD34⁺ or unfractionated human fetal liver cells. Representative flow cytometric analysis profiles are shown. (A) Bone marrow was examined for the presence of human CD45⁺, CD34⁺, and CD19⁺ cells against forward scatter 2 months after transplantation of DKO mice with unfractionated fetal liver cells. The profiles for human CD34⁺ and CD19⁺ cells were determined for cells after gating on human CD45⁺ expression. (B) Human thymocyte development in a reconstituted mouse was evaluated by quantifying human double positive CD4⁺CD8⁺ T cells as well as CD4 and CD8 single positive T cells in the mouse thymus. The profiles of human CD4⁺ and CD8⁺ expressing cells were determined after gating for human CD45⁺ expression. (C) The population of human CD45⁺ cells was detected in the spleen of transplanted mice.

FIG. 3. HIV-infection of hu-DKO mice has reciprocal effects on the levels of peripheral human CD4⁺ and CD8⁺ T cell subsets. Levels of (A) human CD4⁺ and (B) CD8⁺ T cell subsets in the peripheral blood of HIV-infected hu-DKO mice were assessed by flow cytometry before and after intrasplenic injection with HIV-1_{JR-CSF}. Productively infected hu-DKO mice (infectious HIV; $n = 17$) were analyzed along with hu-DKO mice injected with inactivated HIV (inactivated HIV; $n = 5$) and with no HIV (no HIV; $n = 4$). Solid bars represent preinfection levels and shaded bars represent postinfection levels of the T cell subsets. Data are presented as the mean $\pm$ standard deviation for each group with p -values shown. Correlations between the level of HIV infection and the postinfection levels of (C) peripheral CD4⁺ or (D) peripheral CD8⁺ T cells in all HIV-injected hu-DKO mice ($n = 25$). The levels of infection were defined as high infection (TCID₅₀ = 625–3125), low infection (TCID₅₀ = 1–125), and none (TCID₅₀ = 0). Solid squares represent TCID₅₀ and solid diamonds represent postinjection levels of CD4⁺ or CD8⁺ T cells. Data are presented as mean $\pm$ standard deviation. The level of HIV infection inversely correlated with the postinfection peripheral CD4⁺ T cell level ($p = 0.003$ by Pearson correlation). Conversely, the level of HIV infection is positively associated with the postinfection peripheral CD8⁺ T cell level ($p < 0.01$ by Pearson correlation).

($p < 0.01$) between a high level of HIV infection in mouse spleens and an increase in the CD8⁺ T cell population after infection (Fig. 3D). These results demonstrate that the productive HIV infection in hu-DKO mice is associated with CD4⁺ T cell depletion and a reciprocal increase in CD8⁺ T cells in the peripheral blood that recapitulates the characteristic T cell population changes observed in HIV-infected humans.

To determine the effect of HIV infection on lymphoid tissues, the human thymocytes in the mouse thymus were analyzed 8 weeks after HIV injection, and the fractions of single positive CD4⁺, single positive CD8⁺, and double positive CD4⁺CD8⁺ human T cells were assessed in the mouse thymus. The fraction of single positive CD4⁺ cells in the thy-

muses of the productively infected hu-DKO mice ($31.98\% \pm 5.20$, $n = 17$) was significantly lower ($p = 0.01$) than the single positive CD4⁺ population in the uninfected hu-DKO mice ($62.49\% \pm 12.54$, $n = 5$) and markedly lower than the single positive CD4⁺ thymocyte population in hu-DKO mice injected with inactivated HIV ($44.44\% \pm 8.21$, $n = 5$). In contrast, the thymuses of the productively infected hu-DKO mice displayed an increased fraction of single positive CD8⁺ thymocytes ($33.42\% \pm 6.82$) and double positive CD4⁺CD8⁺ thymocytes ($39.21\% \pm 7.25$) as compared to the thymuses of the uninfected hu-DKO mice ($13.05\% \pm 5.34$) and (24.46 ± 15.20), respectively, and the hu-DKO mice injected with inactivated HIV ($18.95\% \pm 2.64$) and ($36.61\% \pm 9.65$), respectively.

IgG plasma IgM humoral responses against HIV-1 in hu-DKO mice

While the presence of human immunoglobulin in the plasma of hu-DKO mice has been reported,^{17,19,23} reports of HIV-1-specific antibodies present in the serum of these mice after HIV infection in this model are limited.²⁰ To evaluate the capacity of hu-DKO mice to produce human immunoglobulins and HIV-specific antibodies after infection, human IgG and IgM in the mouse plasma were measured by ELISA. The plasma of HIV-infected humanized mice contained higher levels of both human IgG and human IgM as compared to uninfected humanized mice (Fig. 4A and B). In addition, IgG specific for gp120 was detected in the plasma of the hu-DKO mice inoculated with HIV (Fig. 4C), but not in uninjected hu-DKO mice, hu-DKO mice injected with inactivated HIV, or control mice injected with HIV. The presence of plasma IgG specific for gp120 correlated significantly ($p < 0.001$) with the

levels of HIV-1 infection in the spleen (Fig. 4D), as mice with high levels of HIV infection contained the highest level of gp120-specific IgG.

Hu-DKO mice infected by the intrasplenic route develop increased levels of HIV infection and HIV-specific antibody production than HU-DKO mice infected by the intraperitoneal or intravenous route

To investigate the effect that the route of infection of hu-DKO mice has on the resultant level of HIV infection and HIV-specific humoral responses, we compared the degree of HIV infection and humoral responses in hu-DKO mice injected with the same dose of HIV viruses (8000 TCID₅₀) either intrasplenically, intravenously, or intraperitoneally.

HIV infection in the hu-DKO mouse spleens was evaluated by flow cytometry for intracellular p24 antigen staining. As shown in Fig. 5A, the fraction of p24 antigen-positive cells in

FIG. 4. Measurement of total human IgM and IgG levels and HIV-1-specific IgG production in the peripheral blood of HIV-infected hu-DKO mice. (A) The total human IgM and (B) total human IgG in plasma of HIV-infected and -uninfected hu-DKO mice ($n = 4$ per group) was quantified by ELISA and presented as $\mu\text{g}/\text{mL}$. Data are shown as the mean $\pm$ standard deviation for each group. Production of total human IgM was statistically significant between the two groups ($p < 0.02$). (C) The level of plasma human anti-gp120 IgG in hu-DKO mice injected with HIV ($n = 18$), inactivated HIV ($n = 6$), and no HIV ($n = 5$), and in control DKO mice injected with HIV (control mice; $n = 4$) was determined by ELISA and is presented as specific absorbance (OD 490 nm). Data shown are the mean $\pm$ standard deviation for each group. (D) Correlation between the level of HIV infection measured in TCID₅₀ (solid squares) and the plasma level of human anti-gp120 IgG (solid diamonds) in the HIV-infected mouse plasma was highly significant ($p < 0.001$ by Pearson correlation). Levels of infection were defined as high infection (TCID₅₀ = 625–3125), low infection (TCID₅₀ = 1–125), and none (TCID₅₀ = 0).

FIG. 5. Detection of HIV p24 antigen in the spleen cells of hu-DKO mice injected with HIV_{JR-CSF}. (A) At 8 weeks postinfection, spleen cells from hu-DKO mice injected with HIV_{JR-CSF} intrasplenicly ($n = 4$), intravenously ($n = 3$), or intraperitoneally ($n = 2$), and hu-DKO mice not injected with HIV (none, $n = 4$) were pooled for each experimental group and stained for human CD45, human CD4, and HIV p24 antigen and analyzed by flow cytometry. After the human lymphocyte population was identified based on forward and side scatter, the cells were gated for human CD45⁺ cell expression and dot plots of p24 antigen staining vs. human CD4 staining are shown. (B) The level of HIV infection in these groups was determined by limiting dilution coculture of mouse splenocytes with activated HIV-naïve donor human PBMCs at 8 weeks postinfection. One week later, the p24 antigen levels in the culture supernatant were measured and TCID₅₀ values were calculated. Data are presented as the mean TCID₅₀ per 1 million splenocytes $\pm$ standard errors for each group.

the spleens of hu-DKO mice inoculated intrasplenicly was about 2-fold and 18-fold higher than splenocytes of hu-DKO mice inoculated intravenously or intraperitoneally, respectively. Flow cytometric analysis indicated that the majority of mononuclear cells containing HIV p24 antigen were human CD45⁺CD4⁺ lymphocytes. Since these were also CD8⁻ (data not shown) it is likely that these represent CD4⁺ T cells whose surface expression of CD4 was reduced by HIV infection, particularly by the effect of Nef.³⁵

We further investigated the extent of HIV infection by quantifying the number of cells in the mouse spleen capable of initiating productive infection by limiting-dilution coculture. Eight weeks after inoculation, while all of the HIV-injected groups were infected, the number of HIV-infected cells in hu-DKO mice intrasplenicly injected with HIV was 12-fold and 7-fold higher than hu-DKO mice inoculated intravenously or intraperitoneally, respectively (Fig. 5B).

The increased level of HIV infection observed in the intrasplenicly inoculated hu-DKO mice was paralleled by increased production of HIV-specific antibodies (Fig. 6A). The level of gp120-specific IgM in the plasma of intrasplenicly inoculated hu-DKO mice was nearly 20-fold or 10-fold higher than that of hu-DKO mice infected intravenously or intraperitoneally, respectively; the plasma of hu-DKO mice infected intrasplenicly had approximately 5-fold or 10-fold

higher levels of gp120-specific IgG than did hu-DKO mice infected intravenously or intraperitoneally, respectively (Fig. 6A). Gag-specific IgM levels were markedly higher in the plasma of intrasplenicly infected hu-DKO mice as compared to intravenously or intraperitoneally infected hu-DKO mice (Fig. 6B). The production of IgG against Gag was barely detectable in intrasplenicly infected hu-DKO mice (Fig. 6B). There was a statistically significant positive correlation between the level of IgM production against gp120 and the level of HIV infection, as well as the level of IgG against Gag and HIV infection (data not shown).

Evaluation of the peripheral blood of HIV-infected individuals has indicated that T cells are activated by HIV infection as evidenced by their increased expression of the CD69 activation marker.^{36–38} We examined whether a similar response was induced by HIV infection in hu-DKO mice by analyzing CD69 expression by peripheral human T cells after HIV infection. Although few CD69⁺ T cells were detected in the peripheral blood of uninfected hu-DKO mice or intraperitoneally infected hu-DKO mice, over 10% of the peripheral T cells expressed CD69 in hu-DKO mice infected intrasplenicly with HIV, which was almost 3-fold higher than the fraction of CD69 expression observed in mice infected intravenously with HIV (Fig. 7). Taken together, these results demonstrated that intrasplenic injection of HIV viruses

FIG. 6. Comparative analysis of HIV-1-specific IgM and IgG production against gp120 and Gag in HIV-injected hu-DKO mice by different routes of injection. At 8 weeks postinfection, HIV-1-specific IgM (solid bars) and IgG (diagonal lined bars) production was determined by ELISA in the plasma of hu-DKO mice injected with HIV_{JR-CSF} intrasplenically ($n=6$), intravenously ($n=2$), or intraperitoneally ($n=2$), and hu-DKO mice not injected with HIV (none, $n=4$). Data presented are mean specific absorbance at 490 nm $\pm$ standard errors of each group for the indicated ELISA assay. (A) IgM and IgG production against gp120. The anti-gp120 IgM production was statistically significant between the intrasplenic and intravenous group ($p=0.03$), and the intrasplenic and no HIV group ($p=0.02$) as denoted (**). (B) IgM and IgG production against Gag.

led to more potent HIV infection in hu-DKO mice than intravenous or intraperitoneal injection, which was associated with increased T cell activation and HIV-specific humoral responses.

Antiretroviral therapy suppresses HIV-1 infection and prevents CD4 depletion in hu-DKO mice

Multidrug therapy combinations such as azidothymidine (AZT), lamivudine, and indinavir have been well established and termed HAART; HAART effectively induces a marked decline in plasma viremia and an increase in the peripheral CD4⁺ T cell population.^{39,40} To determine the utility of using hu-DKO mice as a model to investigate the effectiveness of antiretroviral therapy, we examined the capacity of HAART to inhibit HIV infection. One week after intrasplenic inoculation with HIV-1_{JR-CSF} virus (3600 TCID₅₀), mice were treated with an antiretroviral cocktail consisting of AZT, lamivudine, and indinavir in the drinking water.²⁹ After 7 weeks of treatment, the mice were sacrificed to evaluate the development of productive HIV infection.

All of the untreated hu-DKO mice ($n=4$) were extensively infected with HIV, to the maximum measurable TCID₅₀ value of 3150 HIV-infected cells/10⁶ splenocytes. In contrast, treatment of the HIV-infected hu-DKO mice with HAART ($n=4$) resulted in an almost 300-fold ($p<0.001$) lower number of HIV-infected cells in the mouse spleen (Fig. 8A). The potent inhibition of infection in the spleen cells of HAART-treated hu-DKO mice was associated with markedly lower plasma viral loads with an over 300-fold reduction in plasma HIV-1 RNA levels (69 copies/ml) ($p<0.001$) compared to plasma HIV RNA levels in the untreated group (24,793 copies/ml) (Fig. 8B). HAART-mediated inhibition of HIV infection correlated with preservation of the peripheral CD4⁺ T cell pop-

ulation. The postinfection level of CD4⁺ T cells in the peripheral blood of HAART-treated hu-DKO mice was almost unchanged, compared to a nearly 40% reduction in the CD4⁺ T cell population of the untreated hu-DKO mice (Fig. 8C). This indicates that CD4 depletion caused by HIV infection was prevented by HAART.

Discussion

Humanized mice generated by reconstituting DKO mice with HSCs are an emerging mouse model for studying HIV infection.¹⁸ The severe immunodeficiencies of the DKO mice permit these mice to be extremely tolerant of human HSC transplantation^{16,17} and support the subsequent maturation by the transplanted human HSCs into a large repertoire of human immune cells including T cells, B cells, monocytes, and dendritic cells.^{18,41} Reconstitution of a functional human immune system in this model provides an experimental system that may be employed to evaluate vaccine and therapeutic strategies against HIV infection.

Varying levels of HIV infection have been reported in hu-DKO mice using different doses of HIV virus injected either intraperitoneally^{19,20,22,23} or intravenously²⁴ that were associated with minimal HIV-specific humoral responses. Baenziger *et al.* demonstrated in their study that only 1 out of 25 mice produced IgG, but no IgM, against HIV-1 p34, gp41, p52, p58, and gp120.²⁰ An *et al.* did not detect HIV-1 specific IgG and IgM against viral proteins prepared from virions or recombinant pr55 Gag¹⁹ while Gorantla *et al.* failed to detect any HIV-specific antibodies.²³

We hypothesized that using an alternative approach to infecting the humanized mice, directly introducing HIV into the spleen, which is a lymphoid tissue populated with human CD4⁺ and CD8⁺ T cells, monocytes, and dendritic cells, could

FIG. 7. Activation of T cells in the peripheral blood of HIV-challenged hu-DKO mice. At 6 weeks postinfection, the human T cell population in the peripheral blood was assessed by flow cytometry for the expression of the activation marker, CD69, in hu-DKO mice injected with no HIV (none; $n=4$), and in hu-DKO mice injected with HIV_{JR-CSF} intrasplenically ($n=5$), intravenously ($n=3$), or intraperitoneally ($n=3$). Data presented are means $\pm$ standard errors. **Denotes statistically significant differences between intraspinal and no HIV ($p=0.03$), intraspinal and intraperitoneal ($p=0.049$), intravenous and no HIV ($p<0.01$), and intravenous and intraperitoneal ($p<0.01$).

lead to more potent infection and elicitation of HIV-1-specific humoral responses. Using this approach, we detected plasma human IgG against gp120 following HIV-1 infection. The successful induction of humoral responses may be due in part to the direct inoculation of HIV virus in the spleens of

hu-DKO mice. The route of antigen administration contributes to the variability of immune responses in HIV-1 infection.² Mucosal or lymph node inoculation of SIV antigens was more effective for the induction of immune responses than peripheral infection.^{42,43} Intraspinal inoculation as a route to initiate HIV infection has been demonstrated by us using thyliv-SCID-hu mice^{44,45} and hu-PBL/SPL-SCID mice⁴⁶ and by others in hu-PBL-SCID mice.⁴⁷ In the hu-PBL NOD/SCID mouse model, intraspinal injection with HIV-infected human PBMCs induced HIV-specific antibody production.⁴⁸ Although the production of human immunoglobulins in hu-DKO mice has been reported,^{17,19,20,23} our study is the first report of the production of anti-HIV human immunoglobulins after intraspinal inoculation in this model.

In accordance with several recent studies,^{19,20,22–24} we achieved successful engraftment of human T, B, and myeloid cells in primary and secondary lymphoid tissues in human HSC-transplanted DKO mice and HIV infection after intraspinal inoculation of the humanized mice with HIV-1_{JR-CSF} virus. We demonstrated productive infection by quantifying the number of cells in the spleen capable of initiating productive *in vitro* HIV infection. As reported in other studies of HIV infection in hu-DKO mice, the productive HIV-1 infection was associated with peripheral CD4⁺ T cell depletion, an increase in CD8⁺ T cells, and plasma viremia. We observed a significant inverse relationship between the level of HIV infection and the degree of CD4⁺ T cell depletion and a reciprocal direct correlation with the level of infection and the increase in the level of CD8⁺ T cells.

HIV challenged mice were monitored for total human immunoglobulin levels in peripheral blood. Plasma human IgG and IgM was detected in all hu-DKO mice, with higher levels of plasma IgM than plasma IgG in all of the hu-DKO mice. Plasma human IgG levels were comparable to that detected with hu-DKO mice immunized with tetanus toxoid (TT),¹⁷ and plasma human IgG and IgM levels are comparable to those described by Watanabe *et al.* in the HIV-1-infected NOD/SCID/ $\gamma_c^{-/-}$ model.⁴⁹ Recent studies of HIV-1 infection

FIG. 8. HAART suppresses HIV infection and prevents CD4 depletion in hu-DKO mice. One week after intraspinal HIV injection, HAART consisting of AZT, lamivudine, and indinavir was administered in the drinking water to hu-DKO mice for 7 weeks. (A) HIV infection was quantified by limiting dilution coculture of mouse splenocytes with activated HIV-naive donor human PBMCs. One week later, culture supernatants were tested for HIV p24 antigen levels and TCID₅₀ was calculated for mice in the untreated group ($n=4$) and HAART-treated group ($n=4$). Data are presented as mean TCID₅₀/10⁶ cells $\pm$ standard errors. (B) At 8 weeks postinfection, plasma from the untreated- and HAART-treated groups was collected and pooled, and HIV RNA levels were determined by the bDNA assay. Data are shown as HIV RNA copies/ml. (C) The human CD4⁺ T cell population in the peripheral blood of the untreated and HAART-treated group was measured by flow cytometric analysis before and after infection. Data are presented as the mean percentage reduction of peripheral CD4⁺ T cells $\pm$ standard error for each group.

in hu-DKO mice demonstrated either no production of HIV-1-specific human immunoglobulins^{19,23} or minimal production.²⁰ We detected measurable levels of human IgG gp120 in mouse plasma. We speculate that the direct inoculation of HIV-1 virus into spleens that contain large populations of human dendritic cells may promote better antigen presentation and thereby induce immune responses, as compared to other routes of inoculation used including intraperitoneal and intravenous injection. This was supported by our comparative analysis of HIV infection and immune responses after intrasplenic, intravenous, or intraperitoneal inoculation of hu-DKO mice with HIV; intrasplenic injection of HIV resulted in significantly higher levels of HIV infection than other injection routes and induced the most potent HIV-specific IgG and IgM antibody responses against two HIV proteins, gp120 and Gag. There was a high degree of correlation between the level of plasma anti-gp120 IgG and the level of HIV infection in the mouse spleens as measured by TCID₅₀; mice exhibited a higher level of HIV infection mounting a more potent HIV-1-specific antibody responses. Taken together, these results indicate that functional B cells derived from transferred HSCs develop and are able to produce human immunoglobulins including antigen-specific antibodies, suggesting that effective antigen presentation and recognition can occur in the reconstituted immune tissues of hu-DKO mice.

In addition to evaluating the humoral responses, we also examined the development of cellular immune responses against HIV-1 infection using an interferon (IFN)- γ ELISPOT assay. Unfortunately, as reported by others,^{19,49} after *in vitro* restimulation of T cells with HIV-1 antigen we did not detect antigen-specific T cell responses. It is unclear why T cell immune responses do not develop in hu-DKO mice despite their population with human T cells expressing a broad TCR repertoire in the reconstituted mice.^{16,17,23} This may be due partially to impaired thymic selection for HIV-specific human T cells, impaired development of the lymph node microenvironment,¹⁸ or lack of human cytokines and chemokines required for appropriate T cell expansion.⁴¹ The lack of HIV-specific CD8⁺ T cell responses may also be due to inefficient T cell activation in mouse lymphoid tissues or by HIV-mediated reduction of the population of human CD4⁺ T cells that is required for the expansion of the HIV-specific CD8⁺ T cell population. Further improvements of the model need to be made, including developing approaches to generate higher and consistent engraftment of the mice with human cells, and prolonged and higher levels of HIV infection, to induce more robust humoral responses and cellular immune responses.

In the present work, we have also demonstrated evidence of the efficacy of antiretroviral therapy in this mouse model. The mice treated with a well-established clinical antiretroviral regimen consisting of AZT, lamivudine, and indinavir showed a significant suppression of HIV infection when compared to the untreated animals, as measured by the decreased number of infectious cells and plasma viremia. We did not observe an occurrence of virus rebound since this established treatment completely suppressed the virus infection. Our observation is in contrast with the recent demonstration of viral rebound and the emergence of antiretroviral drug-resistant mutants in hu-DKO mice by Choudhary *et al.*⁵⁰ We speculate that the more potent treatment regimen we used prevented the emergence of resistant isolates. HAART also prevented HIV-induced reduction of the CD4⁺ T cell

population when compared to HAART untreated mice. These results suggest that the hu-DKO mice may recapitulate the effects of antiretroviral therapy seen in humans.

Thus we have successfully demonstrated the production of HIV-specific humoral responses induced by intrasplenic immunization and the efficacy of antiretroviral therapy in hu-DKO mice reconstituted with a human immune system. Our results further support the application of this model to serve as a valuable *in vivo* tool to study the pathogenesis, immunology, and virology of HIV infection, and to promote the development of a therapeutic design against HIV infection.

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Author Disclosure Statement

No competing financial interests exist.

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