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Humanized Rag2$^{-/-}\gamma c^{-/-}$ (RAG-hu) mice can sustain long-term chronic HIV-1 infection lasting more than a year

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ABSTRACT

HIV-1 infection is characterized by life-long viral persistence and continued decline of helper CD4 T cells. The new generation of humanized mouse models that encompass RAG-hu, hNOG and BLT mice have been shown to be susceptible to HIV-1 infection and display CD4 T cell loss. Productive infection has been demonstrated with both R5 and X4 tropic strains of HIV-1 via direct injection as well as mucosal exposure. However the duration of infection in these mice was evaluated for a limited time lasting only weeks post infection, and it is not established how long the viremia can be sustained, and if the CD4 T cell loss persists throughout the life of the infected humanized mice. In the present study we followed the HIV-1 infected RAG-hu mice to determine the long-term viral persistence and CD4 T cell levels. Our results showed that viremia persists life-long lasting for more than a year, and that CD4 T cell levels display a continuous declining trend as seen in the human. These studies provide a chronic HIV-1 infection humanized mouse model that can be used to dissect viral latency, long-term drug evaluation and immune-based therapies.

Introduction

Since HIV-1 causes disease only in the human, a number of humanized mouse models have been developed through the years to study the viral pathogenesis in human cells *in vivo*. In this regard, two conventional human-mouse chimeric models, namely the hu-PBL-SCID mouse with transplanted adult human PBLs (Mosier et al., 1988), and the SCID-hu mouse model with transplanted human thymus and liver tissues (McCune et al., 1988) played an important role in HIV-1 pathogenesis studies using a human hemato-lymphoid system. However, despite many notable successes, some limitations exist. They lack multi-lineage human hematopoiesis and a functional human immune system. Also, these models primarily mimic an acute HIV infection with rapid CD4 T cell loss thus restricting pathogenesis studies to a short-term period lasting only a few weeks (Jamieson et al., 1996; Mosier, 1996).

Improved humanized mouse models have recently been developed that can rectify the above limitations (Manz, 2007; Shultz et al., 2007). These new models include the NOD/SCIDγc$^{-/-}$ and Rag2$^{-/-}\gamma c^{-/-}$ strains reconstituted with human CD34 cells (hNOG and RAG-hu mice). Transplantation of human CD34 hematopoietic stem cells into conditioned neonatal mice leads to de novo multi-lineage human hematopoiesis with the production of T cells, B cells and dendritic cells. Furthermore, an improvement of the standard SCID-hu mouse model involved transplantation of thymic and liver tissues under the kidney capsule of NOD-SCID mice followed by reconstitution with autologous human CD34 cells (BLT mice) (Melkus et al., 2006). Multilineage hematopoiesis with the generation of HIV-susceptible CD4 T cells, macrophages, monocytes, and dendritic cells in addition to B cells with a capacity for primary human immune responses distinguish these newer humanized mouse models from that of previous conventional models (An et al., 2007; Baenziger et al., 2006; Brainard et al., 2009; Gorantla et al., 2006; Kuruvilla et al., 2007; Melkus et al., 2006; Tonomura et al., 2008; Traggiai et al., 2004; Watanabe et al., 2007). A number of groups including ours have demonstrated the utility of these humanized mice as improved models for HIV-1 infection by showing chronic viremia lasting several weeks by both R5 and X4 tropic viral strains, virus replication in a variety of lymphoid and non-lymphoid organs including thymus, lymph nodes, spleen, lung, gut-associated lymphoid tissue, and male and female reproductive tracts. Viral infection leads to gradual CD4 T cell depletion (An et al., 2007; Baenziger et al., 2006; Berges et al., 2006, 2008; Brainard et al., 2009; Choudhary et al., 2009; Denton et al., 2008; Gorantla et al., 2006; Jiang et al., 2008; Kumar et al., 2008; Sun et al., 2007; Van Duyne et al., 2008; Watanabe et al., 2007; Zhang et al., 2006). Furthermore, although not robust enough to be protective, humoral and cellular immune responses against HIV-1 could also be seen to some extent (Baenziger et al., 2006; Brainard et al., 2009; Watanabe et al., 2007). Since human cells populate mucosal tissues such as the gut and vaginal tracts in both RAG-hu mice (Berges et al., 2008; Choudhary et al., 2009; Kwant-

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Mitchell et al., 2009) and BLT mice (Denton et al., 2008; Sun et al., 2007), another innovation with these new humanized mouse models has been the successful mucosal transmission of HIV-1 through both vaginal and rectal routes (Berges et al., 2008; Denton et al., 2008; Sun et al., 2007). In a recent report, RAG-hu mice were also shown to be capable of giving rise to protective human mucosal immune responses when infected by HSV-2 by the vaginal route (Kwant-Mitchell et al., 2009).

HIV-1 infection in the human is life-long and the virus is known to persist even in the presence of aggressive anti-retroviral therapy. Therefore, long-term infection studies in animal models would be of significance to experimentally evaluate the chronic effects of HIV on the human immune system. Despite the above-mentioned important innovations of HIV-1 infection of humanized mice, it is not clear how long the viral infection can be sustained since little data has thus far been reported for long-term HIV-1 infection. Nearly all studies have been terminated by 8–16 weeks post-infection, with the longest-term data available being viremia detected up to 30 weeks post-infection, CD4 T cell loss through 24 weeks post-infection, and *in situ* histological confirmation of virus replication through 12 weeks (Berges et al., 2006). In the current study, we provide data for long-term HIV-1 infection of RAG-hu mice, with viremia measured up to 63 weeks post-infection, CD4 T cell depletion tracked through 51 weeks, and detection of virus replication in lymphoid organs through 70 weeks post-infection. Our findings demonstrate that HIV-1 infection persists for essentially the lifetime of the infected animal, as seen in the human.

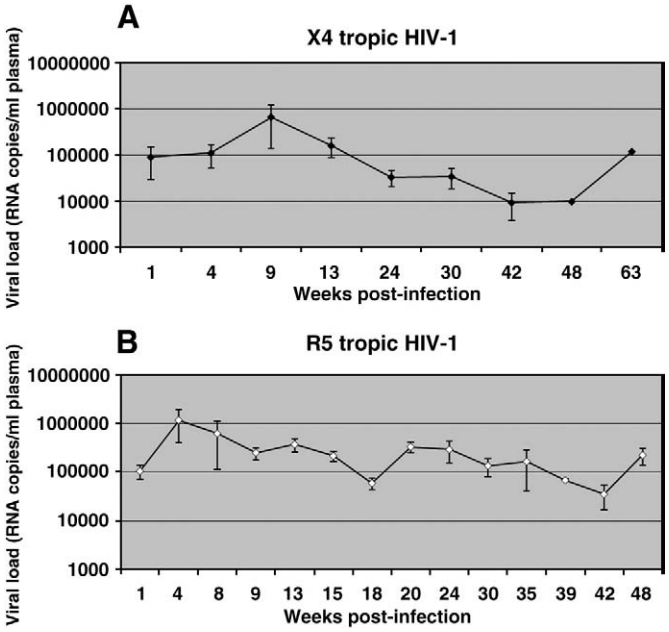

Fig. 1. Long-term viremia following HIV-1 infection of RAG-hu mice. RAG-hu mice infected with either X4 tropic HIV-1 (A) or R5 tropic HIV-1 (B) were bled periodically and HIV-1 viremia was measured in plasma by Q-RT-PCR as described in methods.

Results and discussion

RAG-hu mice sustain long-term chronic HIV-1 infection for more than a year

HIV infected individuals carry the virus life-long since virus susceptible cells such as CD4 T cells are generated on a continuous basis and the ineffective immune response is not able to eliminate the viral infection. Similarly, continued production of HIV-1-susceptible cells in humanized mice (RAG-hu mice) engrafted with hematopoietic stem cells is likely to result in a life-long viral infection, thus allowing the study of the chronic viral effects as seen in HIV-infected humans.

The data presented herein were analyzed in retrospective fashion as we monitored long-term infection in animals from various experimental groups which were infected at different times, including both intraperitoneal and mucosal infection. To determine the duration of persistent HIV-1 infection in RAG-hu mice, we monitored long-term levels of plasma viremia by Q-RT-PCR following infection with either X4 or R5 virus. Our results showed that RAG-hu mice infected with either X4 or R5 HIV-1 maintain detectable viremia for at least 63 weeks post-infection, the last time point analyzed (Fig. 1). The mean peak viremia is about 8×10^5 RNA copies/ml plasma and occurs at about 8–9 weeks post-infection, regardless of virus used or route of infection (intraperitoneal or mucosal). Thereafter, while the infected animals continue to be virus-positive, viral load tends to slowly diminish over time. Long-term viral load data for representative mice (correlated with infecting virus and route of infection) are provided in the supplementary materials (Table S1). In total, a single plasma sample was found to be negative by Q-RT-PCR ($n = 105$), and that animal later tested positive again. Some previous reports were unable to detect HIV-1 viremia in some RAG-hu mice at later time points (Gorantla et al., 2006; Van Duyne et al., 2008). However, the p24 ELISA method was used in these studies as opposed to detection of viral RNA by Q-RT-PCR. A recent study showed detection of viral RNA and provirus in infected mice while p24 was undetectable by ELISA in the same animals (Van Duyne et al., 2008). Therefore use of Q-RT-PCR is clearly more sensitive than p24 ELISA for measurement of HIV-1 viremia in RAG-hu mice.

To further confirm the presence of HIV-1 infected cells during chronic infection, animals infected with either R5 or X4 viruses were sacrificed and histological sections of lymphoid organs were evaluated. HIV-1 RNA was detected by *in situ* hybridization in the bone marrow at 14 months post-infection with X4 virus (Fig. 2B), while none was detected in uninfected bone marrow (Fig. 2A). Similarly, HIV RNA was detected in the mesenteric lymph node at 16 months post-infection with R5 virus (Fig. 2D), but not in uninfected mesenteric lymph node (Fig. 2C). The viral load and *in situ* viral detection data taken together, confirmed that infected mice carry the virus long-term, most likely lifelong.

RAG-hu mice display CD4 T cell depletion during chronic HIV-1 infection

CD4 T cell depletion is a hallmark of HIV infection in the human and similar findings were noted in previous reports in infected

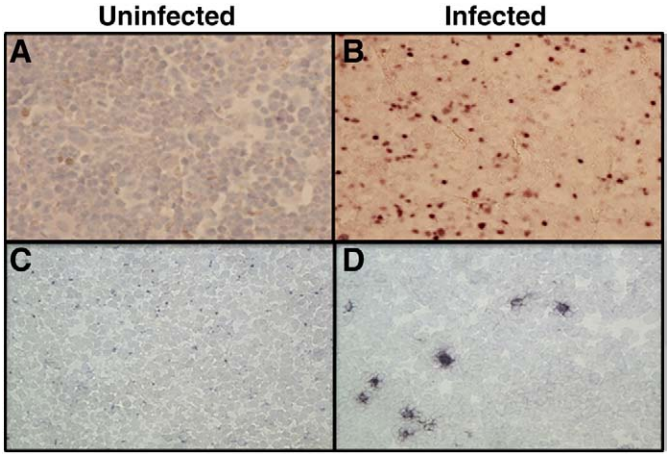

Fig. 2. Detection of HIV-1 infected cells in chronically infected RAG-hu mice. Tissues from HIV-1 infected RAG-hu mice were subjected to *in situ* hybridization to detect viral RNA. Bone marrow at 14 months post-infection with HIV-1 NL4-3 (B) or uninfected bone marrow (A). Mesenteric lymph node at 16 months post-infection with HIV-1 BaL-1 (D) or uninfected lymph node (C). 200× magnification shown in C,D and 100× magnification shown in A,B.

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humanized mice that were followed for about 3 months. CD4 T cell depletion was seen to occur with either of the R5 or X4 HIV-1 strains that use different co-receptors for viral entry. In the present study of chronic infection lasting more than a year, our data showed that there is an initial drop in peripheral blood CD4 T cell counts which occurs around 6 weeks for X4 virus-infected and at 9 weeks for R5 virus-infected animals (Fig. 3). A rebound then ensues, followed by periodic rises and falls of CD4 T cell counts. However, the overall trend is a gradual decrease in CD4 T cell levels, being more severe for X4 virus than for R5 virus consistent with that seen in the human towards the late stages of the disease when the X4 virus predominates (Tersmette et al., 1989). Long-term CD4 depletion data for representative mice (correlated with infecting virus and route of infection) are provided in the supplementary materials (Table S2).

In summary, we have shown that RAG-hu mice infected with HIV-1 sustain chronic viremia with associated CD4 T cell level fluctuations. To our knowledge, this is the first report wherein long-term HIV-1 infection has been followed for more than a year. These findings establish the utility of this humanized mouse model for long-term pathogenesis and therapeutic studies and open new avenues for *in vivo* investigation. For example, viral latency can be evaluated and its elimination can be attempted. Also, long-term suppression of the virus with novel anti-retrovirals currently in the pipeline can be more effectively evaluated *in vivo* and potential drug resistant viruses can be isolated and characterized after prolonged treatment. Furthermore, viral evolution *in vivo* from R5 to X4 virus can be better studied in addition to evaluating chronic immune activation and its possible reversal.

Materials and methods

Generation of humanized Rag2$^{-/-}\gamma c^{-/-}$(RAG-hu) mice

Humanized BALB/c-Rag2$^{-/-}\gamma c^{-/-}$ mice were prepared essentially as described by Traggiai et al. (2004) with the exception that human fetal liver-derived CD34 cells were used for engraftment (Akkina et al., 1994). Human fetal liver-derived CD34 cells were purified and cultured for 1 day in cytokine media containing 25 ng each IL-3, IL-6 and SCF as described previously (Akkina et al., 1994). After irradiation at 350 rads, 2- to 3-day-old neonatal mice were injected intrahepatically with $0.25–1\times10^6$ CD34 cells. Transplanted mice were screened for human cell engraftment at 12 weeks post-reconstitution. Peripheral blood was collected by tail bleed, red blood cells were lysed using the Whole Blood Erythrocyte Lysing Kit (R and D Systems, Minneapolis, MN), and cells were stained with the human pan-leukocyte marker CD45 and FACS analyzed to verify engraftment.

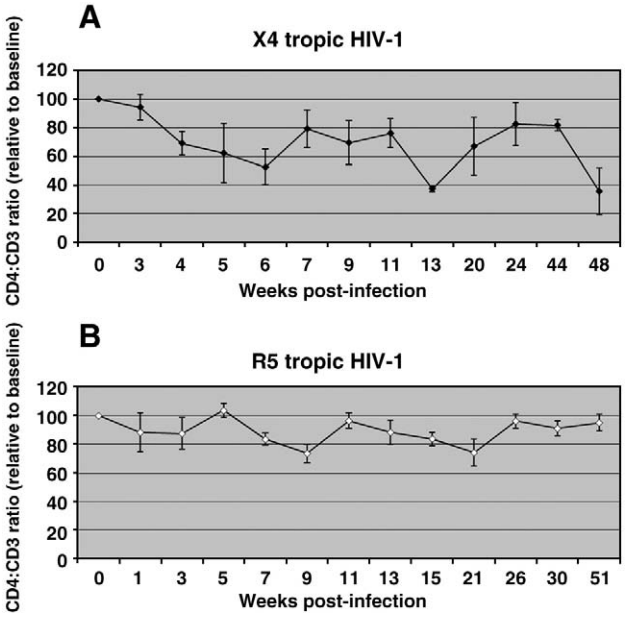

Fig. 3. Long-term CD4 T cell depletion in the peripheral blood of RAG-hu mice. RAG-hu mice infected with either X4 tropic HIV-1 (A) or R5 tropic HIV-1 (B) were bled periodically and examined for CD4 T cell depletion by FACS analysis of peripheral blood cells stained for human CD3 and CD4 markers. CD4:CD3 ratios were determined as described in methods. Data in (A) represent a mean of 4.5 mice evaluated per time point, while (B) represents a mean of 5.7 mice per time point.

HIV-1 infection of humanized Rag2$^{-/-}\gamma c^{-/-}$ mice

Humanized mice were infected either intraperitonially or by mucosal routes (intravaginally or intrarectally). For intraperitonial infections, HIV-1 in a 100 µl volume was injected at least 12 weeks after cell engraftment. Mice received either HIV-1 NL4-3 (1.2×10^5 i.u.) or HIV-1 BaL-1 (0.9×10^5 i.u.), where infectious units were based upon p24 ELISA titers (100 i.u. per 1 ng of p24). For mucosal infection, mice were infected with cell-free HIV-1 strain BaL-1 (R5 tropic) (Berges et al., 2008). Briefly, vaginal infections were performed in a volume of 50 µl (156 $TCID_{50}$ of BaL-1 virus or 203 $TCID_{50}$ of NL4-3) and rectal infections were performed in a volume of 20 µl (BaL-1 virus at 62 $TCID_{50}$; NL4-3 virus at 81 $TCID_{50}$). $TCID_{50}$ titers were determined by limiting dilution and infection of GFP-expressing Ghost R3/X4/R5 cells.

Measurement of viral load in plasma

To detect cell-free HIV by Q-RT-PCR, RNA was extracted from 25 to 50 µl of plasma collected at different times post infection using the QIAamp Viral RNA kit (Qiagen, Valencia, CA). cDNAs were produced with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) using a primer set specific for the HIV LTR sequence, and Q-PCR was performed with the same primer set and a LTR-specific probe (Rouet et al., 2005) using Supermix UDG (Invitrogen, Carlsbad, CA).

Flow cytometry

FACS analysis was performed on EDTA-treated peripheral blood samples. Erythrocytes were lysed using the Whole Blood Erythrocyte Lysing Kit (R and D Systems, Minneapolis, MN) per the manufacturer's protocol. To determine CD3:CD4 ratios, white blood cells were stained for CD3 and CD4 markers and FACS analyzed. CD4 T cell levels were calculated as a ratio of the entire CD3 population ($CD4^+CD3^+$: $CD3^+$). To establish baseline CD4 T cell levels, all mice were analyzed prior to infection and baseline values for each mouse were standardized to 100%. The following antibodies were used: hCD45-FITC, hCD3-PE, hCD4-PECy5 (Caltag).

In situ hybridization for HIV-1 RNA

In situ hybridization was performed on 4-µm formalin-fixed, paraffin-embedded tissue sections from bone marrow or frozen tissue sections from mesenteric lymph node from HIV-1 infected and uninfected RAG-hu mice. At least three sections were evaluated from each tissue. Hybridization was performed using digoxigenin-labeled antisense and sense riboprobes specific for HIV-1 *env* (nt 6411-6752) and detected using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as reported previously (Berges et al., 2005). Sections were lightly counterstained with hematoxylin to enable visualization of nuclei.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.10.034.

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