Regulatory T Cells Prevent Liver Fibrosis During HIV Type 1 Infection in a Humanized Mouse Model

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Human immunodeficiency virus type 1 (HIV-1) disease is associated with aberrant immune activation, and coinfection with hepatitis C virus (HCV) exacerbates hepatic inflammation and fibrosis. However, the role of HIV-1 infection or host immune modulation in liver pathogenesis is not clearly defined. Here, we report that regulatory T (Treg) cells prevent liver immunopathogenesis during HIV-1 infection in a humanized mouse model. In the absence of Treg cells, HIV-1 infection induced liver fibrosis associated with hepatic stellate cell activation, hepatitis, and liver injury. Our findings provide new insight linking Treg cells and liver immunopathogenesis during HIV-1 infection.

Keywords. HIV-1 infection; Treg cells; liver fibrosis; inflammation.

Immune hyperactivation and progressive loss of CD4+ T cells, including regulatory T (Treg) cells, are associated with human immunodeficiency virus type 1 (HIV-1) disease progression [1]. CD4+CD25+ Treg cells play a critical role in modulating the immunopathogenesis of a number of diseases, including autoimmune and infectious diseases [2]. In HIV-infected patients or simian immunodeficiency virus–infected rhesus macaques, Treg cells are accumulated and infected in acutely infected hosts, but numbers are gradually reduced during chronic pathogenic infection [3]. However, the role of Treg cells in HIV-1 infection and pathogenesis is not clearly defined, because of a lack of a robust model. We have recently reported that functional Treg cells are developed in all lymphoid organs in humanized mice. In addition, Treg cells are preferentially infected, and depletion of Treg cells before HIV-1 infection in humanized mice leads to an elevated immune response and reduced HIV-1 replication [4].

Liver disease has become a leading cause of non-AIDS–related mortality because HIV-infected patients continue to live longer because of successful antiretroviral therapy. HIV-1–infected patients are often infected with hepatitis C virus (HCV) because the pathogens share transmission routes [5]. HIV/HCV coinfection leads to accelerated hepatic fibrosis and to higher rates of liver failure and death, compared with HCV monoinfection [5]. Furthermore, HIV-1–infected patients have a significant risk of liver fibrosis, even without hepatitis virus coinfection [6]. Both virologic and immunologic mechanisms have been postulated [5]. However, the role of Treg cells in hepatic immunopathology during HIV-1 infection with or without HCV coinfection is unclear. We hypothesized that Treg cells played a role in modulating liver diseases during HIV-1 infection. We determined the role of Treg cells in liver fibrosis during HIV-1 infection in vivo, using a humanized mouse model.

METHODS

Construction of Humanized Mice

Approval for animal work was obtained from the University of North Carolina Institutional Animal and Use Committee. Rag2−/− IL-2Rγ−/− or NOD-scid IL-2Rγ−/− mice were used to create humanized mice. We constructed humanized mice as previously reported [4]. In brief, human CD34+ hematopoietic stem cells were isolated from liver tissue of human fetuses with a gestational age of 16–18 weeks. Tissue was filtered through a 70-μm cell strainer (BD Falcon, Lincoln Park, NJ) and centrifuged at 150 g for 5 minutes to collect mononuclear cells. Cell viability, measured using Guava Easy Cyte (Millipore, Billerica, MA), generally exceeded 90%. After selection with the CD34 magnetic-activated cell sorting kit (Miltenyi Biotech, Gladbach, Germany), the purity of CD34+ cells was >95%.
Between $0.5 \times 10^6$ and $1 \times 10^6$ CD34+ cells were injected into the liver of each 1–5-day-old mouse, which had been previously irradiated at 400 or 250 rad. Leukocyte populations in >95% of the mice were stably reconstituted with human leukocytes in the blood (percentage of leukocyte population composed of human leukocytes, >10% at 12–14 weeks of age). Humanized mice in each cohort (ie, mice in which leukocyte populations were reconstituted from the same human donor fetal liver tissue) had a similar level of engraftment. All mice were housed at the University of North Carolina–Chapel Hill.

**Treg Cell Depletion by Denileukin Diftitox and HIV-1 Infection in Humanized Mice**

Denileukin difftitox (Ontak; 150 µg/mL in citrate buffer) was provided by Ligand Pharmaceuticals (San Diego, CA). For in vivo treatment, humanized mice with leukocyte populations stably reconstituted with human leukocytes 12–16 weeks after CD34+ cell transfer (percentage of leukocyte population composed of human CD45+ leukocytes, >10%) were injected retro-orbitally (2 days before HIV-1 infection) or intraperitoneally (15 days after infection) with denileukin difftitox at 31 µg/kg of mouse weight diluted in Hank’s balanced salt solution (HBSS). HBSS-injected humanized mice were used as controls. Either denileukin difftitox–injected or HBSS-injected humanized mice were retro-orbitally infected with HIV-R3A stocks which is a CCR5/CXCR4 dual tropic and highly pathogenic virus (1 ng p24/mouse in 50µL). Humanized mice infected with mock stocks were included as control groups. At the time of euthanasia (20 days after infection), blood, spleen, and liver were harvested, and cell numbers were counted by Guava Easy Cyte.

**Flow Cytometry Analysis**

At the time of euthanization, lymphocytes were isolated from the liver tissue by the ACK lysis method. Lymphocytes were stained with anti-human CD45, CD25, CD4, CD8, and CD3 (Biologend, San Diego, CA); mouse CD45 (Invitrogen); and the Live/Dead Fixable dead cell stain kit (Invitrogen). The Cytofix/ Cytoperm kit (BD Bioscience, San Jose, CA) and anti-HIV-1 p24 antibody (Beckman Coulter, Fullerton, CA) were used for intracellular p24 staining. Stained cells were fixed and analyzed by CyAn ADP (Dako, Glostrup, Denmark).

**Immunohistochemistry Staining and Area Quantification**

Paraffin-embedded sections of livers from humanized mice were stained with hematoxylin and eosin, Sirius red/fast green, or primary antibodies. After the incubation of sections with primary antibodies, primary antibodies were detected with Rat and Mouse Double Stain Kit (Biocare Medical, Concord, CA). The slides were developed with the Betazoid DAB chromogen kit or the Vulcan Red chromogen kit 2 (Biocare medical). Slides were observed using an Eclipse E600 microscopy system (Nikon, Garden City, NY) with a 10× or 20× nonoil objective and a 10× ocular lens. Images were captured using OmniVid microscope camera (LW Scientific, Lawrenceville, GA). For area quantification, pictures were taken with a 4× nonoil objective and a 10× ocular lens. Pictures were analyzed by ImageJ (available at: http://rsbweb.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) to quantify positive signals. At least 5 pictures from each liver were used to quantify positive areas.

**Alanine Transaminase (ALT) and Hyaluronic Acid Levels in Plasma**

Blood specimens from humanized mice were obtained at indicated time points, using ethylenediaminetetraacetic acid as an anticoagulant. Plasma was then prepared by centrifugation. Plasma ALT and hyaluronic acid levels were measured by the ALT Color Endpoint Assay kit (Bioo Scientific, Austin, TX) and the hyaluronic acid ELISA kit (Echelon, Salt Lake City, UT), respectively.

**Gene Expression Analysis**

Liver tissue was harvested from each humanized mouse and stored using RNAlater (Qiagen, Germantown, MD). Total RNA was extracted from liver tissue, using the RNasy Plus Mini Kit (Qiagen). Complementary DNA was reverse transcribed using Super Script III reverse transcriptase (Invitrogen). Human- and mouse-specific tissue inhibitor of metalloproteinase 1 (TIMP-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) real-time quantitative polymerase chain reaction (qPCR) primers were designed and described previously [7]. The real-time qPCR primers for human interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), and α-smooth muscle actin (SMA) were previously reported [8, 9]. Gene expression was examined using Thermo Scientific SYBR green real-time PCR reagents (Thermo Scientific, Waltham, MA). GAPDH was used to normalize messenger RNA (mRNA) expression. mRNA expression was shown by either fold induction or arbitrary relative expression.

**Statistical Analysis**

Unpaired 2-tailed Student t tests and 1-way analysis of variance with the Bonferroni multiple comparison test were performed using GraphPad Prism (GraphPad Software, San Diego, CA). A P value of < .05 was considered statistically significant. All data were reported as mean ± SD.

**RESULTS**

We used the interleukin 2–toxin fusion protein denileukin difftitox to deplete CD4+CD25hi Treg cells from humanized mice. A single denileukin difftitox injection significantly decreased CD4+CD25hi Treg cells in blood and lymphoid organs from humanized mice with no significant effect on other human leukocytes, including CD4+CD25− or CD8+ T cells (Supplementary
Figure 1 and the article by Jiang et al [4]). To define the role of Treg cells in hepatitis during HIV-1 infection, we quantified liver proinflammatory cytokine and chemokine expression. We found that expression of the human proinflammatory cytokines IL-6 and TNF-α was increased in the denileukin diftitox group. Their expression was further increased in the HIV-1 plus denileukin diftitox group (Figure 1A and 1B). Interestingly, expression of the human inflammatory chemokines MCP-1 and MIP-1α was highly upregulated by denileukin diftitox alone in the liver (Figure 1C and 1D), and HIV-1 infection showed no significant further induction.

To detect leukocyte infiltration in the liver, we stained liver sections with hematoxylin and eosin and human leukocyte markers. We detected significant leukocyte infiltration only in the liver of the HIV-1 plus denileukin diftitox group (Figure 1E and 1H). The infiltrated leukocytes included human CD45+ cells, CD3+ T cells, and CD68+ macrophages (CD45, CD3, and CD68; Figure 1E). They also included Ki-67+ proliferating cells (Ki-67; Figure 1E) and p24+ HIV-infected cells (Supplementary Figure 2A). Consistently, infiltrated human leukocyte numbers were significantly increased only in the HIV-1 plus denileukin diftitox group (Figure 1F and Supplementary Figure 3A and 2B). These data suggested that Treg cell depletion and HIV-1 infection cooperatively induced inflammatory cytokines/chemokines and hepatitis in the liver.

To detect liver injury associated with hepatitis, we measured ALT levels in the plasma. ALT levels were significantly elevated only in mice from HIV-1 plus denileukin diftitox group 20 days after infection (Figure 2A). These data suggest that Treg cells prevent HIV-1-induced liver damage in humanized mice. We further measured the plasma hyaluronic acid level, which is a marker of hepatic injury and fibrosis. Similar to ALT levels, no significant increase of plasma hyaluronic acid levels was found in recipients of mock stock, mock stock plus denileukin diftitox, and HIV-1. However, we detected significantly elevated hyaluronic acid levels only in the HIV-1 plus denileukin diftitox group 20 days after infection (Figure 2B). Consistent with the elevated liver inflammation and injury, we observed that livers from mice in the HIV-1 plus denileukin diftitox group showed significant pathology (Figure 2C).

To further investigate the liver immunopathogenesis, we stained liver sections with Sirius red/fast green. We observed significant collagen deposition in the parenchymal area only in the livers from mice in the HIV-1 plus denileukin diftitox group (Figure 2D and 2E). Activation of hepatic stellate cell and their transition to myofibroblast is a key step for liver fibrosis development, and activated hepatic stellate cells (or myofibroblasts) express glial fibrillary acidic protein (GFAP) [10] and α-SMA. To determine hepatic stellate cell activation in the liver, we stained liver sections with antibodies specific to human GFAP and α-SMA. We found that GFAP and α-SMA expression was clearly associated with collagen deposition (Figure 2D and 2E and data not shown). We further quantified liver fibrosis by measuring expression of TIMP-1 in the liver. Expression of human (but not mouse) TIMP-1 mRNA in the liver of HIV-1 plus denileukin diftitox group was significantly induced (Figure 2F). Therefore, depletion of Treg cells and HIV-1 infection cooperatively lead to liver inflammation and fibrosis.

**DISCUSSION**

Our findings indicate that Treg cells prevent HIV-1-induced liver fibrosis associated with hepatic stellate cell activation, hepatitis, and liver injury. Consistent with recent reports [11], MCP-1 is likely to play an important role in the fibrogenic process initiated by inflammation in HIV-1–induced liver fibrosis. MCP-1 may serve as both fibrogenic mediator and chemoattractant for leukocytes to the liver. Moreover, a low Treg frequency was observed in HCV-infected patients, compared with healthy controls [12]. Treg cell depletion is likely to induce inflammation and fibrosis in virus-induced liver fibrosis (Supplementary Figure 3C and 3D). However, Treg cells were reported to be infiltrated in HCV-infected liver [12]. It would be interesting to determine the role of intrahepatic Treg cells in liver fibrosis. Interestingly, although HIV-1 p24+ cell counts were reduced in spleens (Supplementary Figure 2B and the article by Jiang et al [4]), HIV-1 p24+ cells significantly infiltrated fibrotic livers (Supplementary Figure 2A). Both HIV-1 and human leukocytes can promote human hepatic stellate cell activation [5, 13, 14]. HIV-1 infection and liver inflammation by Treg cell depletion cooperatively induces liver fibrosis. It will be of great interest to elucidate the molecular mechanism of Treg cells in promoting liver fibrosis during HIV-1 infection in future experiments.

The origin of the human hepatic stellate cells or myofibroblasts [13] in the humanized mouse is currently not clear. Bone marrow–derived cells can contribute significantly to myofibroblast populations during induction of liver fibrosis and cirrhosis [15]. Transdifferentiation of myofibroblasts from bone marrow–derived cells may be induced by HIV-1 infection and Treg cell depletion. Alternatively, these cells may be derived from human liver stem or progenitor cells in the CD34–enriched progenitor populations isolated from fetal livers [13]. Humanized mouse models still have some limitations in the study of liver disease in human hepatic virus infection, including low human cell engraftment and reduced immune activity. In addition, R3A is a highly pathogenic HIV-1 strain. Thus, it is important to confirm the findings with less pathogenic HIV-1 isolates in the future. We have recently developed a novel humanized mouse model that has a human immune system and human hepatic cells [7]. Our findings provide new insight linking Treg cells and liver immunopathogenesis during HIV-1 infection. With this model, we will be able to test the role of...
Figure 1. Regulatory T (Treg) cell depletion and human immunodeficiency virus type 1 (HIV-1) infection lead to hepatitis. Humanized mice received mock stock or HIV-1 2 days after treatment with denileukin diftitox (Ontak; 150 µg/mL in citrate buffer; Ligand Pharmaceuticals [San Diego, CA]). Different groups of mice were euthanized 20 days after infection. A–D, Human interleukin 6 (hIL-6; A), tumor necrosis factor α (hTNF-α; B), monocyte chemotactic protein 1 (hMCP-1; C), and macrophage inflammatory protein 1α (hMIP-1α; D) messenger RNA expression in the liver was analyzed by real-time quantitative polymerase chain reaction. Fold induction relative to values for recipients of mock stock are shown. Seven mice per group were analyzed. **P < .05, by 1-way analysis of variance with the Bonferroni test. Data are mean ± SD. E, Liver sections obtained 20 days after infection were stained with hematoxylin and eosin (HE) or with anti-human CD45, CD3, CD68 (Dako), and anti-Ki-67 (Abcam) antibodies. Pictures were taken at 100× magnification. F, Intrahepatic human leukocyte (CD45) and T-cell (CD3) numbers in different groups of mice are shown. Seven mice per group were analyzed. **P < .05, compared with mock stock, mock stock plus denileukin diftitox, and HIV-1 recipients, as above. Data are mean ± SD.
Figure 2. Depletion of regulatory T (Treg) cells during human immunodeficiency virus type 1 (HIV-1) infection induces liver fibrosis. A and B, Blood was collected 20 days after infection. Plasma alanine aminotransferase (ALT; A) and hyaluronic acid (HA; B) levels were measured. Seven mice per group were analyzed. C, Representative liver morphology from each group of mice at the time of euthanization is shown. D, Liver sections were stained with Sirius red/fast green for collagen detection or with anti-human glial fibrillary acidic protein (GFAP) antibodies (Abcam). Pictures were taken at 100× magnification for all groups and at 200× for the HIV-1 plus denileukin diftitox (Ontak; 150 µg/mL in citrate buffer; Ligand Pharmaceuticals [San Diego, CA]) group. E, Sirius red–positive (left) or GFAP-positive (right) areas in the liver were quantified by ImageJ. At least 5 images from each liver were used to quantify the areas. F, Human and mouse tissue inhibitor of metalloproteinase 1 (TIMP-1) messenger RNA expression in the liver was analyzed by real-time quantitative polymerase chain reaction. Seven mice per group were analyzed. **P < .05 for all analyses, compared with mock stock, mock stock plus denileukin diftitox, and HIV-1 recipients, by 1-way analysis of variance with the Bonferroni test. Data are mean ± SD.
Treg cells in liver diseases induced by HIV/HCV coinfection. Modulating Treg cells may provide a new approach to preventing liver fibrosis in patients coinfected with HIV-1 and HCV.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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J. N. planned, designed, and performed the experiments and wrote the manuscript; M. L. W. and G. I. K. performed the initial design of the experiments and assay; and L. S. conceived the research project, planned and designed the experiments, and wrote the article.

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