RAG2$^{-/-}\gamma_c^{-/-}$ Mice Transplanted with CD34$^+$ Cells from Human Cord Blood Show Low Levels of Intestinal Engraftment and Are Resistant to Rectal Transmission of Human Immunodeficiency Virus[▽]

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Rectal transmission is one of the main routes of infection by human immunodeficiency virus type 1 (HIV-1). To efficiently study transmission mechanisms and prevention strategies, a small animal model permissive for rectal transmission of HIV is mandatory. We tested the susceptibility of RAG2$^{-/-}\gamma_c^{-/-}$ mice transplanted with human cord blood hematopoietic stem cells to rectal infection with HIV. We rectally exposed these humanized mice to cell-free and cell-associated HIV. All mice remained HIV negative as assessed by plasma viral load. The same mice infected intraperitoneally showed high levels of HIV replication. In the gut-associated lymphatic tissue, we found disproportionately smaller numbers of human cells than in other lymphoid organs. This finding may explain the observed resistance to rectal transmission of HIV. To increase the numbers of local HIV target cells and the likelihood of HIV transmission, we treated mice with different proinflammatory stimuli: local application of interleukin-1β, addition of seminal plasma to the inoculum, or induction of colitis with dextran sodium sulfate. These procedures attracted some human leukocytes, but the transmission rate was still very low. The humanized mice showed low levels of human engraftment in the intestinal tract and seem to be resistant to rectal transmission of HIV, and thus they are an unsuitable model for this application.

Millions of people worldwide are infected with human immunodeficiency virus (HIV). Despite improved treatment strategies and efforts at prevention, no end of the pandemic is in view. HIV transmission occurs mostly by sexual intercourse via mucosal routes (11); rectal intercourse carries an especially high risk (32) and thus is considered one of the major risk factors for both homosexual and heterosexual HIV transmission (7). For the rational design of prevention methods such as microbicides, we need detailed knowledge of HIV transmission and in particular the early steps of HIV infection.

HIV research has long been hampered by the lack of a small animal model that recapitulates HIV infection in humans and permits the study of vaginal or rectal transmission. Studies with larger animals can provide important clues to understand transmission and improve the design of trials in humans. Nonhuman primates and, less frequently, cats have been used (1, 8), but these models have significant problems, such as low availability, high costs, and species differences. HIV is a human-specific virus, and nonhuman primates and felines are resistant to HIV. Thus, either simian, simian-human chimeric, or feline immunodeficiency viruses have to be used as an approximation for HIV.

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Immunodeficient mice transplanted with human cells as an animal model for HIV disease have numerous advantages and some shortfalls. Cohort size can be increased significantly, providing more robust statistical validity to experiments. Mice are less costly and labor intensive than larger animals, and, notably, they are susceptible to HIV. Severe combined immunodeficient (SCID) mice transplanted with either human fetal thymus/liver (Thy/Li) or human peripheral blood leukocytes (PBL) (25) are well-established models. The rate of mucosal transmission of HIV in hu-PBL-SCID mice is low and variable (12) and renders this mouse model unsuitable for preclinical studies of microbicides. Significantly, the SCID-hu Thy/Li mouse has never been evaluated for mucosal transmission since human cells are mostly found in the transplanted organ and peripheral engraftment is low (35).

Recently, we and others reported humanized mice as a promising new model for HIV research (2, 3, 5, 15, 37, 42, 44). In these models, transplantation of human hematopoietic CD34$^+$ cells into immunodeficient mice leads to the development of all human lymphoid lineages and the repopulation of lymphoid organs with human cells. These models differ according to the mouse strains used (i.e., NOD/SCID, NOG/SCID, or RAG2$^{-/-}\gamma_c^{-/-}$ mice) and the origins of the human hematopoietic cells transplanted (i.e., CD34$^+$ cells from fetal liver or cord blood). Previously, we showed sustained and disseminated HIV replication in RAG2$^{-/-}\gamma_c^{-/-}$ mice transplanted with cord blood CD34$^+$ cells after intraperitoneal (i.p.) injection of HIV (3). So far, only two studies investigating HIV infection in humanized mice have shown successful HIV infection by the rectal route (4, 37). In these studies, human trans-

plants were obtained from fetal tissue. Aside from any ethical considerations, fetal tissue is not as easily and widely available as cord blood. Furthermore, one study used bone marrow/liver/thymus (BLT) mice, which receive a thymic organ transplant before irradiation and reconstitution with fetal liver-derived hematopoietic stem cells.

In the present study, we examined mucosal transmission of HIV in RAG2$^{-/-}\gamma_c^{-/-}$ mice transplanted with cord blood-derived CD34$^+$ cells. We characterized the engraftment of human cells into the gastrointestinal tract of these humanized mice and determined their susceptibility to rectal transmission of HIV. We challenged mice with cell-free and cell-associated HIV since their relative contributions are not known. Overall, rectal transmission rates were low in all groups of humanized mice, independently of preinfection treatment and inoculation protocols.

(This work was presented in part as a poster at the HIV Pathogenesis Keystone Symposia, Banff, Alberta, Canada, 27 March to 1 April 2008.)

MATERIALS AND METHODS

Generation of humanized mice. Mice were reconstituted as described previously (3, 39). Briefly, newborn RAG2$^{-/-}\gamma_c^{-/-}$ mice were irradiated with 2 × 2 Gy. CD34$^+$ cells were isolated from cord blood with immunomagnetic beads (Milteny Biotec), and 150,000 to 400,000 cells (246,000 ± 60,000) were transplanted into each mouse. Fetal liver-derived CD34$^+$ cells were a kind gift from R. Akkina (Colorado State University). Eight to 12 weeks after transplantation, the levels of blood engraftment were determined by flow cytometry of peripheral blood mononuclear cells (PBMCs) stained for the human panleukocyte marker CD45 in all mice (mean human cells/live cells, 11.56% ± 10.9%). Liver, bone marrow, and spleen engraftment levels were also analyzed in 14 mice. All experiments were approved and conducted according to local guidelines and laws.

Tissue characterization. Formalin-fixed tissue sections from liver and large intestines were stained with hematoxylin and eosin (H&E) or for markers for human CD45 (clones PD7/26 and 2B11 [Dako]), CD4 (clone 1F6 [Novacostra]), and CD68 (clone GG-M1 [Dako]). To control for nonspecific staining, tissue from untransplanted mice was stained as well. Human CD45$^+$ cells were counted at a magnification of ×200 at 10 randomly chosen locations per organ and mouse. In interleukin-1β (IL-1β)-treated mice, cell numbers were evaluated separately for the 1.5 cm most proximal to the anus.

Lymphocytes from the small and large intestines (SI and LI, respectively) were isolated (gut-associated lymphatic tissue [GALT]) (26) sequentially from the intraepithelial and lamina propria compartments of three mice with a mean blood engraftment of 13.7%. Briefly, intestines were opened longitudinally, cut into pieces, and washed five times with cold phosphate-buffered saline (PBS)–2% fetal calf serum (FCS). Then the epithelial layer was dissociated by stirring tissue pieces for 30 min in PBS–2% FCS, 2 mM EDTA, and 2 mM dithiothreitol at 37°C. Supernatants were collected and filtered through 70- and 40-μm cell strainers. Cells were washed twice and kept on ice until analysis by flow cytometry. The remaining tissue was incubated for 15 min in cold RPMI–10% FCS and then stirred at 37°C in RPMI–5% FCS, 100 U/ml collagenase IV, and 10 U/ml DNase I (both from Sigma) to extract lamina propria cells. After 30 and 60 min of digestion, supernatants were collected and fresh digestion medium was added to the tissue pieces after the first collection step. Supernatants were treated as mentioned above.

Proinflammatory treatment with IL-1β. Mice were anesthetized with Ketamine and Rompun (50 and 8 mg/kg of body weight, respectively), and 250 U of recombinant human interleukin IL-1β (Invitrogen) in 25 μl of PBS was applied intrarectally with a pipette 24 h before infection. The mice were suspended head down for 1 min.

Colitis induction. Colitis was induced by cyclic treatment with 1% (wt/vol) dextran sodium sulfate (DSS) (molecular weight, 40,000 [MP Biomedicals]) added to the drinking water for 4 days, followed by 7 days of normal drinking water (21, 43). At the end of the third cycle, colitis was verified in formalin-fixed tissue sections, or the mice were used for HIV challenges. H&E sections of DSS-treated and untreated mice were scored for epithelial changes and inflammatory infiltration as described previously (36).

HIV infections. For cell-associated HIV infections, the CCR5-tropic HIV strain YU-2 was used. For cell-free HIV infections, either YU-2, JR-CSF (CCR5-tropic), NL4-3 (CXCR-4 tropic), or 89.6 (dually tropic) (all prepared according to reference 3) was used. Cell-associated HIV was prepared by infecting CD8$^+$ T-cell-depleted PBMCs pooled from three donors. PBMCs first were stimulated with 2 μg/ml phytohemagglutinin (PHA) for 3 days and then spinoculated with YU-2 at a multiplicity of infection of 0.1 for 2 h at 1,200 × g and 25°C. After the washing, the cells were returned to culture without further addition of PHA. Four days after spinoculation, infection rates were measured by intracellular p24 staining (6.5 to 12.5% positive) and viability was controlled by Trypan blue staining (>90%). Cells were washed twice and suspended in either PBS or seminal plasma. Cell-free virus stock was used undiluted or mixed 1:1 with seminal plasma. Mice received rectally cell-free stock virus at a dose of 2×10^5 (YU-2), 5×10^3 (JR-CSF), 1×10^4 (89.6), or 2×10^5 (NL4-3) 50% tissue culture infective doses ($TCID_{50}$) or 2×10^6 infected PBMCs. Anesthesia and inoculation were performed as described above. Twenty microliters of the respective inoculum was applied per mouse, except for mixtures of cell-free HIV and seminal plasma, which were given in volumes of 40 μl. Mice infected i.p. received 200 μl of cell-free virus, corresponding to 2×10^6 $TCID_{50}$. Plasma viral loads were measured by reverse transcription-PCR (Amplicor; Roche) at 4 to 6 weeks and again at 8 to 12 weeks postinfection. The detection limit was 800 copies/ml.

Tracking of rectally instilled labeled cells. PBMCs were stimulated with PHA, mock spinoculated, and cultured as described above. Cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), washed and mixed in 20 μl of either PBS or seminal plasma, and applied rectally at 2×10^6 cells per mouse. After 2, 4, and 6 h, the mice were euthanized. Intestinal tissue was removed and snap-frozen. Cryosections were ethanol fixed and counterstained with DAPI (4′,6-diamidino-2-phenylindole).

Statistical analysis. GraphPad Prism 5.01 was used for statistical analysis. Data were analyzed by two-tailed t tests—all unpaired, except for naive mice, in which cell numbers from liver and rectum from the same mouse were paired.

RESULTS

Low engraftment efficiency of human cells in the intestinal tract of humanized mice. GALT contributes substantially to the lymphoid system overall, and thus, its detailed characterization is mandatory for estimating its contribution in immunological questions. In humans, GALT contains abundant numbers of CD4$^+$ T cells, the main target cells of HIV. However, little is known about GALT in humanized mice.

We examined the engraftment of human cells into the gut of RAG2$^{-/-}\gamma_c^{-/-}$ mice transplanted with CD34$^+$ cells from human cord blood. Tissue sections of the rectum and the liver of 14 humanized mice were fixed with formalin and stained for the human panleukocyte marker CD45. Isolated cells from the liver, bone marrow, blood and spleen were analyzed for CD45$^+$ cells by flow cytometry. From three additional mice, we isolated GALT cells: intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from the SI and LI. We included three wild-type mice as positive controls for the isolation procedure of the GALT.

Human cell numbers in tissue sections of rectum were significantly lower than in those from liver (Fig. 1A and B). The same pattern was seen in flow cytometric analysis: engraftment levels in the intestines ranged between 0.001 and 0.1%, whereas in other organs examined, they were up to 800 times higher (Fig. 1D). The cellular compositions and the protocols to isolate cells differ substantially, according to tissue. Thus, comparisons between relative cell numbers in organs are difficult to interpret. However, GALT lymphocytes from wild-type mice exhibit large numbers of CD4$^+$ cells in the LI LPL cell fractions (Fig. 1C). Isolation of CD4$^+$ cells from wild-type mice shows that the lack of human cells in the GALT from humanized mice is not due to technical problems.

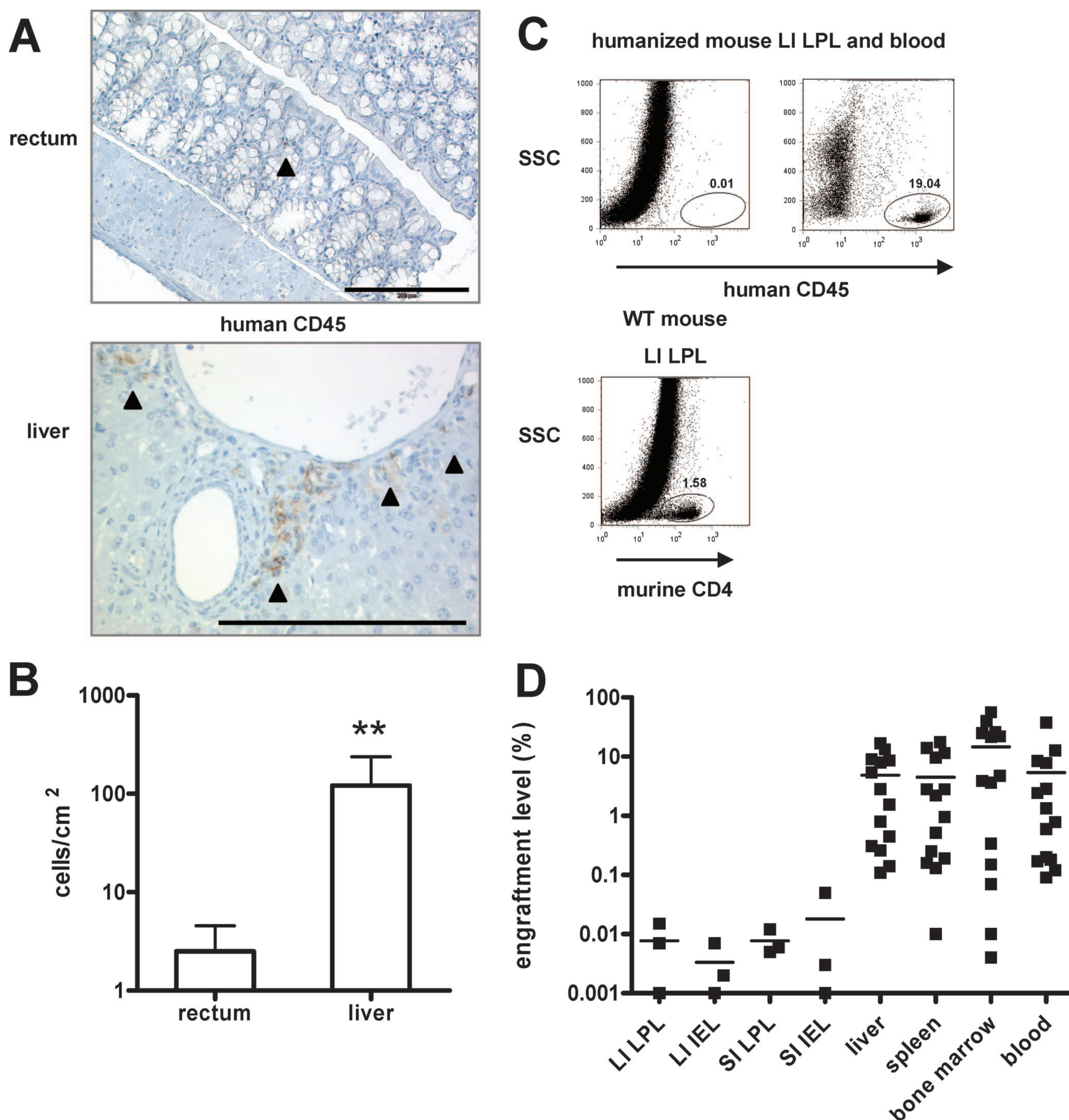

FIG. 1. Engraftment levels were disproportionately lower in the gut of humanized mice than in other tissues. Mice were analyzed for engraftment with human leukocytes by measuring cells positive for the human panleukocyte marker CD45. Tissue sections of rectum and liver of 14 mice were stained for CD45 (A [arrowheads indicate positive cells]), and cells were counted per square centimeter (B [mean and standard deviation; $P = 0.0094$). From three mice, lymphocytes from the intraepithelial space (IEL) and the lamina propria (LPL) from the LI and SI were isolated. Cells from the liver, spleen, bone marrow, and blood were isolated from 14 mice and analyzed by flow cytometry for proportions of human $CD45^+$ cells. Despite high levels of engraftment in the blood, almost no human cells were detectable in the lamina propria of the LI, where the majority of $CD4^+$ cells were found in wild-type (WT) mice (C). Overall, the level of intestinal engraftment was low, whereas in other organs, percentages of human cells were high (D). Bars represent 200 μm.

Naive humanized mice are resistant to rectal transmission of HIV. In body fluids, HIV occurs as free virions (cell free) or is found inside of infected cells (cell-associated virus). The extent of cell-free or cell-associated HIV in rectal transmission is unknown. Infected cells could act as "Trojan horses" to release large numbers of virions upon invasion of mucosal tissue.

We exposed five naive (untreated) humanized mice to cell-free HIV YU-2 at a dose of 2×10^5 $TCID_{50}$ per mouse and

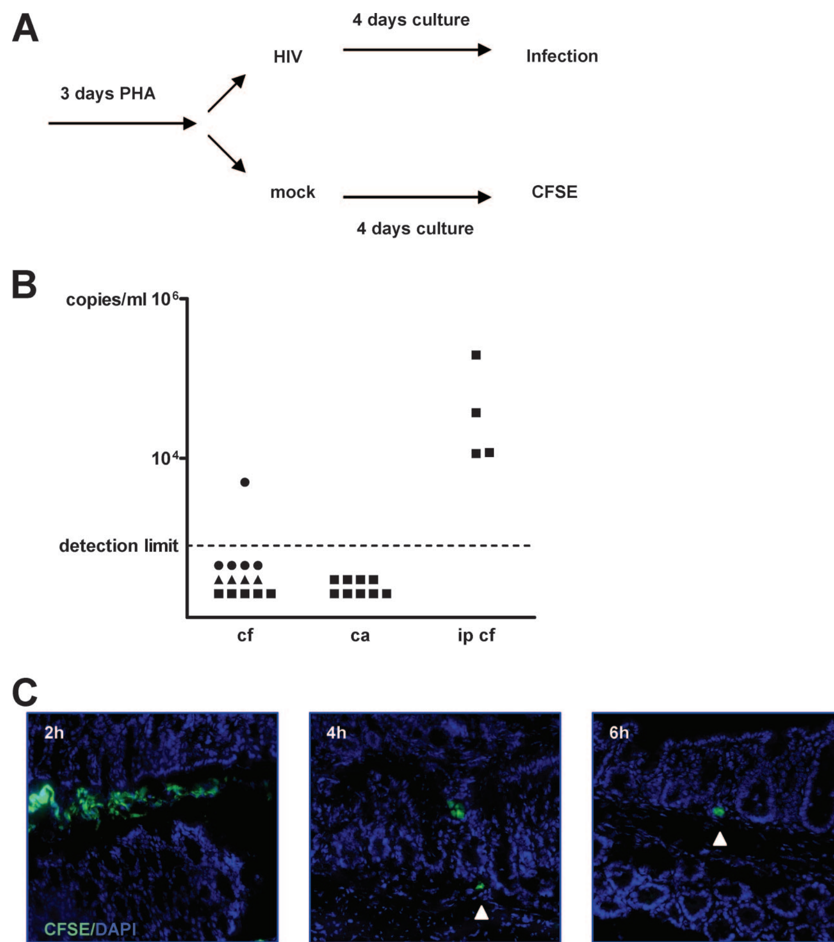

FIG. 2. Rectal transmission of HIV in naive mice was rare. Naive (untreated) humanized mice were exposed rectally to cell-free (cf) or cell-associated (ca) HIV, and plasma viral load was measured 4 to 8 weeks later (Amplicor; Roche). Cell-associated HIV was prepared following a three-step protocol. $CD8^+$-depleted PBMCs from three donors were pooled and PHA stimulated and then spinoculated and cultured for 4 more days (A). Nine mice were inoculated rectally with infected PBMCs; five mice were inoculated with cell-free HIV. Plasma viral load was negative in all mice. Four of the same mice were then inoculated by i.p. injection, and all became HIV positive (B [squares]). Additional mice were reconstituted in parallel either with fetal liver-derived (B [triangles; $n = 4$]) or with cord blood-derived (B [circles; $n = 5$]) $CD34^+$ cells and challenged rectally with cell-free HIV. One of the mice receiving cord blood cells tested HIV positive. A cell migration assay was performed with mock-spinoculated cells. CFSE-labeled cells were applied rectally to three mice. In cryosections, a few single invading leukocytes (arrowheads) were detected (C).

nine mice to cell-associated HIV YU-2 (i.e., 2×10^6 HIV-infected PBMCs [range of HIV-positive PBMCs as verified by intracellular p24 staining, 6.5 to 12.5%]) per mouse. After 4 weeks, all mice were negative for plasma viral load by PCR. We then injected four of the same mice with cell-free HIV YU-2 i.p. These four mice then showed high viral loads, while the remaining rectally exposed mice were still negative 8 weeks after inoculation (Fig. 2B). We included three mice with no detectable human engraftment in the blood to verify whether repopulation with rectally instilled HIV-infected cells would result in HIV infection. These also tested negative for HIV.

It is possible that the origin of the human stem cells used to generate humanized mice could influence intestinal engraftment efficiency and subsequently rectal HIV infection rates. Therefore, we reconstituted nine newborn mice of the same litter with either cord blood-derived or fetal liver-derived $CD34^+$ cells and 12 weeks later exposed them rectally to cell-free HIV YU-2 as described above. One of the five mice which received cord blood cells and none of the four mice reconstituted with liver derived cells tested HIV positive 1 month after challenge (Fig. 2B).

The efficiency of rectal transmission varies between different HIV strains. To exclude that the low rectal transmission rates we observed were a specific feature of YU-2, we tested three other viral strains: JR-CSF, 89.6, and NL4-3. Three groups of six mice received cell-free HIV rectally at a dose of 5×10^3 (JR-CSF), 1×10^4 (89.6), or 2×10^5 (NL4-3) $TCID_{50}$ per mouse. Of the 18 mice challenged, none showed a detectable plasma viral load 4 and 8 weeks after challenge.

We also prepared PBMCs for mock infections. These cells were labeled with CFSE before rectal instillation to track their location subsequent to rectal instillation (Fig. 2A). Only a few single cells had invaded the mucosa after 6 h; most of the inoculum probably was excreted (Fig. 2C).

While infection rates after i.p. injection of HIV were high, we rarely detected HIV transmission after rectal exposure,

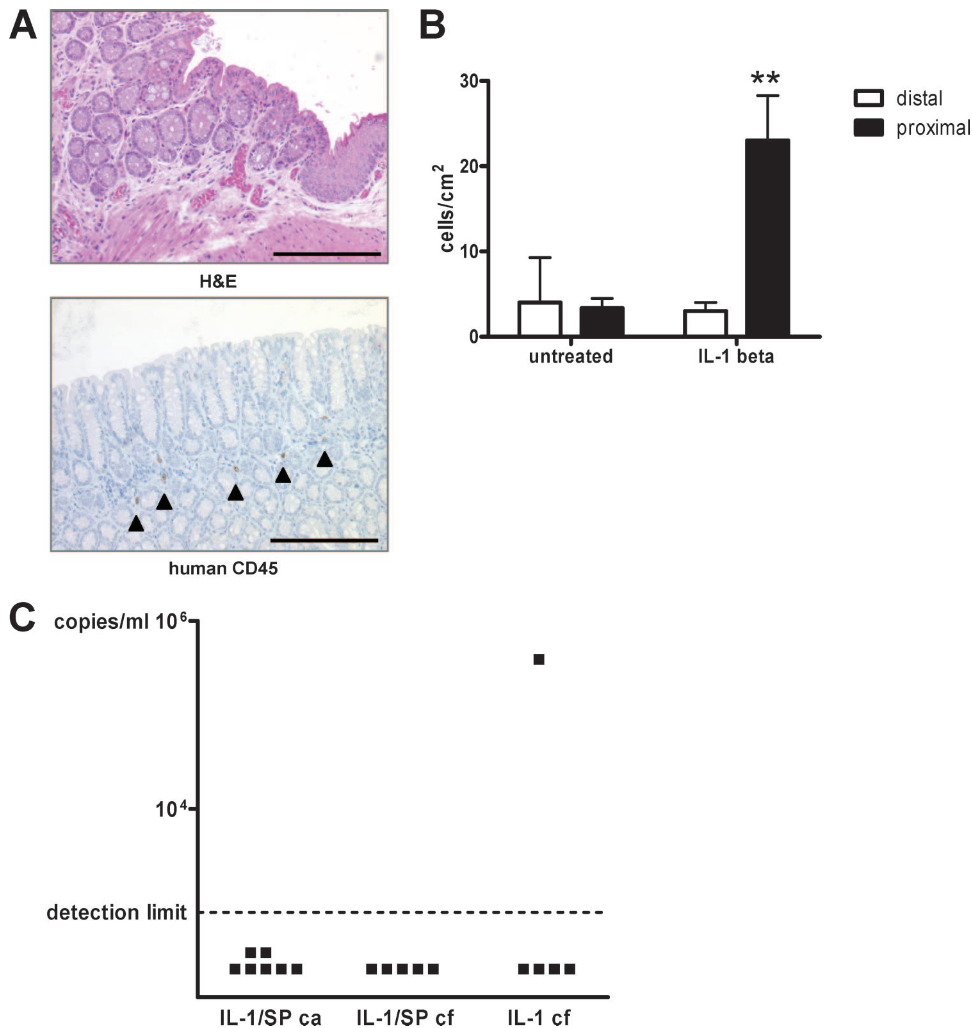

FIG. 3. Rectal transmission of HIV in mice treated with local proinflammatory stimuli. We treated three humanized mice rectally with 250 IU IL-1β and checked for inflammation and infiltration with human $CD45^+$ cells 24 h later (A [arrowheads indicate positive cells]). Rectal mucosa proximal to the application site showed an increase in human cells, whereas cell numbers distal to the application site were as low as those in untreated mice (B [mean and standard deviation, $P = 0.0033$]). Mice treated the same way were then exposed to cell-associated (ca) or cell-free (cf) HIV. In some experiments, seminal plasma (SP) was added to the inoculum. Plasma viral load was measured. One mouse showed viral replication 4 weeks after HIV challenge (C), and the same results were seen 8 weeks postinfection (not shown). Bars represent 200 μm.

either to cell-free or cell-associated HIV. Our finding that instilled cells rarely cross the rectal mucosa can explain the resistance of humanized mice to cell-associated HIV.

Rectal application of IL-1β leads to low levels of infiltration with human cells. IL-1β is a potent proinflammatory cytokine and mediates leukocyte chemotaxis. We speculated that local pretreatment with IL-1β would attract human lymphoid cells to the rectal mucosa and increase HIV transmission. In some experiments, we added human seminal plasma to the inoculum. Seminal plasma may have chemotactic potential (6) and may enhance attachment of HIV to target cells (27). However, semen has many pro- and antiviral factors (10), and its exact role in HIV transmission is still unknown.

We treated three humanized mice rectally with 250 U of IL-1β. After 24 h, we detected inflammatory changes, such as vessel dilatation and cell infiltration, at the site of application (Fig. 3A). Most of the infiltrating cells were murine, but significantly more human cells were found than in untreated mice (Fig. 3A and B). We detected both human CD4- and CD68-positive cells in the rectal mucosa at the site of IL-1β application (data not shown). This observation confirms that IL-1β attracted some human CD4 cells and macrophages, potential HIV targets, to the rectal mucosa of humanized mice.

We also pretreated mice with IL-1β and challenged them 24 h later with cell-associated HIV or cell-free HIV, with or without seminal plasma, at the same doses as the naive mice. Transmission rates were still low (Fig. 3C). Of 12 mice inoculated with cell-free or cell-associated HIV mixed with seminal plasma, none developed viremia, while 1 of 5 mice inoculated with cell-free virus developed viremia.

Additionally, we tracked CFSE-labeled cells after rectal application with the IL-1β pretreatment scheme and addition of seminal plasma to the inoculum. The results were similar to those described for naive mice: only a few single cells crossed

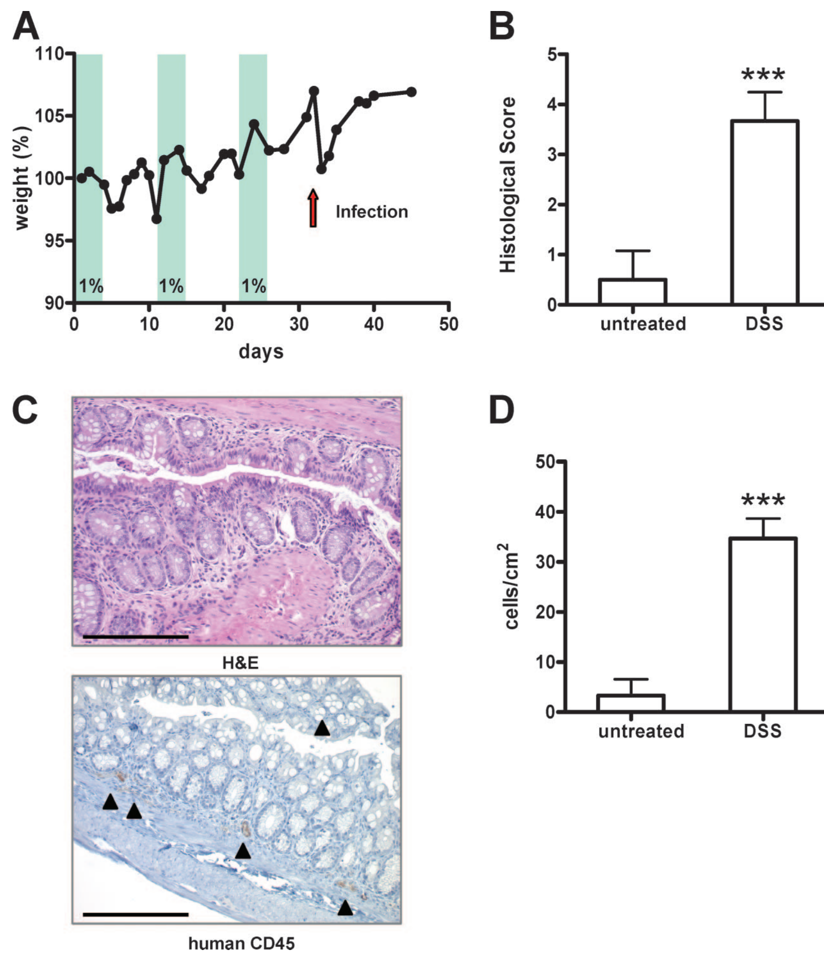

FIG. 4. DSS induces colitis in humanized mice. Twelve humanized mice received three cycles of 1% DSS in normal drinking water. This treatment induced moderate weight changes (A) due to colitis induction. Colitis was confirmed by histological scoring of tissue sections of three mice (B [P = 0.0008]). DSS treatment induced cellular infiltration consisting mostly of murine cells since only a fraction of cells were positive for human CD45 (C [positive cells indicated by black arrowheads]). However, in comparison to untreated mice, human cell numbers were increased after DSS treatment (D [P = 0.0005]). DSS-treated mice were then inoculated with HIV (red arrow in panel A). Bars represent 200 μm.

the rectal epithelial layer and penetrated into the mucosa (data not shown).

Humanized mice are highly susceptible to DSS-induced colitis. Concomitant sexually transmitted diseases are important risk factors for HIV transmission. In particular, mucosal inflammation increases susceptibility to infection by disrupting the epithelial lining and changing the numbers and phenotypes of local target cells (14). We tried to reproduce this situation in our humanized mice by inducing a chronic colitis with DSS (43).

So far, DSS colitis has not been studied in humanized mice. Prior studies established that Rag2$^{-/-}$ mice are susceptible to acute (31) and chronic (17) colitis in the DSS model. In a first experiment, humanized mice treated with 3% DSS in normal drinking water for 4 days lost ≥20% of starting weight and showed severe clinical symptoms. Therefore, we treated the humanized mice with three cycles of 1% DSS interrupted by 7 days of normal drinking water and then monitored their weight and state of health daily (Fig. 4A). Mice tolerated this reduced dose well and suffered only from minimal weight loss. One week after the last DSS administration, we analyzed the large intestines of three mice histologically: tissue sections showed inflammatory changes with alteration of the epithelial layer and infiltration of leukocytes, partly of human origin (Fig. 4B to D). Notably, human cell numbers in the mucosa seen by staining for human CD45 were significantly higher in DSS-treated animals than in animals that received normal drinking water.

To assess the impact of rectal inflammation on HIV transmission rate, we challenged mice with cell-associated HIV 7 days after the last DSS administration. Unexpectedly, 24 h after inoculation, all mice were severely affected, and some did not recover. Mortality was 50% in the first week after infection. The remaining animals recovered completely, and HIV RNA levels were measured 4 and 8 weeks after HIV inoculation. No infection was detectable in all three mice that completed the trial. We conclude that, despite mucosal inflammation, transmission of cell-associated HIV is improbable in humanized mice.

DISCUSSION

Humanized mice are a promising model for large-scale studies of HIV transmission, and their characterization is critical to future studies. Our study describes human engraftment in

GALT of RAG2$^{-/-}\gamma_c^{-/-}$ mice into which have been transplanted cord blood-derived CD34$^+$ cells. We show that, in this particular model, human cell numbers in the GALT are lower than in other lymphatic organs and increase only moderately after proinflammatory treatment. Furthermore, we examined the susceptibility of these humanized mice to rectal transmission of HIV and found a very small rate of rectal transmission of HIV. Only 2 of 56 challenged mice became HIV positive. Thus, this model may have limited value for the study of rectal transmission of HIV.

Two other studies showed successful rectal infections of humanized mice (4, 37), with transmission rates of 6/7 and 11/14, respectively. Sun et al. (37) used NOD/SCID mice transplanted with fetal liver and thymus and later reconstituted with liver-derived CD34$^+$ cells (BLT mice). Berges et al. (4) transplanted RAG2$^{-/-}\gamma_c^{-/-}$ mice with CD34$^+$ cells from fetal liver. Thus, a major difference is that we used cord blood-derived cells. So far, it is not clear which source of hematopoietic stem cells is optimal for the generation of humanized mice. In vitro, the colony-forming efficiency of CD34$^+$ cells derived from fetal liver is higher than that of cord blood CD34$^+$ cells (19). However, in vivo cord blood-derived hematopoietic stem cells produced four times more mature human leukocytes than liver-derived cells, when the same number of stem cells was transplanted into adult NOD/SCID mice (18). When telomere length is used as a measure of replicative capacity of hematopoietic stem cells from fetal liver and cord blood, the differences are minimal (40), indicating that both are suitable for generating humanized mice. However, it is not known to what extent the origin of lymphoid cells may be critical for repopulation of the intestines with human cells. Different expression of cell adhesion molecules (30) could influence homing behavior and subsequent repopulation of lymphoid organs. That, in turn, may explain the lower infection rates we observed in our model using cord blood-derived cells for transplantation. But when we reconstituted mice in parallel either with cord blood-derived or with fetal liver-derived cells, rectal transmission of HIV was not more efficient in the mice which had received liver-derived cells.

Apart from the different origins of CD34$^+$ cells, Berges et al. and we used similar models based on mice with the same genetic background. However, Berges et al. reported higher engraftment levels than the ones we detected. They found blood engraftment levels of almost 90%, whereas the mice used in our study had a mean engraftment of about 11%. But these values cannot be directly compared. Berges et al. measured the percentage of human cells in blood lymphocytes; we measured the percentage of human cells in all blood leukocytes, which include murine cells as well. Since RAG2$^{-/-}\gamma_c^{-/-}$ mice have no B, T, and NK cells of their own, obviously all lymphocytes detected in humanized mice should be of human origin. In our cohort of mice transplanted with liver-derived cells, we saw no differences in engraftment levels compared to engraftment levels in littermates transplanted with cord blood-derived cells. This indicates that the origin of the stem cells probably has no major impact, at least in our hands, and that other factors are influencing HIV transmission rates in humanized mice.

The intestinal microflora plays important roles in GALT development: intestinal bacteria influence the migration pattern of lymphocytes into mucosal sites; in germ-free animals, intestinal lymphocyte numbers are drastically decreased (38). Certainly, the microflora in the gut of mice from different genetic backgrounds and from different animal facilities varies and could influence human engraftment in GALT and subsequently HIV transmission susceptibility. Besides potential differences in gut microflora, especially in BLT mice, gastrointestinal engraftment appears to be significantly better than the GALT engraftment we obtained in RAG2$^{-/-}\gamma_c^{-/-}$ mice. BLT mice have the advantage of a human thymic tissue transplant, which could facilitate T-cell development.

In our humanized mouse model, T-lymphocyte development occurs in the anlage of the murine thymus, which is almost completely repopulated with human cells. Nonetheless, the stroma of this thymus is of murine origin and may have unknown disadvantageous effects on overall lymphoid development. Furthermore, to generate BLT mice, the NOD/SCID mouse strain was used. In contrast to RAG2$^{-/-}\gamma_c^{-/-}$ mice, NOD/SCID mice do not have mutations or loss of γ_c, the common gamma chain in receptors of multiple cytokines, including IL-7. Signaling through the IL-7 receptor is essential for formation of the Peyer's patches' anlagen (28), and humanized mice lacking this important feature may have more difficulties in repopulating the GALT than mice that have Peyer's patches' anlagen.

To enrich for human cells of lymphoid origin in the rectal mucosa in our humanized mouse model, we induced local inflammation. Mucosal inflammation as seen in sexually transmitted diseases is a risk factor for HIV acquisition by increasing numbers of HIV target cells and disturbing epithelial integrity (14). We treated humanized mice with Il-1β rectally 24 h before HIV challenge. In untreated mice, no infections could be seen; in treated mice, 1/17 showed a detectable plasma viral load. Immunohistochemical analysis of Il-1β-treated uninfected mice suggests that, despite an inflammatory response and cell infiltration, human cell numbers were probably still too low to establish infection. It is believed that HIV first infects a local founder population in the lamina propria of the mucosal tissue and amplifies there before systemic seeding and dissemination of the infection (16). In our model, local expansion of infection is difficult with only a few human cells present in the lamina propria. We only observed disseminated infection when mice were injected i.p. with HIV, and the mucosal amplification step was not needed.

To attract human cells more efficiently to the GALT, we treated humanized mice with DSS, a compound widely used in murine models of inflammatory bowel disease. Repeated treatment with DSS leads to a chronic colitis with infiltration of lymphocytes and macrophages (21). After three cycles of DSS treatment of humanized mice, we also saw infiltration of human cells into the rectal mucosa. Moreover, we confirmed colitis induction by monitoring weight changes and finally by histological scoring of rectal tissue. Mice tolerated treatment well up to 1 week after the last DSS administration, when a rapid decline in health occurred and half of the mice had to be euthanized. The HIV challenge could be a reason for the observed health decline. However, all mice were similarly affected, even surviving mice, which remained HIV negative. Notably, in non-DSS-treated mice, the same HIV challenge never elicited any symptoms. We therefore conclude that the

DSS chronic colitis model is not suitable for rendering humanized mice permissive to rectal challenges with HIV.

It is still unknown whether cell-free or cell-associated HIV is preferably transmitted. To develop microbicides or vaccines, it is essential to know whether protection is needed against free virions, infected cells, or both. In simian and feline models, both cell-free and cell-associated virus transmission can be observed with different efficiencies, depending on the experimental design (9, 20, 33). Results from studies in humans are conflicting: both free virions and infected cells are detected in cervicovaginal fluid (34) and semen (41). In cervicovaginal explants, which frequently are used for preclinical microbicide testing, cell-free HIV and cell-associated HIV are infectious (23). Here, we detected only minimal transmigration of rectally applied mononuclear cells into the mucosa both in untreated and Il-1β-treated humanized mice. Further infection experiments with cell-associated HIV confirmed this observation. None of the mice exposed to HIV-infected PBMCs showed systemic viral replication, not even mice that had DSS colitis. Thus, cell-associated HIV transmission is, at least in our model, not more efficient than cell-free HIV transmission.

HIV strains with selective coreceptor use or even more subtle viral variants may differ in their abilities to establish infection by the mucosal route. In humans, CCR5-tropic HIV is transmitted preferably over CXCR4-tropic HIV (24), and even in the group of CCR5 viruses, potential for transmission is diverse. Patients during acute infection show a more homogenous viral population, whereas patients in the chronic phase harbor many distinct variants (13, 29), indicating that only some of the viral variants in the transmitter are passed on. However, the characteristics of HIV variants preferentially transmitted are unknown so far. The CCR5-tropic HIV variant YU-2, which we used in our study for rectal challenge, was first isolated from neural tissue of a child suffering from AIDS (22). There is some uncertainty whether it is easily transmitted by the mucosal route or not. In any case, YU-2 replicates well in humanized mice and establishes disseminated infection after i.p. injection (3), and the three other HIV strains we tested in this study were not more efficient in rectal transmission of HIV. The other two studies (4, 37) demonstrating mucosal transmission of HIV in humanized mice showed that the mice were permissive to CXCR4-tropic HIV infection by the rectal route. However, Sun et al. challenged mice with HIV after mechanical disruption of the epithelial layer. It remains unknown whether the CXCR4-tropic viral strain used would have been transmitted otherwise. So far, we do not know whether the same bottleneck seen in humans for mucosal transmission of HIV exists in humanized mice.

In conclusion, our data indicate that GALT reconstitution in RAG2$^{-/-}\gamma_c^{-/-}$ mice transplanted with CD34$^+$ cells from cord blood is low and these mice seem to be quite resistant to rectal transmission of HIV, even in an inflammatory setting. Their value to study measures preventing mucosal transmission of HIV probably is limited. Further efforts are needed to clarify which mouse strain and transplantation protocol are best suited to generate the optimal humanized mouse.

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