Virology 417 (2011) 154–160

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Superior human leukocyte reconstitution and susceptibility to vaginal HIV transmission in humanized NOD-*scid* IL-2Rγ$^{-/-}$ (NSG) BLT mice

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ARTICLE INFO

Article history:
Received 5 February 2011
Returned to author for revision 28 March 2011
Accepted 24 May 2011

Keywords:
HIV/AIDS pathogenesis
HIV vaginal transmission
Humanized mice
HIV animal models
Hematopoietic stem cells
BLT mice
NOD-*scid* mice
NOD-*scid* IL-2Rγ$^{-/-}$ mice

ABSTRACT

Humanized Bone marrow/Liver/Thymus (BLT) mice recapitulate the mucosal transmission of HIV, permitting study of early events in HIV pathogenesis and evaluation of preexposure prophylaxis methods to inhibit HIV transmission. Human hematopoiesis is reconstituted in NOD-*scid* mice by implantation of human fetal liver and thymus tissue to generate human T cells plus intravenous injection of autologous liver-derived CD34$^+$ hematopoietic stem cells to engraft the mouse bone marrow. In side-by-side comparisons, we show that NOD-*scid* mice homozygous for a deletion of the IL-2Rγ-chain (NOD-*scid* IL-2Rγ$^{-/-}$) are far superior to NOD-*scid* mice in both their peripheral blood reconstitution with multiple classes of human leukocytes (e.g., a mean of 182 versus 14 CD4$^+$ T cells per μl 12 weeks after CD34$^+$ injection) and their susceptibility to intravaginal HIV exposure (84% versus 11% viremic mice at 4 weeks). These results should speed efforts to obtain preclinical animal efficacy data for new HIV drugs and microbicides.

Introduction

The development of small animal models for the study of HIV infection is important for the study of HIV prophylaxis and disease pathogenesis. Mouse models that recapitulate the mucosal transmission of HIV permit the study of early events in HIV pathogenesis and evaluation of preexposure prophylaxis methods to inhibit HIV transmission. Humanized Bone marrow/Liver/Thymus (BLT) mice, developed and reported by Lan et al. (2006) and Melkus et al. (2006), represent such a key model. In these mice, human hematopoiesis is reconstituted in NOD-*scid* mice in which human fetal liver and thymus tissue (Thy/Liv) are implanted under the kidney capsule to produce a thymic organoid that supports the development of human T cells. Three weeks after tissue implantation, mice are irradiated, and purified fetal-liver-derived human CD34$^+$ hematopoietic stem/progenitor cells (HSPCs) are injected intravenously. This method leads to robust reconstitution of human leukocytes in the mouse peripheral blood and lymphoid organs and importantly, BLT mice are susceptible to mucosal HIV challenge by both intrarectal (Denton et al., 2010; Sun et al., 2007) and intravaginal exposure (Denton et al., 2008). An alternative humanized mouse model with robust reconstitution of human leukocytes, the Rag-hu mouse, has been reported to be susceptible to intravaginal and intrarectal HIV challenge by one group of investigators (Berges et al., 2008; Neff et al., 2010) but resistant to intrarectal HIV transmission by another group (Hofer et al., 2008). These mice are not implanted with fetal tissue and are reconstituted as neonates with CD34$^+$ HSPCs injected intrahepatically after irradiation.

In previous work, both NOD-*scid* and NOD-*scid* mice homozygous for a deletion of the IL-2R γ-chain (NOD-*scid* IL-2Rγ$^{-/-}$, also called NSG) (Shultz et al., 2005) have been used as hosts for the BLT model, with no differences reported between the strains in the extent of human immune system reconstitution (Brainard et al., 2009; Denton et al., 2010). In our initial studies with NOD-*scid* mice, however, we observed poor reconstitution and susceptibility to mucosal HIV challenge. We therefore decided to directly compare age-matched NOD-*scid* with NSG mice engrafted with the same human tissues and CD34$^+$ HSPCs, in a strategy similar to that used recently to compare three different IL-2Rγ$^{-/-}$ mouse strains (Brehm et al., 2010). The IL-2R γ-chain is a crucial component of the high-affinity receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 and is required for signaling through these receptors (Shultz et al., 2007; Sugamura et al., 1996). Its absence leads to severe impairment in T- and B-cell development and function,

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doi:10.1016/j.virol.2011.05.013

Table 1
Proportion of viremic BLT mice 4 weeks after intravaginal HIV inoculation.

Cohort	$CD45^+Lin\text{-}1^-CD34^+$ HSPCs injected per mouse	$CD45^+Lin\text{-}1^-CD34^+CD38^-c\text{-}kit^+$ cells injected per mouse	HIV strain ($TCID_{50}$[a] per mouse)	Mouse strain		p value[b]
				NOD-*scid*–BLT (viremic[c]/total)	NSG–BLT (viremic/total)	
A	606,000 autologous	74,000	81A-G (50×10^6)	0/9 (0%)	6/7 (85%)	0.0009
B	206,000 allogeneic	25,000	81A-G (50×10^6)	0/14 (0%)	10/13 (77%)	<0.0001
C	420,000 autologous	54,000	JR-CSF (0.5×10^6)	3/14 (21%)	9/10 (90%)	0.0028
D	310,000 autologous	15,000	JR-CSF (0.5×10^6)	2/9 (22%)	7/8 (88%)	0.0152
Total				5/46 (11%)	32/38 (84%)	<0.0001

[a] $TCID_{50}$: 50% tissue culture infectious doses, as assessed by limiting dilution assay in PHA-activated PBMC with supernatant p24 detection 7 days after inoculation.
[b] p values determined by Fisher's Exact test.
[c] Viremic defined as >1.9 $\log_{10}$ copies HIV RNA per 100 µl of mouse plasma (assay detection limit).

and completely prevents NK-cell development (Cao et al., 1995; DiSanto et al., 1995; Ohbo et al., 1996; Shultz et al., 2007). In four such side-by-side comparisons, we found that NSG mice were far superior to NOD-*scid* mice in both their peripheral blood reconstitution with human leukocytes and their susceptibility to intravaginal HIV exposure.

Results

Four cohorts of BLT mice (A, B, C, and D) were each implanted with human tissues from a single donor into both types of mice in side-by-side comparisons. $CD34^+$ HSPCs were purified from human fetal liver by positive selection, cryopreserved, and injected into irradiated mice 3 weeks after Thy/Liv implantation. Cohorts A, C, and D were injected with HSPCs isolated from the autologous fetal liver; cohort B was injected with allogeneic HSPCs (200,000–600,000 viable $CD45^+Lin\text{-}1^-CD34^+$ HSPCs per mouse) (Table 1). The HSPCs were further evaluated for CD38 and c-kit expression, and the percentage of injected fetal liver cells that were $CD45^+Lin\text{-}1^-CD34^+CD38^-c\text{-}kit^+$, and thus potential long-term SCID-repopulating cells (SRCs) (Dick et al., 1997), was 3–10% (15,000–74,000 SRC per mouse) (Fig. 1).

At the time of HIV inoculation, 12 weeks after $CD34^+$ HSPC injection, NSG–BLT mice had substantially higher levels of human leukocyte reconstitution in peripheral blood than did NOD-*scid*–BLT

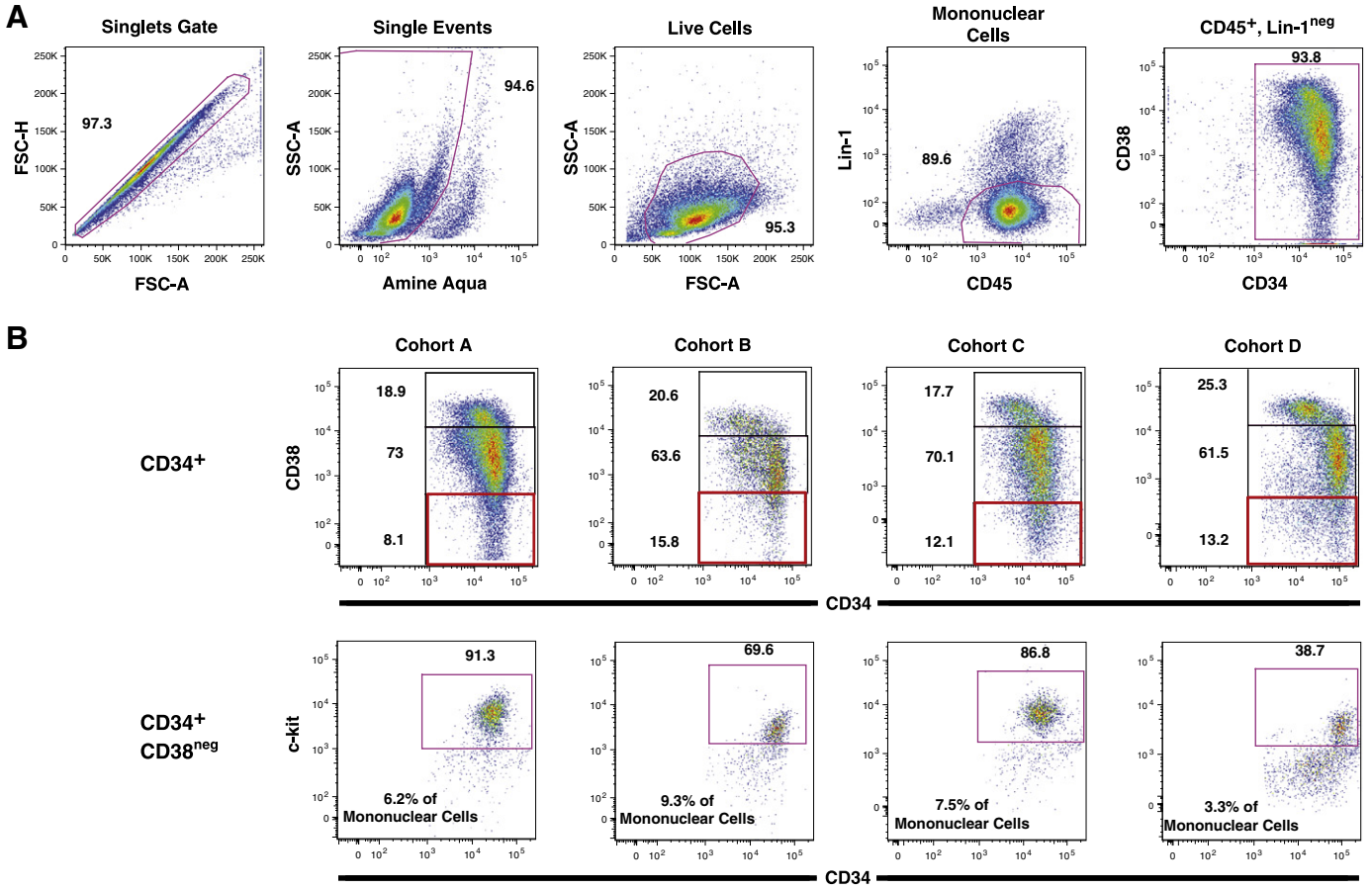
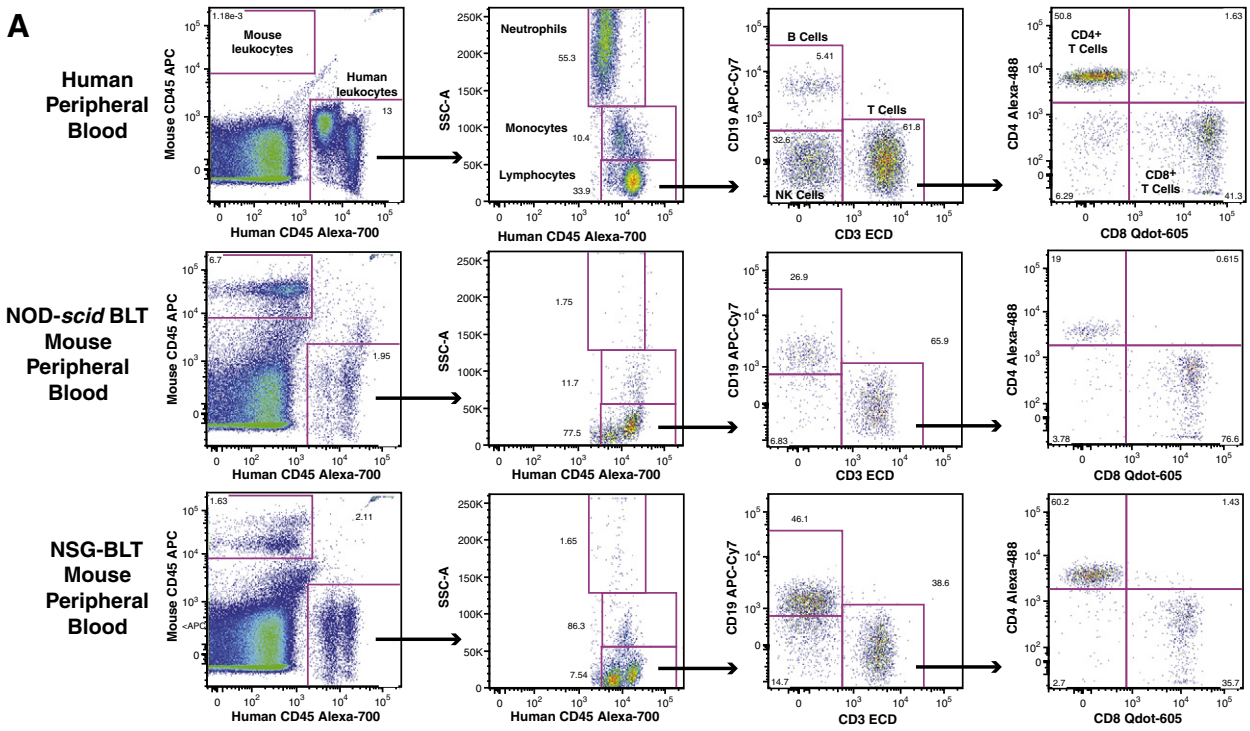
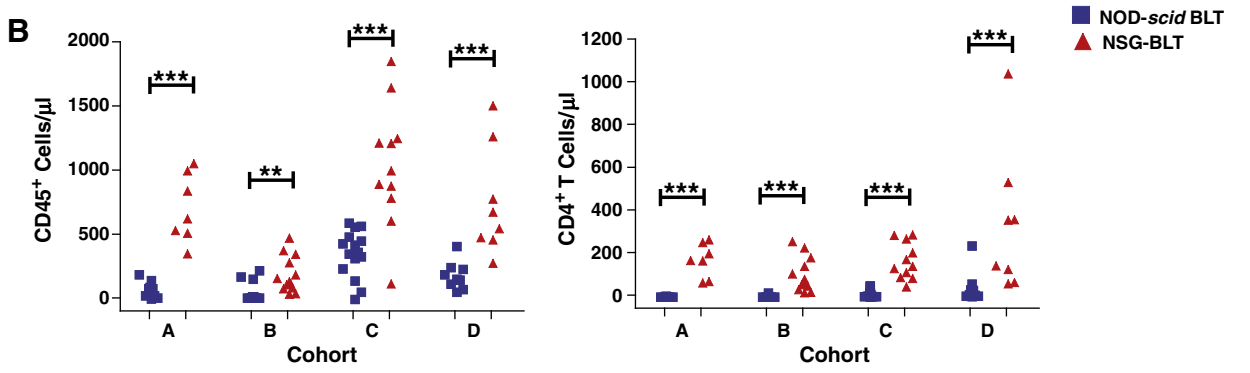
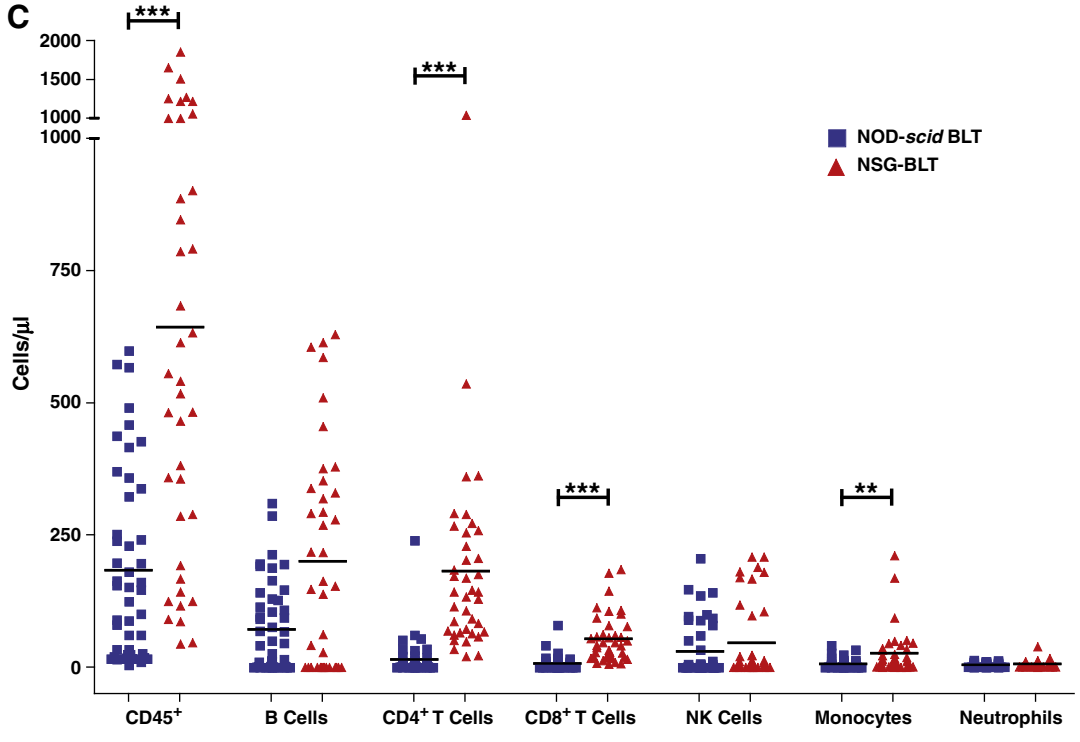


Fig. 1. Phenotypic analysis of CD34-enriched fetal liver cells. (A) Cryopreserved CD34-enriched fetal liver cells used for injection into BLT mice were immunophenotyped by flow cytometry. Isolated fetal liver cells were stained to determine the frequency of $CD34^+$ cells that were $CD45^+$ and Lin-1^-. (B) $CD34^+$ cells were further evaluated for CD38 expression (top) and c-kit expression (bottom). The frequency of $CD45^+Lin\text{-}1^-CD34^+CD38^-c\text{-}kit^+$ (potential long-term SCID-repopulating) cells as a fraction of mononuclear cells is shown.


A
Human Peripheral Blood
NOD-scid BLT Mouse Peripheral Blood
NSG-BLT Mouse Peripheral Blood
Mouse leukocytes
Human leukocytes
Mouse CD45 APC
Human CD45 Alexa-700
SSC-A
Neutrophils
Monocytes
Lymphocytes
CD19 APC-Cy7
CD3 ECD
B Cells
T Cells
NK Cells
CD4 Alexa-488
CD8 Qdot-605
CD4+ T Cells
CD8+ T Cells


B
NOD-scid BLT
NSG-BLT
CD45+ Cells/µl
CD4+ T Cells/µl
Cohort
A
B
C
D


C
NOD-scid BLT
NSG-BLT
Cells/µl
CD45+
B Cells
CD4+ T Cells
CD8+ T Cells
NK Cells
Monocytes
Neutrophils

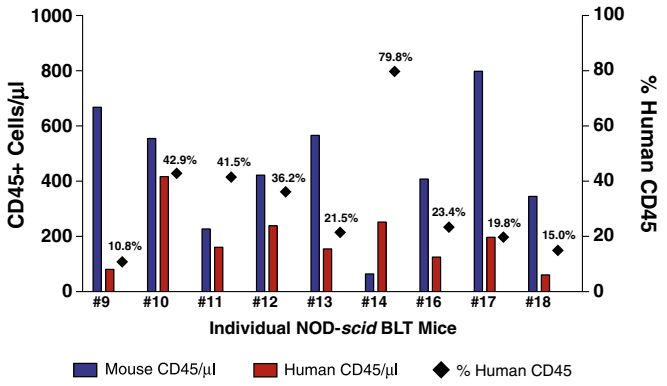


Fig. 3. The percentage of human CD45$^+$ cells in the leukocyte gate was artificially high for mice that were highly leukopenic for mouse leukocytes. For instance, mouse #14 had a high percentage of human CD45$^+$ reconstitution (79.8%) but comparable numbers of human CD45$^+$ cells to mouse #17, which had an apparently low percentage of human CD45$^+$ reconstitution (19.8%). The apparent difference in human reconstitution, expressed as percentage, was largely driven by the large difference in murine CD45$^+$ cells between mouse #14 (64 cells/µl) and mouse #17 (798 cells/µl).

mice (Fig. 2). NSG–BLT mice in all four cohorts had a mean of 13 times more human CD4$^+$ T cells per µl of blood, 8 times more CD8$^+$ T cells, 4 times more monocytes, 3 times more B cells, and similar numbers of human NK cells and neutrophils. The cohort engrafted with allogeneic CD34$^+$ cells (cohort B) had essentially no detectable B cells in the peripheral blood which made their overall reconstitution, as shown by the numbers of CD45$^+$ cells in Fig. 2B, appear lower than for the three cohorts engrafted with autologous CD34$^+$ cells. The numbers of T cells in the peripheral blood was similar for all four cohorts. We have not yet determined whether the lack of B cells in cohort B was the result of using allogeneic CD34$^+$ cells.

We found that the absolute count of these cells was a more useful measure of the level of human reconstitution than was the percentage of human CD45$^+$ cells in the leukocyte gate because the latter value was heavily influenced by the number of mouse CD45$^+$ cells present in the blood. In mice that were highly leukopenic in terms of mouse leukocytes, the percentage of human CD45$^+$ was often artificially high and not reflective of the absolute counts of human CD45$^+$ cells (Fig. 3). For all four cohorts, we obtained a mean of 182 (range of 20–1044) CD4$^+$ T cells per µl in NSG–BLT mice versus 14 (range of 0–240) CD4$^+$ T cells per µl in NOD-*scid* BLT mice. The numbers of HPSCs injected into each mouse were within the range of injected cells (200,000–2.5 × 10^6) reported by Melkus et al. (2006) for NOD-*scid* BLT mice. There was no correlation between the numbers of CD45$^+$Lin-1$^-$CD34$^+$CD38$^-$c-kit$^+$ cells injected and the levels of human CD45$^+$ cells in the peripheral blood 12 weeks after injection for either mouse strain (data not shown).

Most NSG–BLT mice became viremic by 4 weeks after intravaginal HIV exposure, whereas only a few NOD-*scid*–BLT mice became productively infected (Table 1). Viral loads in NSG–BLT mice reached a maximum of 4.7 $\log_{10}$ copies of HIV RNA per 100 µl of plasma, and maximal viral loads in NOD-*scid*–BLT mice were >10-fold lower (Fig. 4). Interestingly, there was no correlation between viral loads 4 weeks after inoculation and numbers of human CD4$^+$ T cells in peripheral blood at the time of inoculation in NSG–BLT mice. NOD-*scid*–BLT mice that were not viremic by 4 weeks after inoculation did not become viremic when followed for up to 12 weeks after intravaginal JR-CSF inoculation, and all successfully infected NSG–BLT mice became viremic by 4 weeks (Fig. 5). The greater susceptibility of NSG–BLT mice to intravaginal HIV infection mice appeared to correlate with the greater numbers of human T cells, including CD4$^+$ T cells, in the vaginal mucosa within both the epithelium and the lamina propria of these mice compared to NOD-*scid*–BLT mice (Fig. 6).

Discussion

The NOD-*scid* xenotransplantation model has been described as the "gold standard" for assessing human hematopoietic stem cell activity, and a number of advancements have been reported to further improve engraftment, such as depletion of NK cells with anti-CD122 Ab, direct intrafemoral injection of HSPCs, and deletion or truncation of IL-2Rγ (McDermott et al., 2010). A recent report directly comparing overall engraftment and multilineage differentiation of lineage-depleted human umbilical cord blood cells after intrafemoral injection showed superior engraftment in the bone marrow and spleen of NSG mice compared to NOD-*scid* mice as well as improved detection of SRCs at limiting dilutions (McDermott et al., 2010). Here, we extend these observations by showing superior peripheral blood reconstitution with multiple classes of human leukocytes and correspondingly greater susceptibility to intravaginal HIV exposure in NSG–BLT mice compared to NOD-*scid*–BLT mice. The BLT mouse model we employed differs in a number of important respects from the SRC-detection model described above, including the presence of a human Thy/Liv organoid under the kidney capsule, intravenous injection of much higher numbers of human fetal liver-derived HSPCs, and successful engraftment defined by large numbers (>50 cells per µl) of human CD4$^+$ T cells in the peripheral blood rather than simple detection of CD45$^+$ cells over isotype control staining in bone marrow. Despite these differences, however, both models show superior human immune reconstitution in NSG mice. Although the precise mechanism by which NSG mice experience more robust reconstitution has not been defined, it is likely to be the consequence of severe defects in NK cell activity resulting from the lack of IL-2R signaling (Shultz et al., 2005). This explanation is supported by the lack of improved human HSPC engraftment in NSG mice treated with an anti-CD122 Ab that inhibits NK cell activity (McDermott et al., 2010). An alternative explanation is that IL-2Rγ$^{-/-}$ murine hematopoietic progenitors in NSG mice cannot compete as efficiently with the injected human HSPCs for bone marrow niches after irradiation, but our preliminary data comparing murine HSPC regrowth in bone marrow of unmanipulated NSG and NOD-*scid* mice after irradiation show no significant differences (data not shown).

We were surprised that so few (11% overall) NOD-*scid* BLT mice became viremic after intravaginal HIV exposure in our study as Denton et al. (2008) reported that 7 of 8 BLT mice (88%) became viremic by 50 days after intravaginal JR-CSF inoculation (with 63% viremic by 28 days). The authors do not specify whether NOD-*scid* or NSG mice were used in that study. In a just-published report by the same group, 13 of 16 total mice (81%) became viremic after intravaginal exposure to JR-CSF (Denton et al., 2011). The authors

Fig. 2. Reconstitution with multiple classes of human leukocytes in peripheral blood. (A) Trucount quantification of peripheral blood leukocytes. The number of events within any gated region was used to calculate the absolute number of cells per µl inside the gate. The calculation uses the known number of gated beads in a ratio with the number of gated events per unit volume of whole blood. Human whole blood control (top panels) was used to set the gates for enumeration of total human and mouse leukocytes (left panel); neutrophils, monocytes, and lymphocytes (second from left); B cells, T cells, and NK cells (third from left); and CD4$^+$ and CD8$^+$ T cells (right panel). Gates set using human whole blood were applied to whole blood from NOD-*scid* BLT and NSG–BLT mice (bottom). (B) The absolute number of human leukocytes (CD45$^+$) and CD4$^+$ T cells was determined by Trucount analysis for each individual cohort (A–D) 12 weeks after injection of fetal liver derived HSPCs. NSG recipient mice from all four cohorts displayed greatly elevated numbers of CD45$^+$ leukocytes and of CD4$^+$ T cells relative to NOD-*scid* mouse recipients. (C) Absolute numbers of CD45$^+$ leukocytes, B cells (CD19$^+$), CD4$^+$ T cells, CD8$^+$ T cells, NK cells (CD56$^+$), monocytes, and neutrophils were combined from all four cohorts of BLT mice. On average, NSG recipients had much greater numbers of each of these human leukocyte lineages than did NOD-*scid* recipients. (**$p<0.01$, ***$p<0.001$, Mann–Whitney *U*-test).

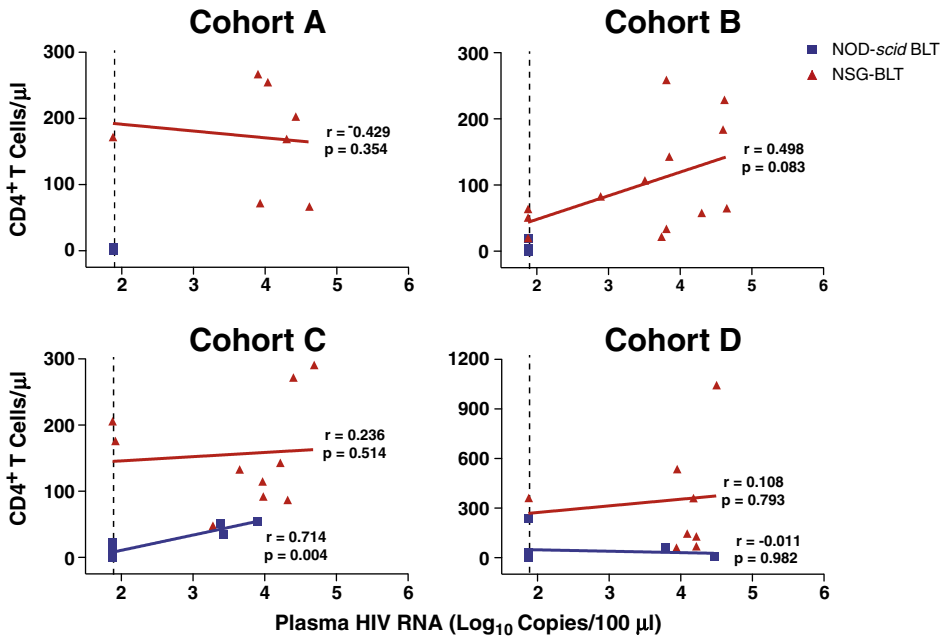


Fig. 4. Overall lack of correlation between human CD4$^+$ T cells in peripheral blood at the time of intravaginal HIV inoculation and HIV viremia 4 weeks later. Cohorts A and B were inoculated with HIV 81A-G, and cohorts C and D were inoculated with JR-CSF. HIV RNA in mouse plasma was quantified by bDNA assay. Data were analyzed by Spearman's rank correlation coefficient and show no correlation between CD4$^+$ T-cell count and HIV viremia, except for NOD-*scid* mice in cohort C.

state that NOD-*scid* or NSG mice were used to prepare BLT mice and do not mention a difference in HIV susceptibility between the strains. In the NOD-*scid* BLT mice that we followed until 12 weeks after inoculation, no additional mice became viremic after the first 4 weeks (Fig. 5). We have no clear explanation for this discrepancy. For cohorts C and D, we inoculated the same HIV isolate (JR-CSF) at an even higher inoculum size (500,000 versus 90,000 $TCID_{50}$) than in the 2008 study. We obtained the NOD-*scid* mice from a different source (Taconic) than reported by Denton et al. (The Jackson Laboratory), but this seems an unlikely explanation for the difference in susceptibility to intravaginal HIV infection in the BLT mice prepared from these animals.

The ultimate goal of our BLT model studies is reproducible susceptibility to intravaginal HIV exposure for the study of early events in HIV pathogenesis as well as for preclinical evaluation of HIV microbicides and oral preexposure prophylaxis. The superior human leukocyte reconstitution and susceptibility to vaginal HIV transmission that we have demonstrated for NSG mice support their use

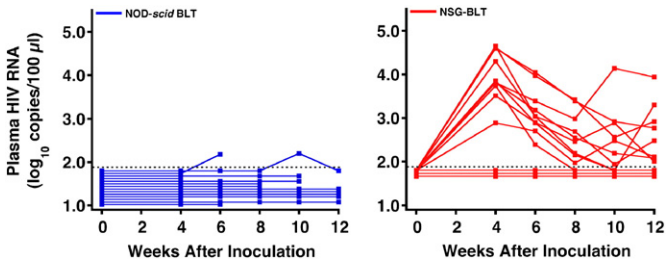


Fig. 5. NOD-*scid*–BLT mice that were not viremic by 4 weeks after inoculation did not become viremic when followed for up to 12 weeks after intravaginal JR-CSF inoculation, and all successfully infected NSG–BLT mice became viremic by 4 weeks after inoculation. Data shown are for all mice in cohort B (14 NOD-scid–BLT mice and 13 NSG mice). Similar results were obtained for cohorts A and C; all mice in cohort D were euthanized for tissue collection at 4 weeks. The dotted line is the limit of HIV RNA detection (1.9 $\log_{10}$ copies per 100 µl mouse plasma).

(as well as the similar IL2R$\gamma^{-/-}$ NOG mouse) (Ito et al., 2002) as the "gold standard" for humanized BLT mice, as has been proposed for their use in human HSPC and cancer stem cell research (McDermott et al., 2010).

Materials and methods

BLT mouse generation

BLT mice were produced as described by Melkus et al. (2006) by implanting 1-mm^3 pieces of human fetal liver and thymus under the kidney capsule of 11–15-week-old female NOD-*scid* (NOD/MrkBomTac-*Prkdc*^{scid}, Taconic) and female NSG mice (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ, The Jackson Laboratory). Each cohort (A, B, C, and D) was implanted with human tissues from a single donor into both types of mice in side-by-side comparisons. CD34$^+$ HSPCs were purified from human fetal liver by collagenase dispersion, isolation of mononuclear cells by centrifugation over NycoPrep 1.077 (Axis-Shield), and magnetic bead selection for CD34$^+$ cells (Miltenyi Biotech). HSPCs were cryopreserved in 90% FBS/10% DMSO and stored in liquid nitrogen until injection into mice 3 weeks after Thy/Liv implantation. Mice were conditioned by 225 cGy γ-irradiation from a cesium-137 source (Gammacell 3000, Best Theratronics) 30 h before HSPC injection into the tail vein. Cohorts A, C, and D were injected with HSPCs isolated from the autologous fetal liver; cohort B was injected with allogeneic HSPCs. A portion of the thawed cells were immunophenotyped by flow cytometry to determine the frequency of CD34$^+$ cells that were CD45$^+$ and Lin-1$^-$ (lineage cocktail 1, BD Biosciences), and 200,000–600,000 of these viable CD45$^+$Lin-1$^-$CD34$^+$ HSPCs were injected into each mouse (Fig. 1 and Table 1). The CD34$^+$ cells were further evaluated for CD38 and c-kit expression, and the percentage of injected fetal liver cells that were CD45$^+$Lin-1$^-$CD34$^+$CD38$^-$c-kit$^+$, and thus potential long-term SCID-repopulating cells (SRCs) (Dick et al., 1997), was 3–10%. Animal protocols were approved by the UCSF Institutional Animal Care and Use Committee.

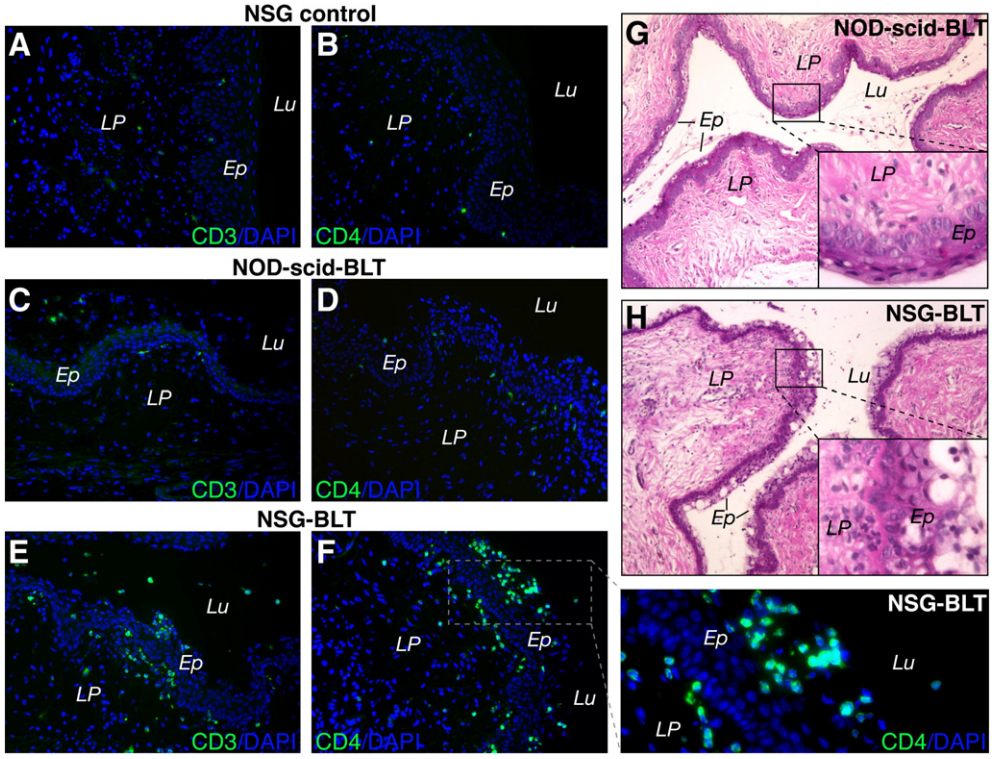


Fig. 6. Formaldehyde-fixed, frozen/OCT-embedded tissue sections of NSG control (A, B), NOD-*scid*–BLT (C, D) and NSG–BLT (E, F) mice immunostained for human CD3 and CD4 with nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI). BLT mice were from cohort D. Original magnification, ×200; inset, ×400. Paraffin-embedded vaginal tissue sections of NOD-*scid*–BLT (*G*) and NSG–BLT (*H*) mice stained with H&E. Original magnification, ×100; inset, ×400. LP, lamina propria, Ep, epithelium, Lu, vaginal lumen.

HIV exposure

Twelve weeks after HSPC injection, peripheral blood was collected from the retroorbital sinus for assessment of human leukocyte reconstitution, and mice were inoculated intravaginally with 20 µl of the R5 HIV molecular clones 81A (Chesebro et al., 1991, 1992; Toohey et al., 1995; Walter et al., 2005) or JR-CSF (Cann et al., 1990; Haltiner et al., 1985; Koyanagi et al., 1987) delivered with an unmodified 200-µl yellow plastic pipette tip with no intended abrasion or trauma. We chose HIV 81A because we and others had studied this clone previously in SCID-hu Thy/Liv mice (Berkowitz et al., 2000; Stoddart et al.); JR-CSF was chosen because Denton et al. (2008) reported this clone to productively infect BLT mice after intravaginal exposure with 90,000 tissue culture infectious units. p81A-4, which contains the V1–V3 *env* regions of Ba-L in an NL4-3 background, was altered by site-directed mutagenesis to match the consensus sequence (and thereby restore sensitivity to the HIV fusion inhibitor T-20) at amino acid position 36 (aspartic acid replaced by glycine) of gp41, creating HIV 81A gp41/D36G (81A-G) (Greenberg and Cammack, 2004; Stoddart et al., 2008). HIV inocula were generated by lipofectamine transfection of 293 T cells with plasmid DNA and concentrated >100-fold by ultracentrifugation through a 30% sucrose cushion for 2 h at 120,000 ×*g*.

Flow cytometry and Trucount enumeration

Human leukocyte reconstitution was assessed by flow cytometry using Trucount tube (BD Biosciences) enumeration to determine the absolute number of human B cells, $CD4^+$ and $CD8^+$ T cells, monocytes, NK cells, and neutrophils per µl of blood (Fig. 2). Human CD45-specific (Caltag) and mouse CD45-specific (BD Biosciences) antibodies were used to separate mouse from human leukocytes. Human $CD45^+$ cells were typed using antibodies specific for CD3 (Beckman Coulter), CD8 (Invitrogen) CD4, CD14, CD19, and CD56 (Biolegend). Data analysis was performed using FlowJo software (TreeStar).

Viral load quantification and histochemistry

Blood was collected 4, 6, 8, 10, and 12 weeks after HIV inoculation for quantification of viral load in plasma by branched DNA assay (VERSANT HIV RNA 3.0, Siemens Healthcare) according to the manufacturer's instructions. Mouse vaginas were fixed in formalin for paraffin embedding and H&E staining or in 4% paraformaldehyde for embedding and freezing in optimal-cutting-temperature (OCT) compound and immunohistochemistry for human CD3 (BD Pharmingen) and CD4 (Abcam).

Acknowledgments

This work was supported in part with federal funds from NIAID, NIH, under contract no. N01-AI-70002. This work was also supported in part by the AIDS Research Institute at UCSF and the Harvey V. Berneking Living Trust. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: p81A-4 (Cat. #11440) from Dr. Bruce Chesebro (Chesebro et al., 1991, 1992; Toohey et al., 1995; Walter et al., 2005) and pYK-JRCSF from Dr. Irvin S. Y. Chen and Dr. Yoshio Koyanagi (Cann et al., 1990; Haltiner et al., 1985; Koyanagi et al., 1987). We thank Leonard Shultz, Dale Greiner, and Paul Black for insightful discussions and Teri Liegler and Vikash Jethwani for technical assistance.

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