Interferon α and HIV Infection Cause Activation of Human T Cells in NSG-BLT Mice

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The development of small animal models for the study of HIV transmission is important for evaluation of HIV prophylaxis and disease pathogenesis. In humanized bone marrow/liver/thymus (BLT) mice, hematopoiesis is reconstituted by implantation of human fetal liver and thymus tissue (Thy/Liv) plus intravenous injection of autologous liver-derived hematopoietic stem/progenitor cells (HSPC). This results in reconstitution of human leukocytes in the mouse peripheral blood, lymphoid organs, and mucosal sites. NOD-scid IL-2R<sup>−/−</sup> (NSG)-BLT mice were inoculated intravaginally with HIV and were monitored for plasma viremia by branched DNA assay 4 weeks later. T-cell activation was determined by expression of CD38 and HLA-DR on human CD4<sup>+</sup> and CD8<sup>+</sup> T cells in mouse peripheral blood at the time of inoculation and at 4 weeks. Additional BLT mice were treated with human IFN-α<sub>2b</sub> (Intron A) and were assessed for T-cell activation. Productive HIV infection in BLT mice was associated with T-cell activation (increases in CD38 mean fluorescence intensity and both frequency and absolute number of CD38<sup>+</sup>HLA-DR<sup>+</sup> T cells) that correlated strongly with plasma viral load and was most pronounced in the CD8<sup>+</sup> T-cell compartment. This T-cell activation phenotype was recapitulated in NSG-BLT mice treated with Intron A. HIV susceptibility correlated with the number of HSPC injected, yet a number of mice receiving the Thy/Liv implant alone with no HSPC injection were also susceptible to intravaginal HIV. These results are consistent with studies linking T-cell activation to progressive disease in humans and lend support for the use of NSG-BLT mice in studies of HIV pathogenesis.
Generalized T-cell activation is one of the hallmarks of progressive HIV disease leading to the onset of AIDS (21). Though many phenotypic markers have been reported to reflect an activated status, the most commonly used are CD38 and HLA-DR. The level of CD38 expression on CD8^+ T cells is an important prognosticator of viral replication, eventual CD4^+ T-cell depletion, and diminished immune function (7, 14-16). The dynamics of the immune response and the level of viral replication that occurs during the acute or early stages of HIV infection have a strong impact on subsequent disease progression. In a prospective study of individuals with early HIV infection, viral replication was positively associated with CD38 expression levels on CD8^+ T cells, which in turn was associated with the rate of CD4^+ T-cell loss carried forward into the chronic stage of infection (7). Elevated levels of CD38 expression on CD8^+ T cells is a strong marker for HIV disease progression and may have better prognostic value than either CD4^+ T-cell count or viral load measurements (16, 21).

Persistent T-cell activation in response to chronic viral infection may occur as an evolutionary remnant of a once-beneficial immunological response. It has been suggested that low-level activation of T cells in response to chronic infections such as HIV leads to functional anergy, in turn leading to reduced numbers of fully activated target cells capable of productively replicating virus (1). Similarly, chronic HIV infection is associated with T-cell exhaustion in which HIV-specific T cells display reduced function associated with the expression of PD-1 (6). Despite significant levels of HIV-
specific CD8$^+$ T cells, high level expression of PD-1 is associated with the maintenance of elevated viral loads. Blockade of the PD-1 pathway by interfering with PD-1L interactions restores T-cell function and results in a reduction in viral load, indicating that immune dysfunction in HIV disease may be reversible. The causes of T-cell activation in HIV-infected subjects have been postulated to include the presence of replicating HIV, the translocation of bacterial lipopolysaccharide (LPS) across disrupted gut lumen (3), and the release of type 1 interferons by innate effector cells (4). Though the presence of either replicating virus or LPS may serve to partially explain persistent T-cell activation in HIV-infected individuals, neither is essential for this activation. Murine models have demonstrated generalized T-cell activation and CD4$^+$ T-cell decline in the absence of infection resulting from chronic CD27 stimulation through transgenic expression of its ligand, CD70, on B cells (35). Persistent T-cell activation, regardless of the cause, can result in immunodeficiency.

Elucidation of the mechanisms of T-cell activation and the subsequent impact on disease progression has been hampered by lack of experimental models. Samples from human subjects are generally not uniform with respect to duration and stage of infection, and aggressive experimental interventions and tissue excisions pose obvious ethical dilemmas. Much has been learned from the study of SIV infection of nonhuman primates; however, limitations exist with respect to differences between SIV and HIV and the largely prohibitive costs of this model. Consequently, much effort has gone into the development of a small animal model of HIV infection (30). Efforts to develop humanized mice have focused on the adoptive transfer of human immune system tissues, cells, or hematopoietic progenitors into immunodeficient recipient mice to render them susceptible to infection with HIV. One such model is the bone...
marrow/liver/thymus (BLT) mouse in which fragments of human fetal liver and thymus
(Thy/Liv) are implanted together under the kidney capsule of recipient mice followed by
the injection of CD34⁺ hematopoietic stem progenitor cells (HSPC) isolated from the
autologous fetal liver (2, 20, 25). The Thy/Liv implant allows for positive and negative
selection of human T cells to occur in autologous human thymus tissue, while injected
HSPC seed the mouse bone marrow to reconstitute human hematopoiesis. This
approach arguably leads to the most comprehensive reconstitution of the human
immune system in a mouse model yet reported, with high levels of multilineage human
cell engraftment.

Here, we have examined T-cell activation parameters on human lymphocytes in
BLT mice infected intravaginally with HIV. Intravaginal HIV inoculation leads to high
levels of viremia in many of these mice, and viremia is strongly correlated with
increased expression of CD38 and HLA-DR on both CD4⁺ and CD8⁺ T cells. This effect
was recapitulated in BLT mice treated with recombinant IFN-α2b (Intron A), suggesting
that T-cell activation in HIV-infected BLT mice and, by extension, HIV-infected human
subjects, may be mediated by IFN-α.
MATERIALS AND METHODS

BLT mice. BLT mice were produced as described previously (20, 25) by implanting 1-mm³ pieces of human fetal liver and thymus under the kidney capsule of 6–8-week-old female NSG mice (NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ, Jackson Laboratories). Each cohort was produced with tissues from a single donor. CD34⁺ HSC were purified from fetal liver and isolated by magnetic bead selection for CD34⁺ cells (Miltenyi), phenotyped cytometrically (34), and cryopreserved until injection into mice 3 weeks after Thy/Liv implantation (Fig. 1A; Table 1).

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. A portion of the thawed cells were immunophenotyped by flow cytometry to determine the frequency of CD34⁺ cells that were CD45⁺ and Lin⁻, and 130,000–880,000 CD34⁺ cells (150,000–1,110,000 total viable cells) were injected into each mouse. The CD34⁺ cells were further evaluated for expression of CD38, c-kit, CD90 (Thy-1), and CD45RA. An estimate of injected fetal liver cells that were hematopoietic stem progenitor cells (HSPC), defined as CD34⁺, Lin⁻ cells that are negative or low for CD38, c-kit⁺, CD90 (Thy-1)⁺ and CD45RA⁻, was determined for each cohort (22).

Animal protocols were approved by the UCSF Institutional Animal Care and Use Committee.

CCR5 genotyping. Genomic DNA was extracted from tissue using the Allprep DNA/RNA Mini Kit (Qiagen). Detection of CCR5-Δ32 by restriction fragment length polymorphism was performed using primers: 5'-CCTGGCTGTCGTCCATGCTG-3' and 5'-CAGCAAGCTGCAAGTCGCGT-3'.
5′-CTGATCTAGAGCCATGTGCACAACTCT-3′. The PCR product was digested with EcoR1, giving two bands of 332 and 403 bp for the homozygous CCR5/CCR5, two bands of 332 and 371 bp for the homozygous CCR5-Δ32/Δ32, and three bands of 332, 371, and 403 bp for the heterozygous CCR5/Δ32 [+/-].

**Intravaginal HIV inoculation.** Twelve weeks after HSC injection, peripheral blood was collected from the retroorbital sinus for assessment of human leukocyte reconstitution, and mice were inoculated intravaginally once with 20,000–4.5 x 10⁶ 50% tissue-culture infectious doses (TCID₅₀) of the R5 HIV molecular clone JR-CSF in 20 µl with no abrasion, trauma, or preconditioning with progesterone (Fig. 1A; Table 1). HIV inocula were generated by lipofectamine transfection of 293T cells with plasmid DNA and concentrated >100-fold by ultracentrifugation over a 30% sucrose cushion for 2 h at 30,000 x g. Blood was collected 4 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.

**Flow cytometry.** Human leukocyte reconstitution was assessed by flow cytometry using Trucount tube (BD Biosciences) enumeration to calculate the absolute number of human B cells, CD4⁺ and CD8⁺ T cells, monocytes, NK cells, and neutrophils per µl of blood. Anti-human CD45-Alexa700 (Caltag) and anti-mouse CD45-APC (BD Biosciences) antibodies were used to differentiate mouse from human leukocytes. Human CD45⁺ cells were typed using antibodies specific for human antigens including CD3-ECD (Beckman Coulter), CD8-Qdot605 (Invitrogen), CD4-Pacific Blue, CD19 APC-Cy7 (to exclude B cells), HLA-DR-FITC, and CD38-PE (Biolegend). Data analysis was performed using FlowJo software version 9.2 (TreeStar).
RESULTS

Experimental design and BLT mouse reconstitution. A total of 137 cell-injected BLT mice in 14 separate cohorts were inoculated intravaginally with HIV and were evaluated for HIV plasma viremia 4 weeks after inoculation (Table 1). Of the 137 inoculated mice, 79 had detectable viral load (>1.8 log_{10} RNA copies/100 μl plasma), while 58 mice had undetectable viral loads. Viremic mice included those from cohort BLT-1012, which was produced with tissues from a heterozygous CCR5 Δ32+/– donor. In this group, 14 of 16 HIV inoculated mice (88%) had detectable viremia (mean of 4.3 log_{10} copies/100 μl), indicating that CCR5 Δ32+/– mice are just as susceptible to infection with R5 HIV JR-CSF as are mice reconstituted with wild-type CCR5 donor tissue. Target cells for HIV infection in this cohort would be expected to express some full-length CCR5 from the wild-type allele, and since these mice displayed no resistance to intravaginal HIV transmission, there must be sufficient expression of the coreceptor to permit viral entry and productive infection of CCR5-positive target cells.

Peripheral blood was stained for polychromatic flow cytometry in Trucount tubes to allow for accurate enumeration of the indicated populations of human leukocytes per μl of whole blood (Fig. 1B). At the time of inoculation (0 weeks), the BLT mice had a mean of 952 human CD45+ cells, 134 CD4+ T cells, 86 CD8+ T cells, and 541 CD19+ B cells per μl of blood. By way of comparison, human peripheral blood control samples (n = 30) had 6,056 CD45+ cells, 808 CD4+ T cells, 527 CD8+ T cells, and 145 CD19+ B cells per μl. In agreement with previous reports on HIV-infected humanized mice (2, 5, 8, 9, 26, 27), we observed a decrease in the number of CD4+ T cells 4 weeks after virus
inoculation. The mean number of CD4\(^+\) T cells/µl increased from 78 to 117 in the 58 aviremic mice and decreased from 174 to 131 in the 79 mice with detectable viral RNA at 4 weeks. Hence, the aviremic mice had a net gain of 39 CD4\(^+\) T cells/µl whereas HIV-infected mice had a net loss 43 cells/ml (\(P < 0.001\), Fig. 1C).

There was wide variation in the number of both human CD45\(^+\) leukocytes and CD4\(^+\) T cells in the peripheral blood across cohorts at the time of inoculation (Table 1 and Fig. 1B). We evaluated the number of CD4\(^+\) T cells present in peripheral blood at the time of inoculation in conjunction with the HIV viral load data obtained 4 weeks later (Fig. 3). In agreement with our previous results (34), there was only weak or, in most cohorts, no correlation between viral loads 4 weeks after inoculation and numbers of human CD4\(^+\) T cells or CD45\(^+\) human leukocytes in peripheral blood at the time of inoculation (Fig. 2).

The autologous fetal liver that was used to isolate CD34\(^+\) cells by magnetic bead selection and the resulting CD34\(^+\) population were extensively phenotyped by flow cytometry to enumerate the number of potential HSPC injected into each BLT mouse. The CD34\(^+\) fraction of cells was analyzed for the expression of lineage markers (Lin-1), CD45, CD38, c-kit, CD90 (Thy-1), and CD45RA with the cells that were Lin-1\(^-\), CD45\(^+\), CD38\(^-\), c-kit\(^+\), CD90 (Thy-1)\(^+\) and CD45RA\(^-\) assumed to be the most enriched for HSPC, as described previously (11, 22). We evaluated both the number of CD34\(^+\) cells and number of HSPC injected per mouse for each cohort and related that to the frequency with which HIV inoculated mice became viremic (Fig. 3 and Table 1). While the total number of CD34\(^+\) cells injected per mouse appeared to have little bearing on intravaginal HIV susceptibility (Spearman correlation \(r = 0.269, P = 0.353\)), the number of injected HSPC correlated strongly with the percentage of mice with detectable
viremia ($r = 0.692, P = 0.006$). Similarly, the mean number of CD4$^+$ T cells/µl in each cohort also correlated with the percentage of mice with detectable viremia ($r = 0.793, P < 0.001$).

**Injection of HSPC is not required for HIV infectivity.** For 12 of the 14 cohorts, groups of 2 or 5 Thy/Liv implanted NSG mice were irradiated and injected with saline rather than autologous HSPC (Table 1). Many of these mice became reconstituted with human T lymphocytes in the peripheral blood and were inoculated intravaginally at the same time as the HSPC-injected mice. In 4 cohorts, these mice had detectable HIV viremia at 4 weeks despite having not been injected with any HSPC (Table 1). Further, the percentage of the mice that became viremic correlated, on a per cohort basis, with the percentage of HSPC-injected BLT mice that became viremic (Spearman correlation $r = 0.795, P = 0.003$, Fig. 3). In the mice that had no CD34$^+$ HSPC injected, all peripheral human cells were necessarily derived from the Thy/Liv implant. Human peripheral blood leukocytes in these mice consisted nearly entirely of CD3$^+$ T cells and were largely devoid of CD19$^+$ B cells and CD14$^+$ monocytes (data not shown). Further, cohorts of BLT mice that had high levels of human cell reconstitution also had high levels of human T cells in the absence of HSPC injection. For example, cohorts BLT-1011 and BLT-1021 had robust numbers of human CD45$^+$ cells (2,142 cells/µl and 561 cells/µl respectively) and CD4$^+$ T cells (257 cells/µl and 308 cells/µl respectively) in HSPC-injected mice, resulting in a high level of HIV infectivity (93% viremic for both cohorts, Table 1). Similarly, 5 mice from each cohort did not receive HSPC, yet still had high levels of peripheral human leukocytes (163 CD45$^+$ cells/µl and 50 CD4$^+$ T cells/µl for BLT-1011, and 292 CD45$^+$ cells/µl and 204 CD4$^+$ T cells/µl for BLT-1021) and had a high percentage of HIV viremia following intravaginal inoculation (60% and 100%, respectively).
respectively). Consequently, high-level immune reconstitution with CD3+ T cells, allowing for HIV infectivity, is likely to reflect the quality and/or functional properties of the implanted thymus and liver tissue and is not necessarily correlated with the number of injected fetal liver-derived HSPC. To summarize this observation, it appears from Fig. 3 that susceptibility to viremia is correlated with the number of HSPC injected as well as the level of CD4+ T cell reconstitution in peripheral blood, but the correlation with injected HPSC cannot hold true for the HIV-viremic mice that were not injected with HSPC. The robust human immune reconstitution in some of the saline-injected mice must have resulted from HPSC present in the liver tissue that was originally implanted. We are now studying this phenomenon more closely to determine whether irradiation and HSPC injection are necessary for intravaginal HIV susceptibility in these mice.

Increased expression of T-cell activation markers in HIV-infected BLT mice.

A large body of previous work has examined generalized T-cell activation in progressive HIV disease (7, 14-16, 21). The expression level of CD38, with or without co-expression of HLA-DR, has been used as a measure of the activation status of CD4+ and CD8+ T cells with higher levels of expression, especially by CD8+ T cells, correlating strongly with loss of CD4+ cells and disease progression (7, 14, 15). Peripheral blood leukocytes from 123 HIV inoculated BLT mice (out of the 137 total inoculated mice) were assessed for activation using polychromatic flow cytometry to measure expression of HLA-DR and CD38 on CD4+ and CD8+ T cells. Matched blood samples at the time of inoculation (0 weeks) and 4 weeks after inoculation were evaluated using the gating scheme shown in Fig. 4.

Of the 123 mice for which we obtained activation data, 66 (54%) had detectable viremia at 4 weeks. Of these, matched activation data from 0 weeks and 4 weeks was
available for 46 mice (Fig. 5A). We observed a significant increase in the expression
level of CD38 and in the frequency and absolute number of CD8\(^+\) T cells expressing
both CD38 and HLA-DR (CD38/HLA-DR\(^+\)) in HIV-viremic mice. This increased
expression of activation markers was only weakly reflected in the CD4\(^+\) T-cell
compartment. HIV viral load in the 66 viremic mice was correlated with the mean
fluorescence intensity (MFI) of CD38 and both the frequency and absolute number of
CD38/HLA-DR\(^+\) cells observed 4 weeks after inoculation (Fig. 5B). The MFI of CD38
correlated strongly with plasma HIV viral load for both CD4\(^+\) (Spearman \(r = 0.326, P =
0.008\)) and CD8\(^+\) T cells (\(r = 0.317, P = 0.010\)). Although the frequency of
CD38/HLA-DR\(^+\) CD4\(^+\) T cells did not correlate with viral load (\(r = 0.166, P = 0.183\)), the
absolute number per \(\mu\)l did correlate with viral load (\(r = 0.413, P = 0.001\)). Both the
frequency (\(r = 0.314, P = 0.010\)) and absolute number (\(r = 0.513, P < 0.001\)) of
CD38/HLA-DR\(^+\) CD8\(^+\) T cells correlated strongly with HIV viral load. That CD8\(^+\) T-cell
activation appears to be more tightly correlated with HIV viral load than CD4\(^+\) T-cell
activation is consistent with previous reports linking HIV disease progression with T-cell
activation where the effect was most pronounced in the CD8\(^+\) T-cell compartment (14,
21, 28).

**Treatment with Intron A causes T-cell activation in BLT mice.** To explore a
mechanism by which HIV may be increasing the activation state of the immune system,
we treated groups of BLT mice with recombinant IFN-\(\alpha\)2b (Intron A). IFN-\(\alpha\) is a
proinflammatory cytokine released by innate immune effector cells that induces
upregulation of a number of IFN-\(\alpha\) stimulated genes and has strong links to HIV
pathogenesis (17). The importance of this cytokine is highlighted by studies of SIV in
nonhuman primates where low levels of T-cell activation have been associated with
nonpathogenic infection and vice versa. In those studies, SIV-mediated IFN-\(\alpha\) release triggered through TLR7 and TLR9 stimulation of plasmacytoid dendritic cells was reduced in nonpathogenic infection of sooty mangabeys and elevated in pathogenic infection of rhesus macaques (23). Further work in our own lab has demonstrated expanded tropism of HIV resulting from IFN-\(\alpha\)-mediated upregulation of CCR5 (33).

More recently, IFN-\(\alpha\) was found to contribute to CD4\(^+\) T-cell loss from HIV-infected fetal thymic organ cultures (31). To assess the effect of IFN-\(\alpha\) on T-cell activation in our model, BLT mice were treated daily with Intron A (10\(^6\), 10\(^5\), and 10\(^4\) IU) for 6 days and T-cell activation was assessed by flow cytometry on day 7. Intron A treatment resulted in elevated expression of CD38 on both CD4\(^+\) and CD8\(^+\) T cells in a dose-dependent manner (Fig. 6A). Similarly, the frequency and absolute number of CD38\(^+\)/HLA-DR\(^+\) cells was elevated in the CD4 compartment, and the number of double-positive cells was increased in the CD8 compartment, all in a dose-dependent manner. The frequency of CD38\(^+\)/HLA-DR\(^+\) CD8\(^+\) T cells in the Intron A-treated mice was not higher than for water-treated controls, but both CD38 MFI and the absolute number of CD38\(^+\)/HLA-DR\(^+\) CD8\(^+\) T cells in peripheral blood were significantly higher in the mice treated with 10\(^6\) IU Intron A. This highlights the importance of multiple endpoint analyses, especially absolute counts, rather than relying on percentages of population of cells for assessment of lymphocyte activation. To ascertain whether T-cell activation was anatomically restricted to the peripheral blood or was diffuse throughout secondary lymphoid tissue, we measured the levels of CD38 and HLA-DR on splenic T cells (Fig. 6B). MFI measurements of CD38 on splenic CD4\(^+\) and CD8\(^+\) T cells mirrored that of PB T cells though unexpectedly, the frequency of CD38\(^+\)/HLA-DR\(^+\) cells was not different among the treatment groups. These results show that the activation phenotype
observed in viremic HIV infected BLT is reproduced by treatment with Intron A, confirming that IFN-α is an important mediator of T-cell activation.
DISCUSSION

Generalized T-cell activation is an established phenomenon in human HIV infection and is implicated in HIV disease progression (7, 15). HIV-infected BLT mice recapitulate the T-cell activation observed in human HIV-infected subjects and may be a useful model to dissect the mechanisms by which HIV infection causes T-cell activation. The NSG mouse is a major improvement over the CB-17-scid and NOD-scid mouse strains for human immune reconstitution because they lack murine NK cell and other innate immune activities that limit the engraftment of human HSPC (30). While NOD-scid mice are superior to CB-17-scid mice for human reconstitution, we (34) and others (24) have shown that NSG mice allow even higher levels of engraftment than NOD-scid mice; moreover, we have shown that NSG mice are far more susceptible to intravaginal HIV transmission than NOD-scid mice in side-by-side comparisons (34). Although such side-by-side comparisons have not been reported for BLT mice generated with and without HSPC injection, the cell injection step was envisioned by Melkus et al. (25) to engraft the mouse bone marrow with human hematopoietic progenitors capable of developing into B cells, NK cells, monocytes, and dendritic cells in addition to the human T cells generated by the Thy/Liv implant. The HSPC injection thus provides a more complete immune reconstitution of the mice which serves to more closely model events occurring in HIV-infected humans.

The T-cell activation we have observed during what can be described as the acute phase of infection in BLT mice highlights the relevance of studying this phenomenon with the aim of establishing an effective prophylaxis. As the study by
Deeks et al. (7) highlights, an immune activation set point is established early in HIV infection that carries forward into the chronic stage and determines the rate of disease progression. This implies that interventions aimed at reducing T-cell activation early in HIV disease may serve to reduce the CD4⁺ T-cell loss that occurs during late stage HIV disease and impede AIDS-related morbidity and mortality. This is further bolstered by a study of HIV-positive “elite controllers”, defined as a population that is able to maintain clinically undetectable plasma viral loads in the absence of therapy, that have significantly lower levels of CD38/HLA-DR⁺ CD8⁺ T cells (28). Here, reduced levels of activation correlate with maintenance of T-cell function, and these subjects rarely progress to AIDS. Our data demonstrate that the T-cell activation observed in HIV-infected human subjects is reproduced in HIV-infected BLT mice, allowing for investigators to address preventive measures in a relevant in vivo model. One such preventive measure has recently been reported in which HIV-infected subjects treated with atorvastatin for an 8-week period displayed significantly reduced numbers of activated CD8⁺ T cells (13). Although statins have been shown to exhibit antiviral activity against HIV, no reduction in viral load was detected. Measurements of CD38 and HLA-DR revealed a reduction in the number of CD8⁺ T cells that express HLA-DR and cells that coexpress CD38 and HLA-DR. By making use of BLT mice, agents with the potential to reduce persistent T-cell activation in the setting of HIV infection may be screened more rapidly to expedite their adoption in a clinical setting.

Our description of T-cell activation following HIV infection is in agreement with previous findings in BLT mice described by Brainard and colleagues (2) who described a 4-fold increase in Ki-67 expressing CD4⁺ and CD8⁺ T cells in matched samples obtained before and 12 weeks after intraperitoneal HIV inoculation. This increase in
Ki-67 coincided with a decrease in CD27 in both the CD4 and CD8 compartments and an increase in CD69, HLA-DR, and perforin expression by CD8+ T cells.

There are few published studies on IFN-α levels in HIV-infected patients. One group reported that when patients were studied during primary HIV infection (on average 2 months after infection) type I IFN was not detectable in the plasma, at least by conventional methods (18, 29), while another group detected IFN-α in the sera of 3 of 9 patients only within the first week of symptomatic primary HIV infection (36). We have not yet assayed specimens from HIV-infected NSG-BLT mice for IFN-α levels but plan to do so in future experiments designed to explore in more detail the role of IFN-α in the model. While we have not formally demonstrated that the release of IFN-α in response to HIV infection is the cause of the elevated T-cell activation observed in the mice, we believe our data support this possibility. IFN-α induces or enhances the expression of more than one hundred genes (10), and a portion of these interferon-response gene (ISG) products may contribute to the lymphocyte activation we observe after Intron A treatment of the mice. IFN-α is one of multiple cytokines and chemokines induced during early HIV infection, including rapid and transient elevations in IL-15, a large increase in inducible protein 10 (IP-10), rapid and more-sustained increases in TNF-α and monocyte chemotactic protein 1, more slowly initiated elevations in IL-6, IL-8, IL-18, and IFN-γ, and a late-peaking (15 days after HIV RNA detection) increase in IL-10 (32).

It is not clear why the increase in CD38 expression was more pronounced on CD8+ T cells than CD4+ T cells, but previous reports show this to be true for HIV-infected individuals. It may be that CD8+ T cells are more susceptible to the activation signals that cause increased expression of CD38 because of their key role in cytotoxic
immune responses. Fewer CD38-expressing CD4+ T cells may have been detected because they were activated and killed by the virus, although the overall loss of CD4+ T cells in the HIV-positive mice 4 weeks after inoculation was modest with a mean reduction of 43 cells per µl (from 174 to 131 CD4+ T cell per µl; Fig. 1C).

CD8+ T-cell activation and subsequent dysfunction has been causally linked to IFN-α, where HIV infection induces increased IFN-α production resulting in increased MHC-I expression (19). This in turn correlates with an increase in the frequency of CD8low T cells that display increased levels of CD38 and are functionally impaired in response to TCR stimulation (12). As such, a prophylactic treatment that limits the effect of IFN-α during the acute phase of the infection may reduce the T-cell activation set point and ultimately delay disease progression. Our future studies include experiments to determine the effect of neutralizing anti-IFN-α antibody on T-cell activation in HIV-infected BLT mice with the goal of examining the feasibility of using this treatment to alter the activation set point during acute infection. Ultimately, if applied to the human setting, this may provide a treatment for delaying or preventing HIV disease progression and the onset of AIDS.
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REFERENCES


TABLE 1. Numbers of injected cells, levels of human leukocyte reconstitution, and proportion of viremic mice in 14 NSG-BLT mouse cohorts inoculated intravaginally with HIV JR-CSF

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Total cells injected per mouse</th>
<th>CD34+ cells injected per mouse</th>
<th>HSPC injected per mouse</th>
<th>Mean human CD45+ cells/µl</th>
<th>Mean human CD4+ cells/µl</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; inoculated (JR-CSF)</th>
<th>Viremic mice (%)</th>
<th>Viremic mice (%) with no HSPC injected</th>
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<tbody>
<tr>
<td>BLT-1010</td>
<td>6.5 x 10^5</td>
<td>4.7 x 10^5</td>
<td>4,900</td>
<td>532</td>
<td>138</td>
<td>1.4 x 10^6</td>
<td>6 of 8 (75%)</td>
<td>0 of 5 (0%)</td>
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<tr>
<td>BLT-1011</td>
<td>5.0 x 10^5</td>
<td>4.3 x 10^5</td>
<td>8,700</td>
<td>2,142</td>
<td>257</td>
<td>0.8 x 10^6</td>
<td>14 of 15 (93%)</td>
<td>3 of 5 (60%)</td>
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<tr>
<td>BLT-1012&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.3 x 10^5</td>
<td>2.7 x 10^5</td>
<td>2,500</td>
<td>1,785</td>
<td>261</td>
<td>1.1 x 10^6</td>
<td>14 of 16 (88%)</td>
<td>2 of 5 (40%)</td>
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<tr>
<td>BLT-1013</td>
<td>4.4 x 10^5</td>
<td>2.7 x 10^5</td>
<td>350</td>
<td>324</td>
<td>94</td>
<td>1.1 x 10^6</td>
<td>7 of 15 (47%)</td>
<td>0 of 5 (0%)</td>
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<td>BLT-1014</td>
<td>2.0 x 10^5</td>
<td>1.6 x 10^5</td>
<td>1,400</td>
<td>520</td>
<td>100</td>
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<td>5 of 7 (71%)</td>
<td>1 of 5 (20%)</td>
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<tr>
<td>BLT-1015</td>
<td>3.4 x 10^5</td>
<td>1.5 x 10^5</td>
<td>1,100</td>
<td>266</td>
<td>9</td>
<td>0.02 x 10^6</td>
<td>2 of 10 (20%)</td>
<td>0 of 2 (0%)</td>
</tr>
<tr>
<td>BLT-1016</td>
<td>1.5 x 10^5</td>
<td>1.3 x 10^5</td>
<td>620</td>
<td>308</td>
<td>10</td>
<td>0.4 x 10^6</td>
<td>3 of 8 (38%)</td>
<td>0 of 2 (0%)</td>
</tr>
<tr>
<td>BLT-1017</td>
<td>3.7 x 10^5</td>
<td>2.6 x 10^5</td>
<td>910</td>
<td>1,141</td>
<td>148</td>
<td>0.03 x 10^6</td>
<td>4 of 8 (50%)</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BLT-1018</td>
<td>7.5 x 10^5</td>
<td>6.0 x 10^5</td>
<td>760</td>
<td>1,195</td>
<td>43</td>
<td>0.02 x 10^6</td>
<td>2 of 9 (22%)</td>
<td>ND</td>
</tr>
<tr>
<td>BLT-1020</td>
<td>4.4 x 10^5</td>
<td>2.3 x 10^5</td>
<td>150</td>
<td>349</td>
<td>129</td>
<td>3.7 x 10^6</td>
<td>1 of 8 (13%)</td>
<td>0 of 5 (0%)</td>
</tr>
<tr>
<td>BLT-1021</td>
<td>1.8 x 10^5</td>
<td>1.6 x 10^5</td>
<td>1,900</td>
<td>577</td>
<td>317</td>
<td>4.5 x 10^6</td>
<td>13 of 14 (93%)</td>
<td>5 of 5 (100%)</td>
</tr>
<tr>
<td>BLT-1022</td>
<td>11.0 x 10^5</td>
<td>8.8 x 10^5</td>
<td>5,800</td>
<td>1,479</td>
<td>140</td>
<td>3.0 x 10^6</td>
<td>5 of 9 (56%)</td>
<td>0 of 5 (0%)</td>
</tr>
<tr>
<td>BLT-1023</td>
<td>2.9 x 10^5</td>
<td>2.4 x 10^5</td>
<td>820</td>
<td>747</td>
<td>188</td>
<td>0.5 x 10^6</td>
<td>3 of 5 (60%)</td>
<td>0 of 5 (0%)</td>
</tr>
<tr>
<td>BLT-1101</td>
<td>3.0 x 10^5</td>
<td>2.2 x 10^5</td>
<td>1,100</td>
<td>339</td>
<td>82</td>
<td>0.5 x 10^6</td>
<td>0 of 5 (0%)</td>
<td>0 of 5 (0%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> TCID<sub>50</sub>: 50% tissue-culture infectious units, as assessed by limiting dilution assay in phytohemagglutinin-activated peripheral blood mononuclear cells with supernatant p24 detection 7 days after inoculation.

<sup>b</sup> CCR5 ∆32<sup>c</sup> cohort susceptible to HIV JR-CSF infection.

<sup>c</sup> ND: Not determined.
**FIGURE LEGENDS**

**FIG. 1.** Experimental design, HIV inoculation, and loss of CD4+ T cells in HIV-infected BLT mice. (A) 14 cohorts of BLT mice (Table 1) were inoculated intravaginally with HIV JR-CSF at 0 weeks (12 weeks after HSPC injection). Peripheral blood of BLT mice was evaluated for human cell reconstitution by Trucount analysis at 0 weeks and 4 weeks after inoculation. (B) The total number of peripheral blood leukocytes (CD45+), lymphocytes (Ly), monocytes (Mo), CD4+ T cells (CD4+), CD8+ T cells (CD8+), and B cells (B) were determined for each cohort by Trucount analysis at the time of inoculation. (C) Significant differences were observed in the number of CD4+ T cells and the change in CD4+ T-cell number between HIV-negative (n = 58) and HIV-positive (n = 79) mice at the time of inoculation and 4 weeks after inoculation. The Mann-Whitney U-test was used for differences between the means.

**FIG. 2.** Overall lack of correlation within cohorts between human CD4+ T-cell reconstitution at the time of inoculation (0 weeks) and plasma HIV viral load obtained 4 weeks later. Trend lines from linear regression are shown, and a two-tailed Spearman correlation (r) was calculated with resulting P values at 95% confidence.

**FIG. 3.** The number of fetal-liver-derived CD34+ cells (top left panel) and HSPC (Lin-1-, CD38-, CD45RA-, CD45+, CD34+, c-kit+, and CD90+ (top right panel) injected into each mouse (~12 weeks in Fig. 1A) is shown with the frequency of HIV-viremic mice 4 weeks after inoculation for the 14 cohorts of BLT mice. The mean number of...
CD4+ T cells/µl in each cohort at the time of inoculation (0 weeks) correlated with the frequency with which BLT mice became viremic (bottom left panel). The frequency with which BLT mice that did not receive injected HSPC became HIV infected following intravaginal inoculation (bottom right panel) correlated with the frequency with which HSPC-injected BLT mice became infected. Trend lines from linear regression are shown, and a two-tailed Spearman correlation ($r$) was calculated with resulting $P$ values at 95% confidence.

FIG. 4. T-cell activation in HIV-infected BLT mice. Peripheral blood was collected from BLT mice at the time of inoculation (0 weeks, middle row) and 4 weeks later (bottom row). Human peripheral blood was used as a staining and gating control (top row). Blood was immunophenotyped for the presence of human leukocytes and further analyzed for the expression of activation markers. Human CD45+ leukocytes were gated by side scatter into lymphocytes (not shown) and further subdivided into CD3+ (T cells) and CD19+ cells (B cells). CD3+ cells were separated into CD4+ and CD8+ T cells and evaluated for CD38 and HLA-DR expression. Shown in the middle and bottom rows is the same BLT mouse at the 0 week and 4 week time point (4.5 log_{10} copies HIV RNA per 100 µl), where an increase in the frequency of CD38/HLA-DR+ cells was observed in the CD4+ T cell and CD8+ T-cell compartments.

FIG. 5. Increased expression of cellular activation markers on lymphocytes from HIV-infected BLT mice. (A) The geometric mean fluorescence intensity (MFI) of CD38 (left panel), as well as the frequency (center panel) and absolute number per µl (right panel) of CD38/HLA-DR+ cells were determined for CD4+ and CD8+ T cells from a
subset of HIV-positive (n = 46) BLT mice at 0 weeks (the time of inoculation) and 4 weeks. P values are shown for two-tailed, nonparametric Mann-Whitney U-test. (B) Activation marker data for HIV-infected mice were correlated with plasma HIV viral load 4 weeks after inoculation. The MFI of CD38 (left panel) on CD4+ and CD8+ T cells correlated strongly with viral load, as did the frequency (center panel) and absolute number (right panel) of CD38+/HLA-DR+ cells. Trend lines from linear regression are shown, and a two-tailed Spearman correlation (r) was calculated with resulting P values at 95% confidence. Data are from cohorts BLT-1012, -1013, -1014, -1015, -1016, -1017, -1018, -1020, -1022, and -1023 as described in Table 1.

FIG. 6. Treatment with Intron A causes increased expression of CD38 and HLA-DR. (A) Groups of 6 BLT mice from the same cohort were treated with 3 different doses of Intron A (10^6, 10^5, and 10^4 IU), beginning 12 weeks after HSPC injection. One group was treated with sterile water. Mice were dosed once daily by intraperitoneal injection for 6 days. On day 7, peripheral blood was phenotyped and examined for expression of CD38 and HLA-DR. The number of CD4+ and CD8+ T cells per μl of whole blood was only slightly elevated in the treated groups (left panel). The MFI of CD38 (left panel), as well as both the frequency and absolute number per μl of CD38/HLA-DR+ cells was determined (center left, center right, and right panels, respectively) for CD4+ T cells (top row) and CD8+ T cells (bottom row). (B) Spleens were collected from groups of Intron A-treated mice as in panel A and were processed to produce single-cell suspensions for immunophenotyping by flow cytometry. The MFI of CD38 was significantly increased in a dose-dependent manner on both splenic CD4+ and CD8+ T cells (left panels), yet there was no difference in the frequency of CD38+/HLA-DR+ cells (right panels).
Statistical significance was determined by Mann-Whitney U-test against the water-treated group. *$P < 0.05$, **$P < 0.01$. 
Figure 1

A

-15 weeks  -12 weeks  0 weeks  4 weeks
Thy/Liv Implant  HSPC Injection  Intravaginal HIV Inoculation  Plasma Viral Load by bDNA
Immunophenotyping and Trucount Analysis  Immunophenotyping and Trucount Analysis

B

BLT-1010  BLT-1011  BLT-1012  BLT-1013  BLT-1014
BLT-1015  BLT-1016  BLT-1017  BLT-1018  BLT-1019
BLT-1020  BLT-1021  BLT-1022  BLT-1023  BLT-1024

C

HIV-1+ BLT Mice  HIV-1- BLT Mice  Change in CD4+ T Cells/mℓ

0 weeks  4 weeks  0 weeks  4 weeks  4 weeks HIV-1+  4 weeks HIV-1-

p = 0.024  p = 0.014  p < 0.001
Figure 2

BLT-1010

BLT-1011

BLT-1012

BLT-1013

BLT-1014

BLT-1015

BLT-1016

BLT-1017

BLT-1018

BLT-1020

BLT-1021

BLT-1022

BLT-1023
Figure 3

CD34+ Cells Injected
HSPC Injected
% Viremic Mice

\[ r = 0.269 \quad p = 0.353 \]

% Viremic Mice

\[ r = 0.692 \quad p = 0.006 \]

% Viremic Mice

\[ r = 0.795 \quad p = 0.003 \]

% Viremic Mice (HSPC Injected)

\[ r = 0.793 \quad p < 0.001 \]

% Viremic Mice (HSPC Injected)

\[ r = 0.795 \quad p < 0.003 \]
Figure 4

A

Human Peripheral Blood Control

BLT mouse 0 weeks (at inoculation)

BLT mouse 4 weeks

Human CD45+ Lymphocytes
CD3+ Lymphocytes
CD4+ T Cells
CD8+ T Cells

CD19 APC-Cy7
CD3 ECD
PEG-Qdot-605
CD38 PE
HLA-DR FITC

Human CD45+ Lymphocytes
CD3+ Lymphocytes
CD4+ T Cells
CD8+ T Cells

CD19 APC-Cy7
CD3 ECD
PEG-Qdot-605
CD38 PE
HLA-DR FITC
Figure 5

A

CD8+ T Cells

CD4+ T Cells

B

CD8+ T Cells

CD4+ T Cells
Figure 6

A

CD4+ T Cells

CD8+ T Cells

B

Spleen CD4+ T Cells

Spleen CD8+ T Cells