MUCOSAL HIV-1 TRANSMISSION IN HUMANIZED MICE

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DEDICATION

To my very patient and loving wife, Janelle.

MUCOSAL TRANSMISSION OF HIV-1 IN HUMANIZED MICE

by

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MUCOSAL TRANSMISSION OF HIV-1 IN HUMANIZED MICE

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HIV-1 infects ~6,800 people each and every day, transmitting predominantly through unprotected sexual contact. On a global scale, vaginal transmission now accounts for more than half of newly acquired HIV-1 infections. In developed countries intrarectal infection represents a major form of HIV-1 transmission. The social and economic toll of this disease has created an urgency to develop and implement novel approaches capable of preventing HIV-1 transmission. Yet this process has been hindered by the lack of adequate small animal models for pre-clinical efficacy and safety testing. Given the importance of mucosal HIV-1 transmission, the susceptibility of humanized mice to intrarectal and intravaginal HIV-1 infection was investigated.

Human lymphocytes, including $CD4^+$ T cells, generated in situ from hematopoietic stem cells reconstitute the gastrointestinal tract and the female reproductive tract of Bone marrow Liver Thymus (BLT) mice. The presence of human $CD4^+$ T cells in these mucosal tissues renders BLT mice susceptible to both intrarectal and intravaginal HIV-1 transmission. Mucosally transmitted HIV-1 disseminates systemically in BLT mice. Effects of disseminated HIV-1 infection include a systemic loss of $CD4^+$ T cells, particularly in gut associated lymphoid tissue, which closely mimics what happens in HIV-1 patients.

The utility of humanized mice to study mucosal HIV-1 transmission is particularly highlighted by the demonstration herein that pre-exposure prophylaxis with antiretroviral drugs can prevent intravaginal HIV-1 transmission. This experimental finding has important implications for the clinical implementation of antiretroviral-based pre-exposure prophylactic measures to prevent the spread of AIDS.

The goal of this dissertation project was to determine the suitability of the BLT mouse to serve as an animal model of HIV-1 transmission and as a model for assessing interventions aimed at preventing HIV-1 transmission. My conclusions are that BLT mice are susceptible to both intrarectal and intravaginal HIV-1 transmission and that pre-exposure prophylaxis with FDA approved antiretroviral drugs does prevent vaginal transmission in BLT mice. Thus, the BLT mouse system is an excellent candidate for pre-clinical evaluation of both microbicides and pre-exposure prophylactic regimens to prevent mucosal HIV-1 transmission.

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LIST OF ABBREVIATIONS/ACRONYMS

APC, allophycocyanin

BLT, Bone marrow – Liver – Thymus humanized mouse

BM, Bone marrow

CD, cluster of differentiation

CIEA, Central Institute for Experimental Animals in Kawasaki, Japan

CTL, cytotoxic T lymphocytes

DC, dendritic cells

df, degrees of freedom

DTT, dithiothreitol

DMEM, Dulbecco's Modified Eagle Medium

EDTA, ethylenediaminetetraacetic acid

ELISA, enzyme linked immunosorbent assay

FACS, fluorescence activated cell sorting

FBS, fetal bovine serum

FITC, fluorescein isothyiocyanate

FRT, female reproductive tract

FTC, emtricitabine

GAG, group antigen

GALT, gut associated lymphoid tissue

GI, gastrointestinal

HIV, human immunodeficiency virus

HLA, human leukocyte antigen

HSC, hematopoietic stem cell(s)

hu, human

IEL, intraepithelial lymphocytes

Ig, immunoglobulin

IL, interleukin

IL2R, interleukin 2 receptor

LI, large intestine

LN, lymph nodes

LPL, lamina propria lymphocytes

LTR, long terminal repeat

µg, microgram

mg, milligram

MHC, major histocompatibility complex

µl, microliter

ml, milliliter

Nef, negative factor

NK, natural killer cells,

NOD/SCID, nonobese diabetic severe combined immunodeficient mice (NOD/*LtSz-Prkdc*^{scid})

NOG, NOD/SCID mice with the IL-2 receptor gamma chain knocked out (CIEA)

PB, peripheral blood

PBL, peripheral blood lymphocyte

PBMC, peripheral blood mononuclear cells

PBS, phosphate buffered saline

PE, phycoerythrin

PHA, phytohemaglutinin

RAG, recombination activating gene

RBC, red blood cell(s)

rpm, revolutions per minute

SCID, severe combined immunodeficient

SD, standard deviation

SEM, standard error of the mean

SHIV, chimeric simian-human immunodeficiency virus

SI, small intestine

SIV, simian immunodeficiency virus

TCIU, tissue culture infectious units

TCR, T cell receptor

TDF, tenofovir disoproxil fumarate

thy/liv, thymus-liver-thymus implant

Thymic Org., implanted thymic organoid

UTSWMC, University of Texas Southwestern Medical Center

CHAPTER ONE
Introduction

A. History – humanized mice in HIV research

Nearly a century has passed since mice were introduced into medical research (Castle and Little 1909). The benefits of mice in medicine are incalculable. Many findings in mice regarding biological processes have easily been applied to humans (i.e. metabolism, respiration and reproduction). Broad disease categories with afflictions that affect both mice and humans are numerous (i.e. cancer, autoimmunity, and immunodeficiency). Some human pathogens also infect mice (i.e. herpes simplex virus-2 and *Neisseria sp.*) (Zeitlin, Hoen et al. 2001). However, other human pathogens are host specific and they cannot infect mice (i.e. human immunodeficiency virus, hepatitis B virus and hepatitis C virus) (Purcell 1994; Chisari and Ferrari 1995; Guidotti, Matzke et al. 1995; Bieniasz and Cullen 2000; Mariani, Rutter et al. 2000; Sun, Soos et al. 2006). Attempts to model these diseases in mice have lead to the development of "humanized mice", or mice that exhibit a human phenotype in some manner.

The first humanized mice were mice genetically altered to express one or more human proteins. Genetically altered mice, collectively known as transgenic mice, entered the public consciousness in 1982 when mice developed from eggs microinjected with metallothionein-growth hormone fusion genes grew larger than normal mice (Palmiter, Brinster et al. 1982; Palmiter 1998). Since that time, volumes of research papers have reported on human disease models in human transgenic mice. An important consideration regarding human transgenic mice is that the human proteins are being expressed by mouse cells. This can have unanticipated and possibly undesirable consequences, particularly in HIV research. In the early days of HIV research, mouse cells were determined to be unable to support HIV replication (Mosier, Gulizia et al. 1988). Over a decade

later, at least a partial explanation as to why mouse cells are refractory to HIV was supplied when multiple post-translational defects in HIV replication were identified in mouse cells (Bieniasz and Cullen 2000). Even after mouse cells were engineered to express human proteins known to be required for HIV replication they were only able to produce ~10,000-fold less virus than a single cycle of infection in human cells (Bieniasz and Cullen 2000). Not knowing where the mouse cell blocks to HIV replication occurred made the generation of a functional human transgenic mouse model for HIV research a very tricky proposition. Given these extreme challenges inherent to a human transgenic mouse model for HIV disease, an alternative strategy was developed in the late 1980s for humanizing mice.

The new strategy of humanizing mice involved immune deficient mice harboring human cells (McCune, Namikawa et al. 1988; Mosier, Gulizia et al. 1988). These models were collectively known as SCID-hu mice (McCune, Kaneshima et al. 1989; McCune, Kaneshima et al. 1991). SCID-hu mice are distinguished from human transgenic mice by the fact that SCID-hu mice are not genetically manipulated to express human proteins. Rather human cells and/or tissues are placed into immunodeficient mice directly from a human source. Different immunodeficient mice are used in these models, all without functional mouse T or B lymphocytes but with varying levels of residual immune function: SCID mice, exhibit essentially a complete innate immune system along with mouse natural killer cell activity; NOD/SCID mice, similar to SCID with much less mouse natural killer cell function; both $Rag2^{-/-}IL2g^{-/-}$ mice and NOD/SCID-$IL2R\gamma c^{(null)\ or\ (-/-)}$ mice, have essentially no mouse natural killer cell activity (Shultz, Ishikawa et al. 2007). Several protocols for the generation of SCID-hu mice exist and are discussed below. Each model's narrative describes how those

particular SCID-hu mice are generated. In addition, contributions made with that SCID-hu model to the field of HIV research are detailed.

B. SCID-hu mice in HIV research

B1. SCID-hu and NOD/SCID-hu CD34$^+$ HSC

A widely used SCID-hu model involves transplanting human CD34$^+$ HSC into either SCID (Bosma, Custer et al. 1983; Dorshkind, Keller et al. 1984; Custer, Bosma et al. 1985) or NOD/SCID (Greiner, Shultz et al. 1995) mice. Despite their popularity, SCID-hu and NOD/SCID-hu CD34$^+$ HSC mice are rarely used in HIV research. However, important technical improvements within this model were adapted to other SCID-hu models that are used for HIV studies. A key finding in this system is that transplantation of NOD/SCID mice with human CD34$^+$ cells results in dramatically higher levels of systemic repopulation with human cells than transplanting SCID mice (Greiner, Hesselton et al. 1998). A limiting aspect of this system is that B cells are the most abundant human cell type in these mice (accounting for up to 90% of human reconstitution in all tissues) and virtually no human T cells can be detected in any of the mouse hematopoietic organs including the mouse thymus (Palucka, Gatlin et al. 2003). Nevertheless, this model has proven to be quite useful to study a variety of important aspects of hematopoeisis, the ontogeny and function of the human immune system in vivo and the pathogenesis of human-specific virus infection (Miyoshi, Smith et al. 1999; Palucka, Gatlin et al. 2003; Islas-Ohlmayer, Padgett-Thomas et al. 2004; Bente, Melkus et al. 2005; Cravens, Melkus et al. 2005); but it is the general lack of human T cells in SCID-hu and NOD/SCID-hu cord blood CD34$^+$ mice that has precluded their use in HIV research.

B2. SCID-hu and NOD/SCID-hu PBL

SCID-hu and NOD/SCID-hu PBL mice are generated when human peripheral blood lymphocytes are injected intraperitonealy into SCID or NOD/SCID mice (Mosier, Gulizia et al. 1988). The SCID-hu PBL model is characterized by a transient repopulation of the host mouse with mature human lymphocytes; there are no hematopoietic stem cells providing regeneration capacity. Functional human B and T cells circulate through SCID-hu and NOD/SCID-hu PBL over a period of 3-4 weeks (Coccia and Brams 1998; Delhem, Hadida et al. 1998; Cao and Leroux-Roels 2000; Santini, Lapenta et al. 2000), but both the B and T lymphocyte repertoires are severely limited in these mice (Saxon, Macy et al. 1991; Garcia, Dadaglio et al. 1997). While present, human lymphocytes are inappropriately distributed in mouse peripheral blood, spleen, liver, bone marrow, lymph nodes, pancreas and kidneys; and the human lymphocytes minimally engraft lymphoid organs in SCID-hu and NOD/SCID-hu PBL mice (Hoffmann-Fezer, Kranz et al. 1992; Murphy, Bennett et al. 1992; Tary-Lehmann and Saxon 1992; Hesselton, Koup et al. 1993; Hoffmann-Fezer, Gall et al. 1993; Martino, Anastasi et al. 1993). SCID-hu and NOD/SCID-hu PBL mice are susceptible to HIV-1 (Mosier, Gulizia et al. 1991) and they have contributed to HIV research on topics ranging from viral cytopathic effects to potential vaccine approaches (Mosier, Gulizia et al. 1993; Mosier, Gulizia et al. 1993).

SCID-hu PBL mice have a demonstrated susceptibility to vaginal transmission of cell-associated HIV-1 (Di Fabio, Giannini et al. 2001). Vaginal transmission in this model requires that the HIV-1 be cell-associated and that the SCID-hu PBL mice be pretreated with progestin to thin their vaginal epithelium (Di Fabio, Giannini et al. 2001; D'Cruz and Uckun 2007). This model has been used with limited success to evaluate the potential of topical microbicides for

preventing vaginal HIV-1 transmission (Khanna, Whaley et al. 2002; Di Fabio, Van Roey et al. 2003), but there remains some uncertainty regarding how these findings relate to human vaginal transmission (Lederman, Offord et al. 2006). It is somewhat surprising that the transiently repopulated SCID-hu PBL model would be considered for use in transmission studies because, as D'Cruz and Uckun recently published, the incidence of vaginal transmission of HIV-1 in the SCID-hu PBL mouse is "low and variable" (D'Cruz and Uckun 2007). Furthermore, Di Fabio, et al. report that the intraperitonealy injected human PBL targeted for HIV-1 infection were not even present in the vagina because they were not able to migrate from the peritoneal cavity into the cervicovaginal mucosa (Di Fabio, Giannini et al. 2001).

A newer iteration of the SCID-hu and NOD/SCID-hu PBL model involves an intraperitoneal transplant of allogeneic human PBL combined with a heterotopic implant of rolled human vaginal tissue within the subcutaneous space on the back of a NOD/SCID mouse (Kish, Budgeon et al. 2001). Interestingly, there was no data shown regarding the infiltration of the allogeneic PBL into the vaginal tissue graft (Kish, Budgeon et al. 2001). Cell-free HIV-1 infection of these mice occurs only when the virus is injected directly into the lumen of the human vaginal graft (Kish, Ward et al. 2003). Otherwise, the characteristics and limitations of the SCID-hu and NOD/SCID-hu PBL model without the human vaginal tissue apply to these mice.

SCID-hu and NOD/SCID-hu PBL mice have been used in attempts to model human vaginal HIV-1 transmission. No published record exists of attempts to model human rectal HIV-1 transmission in this model. Similarly, there are no data regarding the effects of HIV-1 infection on human cells in the gut-associated lymphoid tissues in these mice.

B3. SCID-hu thy/liv

SCID-hu thy/liv mice are generated by implanting human fetal liver and thymus tissue under the kidney capsule of a SCID mouse (McCune, Namikawa et al. 1988; Namikawa, Weilbaecher et al. 1990). In contrast to the lack of human T cells in SCID-hu and NOD/SCID-hu $CD34^+$ HSC mice described above, in the SCID-hu thy/liv mice there is an abundance of human thymocytes. However, virtually all human cells are confined to the thymic organoid that develops after implantation, except for in the spleen where low levels (<1%) of human T cells (and rarely human B cells) can be found (McCune, Namikawa et al. 1988; Vandekerckhove, Krowka et al. 1991; Aldrovandi, Feuer et al. 1993). Thus, SCID-hu thy/liv mice do not have significant systemic repopulation with human T cells and are virtually devoid of human B cells, monocytes/macrophages and dendritic cells.

In the seventeen years since SCID-hu thy/liv mice were described, many advances in HIV research have been made within the limits of this model. The SCID-hu thy/liv model is well suited for the study of HIV effects on thymopoeisis, including the depletion of $CD4^+CD8^+$ thymocytes (Su, Kaneshima et al. 1995; Jamieson, Uittenbogaart et al. 1997). The SCID-hu thy/liv model has been extensively utilized in the pre-clinical evaluation of antiretroviral therapies (McCune, Kaneshima et al. 1990; McCune, Namikawa et al. 1990; Stoddart, Moreno et al. 2000; Stoddart, Bales et al. 2007). Other therapeutic interventions have also been evaluated in SCID-hu thy/liv mice including HIV-1 entry inhibitors and CCR5 antagonists (Stoddart, Rabin et al. 1998; Strizki, Xu et al. 2001). In addition to therapeutics, SCID-hu thy/liv mice have been used to study HIV-1 pathogenesis, such as the in vivo roles for nef, rev and other accessory genes (Aldrovandi and Zack 1996; Valentin, Aldrovandi et al. 1997; Aldrovandi, Gao et al. 1998; Stoddart, Geleziunas et al. 2003). Cellular factors like co-

receptor expression and usage have also been analyzed in SCID-hu thy/liv mice (Jamieson, Pang et al. 1995; Kitchen and Zack 1997; Berkowitz, Alexander et al. 1998; Berkowitz, Beckerman et al. 1998; Berkowitz, van't Wout et al. 1999; Berkowitz, Alexander et al. 2000).

SCID-hu thy/liv mice are susceptible to HIV-1 infection when the virus is injected directly into the thymic organoid. Pathological analyses are limited to the thymic organoid. There is no humanization of mucosal tissues, so the effects of HIV-1 infection on human cells in the gut-associated lymphoid tissues in these mice cannot be evaluated. Similarly, mucosal transmission of HIV-1 in SCID-hu thy/live mice is not possible.

B4. $Rag2^{-/-}IL2R\gamma c^{-/-}$ $CD34^{+}$ HSC

Mice lacking recombinase activating gene 2 (Rag2) and IL-2 receptor (common) gamma chain ($Rag2^{-/-}IL2g^{-/-}$) are severely immunocompromised (Goldman, Blundell et al. 1998; Mazurier, Fontanellas et al. 1999). The Rag2 mutation blocks the maturation of endogenous mouse B and T lymphocytes, due to a defect in V(D)J rearrangement (Shinkai, Rathbun et al. 1992). In addition to the IL-2 receptor, the common gamma chain is utilized by the IL-7, IL-9, IL-12, IL-15 and IL-21 receptors (Leonard 2001). Therefore, the gamma chain mutation results in further immune deficit; specifically there are no functional mouse natural killer cells in mice with this mutation (Goldman, Blundell et al. 1998; Mazurier, Fontanellas et al. 1999).

Traggiai, et al. described a humanization protocol whereby neonatal $Rag2^{-/-}IL2g^{-/-}$ mice are transplanted intrahepatically with human $CD34^{+}$ HSC. $Rag2^{-/-}IL2g^{-/-}$ mice humanized in this manner develop functional human B, T and dendritic cells (Traggiai, Chicha et al. 2004). Human thymocytes are present in

the mouse thymus of humanized Rag2$^{-/-}$IL2g$^{-/-}$ mice where they are presumed to be educated against mouse MHC (Traggiai, Chicha et al. 2004; Manz 2007).

The human T cells of humanized Rag2$^{-/-}$IL2g$^{-/-}$ mice are susceptible to HIV-1 infection both in vivo (Baenziger, Tussiwand et al. 2006; Berges, Wheat et al. 2006; An, Poon et al. 2007; Gorantla, Sneller et al. 2007; Zhang, Kovalev et al. 2007) and ex vivo (Berges, Wheat et al. 2006). The route of exposure used to study HIV-1 infection in humanized Rag2$^{-/-}$IL2g$^{-/-}$ mice was primarily intraperitoneal, with one report using intravenous inoculation of HIV-1 (Zhang, Kovalev et al. 2007). A vaccine of *Haemophilus influenzae* type b conjugated to tetanus toxoid (ActHIB®) was used to immunize humanized Rag2$^{-/-}$IL2g$^{-/-}$ mice and human IgG specific for *Haemophilus influenzae* type b were identified in immunized mouse plasma (4 of 4) (Gorantla, Sneller et al. 2007). However, human immunoglobulins specific for HIV were identified in the plasma of only 1 of 44 examined HIV-1 infected humanized Rag2$^{-/-}$IL2g$^{-/-}$ mice (Baenziger, Tussiwand et al. 2006; An, Poon et al. 2007; Gorantla, Sneller et al. 2007). Humanized Rag2$^{-/-}$IL2g$^{-/-}$ mice are susceptible to HIV-1 infection but descriptions of CD4^{+} T cell depletion were limited to peripheral blood, primary lymphoid organs and secondary lymphoid organs. There are no data regarding the humanization of mucosal tissues or the effects of HIV-1 infection on human cells in the gut-associated lymphoid tissues in these mice. Nor is there any indication that HIV-1 can transmit mucosally in humanized Rag2$^{-/-}$IL2g$^{-/-}$ mice.

B5. NOD/SCID-IL2Rγc$^{-/-}$ CD34^{+} HSC

Two different mouse strains have been developed in which the common gamma chain is absent from NOD/SCID mice: NOD/SCID-IL2Rγc$^{-/-}$ (Ito, Hiramatsu et al. 2002) and NOD/SCID-IL2Rγc^{null} (Cao, Shores et al. 1995). Both

of these mouse strains have been humanized, but by different approaches. Hiramatsu, et al. humanized NOD/SCID-IL2Rγc$^{-/-}$ mice by transplanting 8-12 week old mice with human CD34^{+} HSC and Ishikawa, et al. humanized NOD/SCID-IL2Rγc^{null} mice by transplanting neonates intrahepatically with CD34^{+} HSC (Hiramatsu, Nishikomori et al. 2003; Ishikawa, Yasukawa et al. 2005). In both cases human T and B lymphocytes develop within the mouse. Human thymocytes are present in the mouse thymus of humanized NOD/SCID-IL2Rγc$^{-/-}$ and NOD/SCID-IL2Rγc^{null} mice, where they are presumed to be educated against mouse MHC (Hiramatsu, Nishikomori et al. 2003; Ishikawa, Yasukawa et al. 2005; Manz 2007).

Thus far, only humanized NOD/SCID-IL2Rγc$^{-/-}$ mice have been shown to be susceptible to HIV-1 infection (Watanabe, Ohta et al. 2007; Watanabe, Terashima et al. 2007). The route of exposure used to study HIV-1 infection in humanized NOD/SCID-IL2Rγc$^{-/-}$ mice was intravenous (Watanabe, Ohta et al. 2007; Watanabe, Terashima et al. 2007). Human immunoglobulins specific for HIV were identified in the plasma of 3 of 14 examined HIV-1 infected humanized NOD/SCID-IL2Rγc$^{-/-}$ mice (Watanabe, Terashima et al. 2007). Humanized NOD/SCID-IL2Rγc$^{-/-}$ mice are susceptible to HIV-1 infection but descriptions of CD4^{+} T cell depletion were limited to peripheral blood, primary lymphoid organs and secondary lymphoid organs. There are no data regarding the humanization of mucosal tissues or the effects of HIV-1 infection on human cells in the gut-associated lymphoid tissues in these mice. Nor are there reports of mucosal HIV-1 transmission in humanized NOD/SCID-IL2Rγc$^{-/-}$ mice.

B6. NOD/SCID-hu BLT

Humanized Bone marrow Liver Thymus (BLT) mice represent advancement beyond these models (Melkus 2006; Melkus, Estes et al. 2006; Wege, Melkus et al. In Press). Humanized BLT mice are generated by initially implanting human fetal liver and thymus tissue under the kidney capsule of a NOD/SCID mouse (as with SCID-hu thy/liv) followed by transplant of autologous human fetal liver $CD34^+$ cells (similar to other humanization protocols). NOD/SCID mice were used instead of SCID mice because NOD/SCID mice support significantly higher levels of reconstitution after transplantation with human $CD34^+$ cells, due to lower endogenous mouse NK cell activity (Greiner, Hesselton et al. 1998).

Humanized BLT mice combine the most desirable attributes of multiple humanized mouse models into a single system. Namely, in BLT mice there is human thymic tissue where T cell education occurs and there is complete systemic reconstitution of all major human hematopoietic lineages, including T, B, monocyte/macrophage, dendritic and natural killer cells (Melkus, Estes et al. 2006). The broad repertoire of human T cells in BLT mice can generate human leukocyte antigen class I- and class II-restricted adaptive immune responses to Epstein-Barr virus and are activated by human dendritic cells to mount a potent T-cell immune response to superantigens (Melkus, Estes et al. 2006). Particularly relevant to this dissertation project is the description of human B, T, myeloid and dendritic cells in BLT lungs. Lungs represent a mucosal-associated lymphoid tissue (MALT) and they serve as an internal control for mucosal human reconstitution (Melkus 2006; Melkus, Estes et al. 2006). With this in mind, a hypothesis was formulated that mucosal reconstitution with human lymphoid cells would be systemic; including the gut-associated lymphoid tissue (GALT) and the

female reproductive tract. This hypothesis led to the first aim of this dissertation project.

The first aim of this dissertation project was to evaluate the humanization of the BLT mouse mucosal sites relevant to HIV-1 transmission: the gastrointestinal tract and the female reproductive tract. This aim was accomplished and both of these BLT mouse mucosal sites were shown to be reconstituted with human cells representing the hematopoietic lineages necessary for HIV-1 transmission (T cells, monocyte/ macrophages and dendritic cells), although multiple attempts have yet to yield unequivocal evidence that the dendritic cells observed in BLT mice include human CD1a Langerhans' cells.

The second aim of this project was to determine the suitability of BLT mice to serve as an animal model of mucosal HIV-1 transmission. The presence of human $CD4^+$ T cells in the relevant mucosal tissues render BLT mice susceptible to both intrarectal and intravaginal HIV-1 transmission. Demonstration of efficient mucosal transmission of HIV-1 in BLT mice following either rectal or vaginal inoculation completed this aim. Mucosally transmitted HIV-1 disseminates systemically in BLT mice. Effects of disseminated HIV-1 infection include a systemic loss of $CD4^+$ T cells, particularly in gut-associated lymphoid tissue, which closely mimics what happens in HIV-1 patients. The results from this aim represent a dramatic advancement beyond the vaginal transmission studies in SCID-hu PBL mice (Di Fabio, Giannini et al. 2001; Kish, Budgeon et al. 2001; Khanna, Whaley et al. 2002; Di Fabio, Van Roey et al. 2003; Kish, Ward et al. 2003; D'Cruz and Uckun 2007).

The third aim of this dissertation project was to evaluate the potential of the BLT mouse model for assessing interventions aimed at preventing mucosal

HIV-1 transmission. Pre-exposure prophylaxis with antiretroviral drugs has considerable potential for preventing mucosal HIV-1 transmission, and this approach was utilized here (Veazey, Klasse et al. 2005). The antiretroviral drug combination of emtricitabine and tenofovir disoproxil fumarate (FTC/TDF) was chosen for this project because of potency, daily dosing, and favorable profiles for both toxicity and viral resistance (De Clercq 2007). Pre-exposure prophylaxis with FTC/TDF afforded complete protection of BLT mice from vaginal HIV-1 transmission. The results from this aim demonstrate that the BLT model can be used for pre-clinical efficacy testing of pre-exposure prophylaxis regimens for the prevention of mucosal HIV-1 transmission and serve as evidence for the potential success of this approach aimed at preventing the further spread of AIDS.

CHAPTER TWO
Methods and Materials

A. Animal husbandry

Nonobese diabetic-severe combined immunodeficient (NOD/SCID) mice (The Jackson Laboratories, Bar Harbor, ME) were housed in a specific pathogen-free facility at the Animal Resources Center of UT Southwestern Medical Center (UTSWMC) in accordance with protocols approved by the UTSWMC Institutional Animal Care and Use Committee. Mice were maintained in microisolator cages, fed sterile food and given sterile chlorinated water.

B. Generation of BLT mice

BLT stands for mice that receive a bone marrow transplant of human hematopoietic stem cells, in addition to a tissue implant of human liver and thymus tissue (Advanced Bioscience Resources, Alameda, CA). NOD/SCID mice underwent survival surgery to implant a human fetal thymus/liver/thymus (thy/liv) tissue sandwich beneath their left kidney's capsule. $CD34^+$ cells for the bone marrow transplant were obtained from fetal liver autologous to the tissue used for the thy/liv implants. Preconditioning of NOD/SCID mice to prepare for the bone marrow transplant consisted of a single 325 cGy dose of cesium-137 gamma radiation at least 4 hours prior to the intravenous cell transplant (Melkus, Estes et al. 2006).

Isolation of fetal liver $CD34^+$ cells was as follows. The tissue was incubated at 37°C in complete RPMI [RPMI 1640 medium (Sigma Chemical Co.), 10% fetal bovine serum (FBS) (Mediatech Inc.), 50 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 2 mM glutamine (Sigma), 1 mM collagenase/dispase (Roche, Mannheim), and 0.5 U/mL DNase I (Roche). The

tissue was gently disrupted by cutting it into small pieces and pipetting the mixture up and down every 15 minutes for 1 hour. The cell suspension was filtered through a 70μm cell strainer, and mononuclear cells were isolated by Ficoll gradient separation. $CD34^+$ cells were isolated using immunomagnetic beads (Miltenyi Biotech). Isolated $CD34^+$ cells were then subsequently stained with the mouse anti-human monoclonal antibodies to CD34 (clone 581, Pharmingen) and CD3 (clone HIT3a, Pharmingen) or similarly labeled isotype controls and analyzed by flow cytometry on a FACSCalibur using CellQuest Pro (version 4.0.2; Becton Dickinson) for CD34 expression and residual $CD3^+$ T cells. This protocol yielded greater than 90-95% pure $CD34^+$ cells and less than 0.6% $CD3^+$ T cells. Cells were frozen (-80^o C) in 90% FBS and 10% dimethyl sulfoxide (Fisher) following isolation and stored in liquid nitrogen until transplanted (Melkus 2006; Melkus, Estes et al. 2006).

C. HIV-1 generation and titer

Virus stocks were prepared by first transfecting 293T cells with plasmids containing a proviral clone. The clones used for this work were HIV-1_{LAI} (Alizon, Sonigo et al. 1984; Wain-Hobson, Sonigo et al. 1985) and HIV-1_{JR-CSF} (Koyanagi, Miles et al. 1987). Supernatants from the transfected cells were harvested, filtered through 0.45μm syringe filters, and aliquoted for storage at -80^{o}C. Supernatants were evaluated for p24 concentration by ELISA as described below. Supernatants were titrated to determine actual tissue culture infectious units (TCIU) using P4R5 cells. P4R5 cells have a β-galactosidase reported under control of the HIV-1 LTR. β-galactosidase production is activated by tat produced in HIV infected cells such that X-gal staining results in infected cells turning blue allowing a visualization and enumeration of each infected cell

(Fredericksen, Wei et al. 2002; Wei, Denton et al. 2005). Dr. Bangdong Luke Wei generated and titered a significant portion of the HIV-1 used in this project.

D. Peripheral blood analysis

BLT mouse peripheral blood was analyzed over time as well as post-mortem. Following acquisition, plasma was removed from the whole blood by centrifugation and aspiration. The plasma was collected for viral load, plasma antigenemia and western blot analyses. The volume removed as plasma was replaced with solution B [1 liter Phosphate Buffered Saline (PBS) (Sigma), 5 grams BSA (Sigma), 50 U/ml Penicillin (Sigma), 50mg/ml Streptomycin (Sigma), 1% Citrate Phosphate Dextrose (Sigma)] and the blood was then used for flow cytometry or DNA preparation. For flow cytometry, peripheral blood was stained and washed according the Becton Dickinson's "lyse-wash" protocol where peripheral blood is blocked and stained, and then BD FACS Lysis Solution (Cat. No. 349202) is added to the blood to lyse red blood cells for 5 minutes, diluted with PBS + 2% FBS and centrifuged 5min, 4^{o}C, 1500RPM. The blood is then washed, fixed and ready for analysis on the cytometer (Cravens, Melkus et al. 2005; Melkus, Estes et al. 2006; Sun, Denton et al. 2007). For PCR, peripheral blood was lysed for 10 minutes [RBC lysing buffer: 500 ml dH2O, 4.15 g NH4Cl (Sigma), 0.5 g KHCO3 (Sigma) and 0.019 g EDTA (Sigma)], washed, pelleted and stored at -80^{o}C until DNA preparation.

E. Viral load analysis

Plasma samples from BLT mice were diluted with normal human plasma (Sigma H4522) to a final volume of 240µl. These aliquots were cryptically labeled and provided for clinical analysis by Veripath Laboratories at UTSWMC for the presence of viral RNA (Amplicore, Roche). Results obtained from

Veripath were decoded and the dilutions were factored into the calculations to obtain HIV-1 RNA copies per ml of plasma (Sun, Denton et al. 2007).

F. Plasma antigenemia analysis

The HIV-1 capsid protein, Gag p24, was utilized as a marker for the presence of HIV-1 in plasma of BLT mice or virus rescue samples. HIV-1 p24 was detected using an ELISA kit marketed by Beckman Coulter (Cat. No. 626391) according to the manufacturer's protocol (Fredericksen, Wei et al. 2002; Wei, Denton et al. 2005).

G. Mucosal exposure to HIV-1

Prior to inoculation, mice were anesthetized with sodium pentobarbital. All inoculation procedures were performed using ~$9X10^4$ TCIU and were based on published protocols (Zeitlin, Hoen et al. 2001). Intrarectal inoculations were performed with LAI and intravaginal inoculations were performed with LAI and JR-CSF. Volumes were delivered to the correct orifice using either a blunt 21 gauge needle or a pipette fitted with a filter tip. To simulate receptive rectal intercourse and cause mild abrasion, rectal inoculations were preceded by insertion of an aluminum oxide-coated cylinder (3/32-inch Dremel tool; Small Parts Inc., Miami Lakes, FL) 15 times (Zeitlin, Hoen et al. 2001). Vaginal inoculations were completely atraumatic.

H. Antiretroviral drug preparation and administration

Emtricitabine (FTC) and Tenofovir disoproxil fumarate (TDF) (Gilead, Foster City, CA) were administered intraperitonealy (3.5 mg and 5.2 mg, respectively) once daily for seven consecutive days starting 48 hr prior to intravaginal inoculation with HIV-1 (Frick, Lambe et al. 1994; Naesens, Bischofberger et al. 1998; Stoddart, Bales et al. 2007). To prepare FTC/TDF, a

single Truvada pill was crushed using a mortar and pestle. Then based on published solubility values for each drug, the drugs were dissolved. First, 2.2ml DMSO in 9.0ml distilled water were added to the crushed pill and pipetted several times. This volume was collected into a 50ml conical tube. Then 11.2ml distilled water were added to wash the mortar and pestle. This volume was pipetted several times and added to the previously collected fraction. The complete mixture was vortexed vigorously for 5 minutes, and then centrifuged 5 minutes, 1500 RPM at 4^{o}C. The supernatant was filtered through a 22μm syringe filter aliquoted and stored at 4^{o}C. Given that FTC and TDF are analogs of cytidine and adenosine, respectively, the spectrophotometric adsorbances of these two nucleosides were utilized to calculate the approximate concentration of each drug present in the preparation.

I. Mononuclear cell isolation from mouse tissues

Mononuclear cells from spleens, lymph nodes, and thymic organoids were isolated by disrupting the tissue by mashing it against a 70μm cell strainer using a plunger from a 3ml syringe. The strainers were washed in solution B to obtain a maximum cell yield and red blood cells were lysed using RBC lysing buffer from the spleen and thymic organoid samples. Following lysis and a wash in solution B (spin at 1500 rpm at 4°C for 5 minutes), the cells were resuspended in solution B and placed on ice (Cravens, Melkus et al. 2005; Melkus, Estes et al. 2006).

Bone marrow mononuclear cell isolations were performed similar to those for spleen, lymph node and organoid listed above. The difference for bone marrow isolation was that the bones were crushed with a mortar and pestle, and then filtered through a 70μm cell strainer (no plunger used) (Cravens, Melkus et al. 2005; Melkus, Estes et al. 2006).

To harvest liver mononuclear cells, the liver was diced into small pieces, and the tissue disrupted by passage through a 5 ml syringe. The cell suspension was filtered through a 70μm cell strainer and centrifuged at 1500 rpm at 4°C for 5 minutes. The cells were then resuspended in 5 ml 40% Percoll (Sigma) and cRPMI in a 14 ml round-bottom tube, underlayed with an equal volume of 70% Percoll and cRPMI and spun at 2400 rpm at 20°C for 20 minutes. Lymphocytes were isolated from the 40% -70% interface and washed in cRPMI. Red blood cells were lysed with RBC lysis buffer (if necessary) and the mononuclear cells were washed in solution B (Melkus, Estes et al. 2006).

Lungs were minced into small pieces and treated with collagenase enzyme cocktail (2.5ml RPMI, 6 mg Collagenase D, 50μg DNAse) and incubated for 30 minutes at 37°C. The cell suspension was filtered through a 70μm cell strainer and centrifuged at 1500 rpm at 4°C for 5 minutes. The cells were then resuspended in 5 ml 40% Percoll (Sigma) and cRPMI in a 14 ml round-bottom tube, underlayed with an equal volume of 70% Percoll and cRPMI and spun at 2400 rpm at 20°C for 20 minutes. Lymphocytes were isolated from the 40% -70% interface and washed in cRPMI. Red blood cells were lysed with RBC lysis buffer (if necessary) and the mononuclear cells were washed in solution B (Melkus 2006; Melkus, Estes et al. 2006).

Isolation of mononuclear cells from the gut was similar to previously described protocols (Mysorekar, Lorenz et al. 2002; Das, Augustine et al. 2003). Small and large intestine were flushed with ice cold PBS, cut into ~1 inch segments, and inverted to expose the intraepithelial surface. These segments were washed five times by vigorous shaking in cold PBS, allowed to settle by gravity, then resuspended in 15-25 ml IEL Extraction Medium (freshly made; 3% FBS,

1mM DTT, 1 mM EDTA in PBS). These mixtures were shaken gently for 30 min on a rotating platform at 37^{o}C, then vigorously for 2 min at RT. The IEL supernatants were collected through a 70μm cell strainer. The intestinal segments were thoroughly washed then incubated for 60 min on a rotating platform at 37^{o}C in 15-25ml LPL Digestion Medium (freshly made; 60μg/ml Collagenase D and 10U/ml DNaseI in RPMI-1640). During this incubation, the IEL supernatants were passed over IEL Extraction Medium equilibrated columns of 0.5g of dimethyldichlorosilane-treated glass wool fiber (Fisher) in 10ml syringes. The column flow-through IEL fractions were washed at 1500 RPM for 5 min at 4^{o}C, resuspended in solution B and placed on ice. Following the LPL digestion, the suspensions were shaken mildly for 1 min at RT. The LPL supernatants were collected through a 70μm cell strainer, washed at 1500 RPM for 5 min at 4^{o}C, resuspended in solution B and placed on ice (Sun, Denton et al. 2007).

The female reproductive tract (FRT) single cell suspension protocol was adapted from a published procedure (Keenihan and Robertson 2004). Briefly, the entire reproductive tract (vagina, cervix, uterus, fallopian tubes and ovaries) was minced and then placed in a 50ml conical tube with 2ml of fresh FRT Digestion Media (25units of DNaseI [Roche] plus 2mg of collagenase D [Roche] in RPMI 1640 supplemented with 10% heat inactivated FBS). The samples were shaken gently at room-temperature for 2 hours. Following this incubation, each sample received an addition of 2 ml of EDTA Media ($Ca^{2+}Mg^{2+}$-Free Hanks Buffered Salt Solution supplemented with 5% heat inactivated FBS and 5mM EDTA) and was shaken gently at room-temperature for an additional 20 minutes. The samples were vortexed briefly and then filtered through a 70μm cell strainer, washed at 1500 RPM for 5 minites at 4^{o}C, resuspended in solution B and placed on ice. Live cells from all mononuclear cell isolations were counted using trypan

blue exclusion and used for flow cytometry, virus rescue, or real time PCR analysis.

J. Flow cytometry

The gating strategy for each subset of cells included first gating on human CD45 to identify human cells and to exclude mouse cells from the analysis. Human cells were then analyzed for the specific human leukocyte antigens indicated in the different figures. Flow cytometry data were collected on the same day of harvest mostly using a FACSCanto instrument with Diva software (version 4.1.2; Becton Dickinson) and occasionally using a FACSCalibur instrument with CellQuest Pro software (version 4.0.2; Becton Dickinson). The percentage of human leukocytes ($CD45^+$) and hematopoietic lineages were determined by flow cytometry using antibodies to human hematopoietic markers as described (Gatlin, Melkus et al. 2001; Gatlin, Padgett et al. 2001; Islas-Ohlmayer, Padgett-Thomas et al. 2004; Cravens, Melkus et al. 2005; Melkus, Estes et al. 2006; Sun, Denton et al. 2007). For further characterization, subsets of myeloid and lymphoid cells were stained with appropriate antibodies and analyzed by flow cytometry. Live cells were identified based on their characteristic side scatter versus forward scatter. Subsequently, live human mononuclear cells were identified with mouse anti-human $CD45^+$ (clone HI30, Pharmingen) to determine the percentage of human reconstitution. Dendritic cells were identified using the BD DC Kit gating through mononuclear cells that were lineage negative, HLA DR bright (Lin^{neg}HLA-DR^{++} gate) and further characterized for CD11c (clone B-ly6, Pharmingen) and CD123 (clone 7G3, Pharmingen) expression. Lymphocytes were gated through human $CD45^+$ cells and CD19 (clone HIB19, Pharmingen) for B cells, $CD3^{neg}$ $CD33^{neg}CD56^+$ (clones HIT3a, P67.6 and B159, respectively; Pharmingen) for natural killer cells. NK cells were identified as mature or immature based on CD16 (clone 3G8, Pharmingen) expression levels. $CD3^+$ T

cells ($CD45^+CD3^+$ gate) were further analyzed for CD4 (clone SK3, BD) and CD8 (clone SK1, BD) subsets, for naive versus memory T cell subsets using CD45RA (clone HI100, Pharmingen) and CD27 (clone MT-271, Pharmingen) and for expression of the chemokine receptors CXCR4 (clone 12G5, Pharmingen) and CCR5 (clone 3A9, Pharmingen). Small intestine $CD8^+$ T cells were also analyzed for CD8α (clone SK1, BD) and CD8β (clone 2ST8.5H7, Immunotech) (Sun, Denton et al. 2007).

K. Immunohistochemistry and in situ hybridization

Immunohistochemical staining and in situ hybridization analysis were performed as described (Li, Duan et al. 2005; Estes, Li et al. 2006; Melkus, Estes et al. 2006; Sun, Denton et al. 2007). Tissues were collected and placed in fresh phosphate buffered 4% paraformaldehyde for 4 to 6 hours, washed with 70% ethanol and stored in 70% ethanol until embedded in paraffin. Immuno-histochemistry was performed using a biotin-free polymer approach (MACH-3™; Biocare Medical) to analyze all BLT tissues. Immunohistochemistry was performed on 5μm tissue sections mounted on glass slides, dewaxed and rehydrated with PBS (pH 7.4). Antigen retrieval was performed by heating sections in 1X EDTA Decloacker™ reagent (Biocare Medical) in a 95^{o}C water bath for 20 minutes followed by cooling to room temperature. Nonspecific Ig-binding sites were blocked with Blocking Reagent (Biocare Medical) for 60 minutes at room temperature. Endogenous peroxidase was blocked with 3% (v/v) hydrogen peroxide in PBS (pH 7.4). Primary antibodies were diluted in 10% Blocking Reagent in 1,3,5-trinitrobenzene and incubated overnight at 4^{o}C. The sections were then analyzed using either the mouse or rabbit MACH-3™ polymer systems (Biocare Medical) according to the manufacturer's instructions and developed with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). Sections were counterstained with Harris Hematoxylin (Surgipath, Richmond), mounted in

Permount (Fisher Scientific) and examined by light microscopy. Primary antibodies used for immunohistochemistry were mouse anti-human CD3 (clone F7.2.38, DakoCytomation), anti-human CD4 (clone 1F6, Novocastra Laboratories), anti-human CD11c (clone 5D11, Novocastra Laboratories), anti-human CD68 (clone KP1, DakoCytomation) and mouse anti-human HLA-DR (clone LN3, Lab Vision/Neomarkers). Isotype matched negative control antibodies used were mouse IgG1 (clone X 0931, Dako Cytomation), mouse IgG2a (clone X 0943, Dako Cytomation), goat ChromPure IgG (Jackson Immuno-Research) and rabbit ChromPure IgG (Jackson Immuno- Research). HIV-1 RNA was detected in cells by in situ hybridization in formalin-fixed and paraffin-embedded tissues. Sections were cut and adhered to silanized slides. After de-paraffinization in xylene, rehydration in PBS and permeabilization by treating the sections with hydrochloric acid, digitonin and proteinase K, the sections were acetylated and hybridized to ^{35}S-labelled HIV-1 specific riboprobes. After washing and digestion with RNases, sections were coated with nuclear track emulsion, exposed, developed and counterstained with Giemsa.

L. Virus rescue

Rescue of infectious virus from different tissues was performed by co-culture with phytohemaglutinin (PHA) activated peripheral blood mononuclear cells (PBMC) from HIV seronegative donors and viral spread was monitored by determining p24 levels in the culture supernatant. PBMC were activated for 48-72 hours prior to co-culture with cells isolated from BLT mice. Activation was elicited by the addition of 1μg/ml PHA to RPMI 1640 supplemented with 10% heat-inactivated FBS, 100U of penicillin/ml, 100μg of streptomycin/ml, 2mM L-glutamine and 1% sodium pyruvate. Following activation, cells isolated from BLT mice were added to the activated PBMC in approximately a 1:1 ratio and incubated in RPMI 1640 supplemented with 20% heat-inactivated FBS, 100 units

of penicillin/ml, 100µg of streptomycin/ml, 2mM L-glutamine, 20µg/ml Fungizone and 20U/ml interleukin-2. Media was removed at regular intervals for p24 ELISA analysis and replaced with equal volumes of fresh media (Sun, Denton et al. 2007).

M. Real-time PCR

Quantitative real-time PCR for viral DNA was performed using Assays-on-Demand™ in a 7500 Fast instrument (sensitivity: 5 copies of JR-CSF; SDS software version 1.3.1.22; Applied Biosystems, Foster City, CA) following the manufacturer's protocol for universal cycling conditions. ABI custom TaqMan® reagents were: Forward primer: 5'-ATCAAGCAGCTATGCA AATGCT-3'; Reverse primer: 3'- CTGAAGGGTACTAGTAGTCCCTG CTATGTC-5'; and MGB probe: 5'-TCAATGAGGAAGCTGCAGAA-3'. DNA for real-time PCR was prepared from lysed peripheral blood or from mononuclear cells isolated from tissues. Lysed peripheral blood was prepared by digestion in proteinase K, vigorously shaking at 56^{o}C for 60 minutes. The enzyme was inactivated by 20 minute incubation while vigorously shaking at 95^{o}C. Samples were frozen for a minimum of 30 minutes then centrifuged at 14,000 for 1 minute. The supernatant was then collected and stored at -20^{o}C for PCR analysis. This maintains the highest yield of DNA from a small volume of blood (Gatlin, Padgett et al. 2001). DNA from cells was prepared according to Qiagen provided protocols using the QiaAMP DNA Mini kit (Cat. No. 51304).

N. Statistical analyses

Data shown as box-plots to be interpreted as follows: the line in the middle is the median; half the values were higher, half lower; the box extends from the 25th to the 75th percentiles; the error bars extend down to the lowest

value and up to the highest value. Bar graph data shown as mean and SEM. Statistical analysis using Student's t test, linear regression comparison and the Kaplan-Meyer plot were performed using Prism v. 4 (Graph Pad Software, Inc., San Diego, CA).

CHAPTER THREE

Results

HUMAN IMMUNE CELLS MIGRATE TO AND POPULATE BLT MOUSE INTESTINES RENDERING THESE HUMANIZED MICE SUSCEPTIBLE TO INTRARECTAL HIV-1 TRANSMISSION*

A. Introduction

In the early stages of HIV infection of humans, the gut-associated lymphoid tissue (GALT) is a major site of virus replication; and subsequent depletion of $CD4^+$ T cells in GALT is a defining event in the course and outcome of disease (Brenchley, Schacker et al. 2004; Veazey and Lackner 2005). As there currently are no small animal models to study HIV-1 infection and pathogenesis in the intestines, macaques inoculated with simian immunodeficiency virus (SIV) or SIV/HIV chimeric viruses (SHIV) are the principal surrogate animal model to study HIV infection and GALT pathogenesis (Pauza, Horejsh et al. 1998). The hypothesis that BLT mice could function as a small animal model for HIV GALT pathogenesis was formulated based on the fact that in BLT mice there is systemic human reconstitution with all human lymphoid lineages (Melkus 2006; Melkus, Estes et al. 2006).

In this chapter, the in vivo repopulation of the BLT mouse GALT with human hematopoietic cells derived from transplanted human stem cells is shown. Demonstration of the susceptibility of BLT mice to intrarectal infection by a single exposure to cell free HIV-1 is also shown. Intrarectal HIV-1 infection of BLT mice results in systemic infection and the production of HIV-specific human

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antibodies. $CD4^+$ T cell depletion was observed throughout the mice, including in the intestines of HIV infected BLT mice. Humanization of BLT mouse GALT, intrarectal HIV-1 infection of BLT mice and HIV-1-induced BLT GALT pathogenesis each represent significant advances that will facilitate the clinical implementation of microbicides and prophylactics to prevent HIV transmission and therapeutic interventions aimed at GALT reconstitution following HIV infection.

B. Human hematopoietic cells populate BLT mouse intestines

B1. Human lymphocytes in BLT GALT

To determine if human lymphoid cells encounter adequate signals to migrate into and repopulate the mouse gastrointestinal tract of NOD/SCID mice, the human hematopoietic cells in different parts of the intestines from BLT mice were characterized by isolating lamina propria and intraepithelial lymphocytes (LPL and IEL) from large and small intestine. In all cases the gating strategy for flow cytometric analysis first identified human lymphoid cells based on their expression of the human leukocyte common marker CD45 and then by the appropriate lineage marker (i.e. CD3 or CD19) followed by the specific subset (i.e. CD4 and/or CD8). All portions of the mouse GALT were reconstituted with human hematopoietic cells, albeit at different levels (Figure 1). Human $CD19^+$ B and $CD3^+$ T cells were identified in all fractions with single positive CD4 and CD8 human T cells representing the majority of human lymphoid cells in all portions of the reconstituted GALT.

B2. Human T cells in BLT GALT exhibit a memory phenotype

Phenotypic characterization of the T cells present in the different GALT fractions indicated that the majority of both $CD4^+$ and $CD8^+$ human T cells were either $CD45RA^{neg}CD27^+$ central memory cells or $CD45RA^{neg}CD27^{neg}$ effector

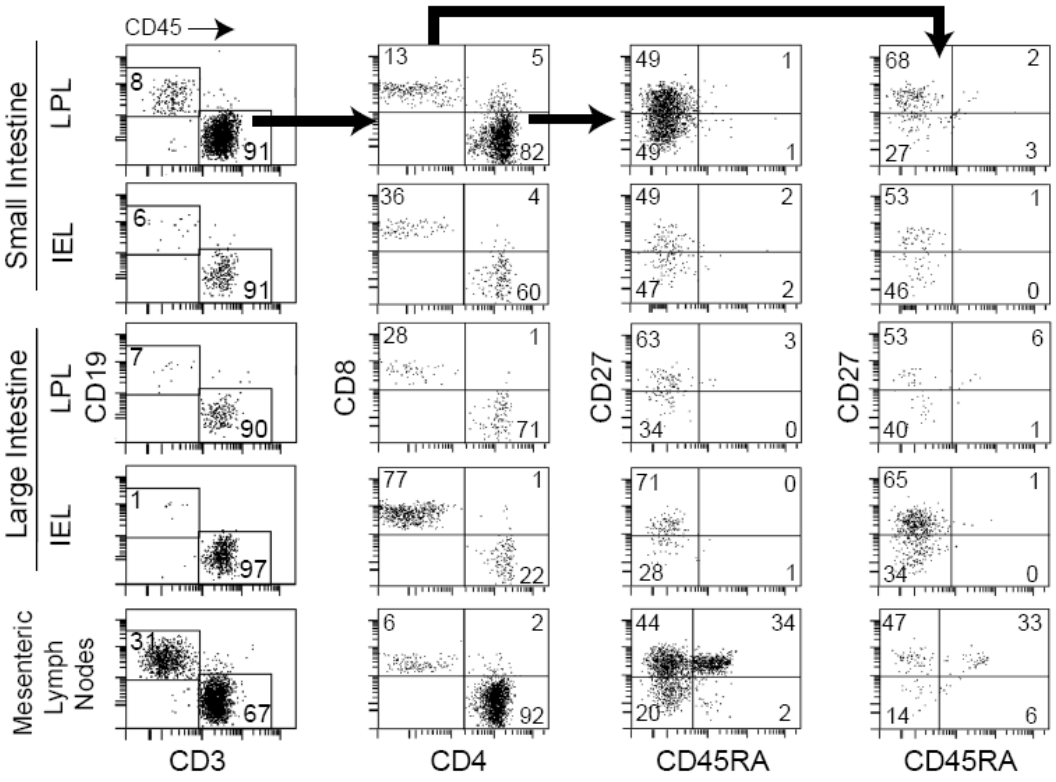

Figure 1. Human reconstitution in the small and large intestines of BLT mice.

BLT intestines are reconstituted with human hematopoietic cells including B and T lymphocytes (left column). Intestinal human T lymphocytes are mostly $CD4^+$ (2^{nd} column from the left) and they exhibit a memory phenotype (both columns to the right). The small and large intestines, along with the mesenteric lymph nodes, were harvested approximately 24 weeks post transplant. Intestinal epithelial and lamina propria lymphocytes (IELs and LPLs, respectively) were isolated from both large and small intestines. Human hematopoietic cells were identified with anti-human CD45 antibodies and characterized for expression of lineage and differentiation specific markers as indicated in the different panels. Human cells in this analysis were sequentially gated as $CD45^+ \rightarrow CD3^+$ or $CD19^+$. $CD45^+CD3^+$ cells were analyzed for $CD4^+$ or $CD8^+$ expression (indicated with a dark arrow between first and second panels). $CD4^+$ or $CD8^+$ cells were further analyzed for CD27 and CD45RA expression (indicated by the short dark arrow between the second and third row of panels for $CD4^+$ cells and a large dark arrow between the second and fourth row of panels for $CD8^+$ cells).

memory cells (Figure 1). Since mesenteric lymph nodes are the secondary lymphatic tissues that drain the GI tract and they play a major role in virus dissemination after intrarectal HIV infection, human reconstitution in these important lymph nodes was evaluated. It was determined that mesenteric lymph nodes were also reconstituted with human T and B cells. In contrast to intestinal LPL and IEL, mesenteric LN contained populations of naïve ($CD45RA^+CD27^+$) T cells (Figure 1).

B3. $CD8\alpha\alpha^+$ T cells present in the small intestine of BLT mice are a human GALT-specific lymphocyte population present in BLT GALT.

In addition to single positive $CD4^+$ and $CD8^+$ T cells, intraepithelial and lamina propria $CD4^+CD8^+$ double positive human T cells were found in the BLT small intestine (Figure 1). The CD8 molecule expressed on human double positive T cells present in the lamina propria was predominantly the CD8α chain (Figure 2). In contrast, the CD8 single positive cells in the small intestine expressed both the CD8α and CD8β chains (Figure 2). These observations are fully consistent with what has been described for human gut (Abuzakouk, Carton et al. 1998; Carton, Byrne et al. 2004). Also consistent with what has been found in human gut, DC represent a small proportion of the human lymphoid cells present and lineage negative $HLA\text{-}DR^{bright}$ $CD11c^+$ DC were most prominent in the small intestine IEL and LPL fractions (Figure 3) (Bell, Rigby et al. 2001). Similar to DC, human NK cells were present in BLT small intestines in small numbers (Figure 4).

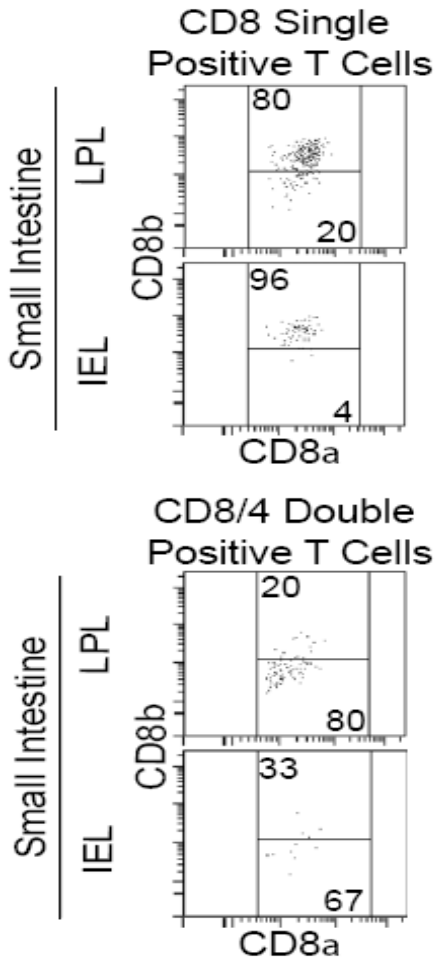

Figure 2. CD8αα$^+$ T cells present in the small intestine of BLT mice are a human GALT-specific lymphocyte population present in BLT GALT. Differential expression of CD8α and CDβ chains was evaluated in small intestine IEL and LPL CD8$^+$ and CD4$^+$CD8$^+$ cells. Note that double positive GALT T cells predominantly express CD8αα homodimers, as in humans (Abuzakouk, Carton et al. 1998; Carton, Byrne et al. 2004). Human T cells in this analysis were sequentially gated as CD45$^+$→CD3$^+$.

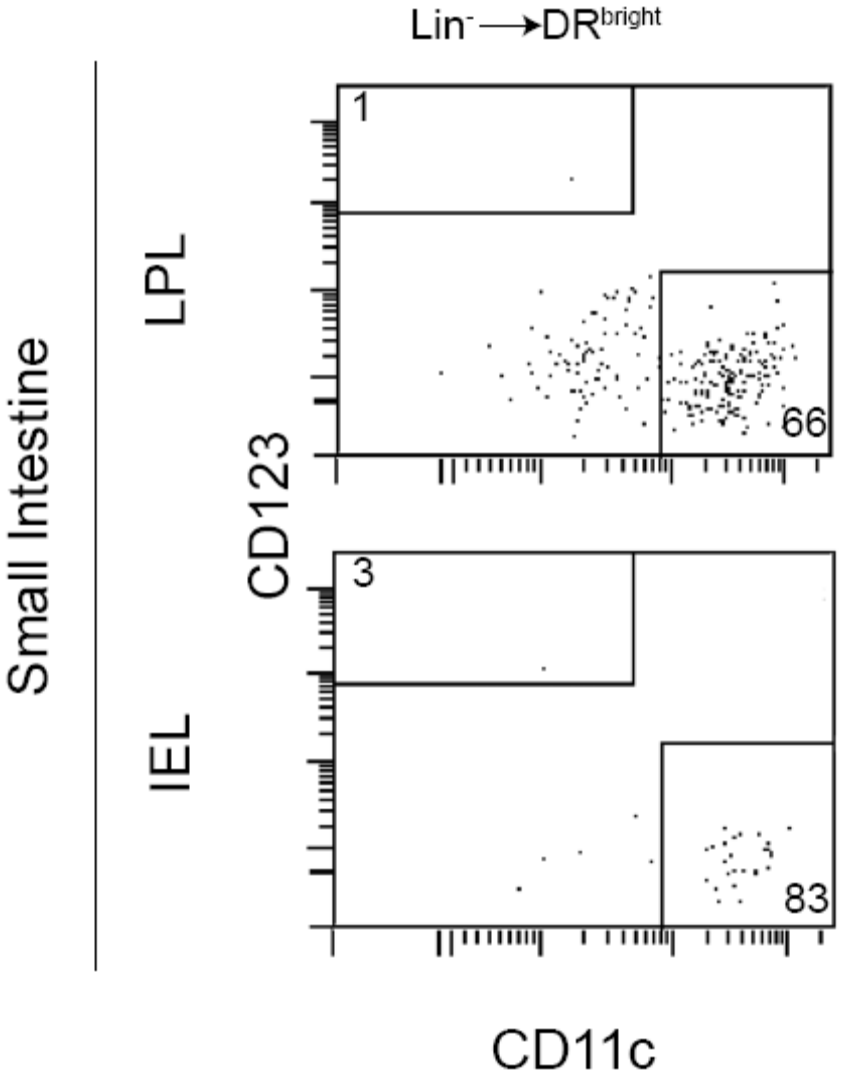

Figure 3. CD11c^{+} human dendritic cells are present in the small intestine of BLT mice.
DC represented a small proportion of the human lymphoid cells present when small intestine IEL and LPL fractions were evaluated for their presence. CD11c^{+} DC were most prominent. In BLT mouse small intestine there were no or few CD123^{+} DCs. DC were defined as lineage negative, HLA-DR^{bright} CD11c, or CD123 positive.

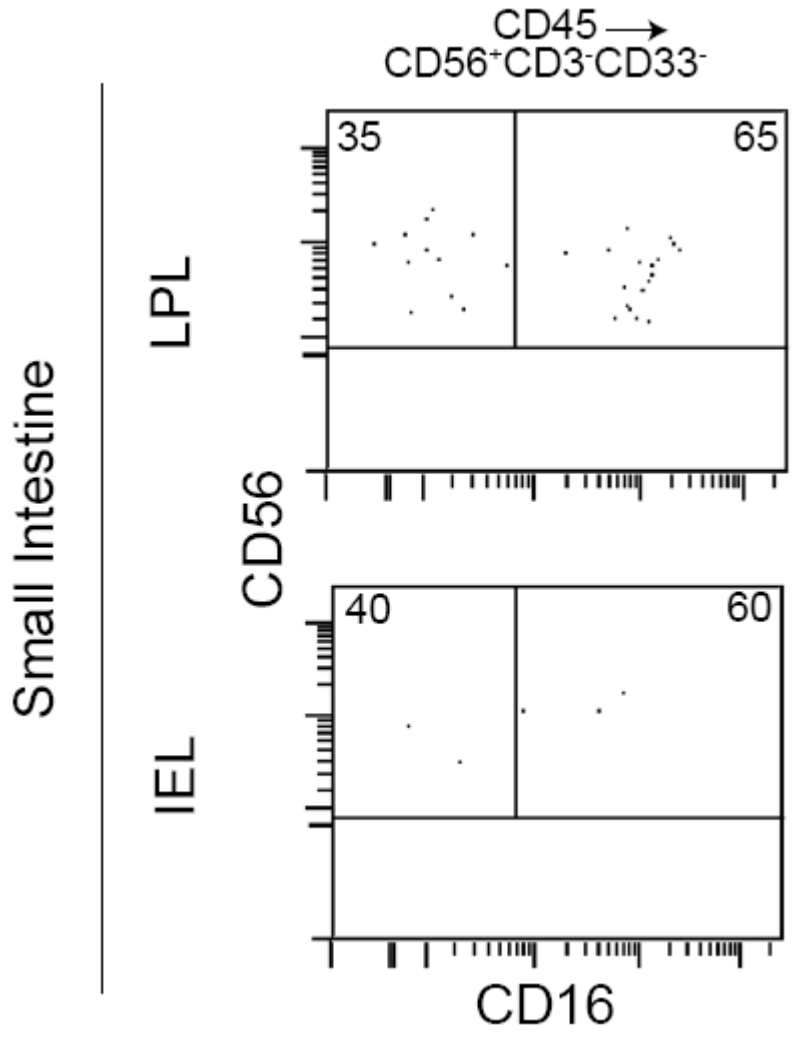

Figure 4. Human natural killer cells are detectable in the small intestine of BLT mice.
Small intestine IEL and LPL fractions contain human NK cells. Mature and immature NK cells represented a very small proportion of the human lymphoid cells present. NK cells were defined as $CD45^{+}CD56^{+}CD3^{neg}CD33^{neg}$. Mature NK cells expressed higher levels of CD16.

B4. HIV-1 co-receptor expression on human T cells in BLT mouse GALT

Co-receptor expression in human $CD4^+$ cells is the major determinant of HIV tropism in vivo (Lederman, Penn-Nicholson et al. 2006; Ray and Doms 2006). Analysis of $CD4^+$ T cells present in the GALT of BLT mice indicated differential expression of CXCR4 and CCR5. The proportion of $CD4^+$ T cells expressing CXCR4 was lower in all fractions of the GALT when compared to the mesenteric lymph nodes ($p=0.006$) (Figure 5). In contrast, the proportion of cells expressing CCR5 was higher in all fractions of the GALT, again when compared to the mesenteric LN ($p=0.03$) (Figure 6). Analysis of CCR5 expression on T cells in the gut with those from other lymphoid and non lymphoid tissues demonstrated that the highest proportion of $CD4^+CCR5^+$ T cells was found in the GALT (Figure 6). Similar results were observed with $CD8^+$ T cells (Figures 5 and 6).

B5. Immunohistochemistry shows human cells in BLT mouse GALT

The small and large intestines of BLT mice were also analyzed for the presence of human hematopoietic cells by immunohistochemistry at approximately 24 weeks post transplant. The positive staining observed indicated an abundant accumulation of human hematopoietic cells in both the small and the large intestines that included human $CD4^+$ cells, $CD11c^+$ dendritic cells, $CD68^+$ monocyte/macrophages and HLA-DR^+ antigen presenting cells (Figure 7). These results are consistent with what has been previously observed in human and macaque GALT (Anton, Elliott et al. 2000; Cranston, Anton et al. 2000; Veazey, Mansfield et al. 2000).

Together, these data establish the reconstitution of the GALT of BLT mice with all the human hematopoietic cells relevant to mucosal HIV transmission. Furthermore, phenotypic analysis of GI and mesenteric LN demonstrates that they

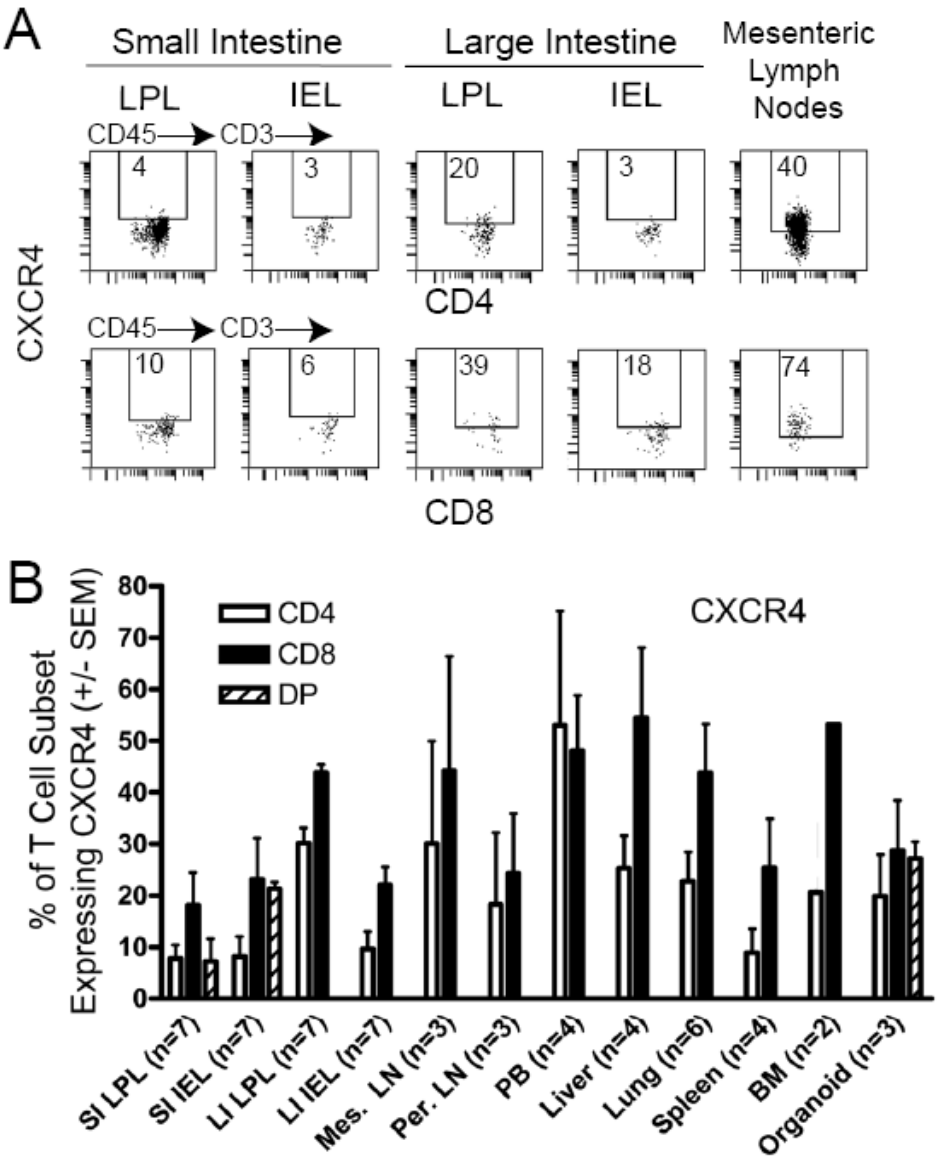

Figure 5. CXCR4 chemokine receptor expression in BLT mice.
Panel **A)** shows a comparison of the levels of the HIV-1 co-receptor CXCR4 expression in human $CD4^+$ and $CD8^+$ T cells isolated from the large and small intestine and the mesenteric lymph nodes of BLT mice. **B)** Distribution of CXCR4 co-receptor expression in human T cell subsets in gut, lymphoid and non-lymphoid tissues of BLT mice. Shown are averages of the indicated replicates with their respective error bars (+/- SEM). $CD45^+CD3^+CD4^+$ or $CD45^+CD3^+CD8^+$ cells were analyzed for expression of CXCR4.

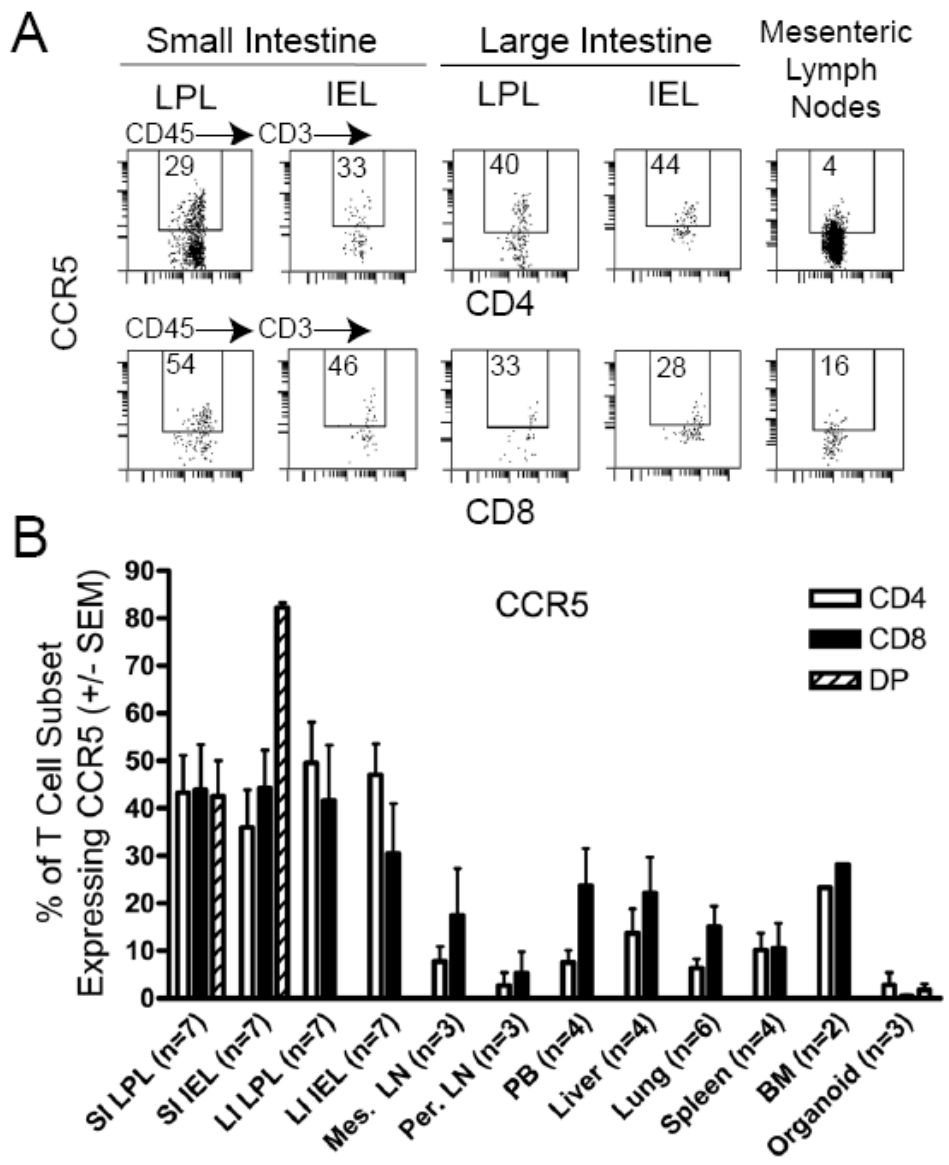

Figure 6. CCR5 chemokine receptor expression in BLT mice.
Panel **A)** shows a comparison of the levels of the HIV-1 co-receptor CCR5 expression in human $CD4^+$ and $CD8^+$ T cells isolated from the large and small intestine and the mesenteric lymph nodes of BLT mice. **B)** Distribution of CCR5 co-receptor expression in human T cell subsets in gut, lymphoid and non-lymphoid tissues of BLT mice. Shown are averages of the indicated replicates with their respective error bars (+/- SEM). $CD45^+CD3^+CD4^+$ or $CD45^+CD3^+CD8^+$ cells were analyzed for expression of CCR5.

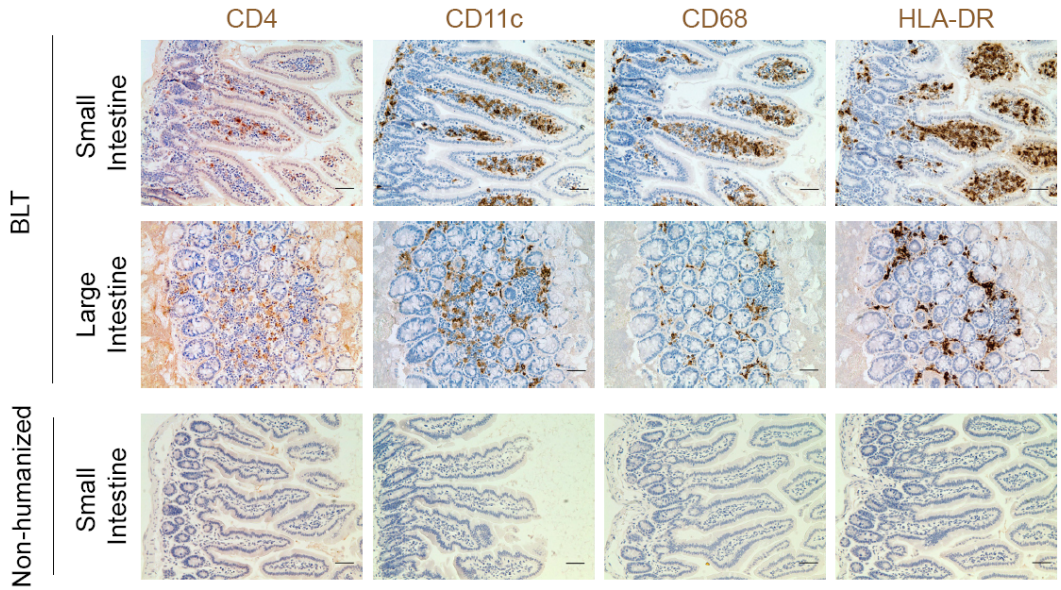

Figure 7. Immunohistocahemical analysis demonstrating the presence of human hematopoietic cells in BLT mouse GALT.
A representative BLT mouse small and large intestine (top) exhibits cells expressing human CD4 (T cells, monocytes and macrophages), CD11c (myeloid cells, subset of dendritic cells), CD68 (monocytes and macrophages) and HLA-DR (antigen presenting cells; B cell, activated T cells, DC and some macrophages). A non-humanized NOD/SCID mouse was stained in parallel with the same antibodies to demonstrate the specificity of the human-specific antibodies (bottom). Bars indicate 25μm. (Immunohistochemistry performed by Dr. Jacob Estes)

are reconstituted in a manner that closely resembles that of normal human and macaque tissues.

C. HIV-1_{LAI} infection in BLT mice

C1. Rectal transmission of HIV-1_{LAI} in BLT mice

A successful small animal model of mucosal transmission of HIV-1 would be a significant gain in the field of HIV research. Therefore, the susceptibility of the BLT mice to infection by HIV-1 administered intrarectally was determined. Both non-humanized control (n=4) and humanized BLT mice (repopulated with human cells, n=7) were inoculated intrarectally with a single dose of cell-free HIV-1_{LAI} (Alizon, Sonigo et al. 1984; Wain-Hobson, Sonigo et al. 1985) and subsequently monitored for evidence of infection using assays for viral load and HIV antigenemia in the plasma. None of the LAI inoculated non-humanized control mice showed any evidence of infection as defined by an absence of plasma antigenemia, an absence of plasma viral load and a lack HIV-1 RNA positive cells by in situ hybridization. Similarly, none of the uninfected humanized BLT control mice showed evidence of infection (n=4). In contrast, viral RNA and HIV antigenemia (capsid p24) were evident in the plasma of 6/7 LAI inoculated BLT mice (Figure 8). These results demonstrate the striking susceptibility of BLT mice to infection by HIV-1 administered intrarectally. Furthermore, human HIV specific antibodies (IgG) were found in the plasma of three of four infected BLT mice tested (Figure 9). The lower diversity of HIV proteins recognized by BLT mouse plasma relative to the control patient plasma could reflect a deficiency in B cell diversity within BLT mice. Knowing that potent immunization of BLT mice with sheep red blood cells leads to B cell–containing follicular-like structures in BLT spleen and more organized T-cell and B-cell zones developed in BLT lymph nodes (Melkus, Estes et al. 2006); an

alternative explanation for low antibody diversity is that germinal centers in BLT mice are incompletely formed in the absence of sufficient immunological stimulation with poor lymphocyte interaction and low antibody diversity being the consequence (Thorbecke, Amin et al. 1994). Perhaps pre-treating BLT mice with an immunization of sheep red blood cells then performing the HIV inoculations will result in a more robust anti-HIV antibody response.

C2. Rectally transmitted HIV-1_{LAI} depletes BLT peripheral blood $CD4^+$ T cells

The appearance of viral RNA in the plasma of rectally infected BLT mice (Figure 8) preceded or coincided with a decline in peripheral blood human $CD4^+$ T cells (Figure 10). This decline eventually resulted in an almost complete depletion of human $CD4^+$ T cells from peripheral blood (Figures 10 and 11). Parallel to the decline of $CD4^+$ T cells there was an increase in the percentage of human $CD8^+$ T cells that by 8 weeks post-inoculation represented the vast majority of the human T cells in the periphery of infected humanized BLT mice (Figures 10 and 11). These results demonstrate the striking susceptibility of BLT mice to infection by HIV-1 administered intrarectally.

C3. Rectal HIV-1_{LAI} infection results in virus dissemination in BLT mice

Peripheral blood serves as the most accessible site to monitor HIV replication but the lymphatic tissues are the principal sites of virus production, persistence, $CD4^+$ T cell depletion, and pathology (Haase 1999). Accordingly, different organs from intrarectally infected BLT mice (8 weeks post inoculation) were harvested and virus dissemination was examined. Replication competent HIV-1 was recovered from cells isolated from spleen, thymic organoid and bone marrow obtained from LAI infected BLT mice (Figure 12). Moreover, productively infected (HIV RNA^+) cells in lymph nodes, spleen and thymic organoid were identified and productively infected cells in lung, large/small intestines, and the male/female reproductive tracts were shown (Figure 13).

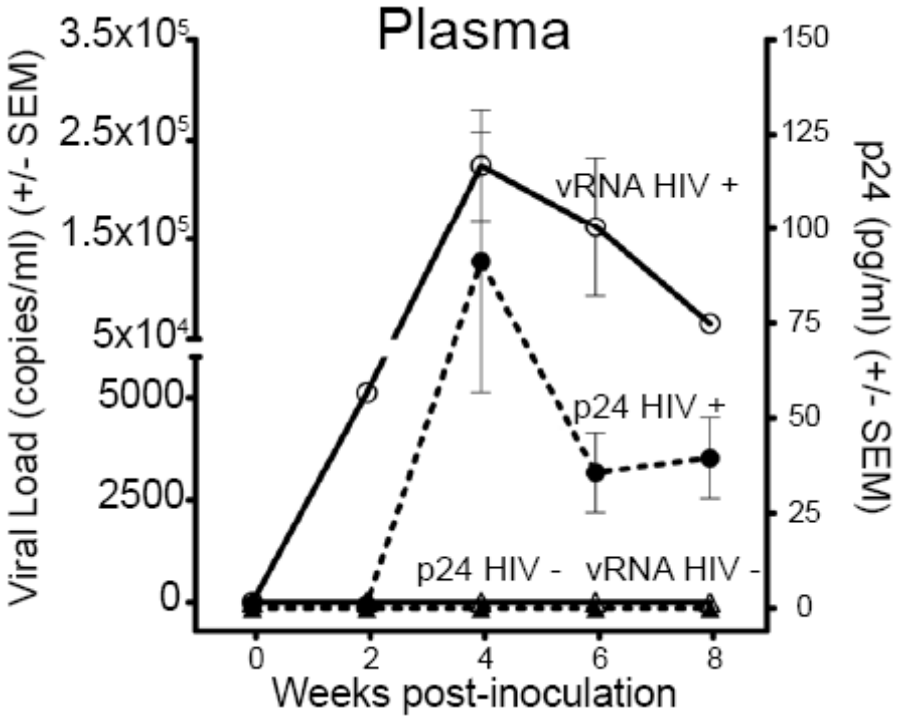

Figure 8. Plasma viremia and antigenemia detected in BLT mice after intrarectal HIV-1_{LAI} exposure.
Plasma from intrarectally infected BLT (circles) or mock infected control BLT mice (triangles) was analyzed for RNA viral load (copies per ml plasma, open symbols) and virus antigenemia (pg of p24 per ml plasma, closed symbols). (Viral RNA PCR performed by Dr. Deborah Payne and Jeannelle Vaughn of Veripath Laboratories, UT Southwestern Medical Center; p24 ELISA performed by Dr. Zhifeng Sun)

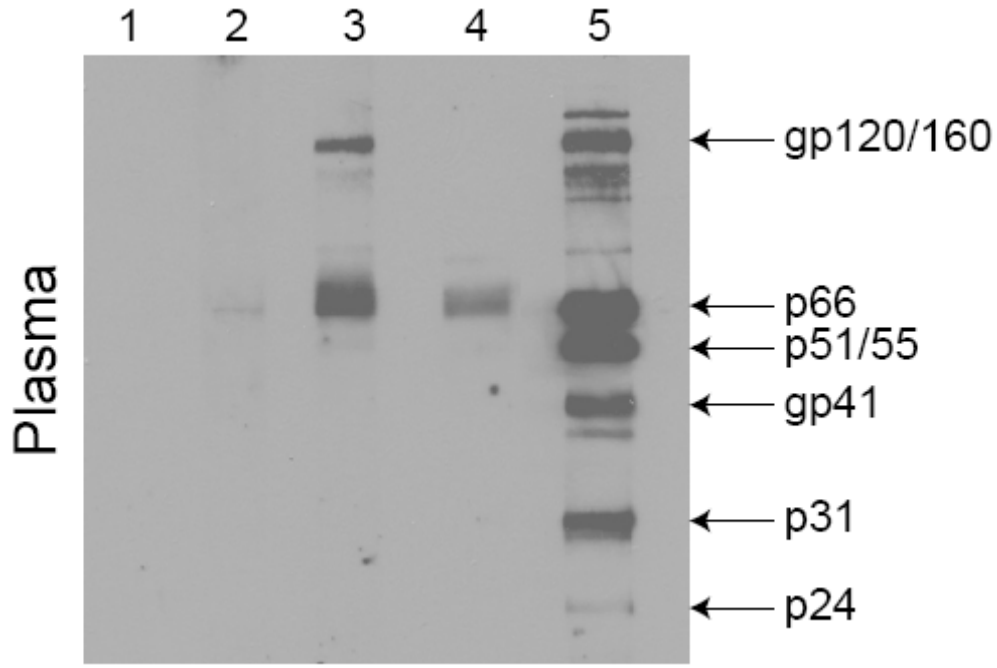

Figure 9. Human anti-HIV-1 IgG was detected in plasma from BLT mice rectally infected with HIV-1_{LAI}.
Western blot analysis of plasma from infected mice shows the presence of human IgG antibodies specific for HIV proteins. Lane 1, plasma from a control mouse. Lanes 2-4, plasma samples from 3 HIV infected mice. Lane 5, plasma from an HIV infected individual. The position of the different HIV proteins in the gel is indicated on the right. (Western blot analysis performed by Dr. Bangdong Luke Wei; Dr. Donald Sodora provided HIV^+ control plasma)

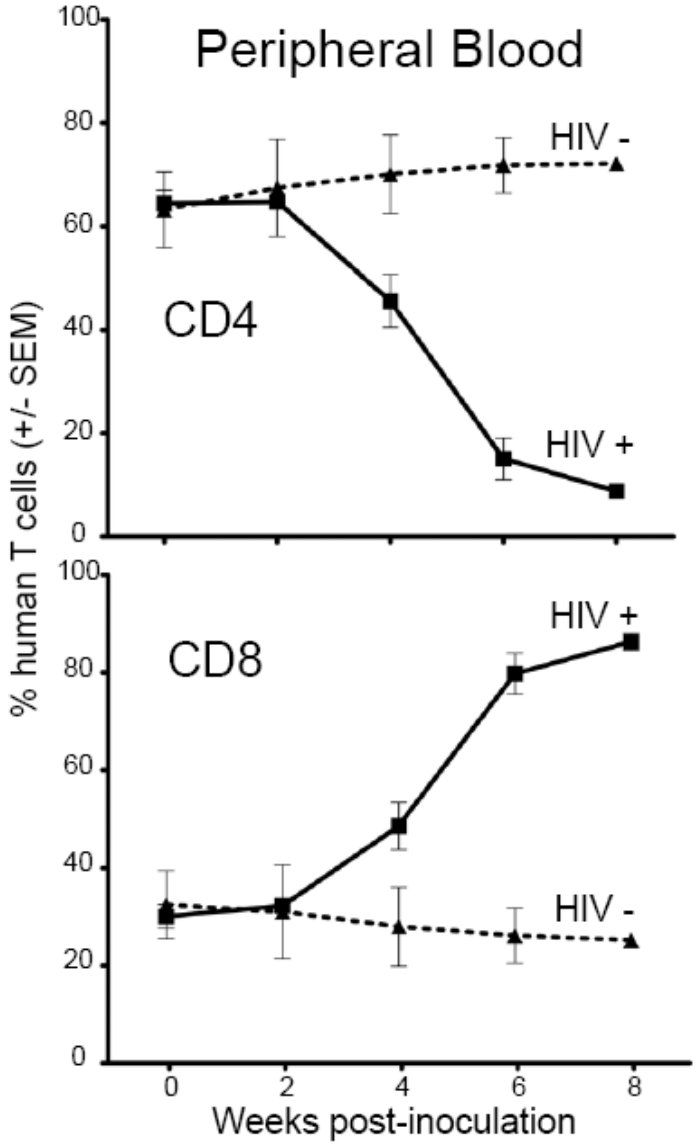

Figure 10. Rectal HIV-1_{LAI} infection in BLT mice results in a rapid loss of $CD4^+$ T cells from peripheral blood.
$CD4^+$ T cells (top) are depleted from the peripheral blood of LAI infected BLT mice (squares). CD4+ T cell levels remained stable in the absence of HIV-1 infection (triangles). A proportional increase in $CD8^+$ T cell levels (bottom) occurred as LAI depleted CD4+ T cells from peripheral blood. (HIV^{neg}: n=4 and HIV^{pos}: n=6)

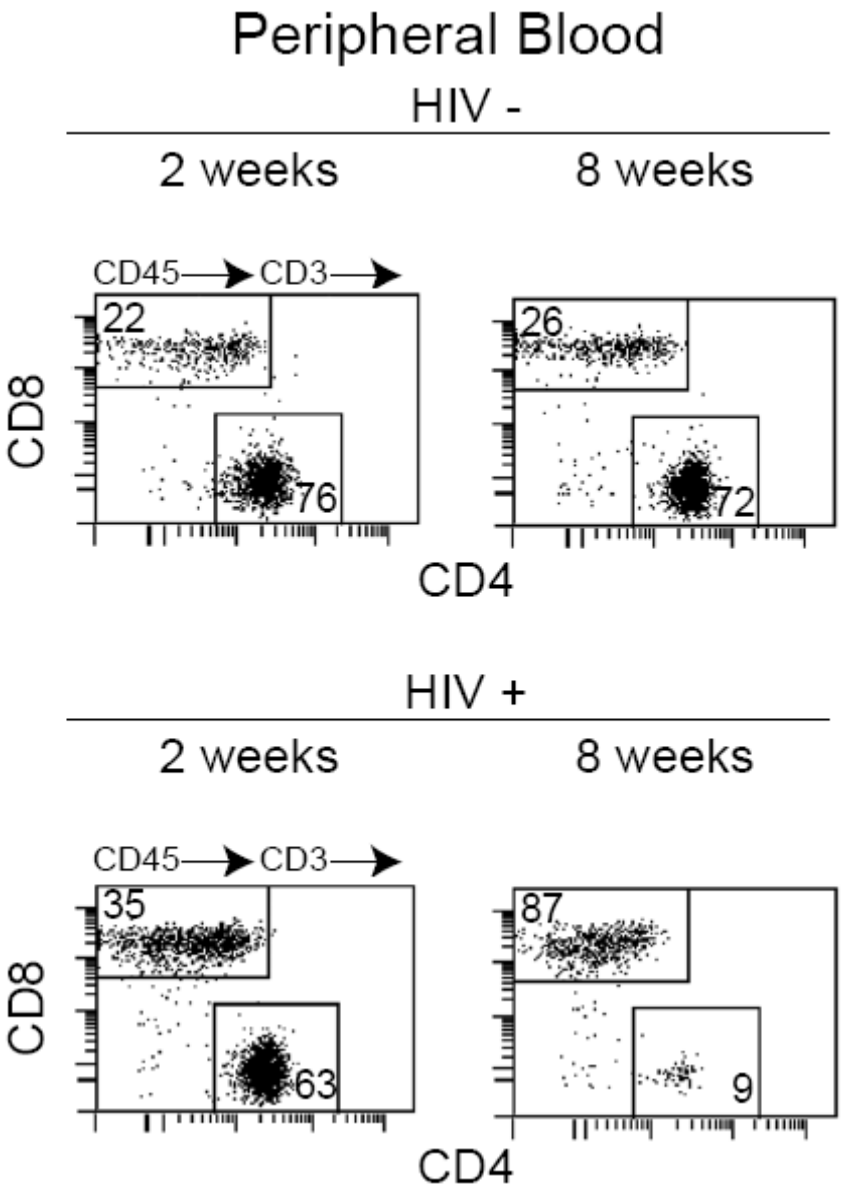

Figure 11. HIV-1_{LAI} rectally inoculated into BLT mice depletes human $CD4^+$ T cells in peripheral blood.
Flow cytometry data of the peripheral blood from a representative BLT mouse's peripheral blood at 2 time points separated by 6 weeks shows that human T cell levels in BLT mouse remain relatively stable over time in the absence of HIV-1 infection (top). Human T cell flow cytometry data of the peripheral blood from a representative infected BLT mouse two and eight weeks post intrarectal inoculation with LAI shows depletion of human $CD4^+$ T cells (bottom). The gating strategy was human $CD45^+ \rightarrow CD3^+$ and then CD4 and CD8.

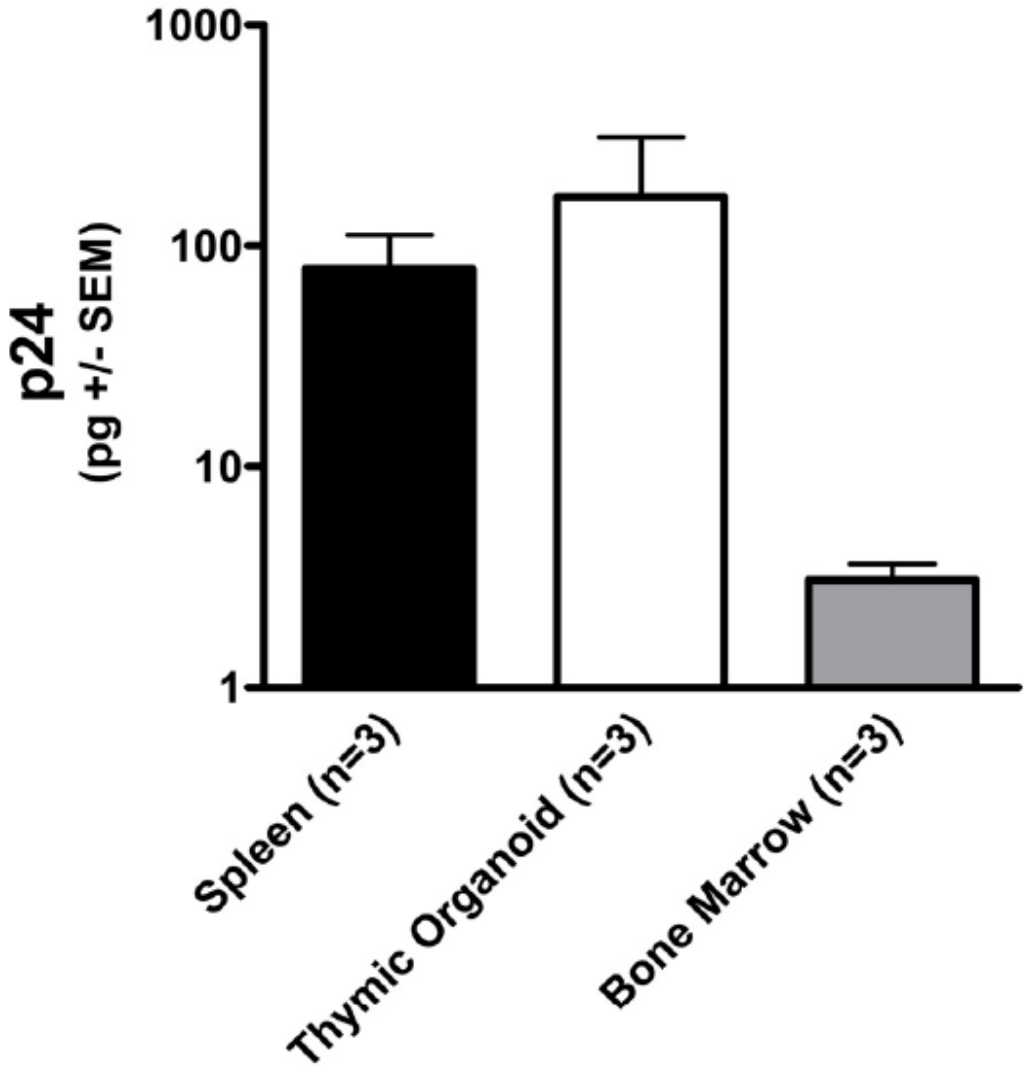

Figure 12. Replication competent virus was rescued from tissues isolated from BLT mice rectally infected with HIV-1_{LAI}.
Mononuclear cells isolated from the spleen, thymic organoid and bone marrow of LAI infected mice (n=3) were co-cultured with PHA and IL-2 activated human peripheral blood mononuclear cells isolated from a normal donor. Virus replication was measured using the presence of p24 in the supernatant as a surrogate marker. (Dr. Zhifeng Sun participated in this analysis)

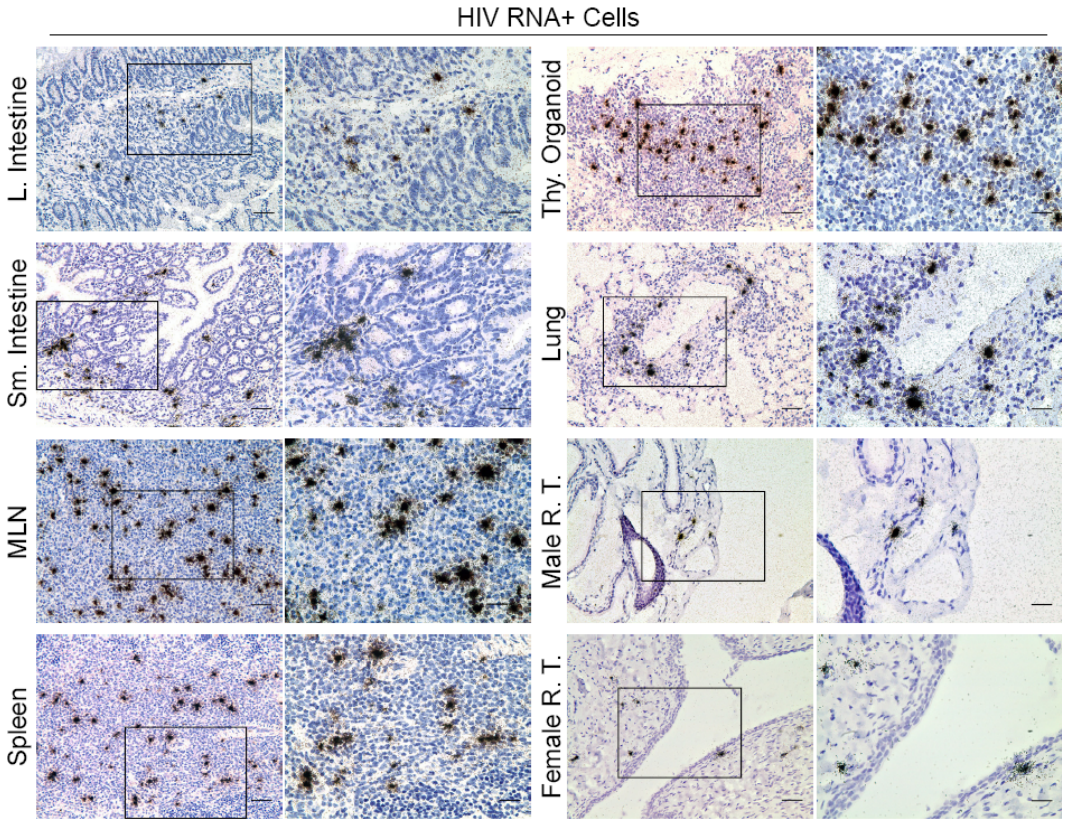

Figure 13. BLT mouse tissues exhibit systemic dissemination of HIV-1_{LAI} following intrarectal exposure.
In situ hybridization analysis of different tissues from infected BLT mice shows the presence of cells productively infected with HIV-1. Autoradiograph images were obtained using brightfield microscopy. Boxes indicate areas shown at higher magnification on the right and bars indicate 25μm and 12.5μm respectively. (In situ hybridization performed by Dr. Jacob Estes)

C4. Rectally transmitted HIV-1_{LAI} depletes $CD4^+$ T cells in BLT mouse tissues, including in GALT

Most productively infected HIV RNA^+ cells in BLT mice were human T cells defined by a combination of immunohistochemistry and in situ hybridization (Figure 14). Flow cytometry was utilized to determine the population of T cells being affected. A dramatic depletion of human $CD4^+$ T cells was observed in bone marrow, human thymic tissue, spleen, and lymph nodes of infected BLT mice (Figures 15 and 17). In addition, there was extensive depletion of human $CD4^+$ T cells from lung and liver (Figures 15 and 17). Since depletion of $CD4^+$ T cells from the GALT is a major consequence of HIV infection, the numbers of human lymphoid cells present in both small and large intestine after infection was characterized. When compared to uninfected BLT mice, there was a dramatic reduction in the levels of human $CD4^+$ T cells in the large and small intestine and in the mesenteric LN of HIV infected mice ($p<0.0001$) (Figures 16 and 17). However, there is an unanswered question regarding the X4-tropism of LAI and the relative levels of CXCR4 expression in GALT compared to other tissues (Figure 5); specifically, why are the GALT $CD4^+$ T cells depleted if there is undetectable CXCR4 expression on the surface of the majority of these cells? Two possible explanations are: 1) even though CXCR4 is not detected by flow cytometry at high levels in BLT GALT, there is sufficient CXCR4 on the $CD4^+$ T cells for LAI to gain entry into the targeted cells (Hori, Sakaida et al. 1998) and 2) the inflammatory environment created by the infection could lead to cytokine release within the GALT such that CXCR4 expression levels are elevated on $CD4^+$ T cells making them susceptible to LAI infection (Juffermans, Paxton et al. 2000). Nevertheless, these data are similar to data obtained from both human and macaque analyses showing a dramatic loss of $CD4^+$ T cells within this

compartment due to HIV or SIV infection, respectively (Brenchley, Schacker et al. 2004; Li, Duan et al. 2005).

The percentages of $CD8^+$ T cells increased throughout LAI infected BLT mice, except in the small intestine. Surprisingly, very few human $CD8^+$ T cells were found in the small intestine LPL and IEL fractions of HIV-infected mice (Figure 16 and 17). The proportion of $CD8^+$ T cells expressing CXCR4 in mesenteric LN and large intestine decreased whereas the levels of $CD8^+CCR5^+$ human T cells increased after HIV infection compared to these same tissues in uninfected mice (Figure 18). The most dramatic change in co-receptor expression was noted in the mesenteric lymph nodes, which contained very high levels of HIV-infected cells (Figure 18) and where virtually all $CD8^+$ human T cells expressed high levels of CCR5 (Figure 18). Consistent with what is seen in human infections (Brenchley, Schacker et al. 2004), the human T cells remaining in the BLT mouse gastrointestinal tract after infection had an effector memory phenotype ($CD27^{neg}$ $CD45RA^{neg}$) (Figure 19) (De Rosa, Herzenberg et al. 2001).

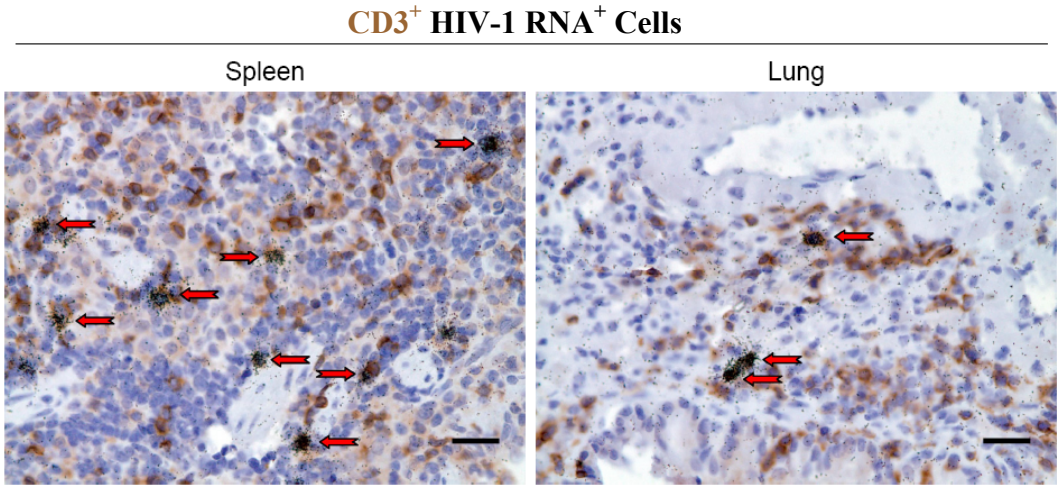

Figure 14. Human $CD3^+$ cells are productively infected with $HIV\text{-}1_{LAI}$ following intrarectal exposure.
$CD3^+$ cells (brown) producing HIV-1 RNA (black) are indicated by red arrows. These data were generated by in situ hybridization of HIV-1 RNA combined with immunocytochemical analysis for human CD3 expression in spleen and lung of BLT mice. Bars represent 12.5µm. (Analysis performed by Dr. Jacob Estes)

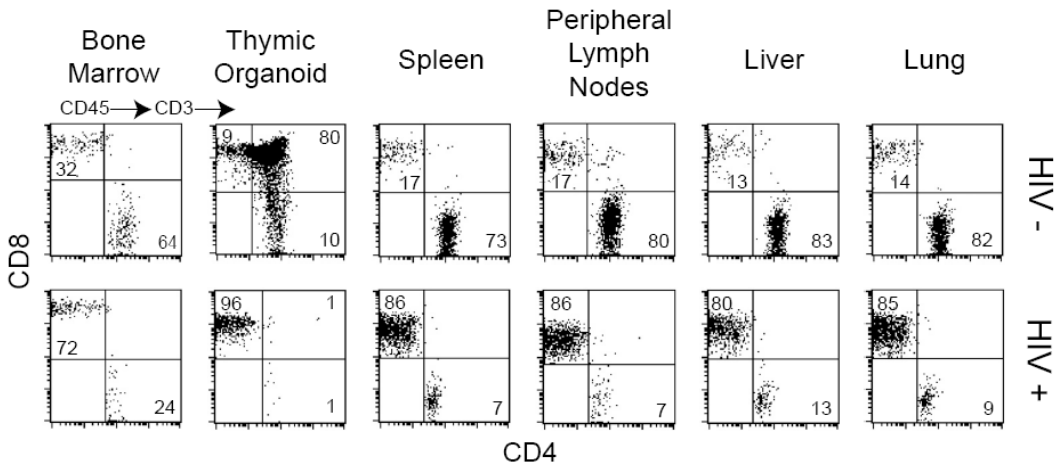

Figure 15. HIV-1_{LAI} depletes human $CD4^+$ T cells throughout rectally infected BLT mice.
Comparison of T cell levels between naive (top) and LAI infected (bottom) BLT mouse tissues reveals a LAI mediated depletion of $CD4^+$ T cells, including double positive thymocytes. The gating strategy was human $CD45^+ \rightarrow CD3^+$ and then CD4 and CD8. Analysis performed 8 weeks post-inoculation. (Florence Othieno assisted with this analysis)

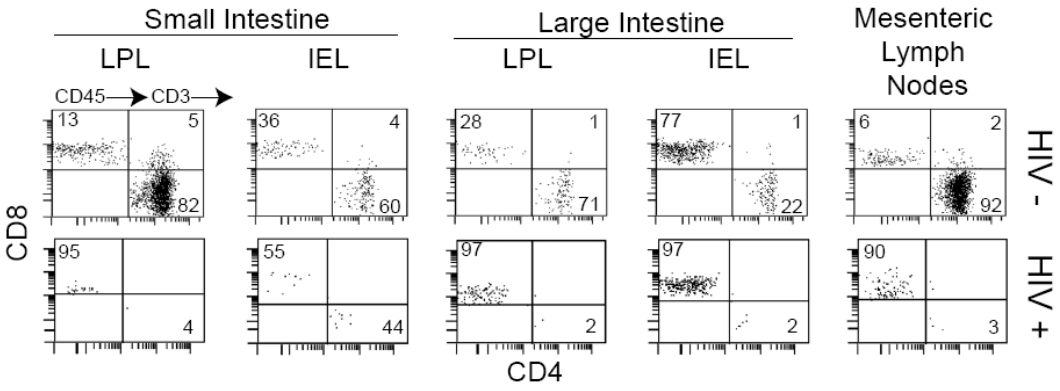

Figure 16. HIV-1_{LAI} depletes human $CD4^+$ T cells from the GALT of rectally infected BLT mice.
Comparison of T cell levels between naive (top) and LAI infected (bottom) BLT mouse GALT show LAI induced depletion of GALT human $CD4^+$ T cells. The gating strategy was human $CD45^+ \rightarrow CD3^+$ and then CD4 and CD8.

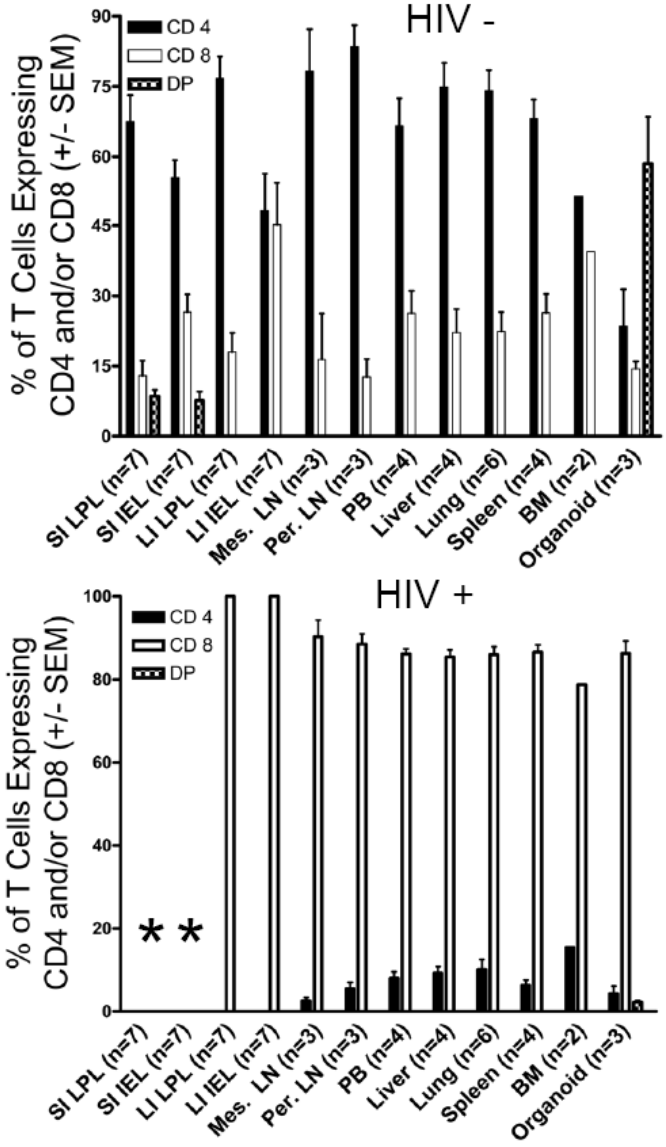

Figure 17. HIV-1_{LAI} depletes human $CD4^+$ T cells inducing a proportional increase in human $CD8^+$ T cells throughout rectally infected BLT mice. Comparison of T cell levels between naive (top) and LAI infected (bottom) BLT mouse tissues reveals a LAI mediated depletion of $CD4^+$ T cells along with an increase in $CD8^+$ T cells. The asterisks indicate a virtual absence of human cells in the small intestine following LAI infection. The gating strategy was human $CD45^+ \rightarrow CD3^+$ and then CD4 and CD8. Bars represent averages of the indicated number of mice (+/- SEM).

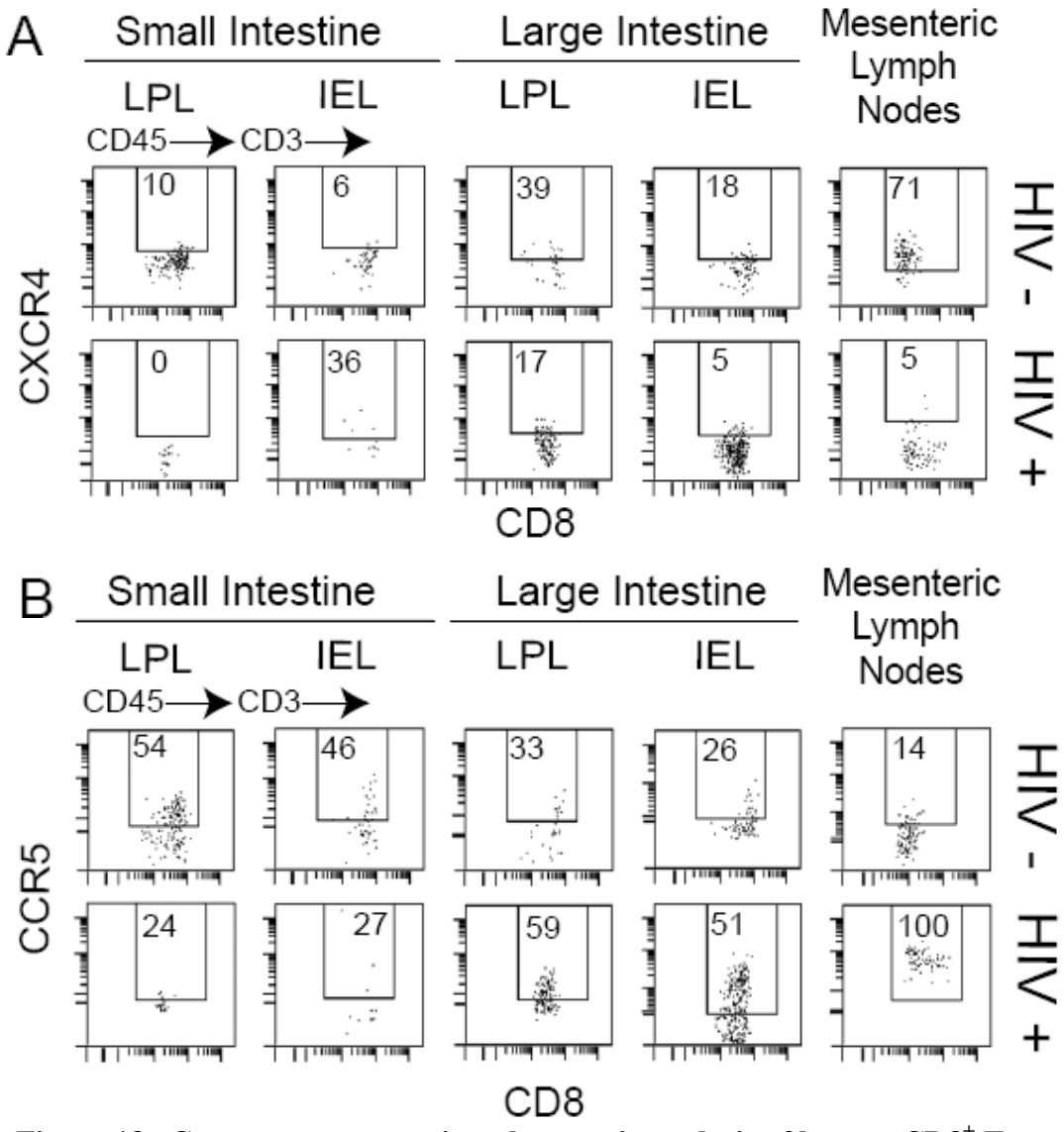

Figure 18. Co-receptor expression phenotypic analysis of human CD8$^+$ T cells following rectal HIV-1$_{LAI}$ infection.

Co-receptor expression in human CD8$^+$ T cells after LAI infection. Note the reduction in CXCR4 and increase in CCR5 on mesenteric lymph node and large intestine CD8$^+$ T cells in response to LAI infection. Cells were isolated from gut tissue 8 weeks post infection and from a non-infected control BLT mouse. Cells were then analyzed for CXCR4 and CCR5 expression. The gating strategy was CD45$^+$CD3$^+$CD8$^+$ and then CXCR4 or CCR5.

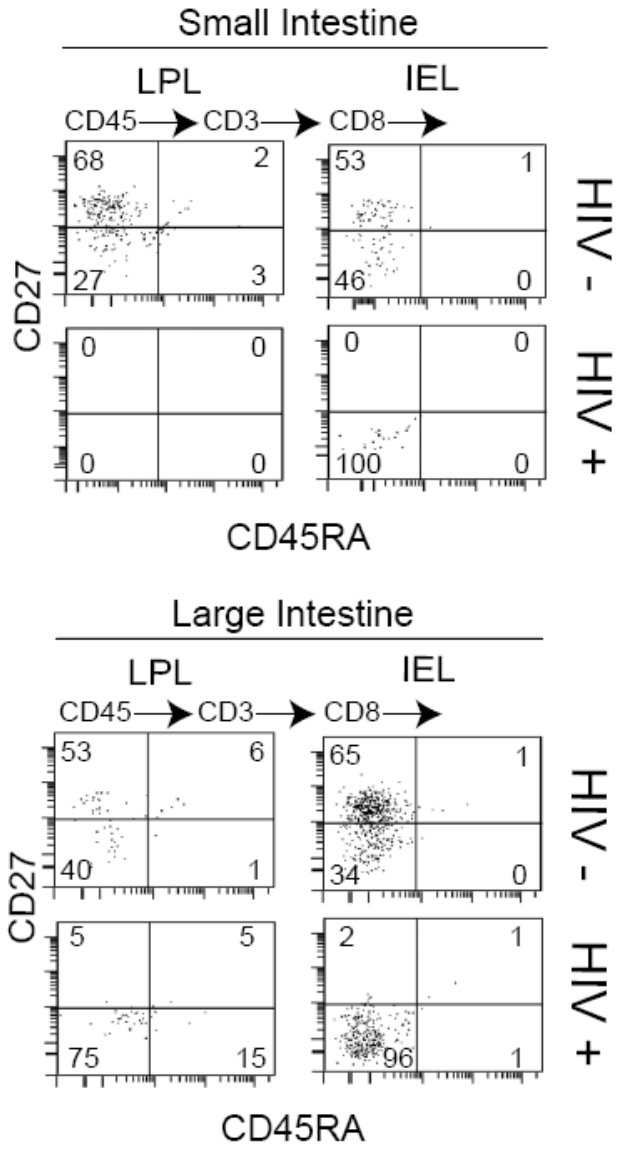

Figure 19. Human CD8$^+$ T cells in BLT GALT exhibit an effector memory phenotype following rectal HIV-1$_{LAI}$ infection.
CD8+ T cells isolated from BLT GALT following LAI infection were essentially all CD27^{neg}CD45RA^{neg} effector memory CD8$^+$ T cells. Note the small number of CD8$^+$ T cells isolated from small intestines of LAI infected BLT mice. The gating strategy was CD45$^+$CD3$^+$CD8$^+$ and then CD27 and CD45RA.

D. Discussion

Lymphocyte migration into effector tissues like the gut is the result of a series of poorly understood but highly complex interactions between cell adhesion molecules, integrins, chemokines and chemokine receptors. The data presented here demonstrate the high degree of compatibility between the NOD/SCID mouse and human systems resulting in the appropriate repopulation of the mouse gut tissue with human lymphoid cells. Human reconstituted in BLT gut closely resembles human gut descriptions in the literature (Abuzakouk, Carton et al. 1998; Carton, Byrne et al. 2004). Perhaps the most telling features were the presence of human $CD4^+CD8\alpha\alpha^+$ cells, a T cell subset known to exist only in GALT, and the presence of human lymphocytes (T and B), macrophages and DC found distributed throughout the effector lamina propria in BLT mice suggesting that these mice have largely "normal-human" GALT. However, some differences were also noted. For example, there was heterogeneity in the repopulation of human cells within the lamina propria of each BLT mice, with some portions of the gut demonstrating lower repopulation. In addition, there were somewhat higher percentages of $CD4^+$ T cells in BLT small intestine IEL compared to normal human gut and no γδ T cells were identified in BLT GALT.

Despite these differences and based on the remarkable similarities observed between the GALT of BLT mice and humans, the susceptibility of BLT mice to rectal HIV transmission was determined. A clear understanding of the molecular basis of mucosal HIV transmission has been hindered to a significant extent by the lack of adequate systems that recapitulate the events taking place during human infection. These results show remarkable similarities between intrarectally HIV infected BLT mice, HIV-infected humans and SIV-infected macaques including the devastating effects to the GALT (Guadalupe, Reay et al.

2003; Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004; Li, Duan et al. 2005; Mattapallil, Douek et al. 2005).

Because of the limited species tropism of HIV, there are few models where potential clinical interventions can be evaluated and where pathogenesis can be studied. The susceptibility of BLT mice to one cell-free intrarectal HIV-1 exposure points to the great potential of this system to study mucosal HIV transmission and prevention. In addition, the $CD4^+$ T cell depletion and disease defining pathology observed in this system demonstrate the utility of this small animal model to address key questions regarding HIV-induced pathogenesis. Furthermore, the AIDS-like pathology and depletion of the BLT GALT after HIV-1 infection highlight the model's usefulness to evaluate therapeutic interventions aimed at GALT reconstitution that might result in the possible reestablishment of a functional immune system in humans.

CHAPTER FOUR

Results

ANTIRETROVIRAL PRE-EXPOSURE PROPHYLAXIS PREVENTS VAGINAL TRANSMISSION OF HIV-1 IN HUMANIZED BLT MICE

A. Introduction

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) is predominantly transmitted by unprotected sexual contact (WHO-UNAIDS 2006). Currently, women worldwide account for more than half of the estimated 6,800 newly acquired infections every day with a majority of those transmissions occurring via the vaginal route (WHO-UNAIDS 2006; WHO-UNAIDS 2007). Therefore, it is critical that strategies to prevent vaginal transmission of HIV are developed and implemented.

Development and efficacy testing of microbicides and other preventive strategies such as antiretroviral pre-exposure prophylaxis necessitate animal models (Veazey, Klasse et al. 2005; Derdelinckx, Wainberg et al. 2006; Gupta and Klasse 2006; Lederman, Offord et al. 2006; Subbarao, Otten et al. 2006). Currently, the only surrogate animal model used to study intravaginal HIV transmission is infecting macaques with simian immunodeficiency virus (SIV) or SIV/HIV (SHIV) chimeric viruses (Miller, Alexander et al. 1989; Pauza, Horejsh et al. 1998). This model does not support HIV-1 replication. Yet, it recapitulates many aspects of the human infection and was used recently in elegant experiments to demonstrate protection of macaques from vaginal SHIV transmission by topical and orally delivered CCR5 inhibitors (Lederman, Veazey et al. 2004; Veazey, Klasse et al. 2005; Veazey, Springer et al. 2005).

There is significant need to develop other animal models to investigate measures to prevent intravaginal HIV-1 transmission because a model of transmission with the human virus and human cells would complement the macaque model in HIV research. BLT mice have the potential to fill this need as they exhibit complete systemic reconstitution of all major human hematopoietic lineages, including T, B, monocyte/macrophage, dendritic and natural killer cells (Melkus, Estes et al. 2006). Particularly relevant to this study is the extensive reconstitution within the gut of BLT mice (Melkus, Estes et al. 2006; Sun, Denton et al. 2007) (Chapter 3). With this particular finding in mind, hypotheses were formulated that mucosal reconstitution with human lymphoid cells would include the female reproductive tract and this reconstitution would render female BLT mice susceptible to intravaginal HIV-1 infection. The study presented in this chapter was designed to conclusively address these hypotheses. The aims were: (1) to characterize the reconstitution of the FRT with human lymphoid cells, (2) to test the susceptibility of BLT mice to viral transmission following a single intravaginal exposure to cell-free HIV-1; (3) to characterize systemic pathogenic effects of HIV-1 transmitted intravaginally and disseminated throughout BLT mice, including effects in the gut-associated lymphoid tissue (GALT); and (4) to utilize this small animal model to conduct pre-clinical evaluation of antiretroviral pre-exposure prophylaxis for intravaginal HIV-1 transmission.

B. Human lymphocytes populate the BLT mouse female reproductive tract

The FRT represents a highly specialized and complex anatomical site where initial infection occurs following intravaginal exposure (Hu, Gardner et al. 2000; Miller, Li et al. 2005; Hladik, Sakchalathorn et al. 2007). Therefore, immunohistochemistry and flow cytometry were used to determine if human lymphocytes and other cells important for HIV-1 infection were present in the

vagina, ectocervix, endocervix and uterus after reconstitution of BLT mice with human HSC. Populations of human cells necessary for HIV-1 infection ($CD4^+$ T cells, macrophages and dendritic cells) were abundant throughout the FRT of BLT mice (Figures 20 and 21). Specifically, human $CD4^+$ cells were distributed throughout the FRT. Also, human $CD68^+$ monocyte/ macrophage cells, clusters of human $CD11c^+$ dendritic cells and HLA-DR^+ antigen presenting cells were identified throughout the FRT. In addition, HIV-1 co-receptor expression was detected on the $CD4^+$ T cells of the BLT mouse FRT (Figure 21). Together these remarkable data establish that in situ differentiated human hematopoietic cells are capable of migrating into and reconstituting the FRT of BLT mice with the human cells relevant to mucosal HIV-1 transmission (Givan, White et al. 1997; Johansson, Rudin et al. 1999; Poonia, Wang et al. 2006).

C. Vaginal transmission of CXCR4-tropic HIV-1_{LAI} in BLT mice

Once the presence of HIV-1 target cells in the FRT of BLT mice was established, vaginal HIV-1 challenge became a priority. HIV-1_{LAI} was the first isolate used for an intravaginal inoculation. LAI uses CXCR4 for its co-receptor and is a highly pathogenic laboratory isolate. Three female BLT mice were challenged intravaginally with LAI and all three of the mice became infected (3 of 3). Virus dissemination and depletion of $CD4^+$ T cells, including in peripheral blood, following vaginal LAI infection mirrored the results presented in Chapter 3 for rectal LAI infection (Figures 22 and 23).

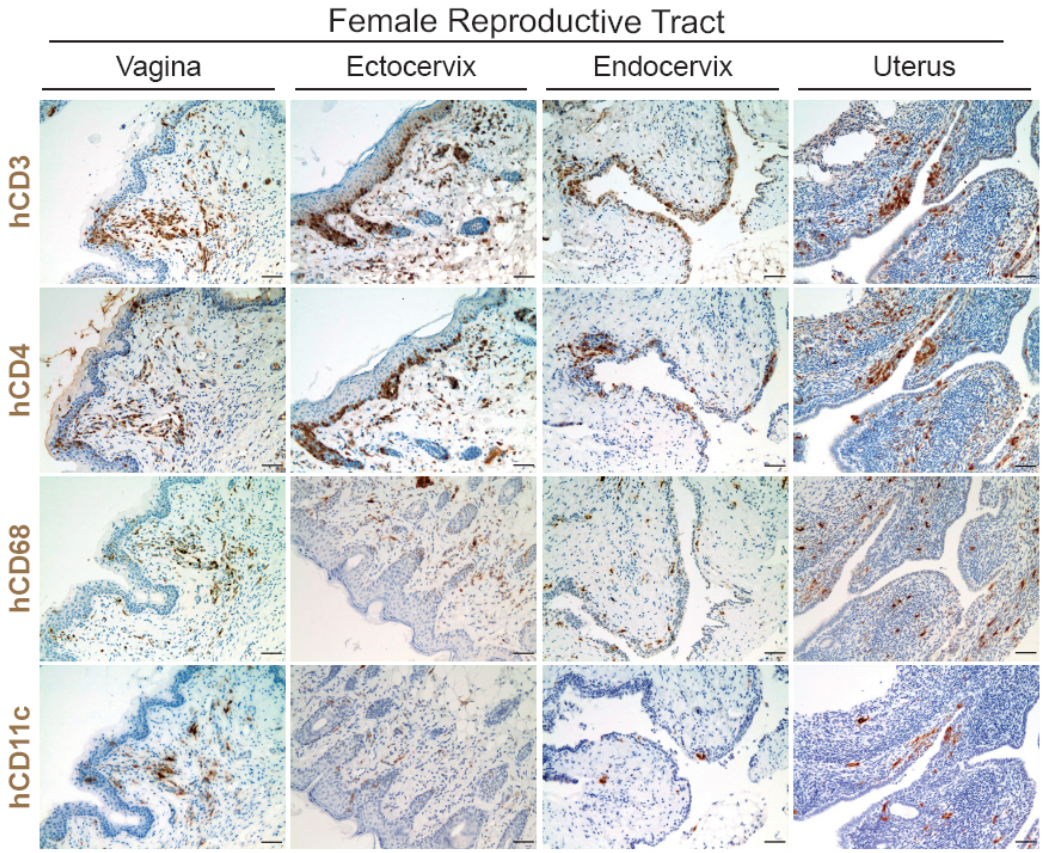

Figure 20. Reconstitution of the female reproductive tract of BLT mice with human hematopoietic cells.
Immunohistochemical analysis of the vagina, ectocervix, endocervix and uterus of female BLT mice showing the presence of human hematopoietic lineages (brown cells) (bars = 25 μm). Robust reconstitution with cells relevant to HIV-1 infection (including human T cells, monocyte/macrophages and dendritic cells) was observed in each compartment of the FRT of BLT mice. This data demonstrates the efficient repopulation of these important mucosal sites.
(Immunohistochemistry performed by Dr. Jacob Estes)

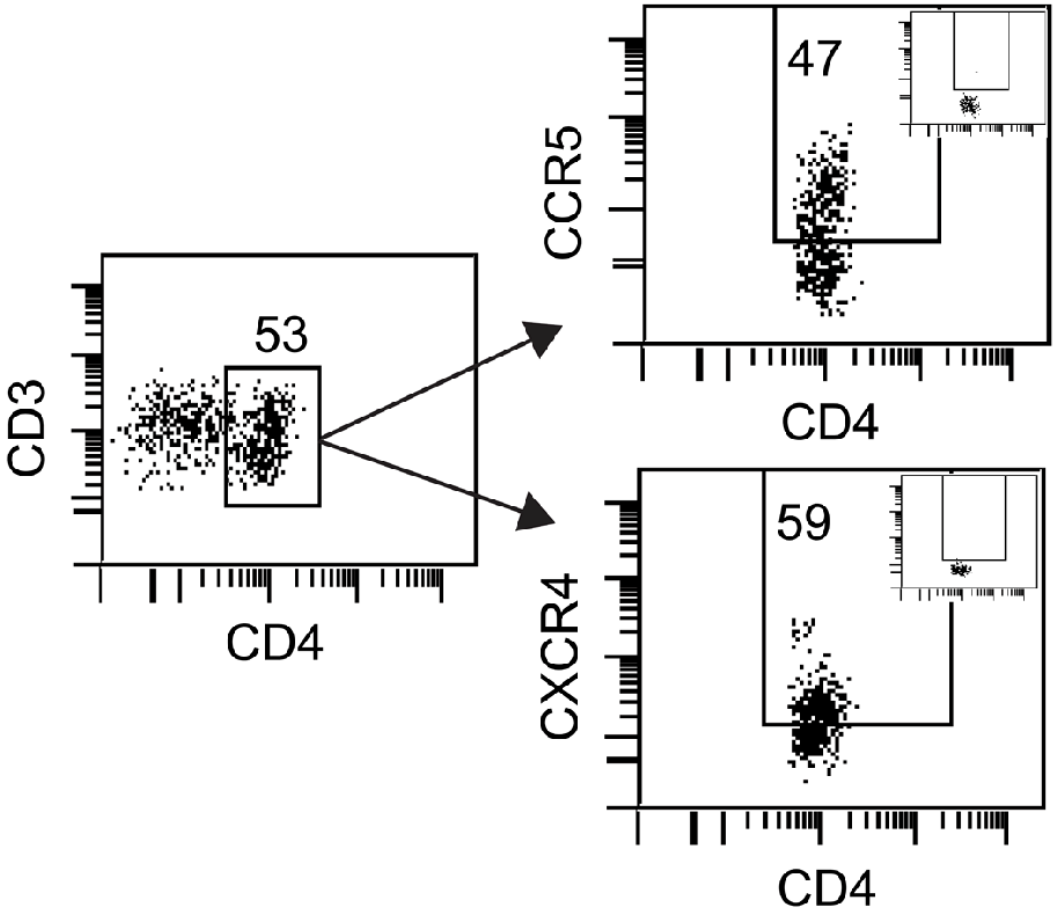

Figure 21. Human $CD4^+CXCR4^+$ and $CD4^+CCR5^+$ T cells were identified in the female reproductive tract of BLT mice.
The presence of human $CD4^+$ T cells in BLT FRT is shown in the left panel. HIV-1 co-receptor expression on these $CD4^+$ T cells is shown in the right two panels. CCR5 (top) and CXCR4 (bottom) were both observed on FRT $CD4^+$ T cells. The vagina, ectocervix, endocervix and uterus were processed together as the FRT for this analysis. Small inset boxes show isotype control stains used to set the gate positions.

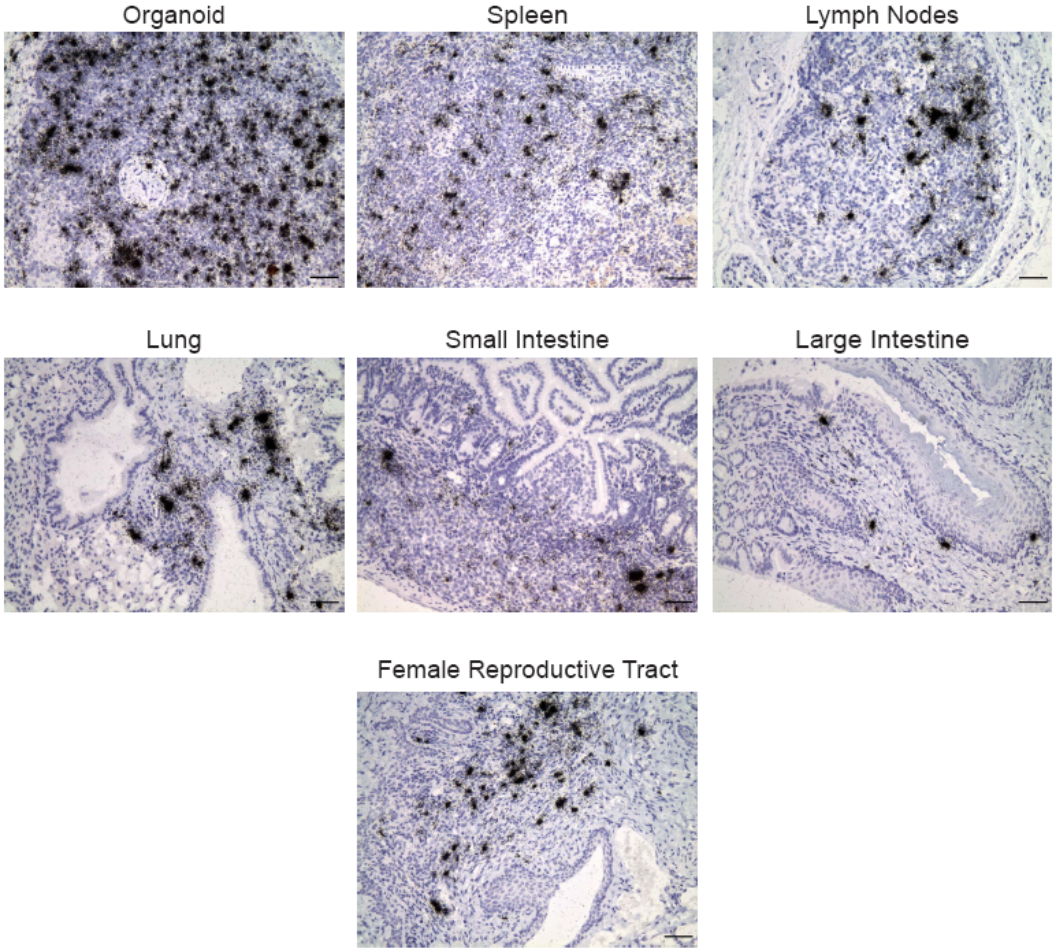

Figure 22. HIV-1_{LAI} disseminates throughout BLT mice following vaginal inoculation. (CXCR4-tropic isolate)
In situ hybridization analysis of different tissues from vaginally infected BLT mice shows the presence of cells productively infected with HIV-1. Autoradiograph images were obtained using brightfield microscopy. Tissues analyzed were harvested 36 days following vaginal LAI inoculation. Bars indicate 25μm. (In situ hybridization performed by Dr. Jacob Estes)

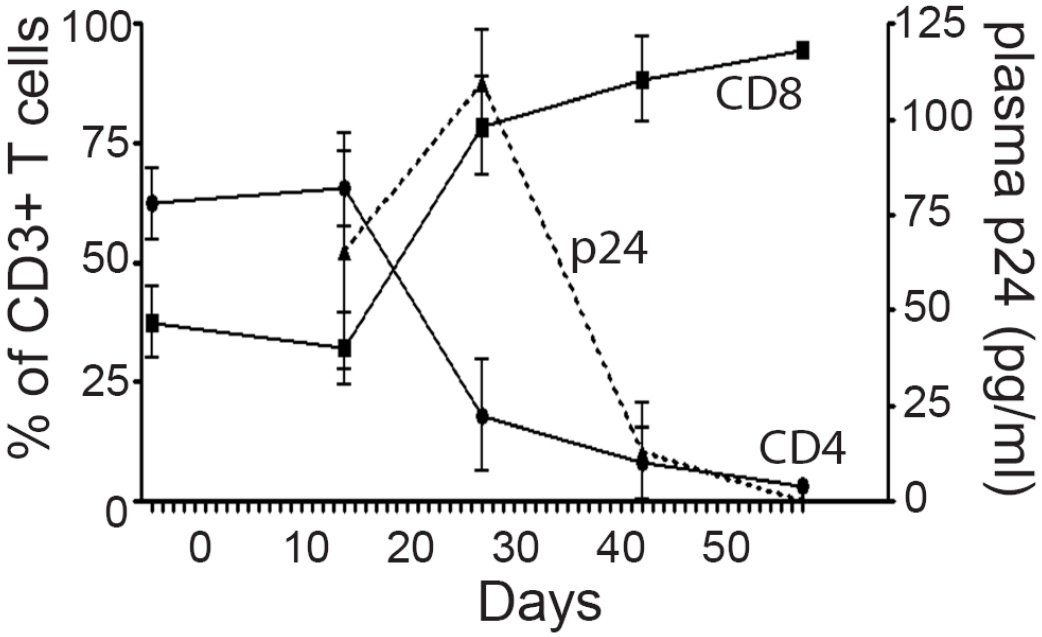

Figure 23. Vaginal HIV-1_{LAI} infection results in plasma antigenemia and rapid $CD4^+$ T cell loss in peripheral blood of BLT mice. (CXCR4-tropic isolate) Rapid depletion of human $CD4^+$ T cells (circles) from the peripheral blood of infected BLT mice that were inoculated with LAI intravaginally on Day 0. There was a concomitant $CD8^+$ T cell (squares) increase proportional to the loss of CD4+ T cells. Plasma from intravaginally infected BLT mice (triangles) was analyzed for virus antigenemia. Shown are the means +/- SEM for all 3 LAI vaginally infected mice. Gating strategy for flow cytometric analysis: live cells → human CD45 → human CD3 →human CD4 or CD8. (p24 ELISA performed by Dr. Zhifeng Sun)

D. Vaginal transmission of CCR5-tropic HIV-1_{JR-CSF} in BLT mice

D1. Vaginal HIV-1_{JR-CSF} transmission in BLT mice is highly efficient and can be prevented by pre-exposure prophylaxis with antiretroviral drugs

The susceptibility of BLT mice to the transmission of HIV-1 administered intravaginally was also determined. Prior to HIV-1 exposure, the peripheral blood of all BLT mice to be used in this study (8 to 12 weeks post transplant) was analyzed. Based on hCD45 expression it was determined that, on average, slightly more than half (51.9% ±7.2%) of all circulating peripheral blood cells were of human origin. BLT mice (n=8) were inoculated with a single dose of cell-free HIV-1_{JR-CSF} (Koyanagi, Miles et al. 1987). BLT mice that did not receive HIV-1 (n=6) were used as naive controls. In addition, intravaginal HIV-1 transmission in BLT mice administered a seven-day course of antiretroviral drugs (n=5) was determined. Emtricitabine and tenofovir disoproxil fumarate (FTC/TDF) were used because of potency, daily dosing, and favorable profiles for both toxicity and viral resistance (De Clercq 2007). FTC/TDF was administered two days prior to intravaginal inoculation, 3 hours prior to inoculation, and for four days post inoculation. Whereas 88% (7/8) of BLT mice inoculated with HIV-1 became infected, none of the animals (0/7) that received FTC/TDF showed evidence of infection (Chi square=9.2, df=1, p=0.002) (Figures 24 and 25).

Neither naive nor FTC/TDF treated BLT mice showed any evidence of plasma antigenemia (Figure 26A&C). In contrast, HIV-1 antigenemia was evident in the plasma from 7/8 intravaginally inoculated BLT mice as early as two weeks post-infection (Figure 26B). Infection was corroborated by determining the viral load in the plasma of infected BLT mice. On average, $5.0x10^5(\pm 1.5x10^5)$ copies of RNA were detected per ml of plasma from the infected mice (Figure 25).

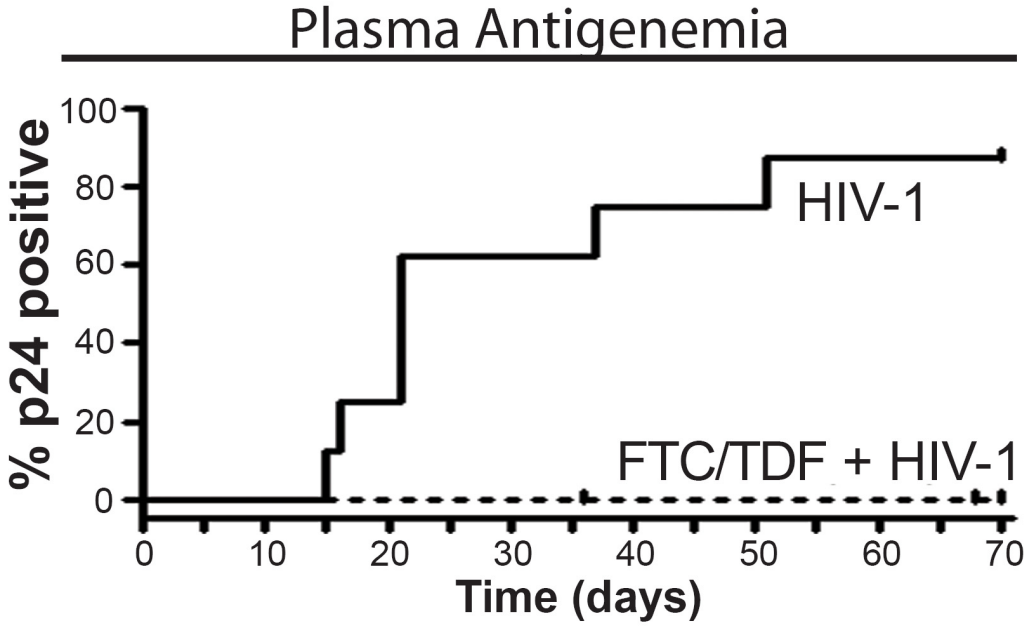

Figure 24. Plasma antigenemia is not detected in BLT mice given pre-exposure prophylaxis with intravaginal HIV-1$_{JR-CSF}$ exposure. (CCR5-tropic isolate)

This Kaplan-Meyer plot shows the time course to plasma antigenemia conversion following intravaginal JR-CSF exposure in BLT mice with or without the 7-day pre-exposure regimen of FTC/TDF. (p24 ELISA performed by Dr. Zhifeng Sun)

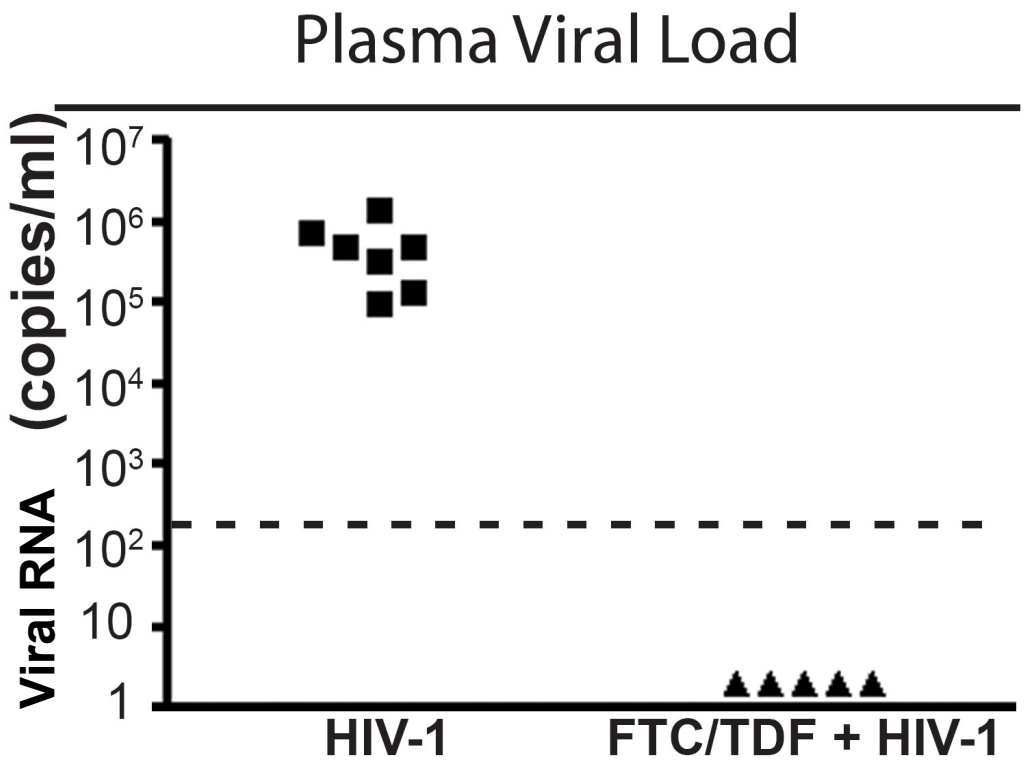

Figure 25. Plasma viral RNA is not detected in BLT mice given pre-exposure prophylaxis with intravaginal HIV-1_{JR-CSF} exposure.
Plasma from the 7 infected BLT mice and 5 of the FTC/TDF + JR-CSF mice were tested for the presence of viral RNA. Data presented depict the initial positive viral RNA value for each mouse examined. The dashed line indicates the limit of detection for this assay. Four FTC/TDF + JR-CSF mice were negative for viral RNA at 28, 55, and 70 post-exposure; one FTC/TDF + JR-CSF mouse was negative for viral RNA at time of kill (36 days post-exposure) (a single time point for each mouse is depicted). (Viral RNA PCR performed by Dr. Deborah Payne and Pei Irwin of Veripath Laboratories, UT Southwestern Medical Center)

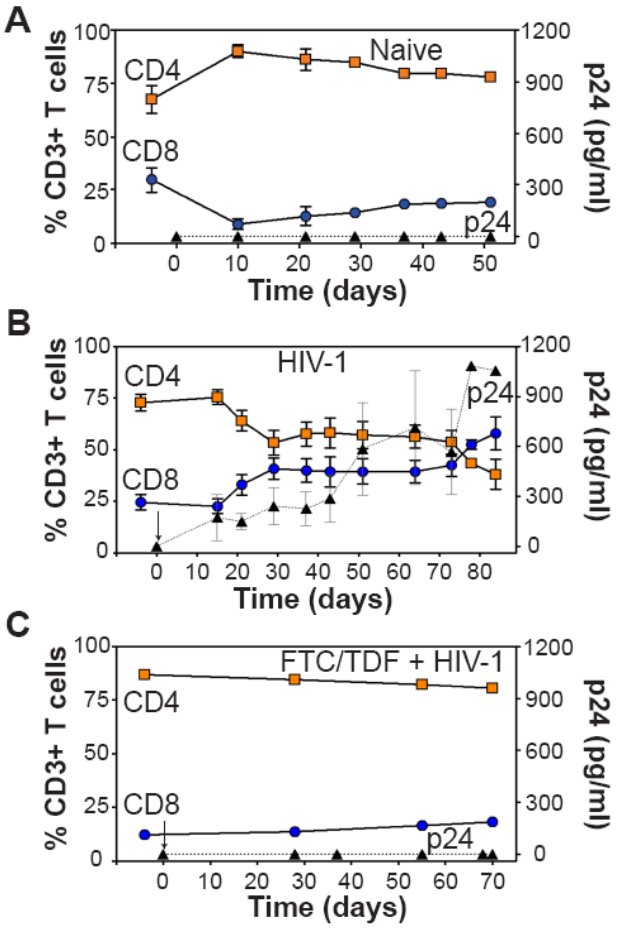

Figure 26. Peripheral blood CD4+ T cells are retained in BLT mice given pre-exposure prophylaxis with intravaginal HIV-1$_{JR-CSF}$ exposure.
A-C) Shown are the levels of human CD4$^+$ (orange squares) and CD8$^+$ (blue circles) T cells in peripheral blood as well as the levels of virus antigenemia (black triangles) in plasma for (**A**) naive control (n=6), (**B**) JR-CSF infected (n=7) and (**C**) pre-exposure FTC/TDF treated animals (n=5). In part B, note that in 7 of 8 tested BLT mice a single exposure to JR-CSF led to intravaginal transmission and an initial drop, with subsequent stabilization, in the levels of peripheral blood CD4$^+$ human T cells. In contrast, no changes were observed in either the naive control (A) or BLT mice that received FTC/TDF for pre-exposure prophylaxis (C). In (B) and (C) day 0 is the day of inoculation and is indicated by an arrow. Gating strategy for flow cytometric analysis: live cells → human CD45 → human CD3 →human CD4 or CD8. (p24 ELISA performed by Dr. Zhifeng Sun)

D2. Vaginally transmitted HIV-1$_{JR-CSF}$ reduces CD4$^+$ T cells levels in BLT mice

The appearance of HIV-1 in the plasma of infected mice preceded or coincided with a decline in peripheral blood human CD4$^+$ T cells (Figure 26). The levels of CD4$^+$ T cells dropped by 30% during the first three weeks post infection and remained relatively constant for 7 weeks at which point there was a further 20% decline and an inversion of the ratio of CD4/CD8 cells (Figure 26). Parallel to the decline of CD4$^+$ T cells there was an increase in the percentage of human CD8$^+$ T cells in the periphery of infected BLT mice, which by 11 weeks post-infection represented 60% of all the CD3$^+$ cells in the periphery (Figure 26). To eliminate the possibility that the lack of HIV-1 infection in FTC/TDF treated mice resulted from the absence of cells that could be infected by HIV-1 within the mucosal portal of entry; the FRT of FTC/TDF treated mice were examined for human CD4$^+$ cells. The presence of CD4$^+$ human cells in the vagina of inoculated mice that received pre-exposure prophylaxis with FTC/TDF rules out a lack of hematopoietic reconstitution of the FRT as responsible for the lack of infection (Figure 27). Together these results demonstrate the striking susceptibility of BLT mice to infection by HIV-1 administered intravaginally and highlight the extensive similarity in the course of HIV-1 infection in peripheral blood between BLT mice, humans and rhesus macaques (infected with R5-tropic SHIV) including plasma viremia and CD4$^+$ T cell depletion from peripheral blood (Lu, Brosio et al. 1996; Harouse, Gettie et al. 1999; Hsu, Ho et al. 2005). Perhaps more importantly, these data demonstrate that pre-exposure prophylaxis affords complete protection to BLT mice from vaginal HIV-1 transmission.

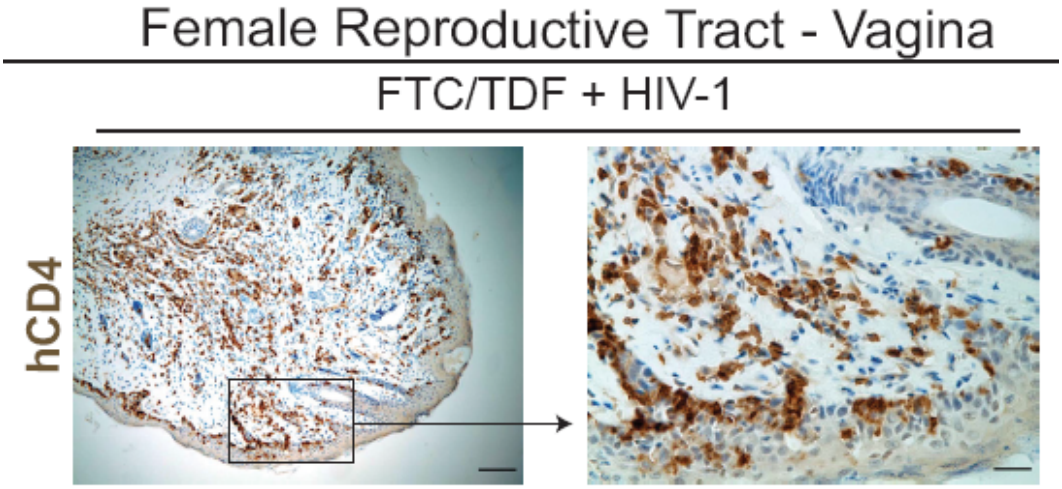

Figure 27. Immunohistochemical staining for human $CD4^+$ cells within the vagina of a representative FTC/TDF treated mouse.
These sections demonstrate the continued presence of $CD4^+$ T cells in this tissue to eliminate a lack of HIV-1 targets cells explaining the protection offered by FTC/TDF pre-exposure prophylaxis (left panel bar = 50 um; right panel bar = 12.5 um; box indicates region magnified in subsequent image).
(Immunohistochemistry performed by Dr. Jacob Estes)

The systemic effects of HIV-1 infection in humans are inherently difficult to study. Yet, the systemic repopulation of BLT mice with human lymphocytes facilitates the evaluation of the effects of HIV-1 infection in relevant internal organs. Since $CD4^+$ T cell depletion is a hallmark of HIV-1 infection, the levels of these cells throughout the body of naive vs. HIV-1 infected vs. pre-exposure FTC/TDF treated BLT mice was determined. No statistical difference was observed when $CD4^+$ T cell levels in naive and pre-exposure FTC/TDF treated BLT mice were compared ($Mean_1$-$Mean_2$ (M_1-M_2)=1.2 ± 8.8, t=0.13, df=65, p=0.90). However, when comparing HIV-1 infected vs FTC/TDF treated and HIV-1 exposed mice, statistically significant reductions in $CD4^+$ T cells were noted in peripheral blood (M_1-M_2=-49 ± 13, t=3.8, df=5, p=0.012), bone marrow (M_1-M_2=-52 ± 4.1, t=13, df=5, p<0.001), spleen (M_1-M_2=-36 ± 4.7, t=7.5, df=5, p<0.001), lymph nodes (M_1-M_2=-28 ± 7.4, t=3.7, df=5, p=0.013), liver (M_1-M_2=-34 ± 11, t=3.2, df=5, p=0.024) and lung (M_1-M_2=-45 ± 8.1, t=5.6, df=5, p=0.003) in HIV-1 infected mice; no significant difference was noted in the thymic organoid (M_1-M_2=1.8 ± 4.5, t=0.40, df=5, p=0.70) (Figure 28). Together with the reduction in the levels of $CD4^+$ human T cells there was a concomitant statistically significant increase in the levels of $CD8^+$ human T cells comparing HIV-1 infected vs FTC/TDF treated and HIV-1 exposed mice in all tissues tested including peripheral blood (M_1-M_2=45 ± 13, t=3.4, df=5, p=0.019), bone marrow (M_1-M_2=46 ± 3.5, t=13, df=5, p<0.001), thymic organoid (M_1-M_2=26 ± 9.9, t=2.7, df=5, p=0.045), spleen (M_1-M_2=29 ± 2.8, t=10, df=5, p<0.001), lymph nodes (M_1-M_2=27 ± 7.8, t=3.4, df=5, p=0.019), liver (M_1-M_2=34 ± 10, t=3.4, df=5, p=0.019) and lung (M_1-M_2=40 ± 6.5, t=6.2, df=5, p=0.002) (Figure 28).

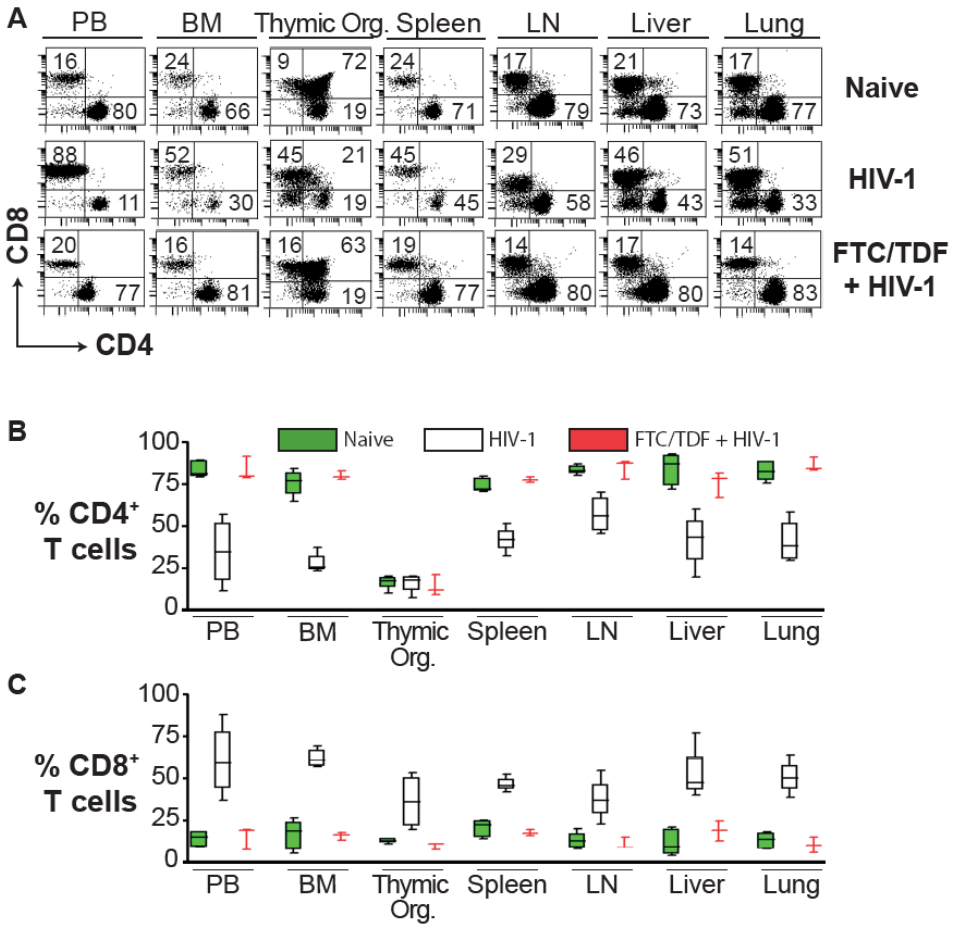

Figure 28. Systemic CD4$^+$ T cell loss resulting from intravaginal HIV-1$_{JR-CSF}$ infection in BLT mice.

A) Comparison of the levels of CD4$^+$ or CD8$^+$ human T cells in the indicated tissues in representative BLT mice that were either naive, JR-CSF infected or that received FTC/TDF for pre-exposure prophylaxis prior to exposure to JR-CSF. Note the JR-CSF induced reduction in CD4$^+$ T cells throughout and the loss of double positive CD4$^+$CD8$^+$ thymocytes. **B-C**) Box plots depicting the levels of CD4$^+$ (B) or CD8$^+$ (C) T cells in the indicated tissues for naive (green), HIV-1 infected (white) and FTC/TDF treated plus HIV-1 exposed (red) BLT mice. In these plots, the boxes extend from the first to the third quartiles, enclosing the middle 50% of the data. The middle line within each box indicates the median of the data, whereas the vertical line extends from lowest to the highest values. Data from naive, HIV-1 or FTC/TDF treated plus HIV-1 exposed mice were not collected on same day. Naive (n=5), HIV-1 infected (n=4) and FTC/TDF + HIV-1 (n=3). Gating strategy for flow cytometric analysis: live cells → human CD45 → human CD3 →human CD4 or CD8.

D3. Vaginally transmitted HIV-1$_{JR-CSF}$ targets human CD4$^+$CCR5$^+$ T cells

CCR5 co-receptor expression levels on human lymphocytes vary by tissue, with lower levels on peripheral blood, bone marrow, thymus, spleen and lymph node lymphocytes and higher levels in liver, lung and gut-associated lymphoid tissue (GALT) (Anton, Elliott et al. 2000; Cranston, Anton et al. 2000; Veazey, Mansfield et al. 2000; Sun, Denton et al. 2007). Comparison of HIV-1 infected vs FTC/TDF treated and HIV-1 exposed mice demonstrated a significant reduction of CD4$^+$CCR5$^+$ T cells in BLT liver (M_1-M_2=-15 ± 2.1, t=7.5, df=5, p<0.001) and lungs (M_1-M_2=-7.3 ± 0.77, t=9.4, df=5, p<0.001); no significant difference was noted in the peripheral blood (M_1-M_2=0.83 ± 2.1, t=0.40, df=5, p=0.71), bone marrow (M_1-M_2=-0.33 ± 2.1, t=0.16, df=5, p=0.88), thymic organoid (M_1-M_2=-1.1 ± 0.73, t=1.5, df=5, p=0.19), spleen (M_1-M_2=-1.4 ± 0.59, t=2.4, df=5, p=0.060) or lymph nodes (M_1-M_2=-1.1 ± 0.47, t=2.3, df=5, p=0.069) (Figure 29). Indicative of a heightened state of immune activation, a dramatic increase was observed in the levels of human CD8$^+$CCR5$^+$ T cells in all tissues in response to HIV-1 infection between HIV-1 infected vs FTC/TDF treated and HIV-1 exposed mice in peripheral blood (M_1-M_2=52 ± 16, t=3.3, df=5, p=0.022), bone marrow (M_1-M_2=35 ± 7.2, t=4.9, df=5, p=0.005), thymic organoid (M_1-M_2=23 ± 5.0, t=4.5, df=5, p=0.006), spleen (M_1-M_2=24 ± 9.3, t=2.6, df=5, p=0.048), lymph nodes (M_1-M_2=23 ± 7.8, t=3.0, df=5, p=0.031), liver (M_1-M_2=33 ± 12, t=2.7, df=5, p=0.043) and lung (M_1-M_2=22 ± 8.5, t=2.6, df=5, p=0.050) (Figure 30). Thus, HIV-1 infection altered the proportions of CCR5$^+$ T lymphocytes throughout BLT mice.

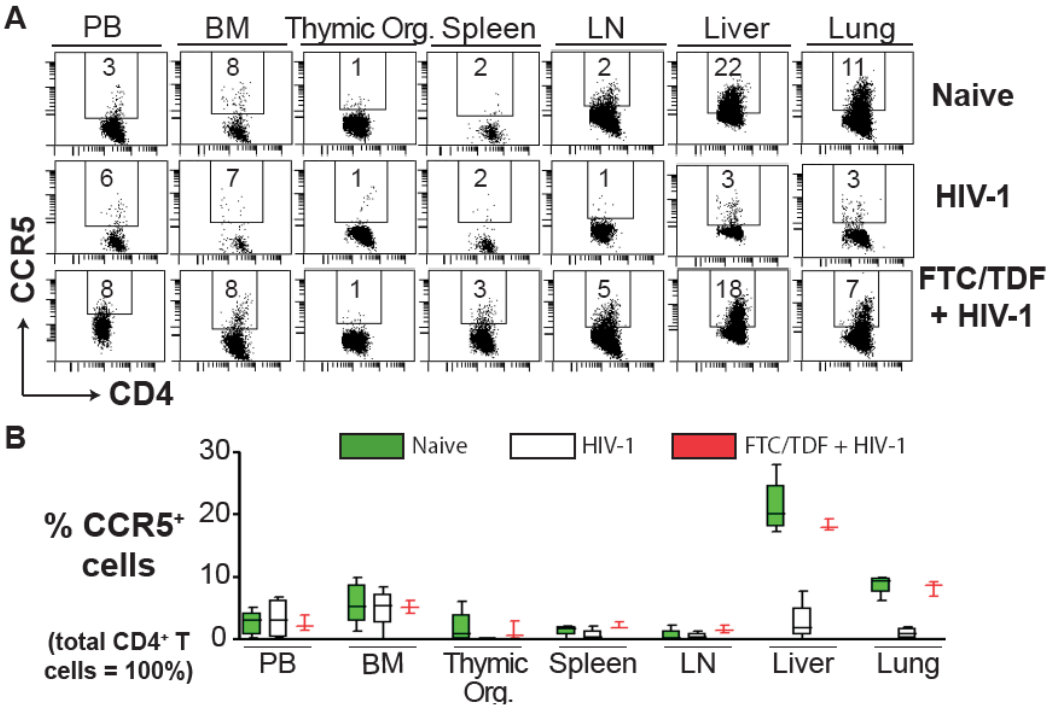

Figure 29. Lower $CD4^+CCR5^+$ human T cell levels result from vaginal HIV-$1_{JR\text{-}CSF}$ infection in BLT mice.

A) Comparison of the levels of human $CD4^+CCR5^+$ T cells in the indicated tissues in a representative naive BLT mouse, a JR-CSF infected and a JR-CSF exposed BLT mouse that received FTC/TDF for pre-exposure prophylaxis. Liver and lung were the examined tissues with the greatest constitutive CCR5 expression and they both showed significant loss of $CD4^+CCR5^+$ T cells due to HIV-1 infection. **B**) Box plot depicting the levels of $CD4^+CCR5^+$ T cells in the indicated tissues for naive (green), HIV-1 infected (white) and FTC/TDF treated plus HIV-1 exposed (red) BLT mice. In the box plots, the boxes extend from the first to the third quartiles, enclosing the middle 50% of the data. The middle line within each box indicates the median of the data, whereas the vertical line extends from lowest to the highest values. Naive (n=5), HIV-1 infected (n=4) and FTC/TDF + HIV-1 (n=3). Gating strategy for flow cytometric analysis: live cells → human CD45 → human CD3 →human CD4 or CD8 → CCR5.

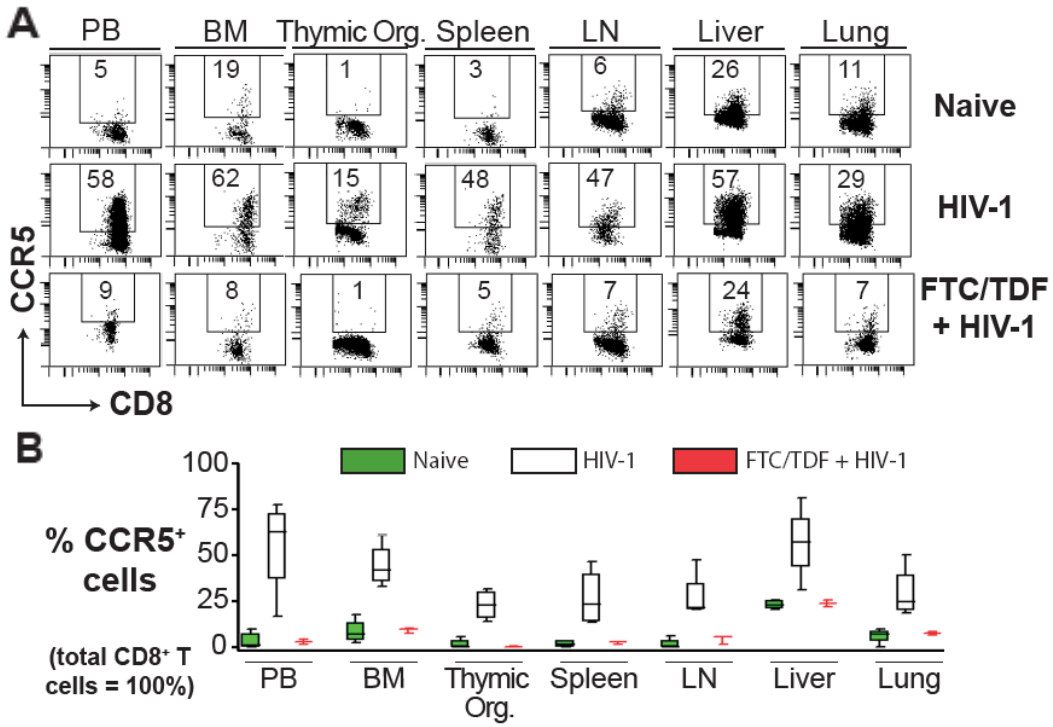

Figure 30. Increased $CD8^+CCR5^+$ human T cell levels resulting from vaginal HIV-$1_{JR\text{-}CSF}$ infection in BLT mice.

A) Comparison of the levels of human $CD8^+CCR5^+$ T cells in the indicated tissues in representative naive, JR-CSF infected and FTC/TDF treated BLT mice. All tissues examined showed increases in $CD8^+CCR5^+$ T cells resulting from JR-CSF infection of BLT mice. **B**) Box plot depicting the levels of $CD8^+CCR5^+$ T cells in the indicated tissues for naive (green), HIV-1 infected (white) and FTC/TDF treated plus HIV-1 exposed (red) BLT mice. In the box plots, the boxes extend from the first to the third quartiles, enclosing the middle 50% of the data. The middle line within each box indicates the median of the data, whereas the vertical line extends from lowest to the highest values. Naive (n=5), HIV-1 infected (n=4) and FTC/TDF + HIV-1 (n=3). Gating strategy for flow cytometric analysis: live cells → human CD45 → human CD3 →human CD4 or CD8 → CCR5.

D4. Vaginal HIV-1$_{JR-CSF}$ transmission lowers CD4$^+$ T cells level in BLT GALT

The GALT is a major site of HIV-1 replication and CD4$^+$ T cell depletion during HIV disease in humans (Brenchley, Schacker et al. 2004). Therefore, intraepithelial (IEL) and lamina propria (LPL) lymphocytes from both small and large intestine (SI and LI, respectively) of BLT mice were analyzed. Consistent with what has been observed during the course of human HIV-1 infection, a dramatic reduction was observed in CD4$^+$ T cells in the SI IEL (M_1-M_2=56 ± 8.0, t=7.0, df=6, p<0.001), SI LPL (M_1-M_2=40 ± 4.9, t=8.3, df=6, p<0.001), LI IEL (M_1-M_2=23 ± 5.3, t=4.3, df=6, p=0.005), and LI LPL (M_1-M_2=49 ± 9.3, t=5.2, df=6, p=0.002) (Figure 31). As described above for liver and lung, HIV-1 infection resulted in a significant reduction of CD4$^+$CCR5$^+$ T cells in BLT mouse SI IEL (M_1-M_2=21 ± 4.1, t=5.1, df=6, p=0.002), SI LPL (M_1-M_2=22 ± 8.5, t=2.6, df=6, p=0.042), LI IEL (M_1-M_2=38 ± 8.9, t=4.3, df=6, p=0.005), and LI LPL (M_1-M_2=48 ± 4.4, t=11, df=6, p<0.001) (Figure 32). A statistically significant reduction was also observed in the levels of CD4$^+$ effector memory T cells (CD45RA^{neg}CD27^{neg}) from the SI IEL (M_1-M_2=41 ± 11, t=3.7, df=6, p=0.010) and SI LPL (M_1-M_2=36 ± 12, t=3.1, df=6, p=0.021) of infected BLT mice (Figure 33). These findings are in agreement with studies in humans and macaques regarding memory T cell loss in GALT by HIV-1 and SIV/SHIV (Guadalupe, Reay et al. 2003; Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004; Li, Duan et al. 2005; Mattapallil, Douek et al. 2005) and highlight the usefulness of the BLT model for studying HIV-1 pathogenesis, particularly in GALT.

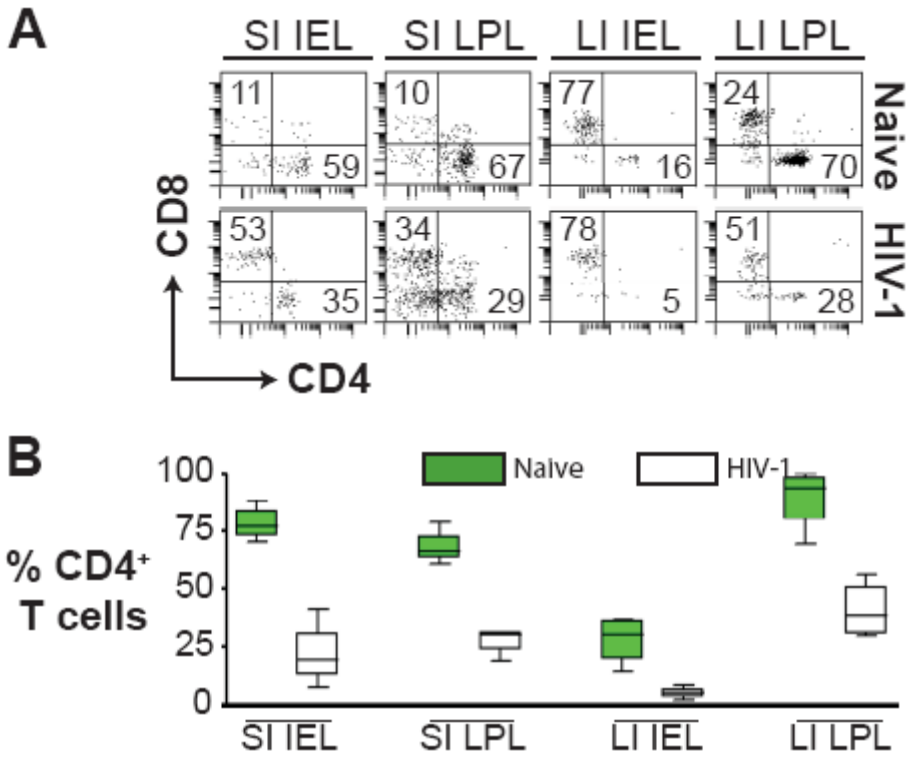

Figure 31. Human $CD4^+$ T cell levels are reduced in BLT GALT as a result of vaginal HIV-$1_{JR\text{-}CSF}$ infection.
A) Comparison of the levels of $CD4^+$ or $CD8^+$ human T cells in the GALT of representative naive and JR-CSF infected mice. **B)** Box plot depicting the levels of GALT $CD4^+$ T cells for naive (green) and HIV-1 infected (white) BLT mice. In the box plots, the boxes extend from the first to the third quartiles, enclosing the middle 50% of the data. The middle line within each box indicates the median of the data, whereas the vertical line extends from lowest to the highest values. Naive (n=4), HIV-1 infected (n=4). Gating strategy for flow cytometric analysis: live cells → human CD45 → human CD3 →human CD4 → CCR5, CD27 or CD45RA.

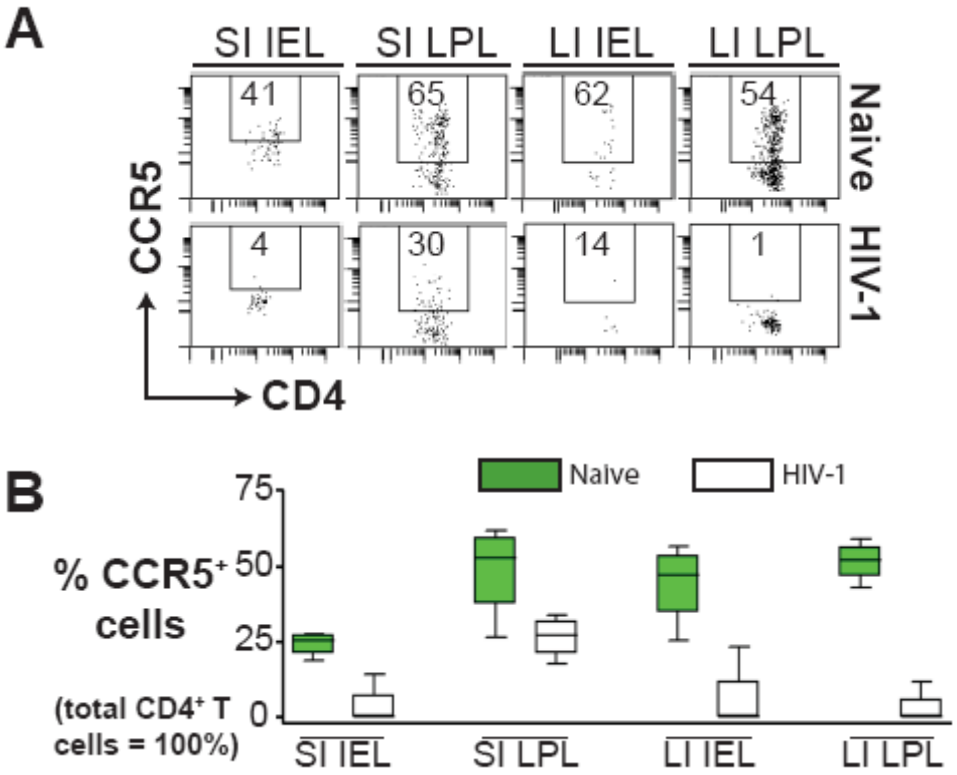

Figure 32. Human $CD4^+CCR5^+$ T cells levels are reduced in BLT GALT as a result of vaginal HIV-1_{JR-CSF} infection.
A) Comparison of the levels of human $CD4^+CCR5^+$ T cells in the GALT of naive and JR-CSF infected BLT mice. Significantly fewer GALT $CD4^+$ T cells had detectable CCR5 expression levels following HIV-1 infection. **B)** Box plot depicting the levels of $CD4^+CCR5^+$ T cells in the GALT for naive (green) and HIV-1 infected (white) BLT mice. In the box plots, the boxes extend from the first to the third quartiles, enclosing the middle 50% of the data. The middle line within each box indicates the median of the data, whereas the vertical line extends from lowest to the highest values. Naive (n=4), HIV-1 infected (n=4). Gating strategy for flow cytometric analysis: live cells → human CD45 → human CD3 →human CD4 → CCR5, CD27 or CD45RA.

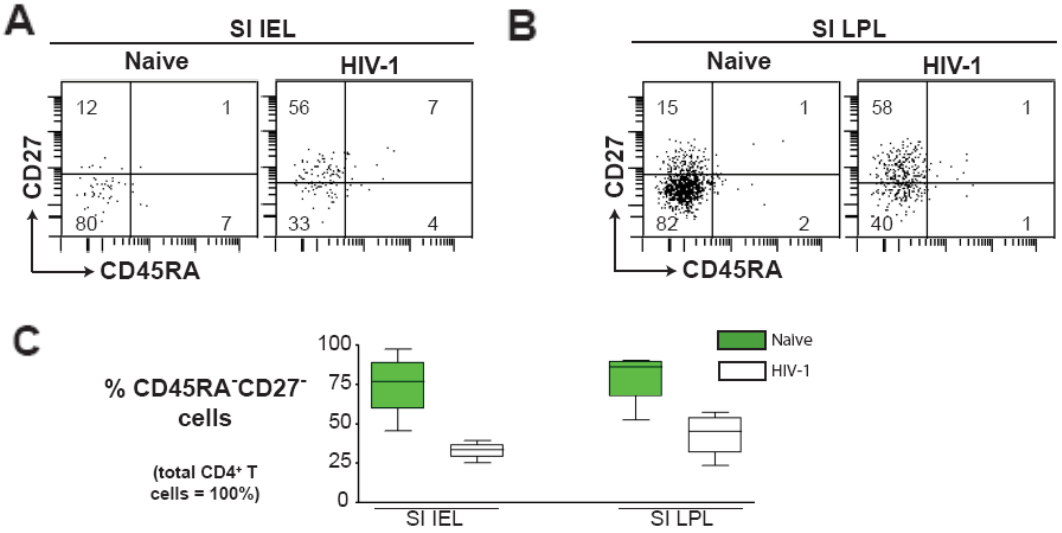

Figure 33. Human $CD4^{+}CD27^{neg}CD45RA^{neg}$ effector memory T cells are reduced in BLT small intestine following vaginal HIV-$1_{JR\text{-}CSF}$ infection.
A) Comparison of the levels of human $CD4^{+}$ effector memory T cells in the small intestine intra-epithelial (**A**) and lamina propria (**B**) lymphocyte compartments of representative naive and JR-CSF infected BLT mice. HIV-1 infected BLT mice have statistically fewer effector memory $CD4^{+}$ T cells present in their small intestines. **C)** Box plot depicting the levels of $CD45RA^{neg}CD27^{neg}$ effector memory T cells in the small intestine of naive (green) and HIV-1 infected (white) BLT mice. In the box plots, the boxes extend from the first to the third quartiles, enclosing the middle 50% of the data. The middle line within each box indicates the median of the data, whereas the vertical line extends from lowest to the highest values. Naive (n=4), HIV-1 infected (n=4). Gating strategy for flow cytometric analysis: live cells → human CD45 → human CD3 →human CD4 → CCR5, CD27 or CD45RA.

D5. Confirmation that antiretroviral pre-exposure prophylaxis prevents vaginal HIV-1$_{JR-CSF}$ transmission

To confirm the lack of infection in FTC/TDF treated animals, three additional approaches were utilized. DNA isolated from cells obtained from different organs of either HIV-1-infected or FTC/TDF-treated BLT mice was analyzed by quantitative real-time PCR for HIV-1 viral DNA. While the tissues from HIV-1-infected mice were clearly positive for viral DNA, samples from pre-exposure FTC/TDF-treated mice were consistently negative (Figure 34). Cells isolated from multiple organs of HIV-1-infected or FTC/TDF-treated BLT mice were co-cultured with PHA/IL2 activated peripheral blood lymphocytes from a seronegative donor. Virus was readily rescued from cells isolated from tissues obtained from the HIV-1 infected mice (Figure 35). In contrast, no virus was rescued from any of the tissues obtained from the BLT mice treated with FTC/TDF. Lastly, in situ hybridization was utilized to determine the presence of productively infected cells in HIV-1 infected or FTC/TDF treated BLT mice. Productively HIV-1 infected cells were readily observed in tissues from the HIV-1 infected BLT mice (Figure 36). In contrast, no productively infected cells were found in any of the tissues from the FTC/TDF treated mice. These results verify the complete protection afforded by this pre-exposure prophylaxis approach to the prevention of intravaginal transmission of HIV-1.

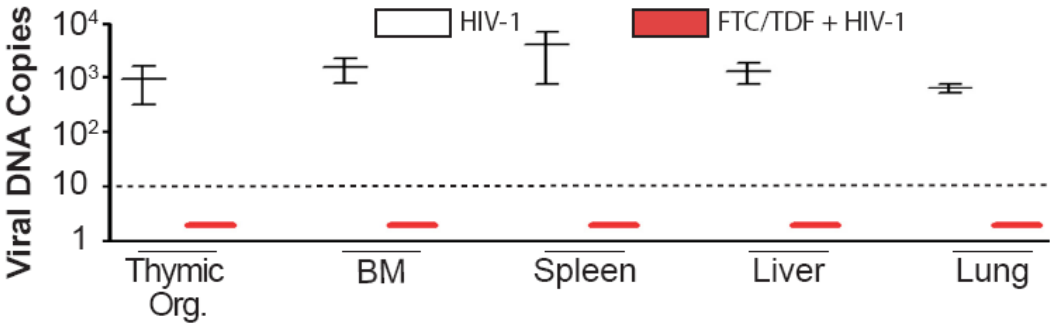

Figure 34. Real-time PCR analysis did not detect HIV-1 DNA in BLT mice given pre-exposure prophylaxis with intravaginal HIV-$1_{JR\text{-}CSF}$ exposure. Box plot depicting real-time PCR levels of JR-CSF DNA in the indicated tissues for HIV-1 infected (white) and FTC/TDF treated plus HIV-1 exposed (red) BLT mice. (Viral DNA copies per million $CD4^+$ T cells shown.) HIV-1 infected (n=2) and FTC/TDF + HIV-1 (n=4). In the box plots, the middle line indicates the median of the data, whereas the vertical line extends from lowest to the highest values. Dashed lines indicate the limit of detection for the assay. (Ty Troutman assisted with the real-time PCR)

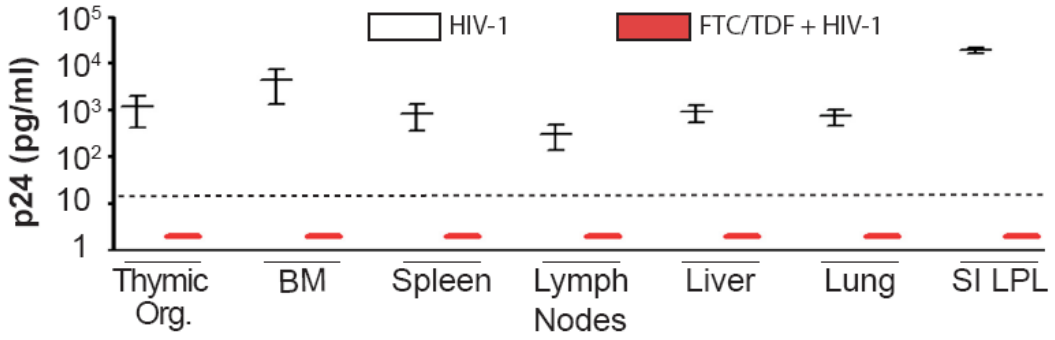

Figure 35. Virus rescue analysis did not detect replication competent HIV-1 in BLT mice given pre-exposure prophylaxis with intravaginal HIV-1_{JR-CSF} exposure.

Box plot depicting virus rescue results from HIV-1 infected (white) and FTC/TDF treated plus HIV-1 exposed (red) BLT mice. Virus rescue data expressed as pg/ml of p24 per $1x10^5$ $CD4^+$ T cells co-cultured with PHA/IL2 activated peripheral blood lymphocyte from a seronegative donor. HIV-1 infected (n=2) and FTC/TDF + HIV-1 (n=4). In the box plots, the middle line indicates the median of the data, whereas the vertical line extends from lowest to the highest values. Dashed lines indicate the limit of detection for the assay. (Dr. Elisa Fleming assisted with the virus rescue)

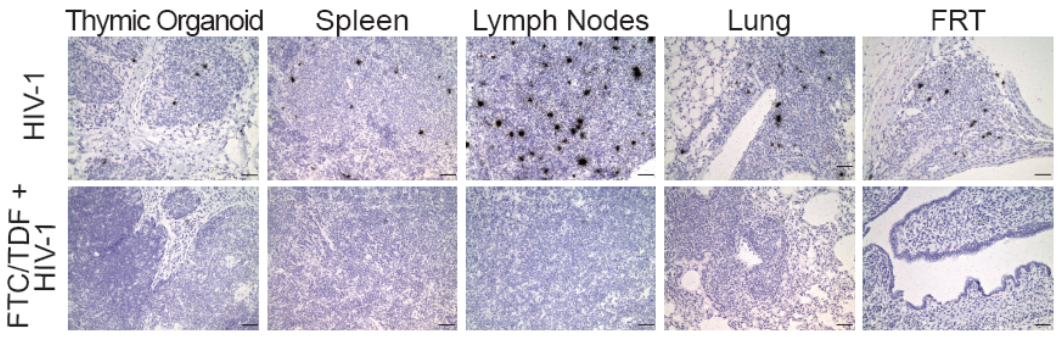

Figure 36. In situ hybridization analysis did not detect HIV-1 RNA in BLT mice given pre-exposure prophylaxis with intravaginal HIV-1$_{JR-CSF}$ exposure. In situ hybridization analysis to determine the presence of productively HIV-1 infected cells in the indicated tissues from JR-CSF infected or FTC/TDF treated BLT mice (bars = 50 μm). Note the lack of HIV-1 in the BLT mice that received pre-exposure prophylaxis with FTC/TDF. HIV-1 infected (n=4) and FTC/TDF + HIV-1 (n=3). (In situ hybridization performed by Dr. Jacob Estes)

E. Discussion

The present study demonstrates efficient intravaginal HIV-1 transmission in BLT mice that results in a systemic human $CD4^+$ T cell reduction and a loss of GALT effector memory human $CD4^+$ T cells, as has been observed in humans (Guadalupe, Reay et al. 2003; Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004; Li, Duan et al. 2005; Mattapallil, Douek et al. 2005). In addition, evidence of the effectiveness of antiretrovirals for pre-exposure prophylaxis to prevent intravaginal HIV-1 transmission is provided.

Antiretroviral drugs have considerable potential to inhibit HIV-1 transmission when used for pre-exposure prophylaxis (Veazey, Klasse et al. 2005). The expectation for pre-exposure prophylaxis is that any given drug(s) taken or administered appropriately can prevent HIV infection (Derdelinckx, Wainberg et al. 2006). There is as yet no clinical evidence for the effectiveness of this approach (Youle and Wainberg 2003; Grant, Buchbinder et al. 2005; Liu, Grant et al. 2006; Cohen, Gay et al. 2007). However, precedent for the administration of antiretrovirals to large populations of individuals at high risk for infection is exemplified by the widespread use of Nevirapine for the prevention of vertical HIV transmission between mother and child (Guay, Musoke et al. 1999; Dao, Mofenson et al. 2007). Similarly, if proven safe and effective, pre-exposure prophylaxis together with other behavioral interventions could provide protection to men and women at risk of HIV infection by preventing horizontal HIV transmission. Therefore, it is critical to evaluate new prevention methods aimed at the populations at highest risk. Despite the urgency to develop and implement novel approaches capable of preventing HIV transmission, this process has been hindered by the lack of adequate animal models readily available for pre-clinical efficacy and safety testing (Stone and Jiang 2006).

The possibility that BLT mice might serve as a highly efficient, relatively fast and cost-effective small animal model of intravaginal HIV-1 infection was examined. The female reproductive tract of BLT mice is populated with in situ generated human cells critical for the transmission and dissemination of HIV-1 and a single intravaginal exposure to HIV-1 results in infection in 88% of the exposed BLT mice demonstrating their susceptibility to vaginal transmission. These observations distinguish the BLT system (with its self-renewing, hematopoietic stem cell based systemic human reconstitution, including throughout the female reproductive tract) from SCID mice injected with human peripheral blood lymphocyte (SCID-hu PBL) for vaginal HIV-1 transmission (Mosier, Gulizia et al. 1991; Di Fabio, Giannini et al. 2001) (Chapter 1). The systemic nature of BLT human reconstitution facilitated examination of the pathogenic effects caused by infection in BLT mice. These analyses revealed that HIV-1 disseminates from the vaginal mucosa to cause systemic $CD4^+$ T cell loss including GALT $CD4^+$ effector memory T cell loss, as in humans (Brenchley, Schacker et al. 2004). Thus, BLT mice represent a useful model for HIV-1 intravaginal transmission, systemic spread and pathogenesis.

The fact that BLT mice are susceptible to intravaginal HIV-1 infection was utilized to demonstrate that this system is uniquely well-suited for the pre-clinical evaluation of pre-exposure prophylactic regimens to prevent intravaginal HIV-1 transmission. The results show that the BLT model can serve as a relatively fast and simple system to test whether pre-exposure prophylaxis can prevent vaginal HIV-1 transmission. Using this system it was found that FTC/TDF can afford complete protection from vaginal HIV-1 transmission. These results suggest that the BLT model could also be suitable for testing topical microbicides.

As with all animal models of human disease, there are limitations to this study. While these findings are consistent with findings from non-human primate research regarding the potential of pre-exposure prophylaxis to prevent HIV-1 transmission, neither model has been shown to predict efficacy or safety in humans. This is due to the lack of data from similar pre-exposure prophylaxis in humans. Therefore, an important limitation is that the BLT model currently has no known predictive value for clinical medicine. It will be essential that ongoing human clinical trial data be compared to this and future BLT studies for validation. Variables between BLT mice and humans include possible differences in drug concentrations, in adherence, in renal and liver biology, virus dosage and co-infections with viruses like hepatitis B virus. While many aspects of HIV GALT pathogenesis are recapitulated in BLT mice, it has not been determined if there is a direct and/or indirect pathologic effect of HIV-1 on enterocytes, as seen in humans. Many of these limitations can be addressed in future studies. In the interim, these data show that there is significant potential for antiretrovirals in general and FTC/TDF in particular to function as a pre-exposure prophylaxis measure against the spread of HIV/AIDS in humans.

The number of infected women worldwide has increased to almost 15.4 million (WHO-UNAIDS 2006; WHO-UNAIDS 2007). As a coital independent prevention measure, antiretroviral pre-exposure prophylaxis and/or topical microbicides could provide women with a powerful tool to protect themselves from infection. However, any candidate drug(s) must be safe, especially in individuals without disease, and efficacious (Derdelinckx, Wainberg et al. 2006). In addition, in order to be successful it must be easy to use (Derdelinckx, Wainberg et al. 2006). The combination of FTC/TDF appears to meet the criteria for drugs to be used for pre-exposure prophylaxis (De Clercq 2007). In addition, it is one of the few drug combinations that can be administered once daily without

food restrictions. In this chapter pre-clinical evidence regarding the potential efficacy of antiretroviral pre-exposure prophylaxis in humans is provided. These results should provide further impetus for the continued implementation of clinical trials using oral antiretroviral pre-exposure prophylaxis.

CHAPTER FIVE

Conclusions and Recommendations

A. Conclusions

There are few models where potential clinical interventions against HIV-1 disease can be evaluated and where pathogenesis can be studied, due to the limited species tropism of the virus. Mice represent an outstanding model to study a variety of aspects related to numerous virus infections. However, mice are refractory to HIV infection and cannot support HIV replication (Bieniasz and Cullen 2000; Mariani, Rutter et al. 2000; Sun, Soos et al. 2006). Using humanized mice to address this need for model systems is attractive because they allow in vivo studies of HIV-1 infection of human cells. The goal of this dissertation project was to determine the suitability of BLT mice to serve as an animal model of HIV-1 transmission and as a model for assessing interventions aimed at preventing HIV-1 transmission. Multiple steps were required to achieve this goal. First, humanization of the gastrointestinal tract and female reproductive tract had to be demonstrated. Then, both intrarectal and intravaginal HIV-1 transmission had to be shown. Finally, an intervention against HIV-1 transmission had to be evaluated.

Prior to HIV-1 exposure, the presence of HIV-1 target cells in the relevant mucosal tissues of BLT mice was demonstrated. Data presented in Chapter 3 show the extensive human reconstitution of the small intestine, large intestine and mesenteric lymph nodes of BLT mice. The small and large intestines (SI and LI, respectively) were analyzed separately. For flow cytometric analysis, cells from these two organs were obtained from either the intraepithelial layer or the lamina propria. Human intraepithelial and lamina propria lymphocytes (IEL and LPL,

respectively) were found in both the SI and LI of BLT mice. The human IEL and LPL of both the SI and LI were predominantly $CD3^+$ T cells, but $CD19^+$ B cells were also identified. $CD4^+$ T cells were present at higher percentages than $CD8^+$ T cells in SI LPL, SI IEL, and LI LPL; this ratio was reversed in the LI IEL fraction. In these four fractions, the T cells exhibited a memory phenotype with virtually no naive T cells detected. HIV-1 co-receptor expression in BLT GALT was examined; both CXCR4 and CCR5 were found to be expressed on gut T lymphocytes. These observations agree with data from human small intestine terminal ileum biopsies (Brenchley, Schacker et al. 2004). In addition to SI and LI, mesenteric lymph nodes were examined for human reconstitution, as these lymph nodes represent the immunological conduit connecting the intestines with the rest of the body. BLT mouse mesenteric lymph nodes contained both human B and T lymphocytes. Within the T cell compartment, there were more $CD4^+$ T cells than $CD8^+$ T cells and, in contrast to GALT, populations of naive T cells were observed in mesenteric lymph nodes.

One salient aspect of the GALT analysis was finding a rare subpopulation of T cells within the small intestine of BLT mice that are phenotypically similar to T cells found in human small intestine (Abuzakouk, Carton et al. 1998; Carton, Byrne et al. 2004). In humans, $CD8\alpha\alpha^+$ T cells are found in the small intestine, mostly in the lamina propria, and typically are also positive for CD4 (Abuzakouk, Carton et al. 1998; Carton, Byrne et al. 2004). The intricate balance of $CD8\alpha\alpha$ homodimers on CD4CD8 double positive small intestine T cells with $CD8\alpha\beta$ heterodimers on CD8 single positive BLT small intestine T cells is a reflection of the appropriate distribution of human lymphocytes that occurs in BLT mice. This was an important observation suggesting that there is a specific population of

human GALT T cells in the BLT mouse intestines rather than simply transient human cells in the BLT GALT.

Lymphocyte migration into effector tissues like the gut is the result of a series of poorly understood but highly complex species-specific interactions between cell adhesion molecules, integrins, chemokines and chemokine receptors. Yet, the distribution of human lymphocytes observed in the BLT mice is very similar to human gut (Abuzakouk, Carton et al. 1998; Carton, Byrne et al. 2004). In addition to the flow cytometry data discussed above, immunohistochemical analysis showed lymphoid follicular aggregates populated with human lymphocytes (T and B), macrophages and DCs in the small and large intestines. Despite the extensive similarity, some differences were also noted. There was heterogeneity in the repopulation of human cells within the lamina propria of each BLT mouse, with some portions of the gut demonstrating lower human repopulation. In addition, there were somewhat higher percentages of $CD4^+$ T cells in BLT small intestine IEL compared to normal human gut and no γδ T cells were identified. Data presented in Chapter 3 demonstrate that BLT GALT has many of the characteristics of human GALT; specifically, cells necessary for HIV-1 infection are present throughout the small and large intestines.

To further characterize the tissues relevant to mucosal HIV-1 transmission, data are presented in Chapter 4 that demonstrate the extensive reconstitution of the female reproductive tract of BLT mice with human lymphocytes. Individual regions of the FRT were examined by immunohistochemistry (vagina, ectocervix, endocervix and uterus) and flow cytometry was used to examine human reconstitution of BLT mouse FRT as a whole. Immunohistochemistry showed human $CD4^+$ cells, human $CD68^+$ monocyte/ macrophage cells and clusters of human $CD11c^+$ dendritic cells

throughout the FRT. CXCR4 and CCR5 expression on the $CD4^+$ T cells of the BLT mouse FRT was shown by flow cytometry. The FRT represents a highly specialized and complex anatomical site where initial infection occurs following intravaginal exposure (Hu, Gardner et al. 2000; Miller, Li et al. 2005; Hladik, Sakchalathorn et al. 2007) and data presented in Chapter 4 establish that in situ differentiation of human HSC leads to reconstitution of the FRT of BLT mice with all the human hematopoietic cells relevant to vaginal HIV-1 transmission (Givan, White et al. 1997; Johansson, Rudin et al. 1999; Poonia, Wang et al. 2006).

An understanding of the molecular basis of mucosal HIV transmission has been hindered to a significant extent by the lack of adequate systems that recapitulate the events taking place during human infection. Therefore, the presence of HIV-1 target cells in the gastrointestinal and female reproductive tracts of BLT mice provided impetus to determine the susceptibility of these mice to HIV-1 following either rectal or vaginal exposure. BLT mice were exposed a single time to HIV-1 either rectally or vaginally. Following exposure, mice were typically bled bi-weekly and the peripheral blood analyzed for indications that mucosal HIV-1 transmission had occurred. Transmission was confirmed by the presence of HIV-1 RNA or HIV-1 proteins in plasma. Plasma viral RNA was frequently detected as early as 2 weeks post-exposure and plasma antigenemia was typically detectable by 4 weeks post-exposure. Rectal transmission of HIV-1_{LAI} occurred in 6 of 7 BLT mice tested, vaginal transmission of HIV-1_{LAI} occurred in 3 of 3 BLT mice tested and vaginal transmission of HIV-$1_{JR\text{-}CSF}$ occurred in 7 of 8 BLT mice tested. The susceptibility of BLT mice to one intrarectal or intravaginal HIV-1 exposure points to the great potential of this system to study mucosal HIV transmission.

The systemic nature of human lymphoid reconstitution of BLT mice facilitated examination of the pathogenic effects caused by HIV-1 infection. These analyses revealed that HIV-1 disseminates from the rectal or vaginal mucosa to cause systemic $CD4^+$ T cell loss including GALT $CD4^+$ effector memory T cell loss, as in humans (Brenchley, Schacker et al. 2004). Virus dissemination was demonstrated by in situ hybridization, virus rescue and real-time PCR. Both LAI and JR-CSF disseminated from the location of inoculation throughout the animal and into the tissues analyzed. These results show remarkable similarities in viral pathogenesis between intrarectally or intravaginally HIV-1 infected humanized mice, HIV-1 infected humans and SIV infected macaques (Guadalupe, Reay et al. 2003; Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004; Li, Duan et al. 2005; Mattapallil, Douek et al. 2005). These similarities include systemic $CD4^+$ T cells loss and the devastating effects of HIV-1 to the GALT.

Regardless of the HIV-1 isolate used to infect BLT mice, the appearance of virus in the peripheral blood occurred within a similar timeframe. However, the isolate used did affect the pathogenesis observed as differences in the outcome of infection between the CXCR4-tropic LAI and the CCR5-tropic JR-CSF were noted. The most prominent difference being the extent to which $CD4^+$ T cells were depleted. After LAI infection, the number of $CD4^+$ T cells present was very low in all tissues examined and the GALT was essentially devoid of any $CD4^+$ T cells by eight weeks post-exposure. This ablation of $CD4^+$ T cells by LAI contrasts rather sharply with JR-CSF pathogenesis. Over a twelve week period, the peripheral blood levels of $CD4^+$ T cells dropped approximately by half. Similarly, the levels of $CD4^+$ T cells in other tissues examined were significantly lower, but $CD4^+$ T cells remained present. Further analysis demonstrated that the loss of $CD4^+$ T cells caused by JR-CSF appears to be limited to $CD4^+CCR5^+$ T

cells. This was most evident in the tissues with the highest CCR5 expression levels (liver, lung and the intestines). Another difference was the kinetics $CD4^+$ T cell depletion from peripheral blood. During LAI infection the loss of $CD4^+$ T cells in peripheral blood was precipitous, but during JR-CSF infection $CD4^+$ T cell levels became relatively stable following the initial drop (Figure 37). A third isolate dependent difference was observed in the GALT. The massive depletion caused by LAI precluded an analysis of the small intestine $CD4^+$ memory T cells following infection. In the case of JR-CSF infection $CD4^+$ effector memory T cells were significantly reduced in the small intestine, as in humans (Brenchley, Schacker et al. 2004). Overall, these data reveal that BLT mice represent a useful model for mucosal HIV-1 transmission, systemic spread and pathogenesis. Furthermore, the AIDS-like pathology and depletion within the BLT GALT after HIV-1 infection highlights this model's usefulness to evaluate therapeutic interventions aimed at GALT reconstitution that might result in the possible reestablishment of a functional immune system in humans.

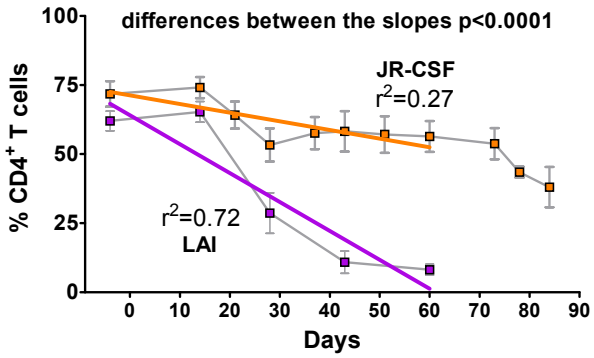

Figure 37. $CD4^+$ T cells depletion kinetics following mucosal transmission of HIV-1_{LAI} or HIV-$1_{JR\text{-}CSF}$. Linear regression of $CD4^+$ T cell levels following LAI or JR-CSF infection (Day 0) in BLT mice. The regression lines show more rapid peripheral blood $CD4^+$ T cell depletion by LAI.

In the absence of an effective vaccine or topical microbicide, alternative preventative measures are desperately needed to help block the spread of AIDS. Therefore, it is critical to evaluate new prevention methods aimed at the populations at highest risk. Despite the urgency to develop and implement novel

approaches capable of preventing HIV transmission, this process has been hindered by the lack of adequate animal models available for pre-clinical efficacy and safety testing (Stone and Jiang 2006). Antiretroviral drugs have considerable potential for preventing HIV-1 transmission as a coital independent prevention measure (Veazey, Klasse et al. 2005). If proven safe and effective, pre-exposure prophylaxis together with other behavioral interventions could provide protection to men and women at risk of HIV infection by preventing transmission. The fact that BLT mice are susceptible to mucosal HIV-1 transmission was utilized to demonstrate that this system is uniquely well-suited for the pre-clinical evaluation of pre-exposure prophylactic regimens to prevent mucosal HIV-1 transmission. My results show that FTC/TDF can afford complete protection of BLT mice from vaginal HIV-1 transmission. These results demonstrate that the BLT model can be used for pre-clinical efficacy testing of pre-exposure prophylaxis regimens for the prevention of mucosal HIV-1 transmission. These results also serve as pre-clinical evidence for the potential success of this approach aimed at preventing the further spread of AIDS. Therefore, these results should provide momentum for continued translational research towards implementation of clinical trials using oral antiretroviral pre-exposure prophylaxis particularly in parts of the world with highest HIV prevalence where pre-exposure prophylaxis would be most beneficial and cost effective (Derdelinckx, Wainberg et al. 2006; De Clercq 2007).

Regarding this project's goals of determining the suitability of BLT mice to serve as an animal model of HIV-1 transmission and as a model for assessing interventions aimed at preventing HIV-1 transmission, my conclusions are that BLT mice are susceptible to both intrarectal and intravaginal HIV-1 transmission and that pre-exposure prophylaxis with FDA approved antiretroviral drugs does prevent vaginal transmission in BLT mice.

B. The emerging field of HIV infection of humanized mice

HIV research in humanized mice had entered a "Renaissance" period. In recent years, new humanization protocols have been shown to yield humanized mice with sustained, systemic and robust levels of human cells relevant to HIV infection, including T cells (Shultz, Ishikawa et al. 2007). To date, three of these different systems have been used to study HIV and this discussion will be limited to these three systems. The common aspect of the new humanization protocols is transplantating human hematopoietic stem cells (HSC) into one of three immunodeficient mouse strains.

Ito, et al. transplanted adult nonobese diabetic-severe combined immunodeficient IL-2 receptor gamma chain null (NOG; Central Institute for Experimental Animals-CIEA; Kawasaki, Japan) mice with human $CD34^+$ HSC and Hiramatsu, et al. showed that functional human T cells develop in NOG mice humanized by the protocol Ito, et al. described (Ito, Hiramatsu et al. 2002; Hiramatsu, Nishikomori et al. 2003). Humanized NOG mice have been infected intravenously with CXCR4- and CCR5-tropic HIV-1 (Watanabe, Ohta et al. 2007; Watanabe, Terashima et al. 2007). $CD4^+$ T cells were depleted in peripheral blood and 3 of 14 mice tested positive for HIV-1 specific antibodies in plasma (Watanabe, Terashima et al. 2007).

Traggiai et al. transplanted 1 day old Rag2 and IL-2 receptor gamma chain double knock-out mice ($Rag2^{-/-}IL2g^{-/-}$; knockout is on BALB/c background; not commercially available) with human $CD34^+$ HSC. These mice go on to develop B, T and dendritic cells (Traggiai, Chicha et al. 2004). Humanized $Rag2^{-/-}IL2g^{-/-}$ mice have been used by several groups to study CXCR4- and CCR5-tropic HIV-1 infection. Humanized $Rag2^{-/-}IL2g^{-/-}$ mice have been inoculated with HIV-1 intraperitonealy (Baenziger, Tussiwand et al. 2006; Berges, Wheat et al. 2006;

An, Poon et al. 2007; Gorantla, Sneller et al. 2007) and intravenously (Zhang, Kovalev et al. 2007). $CD4^+$ T cells were depleted in peripheral blood but HIV-1 specific antibodies in plasma were rare. Of 25 humanized $Rag2^{-/-}IL2g^{-/-}$ plasma samples tested; only a single plasma sample had human IgG against HIV, as detected by western blot analysis (Baenziger, Tussiwand et al. 2006). Similarly, in a separate study using humanized $Rag2^{-/-}IL2g^{-/-}$ mice researchers failed to identity HIV-1 specific human antibodies by enzyme immunoassay or western blot from any of their infected mice (n=11) (Gorantla, Sneller et al. 2007). A third group studying humanized $Rag2^{-/-}IL2g^{-/-}$ mice was also unable to detect HIV specific antibodies by western blot (n=8) (An, Poon et al. 2007).

Our group described a humanization protocol whereby nonobese diabetic-severe combined immunodeficient mice (NOD/SCID) mice are made to develop a functional human immune system, components of which include B, T, dendritic, NK and myeloid cells (Melkus, Estes et al. 2006). Melkus, et al. used a two-step humanization protocol where fetal thymus and liver were implanted under the NOD/SCID mouse kidney capsule, followed by a bone marrow transplant of autologous fetal liver $CD34^+$ HSC into implanted mice (BLT mice). The route of HIV-1 transmission used to infect BLT mice was either intrarectal (Sun, Denton et al. 2007) or intravaginal (Denton, Estes et al. In Press). $CD4^+$ T cell depletion was extensive, including GALT, in BLT mice following HIV-1 infection. Plasma samples from 3 of 4 HIV-1 infected BLT mice were positive for human anti-HIV IgG by Western blot (Sun, Denton et al. 2007).

The pathological effects of HIV-1 infection in the intestines have been extensively studied in recent years and this effector mucosal site has been shown to be profoundly affected by HIV/SIV infection within the first few weeks of transmission in humans or macaques, respectively (Guadalupe, Reay et al. 2003;

Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004; Li, Duan et al. 2005; Mattapallil, Douek et al. 2005). BLT mice have a demonstrated ability to support mucosal CXCR4- and CCR5-tropic HIV-1 transmission. The BLT studies evaluate the effects of HIV-1 infection within mucosal sites in humanized mice (Sun, Denton et al. 2007; Denton, Estes et al. In Press). In the BLT model, in situ hybridization for HIV-1 RNA has shown productively infected cells in the female reproductive tracts, small and large intestines and lungs. Disseminated HIV-1 infection of BLT mice results in systemic $CD4^+$ T cell loss including GALT $CD4^+$ effector memory T cell loss, as in humans (Brenchley, Schacker et al. 2004). Unfortunately, comparable data have not been presented for the humanized NOG and humanized $Rag2^{-/-}IL2g^{-/-}$ HIV-1 infection models.

A practical difference between these models is that NOD/SCID mice do not live as long as NOG or $Rag2^{-/-}IL2g^{-/-}$ mice. NOD/SCID mice typically live for less than one year, whereas human engraftment in NOG and $Rag2^{-/-}IL2g^{-/-}$ mice has been documented for over a year (Shultz, Schweitzer et al. 1995; Berges, Wheat et al. 2006; Watanabe, Ohta et al. 2007). Also, the humoral immune response data for each model mentioned above can only be considered as preliminary. More work is needed to establish the scope of the human humoral immune response to HIV-1 in humanized mouse models.

BLT mice meet or exceed the NOG and $Rag2^{-/-}IL2g^{-/-}$ humanized mouse models with respect to criteria related to human reconstitution and usefulness to HIV research. Such criteria include: consistent mucosal reconstitution including human cells expressing mucosal tissue-specific phenotypes, mucosal HIV transmission, therapeutic and prophylactic intervention approaches, human thymic epithelial layer for T cell education, robust antiviral B and T cell responses, robust innate responses to bacterial proteins, consistent systemic

(including GALT) human reconstitution and the mice used are commercially available.

C. The BLT model in HIV-1 research – the future

Making a vaccine for HIV has been extraordinarily difficult. The best system for pre-clinical evaluation of vaccines has been SIV infection of macaques. There is a major limitation to the SIV/macaque system. Namely, epitopes in the vaccines must be matched to the challenge virus. In other words, the immunogenicity of SIV, not HIV, epitopes is being tested (Koff, Johnson et al. 2006; De Boer 2007). If determining protective immunodominant epitopes is a goal of the vaccine effort (McMichael and Hanke 2003), then humanized mice can aid the effort as they can be infected with HIV. However, if humanized mice are used in vaccine research, viral epitope processing and presentation should occur in the context of human MHC, not mouse MHC. Otherwise, extrapolation of the results to the human population will be difficult. Immunodominant epitopes displayed in mouse MHC to human TCR may have little or no relation to immunodominant epitopes displayed in human MHC to human TCR. This topic raises a key difference between other humanized mouse models compared to the BLT system. That difference is the presence of human thymic stroma in BLT mice. The human thymus allows for the education of human thymocytes on human MHC. The demonstration of a human MHC restricted CTL response to EBV in BLT mice shows that human T cell education is occurring on human MHC in BLT mice (Melkus, Estes et al. 2006). Currently there is no definitive data on the biology of T cell selection in other humanized mouse models. However, "human thymocytes should be positively selected on mouse (MHC), whereas negative selection could occur on both mouse and human MHC" (Manz 2007). This presumption is based on the fact that no human thymic stroma is

present upon which selection could occur (Yahata, Ando et al. 2002; Traggiai, Chicha et al. 2004; Manz 2007). Until human MHC presentation to human TCR is demonstrated in other humanize mice models, in my opinion the BLT model is the clear best choice for any vaccine studies to be performed in humanized mice.

BLT mice are well-suited to address a wide range of important topics in HIV research. The fact that mucosal transmission readily occurs in BLT mice allows research into virological or molecular determinants of mucosal virus transmission. Mutant viruses can be evaluated in BLT mice to determine which in vitro functions described for different proteins are necessary for in vivo pathogenesis. Such experiments would help sift through the enormous volumes of in vitro virological data about HIV in order to determine what functions are actually required by the virus in vivo.

Following are just a few questions I would like to see addressed with BLT mice:

- Do topical microbicides prevent mucosal HIV-1 transmission? With multiple isolates?
- What if the V3-loops of LAI and JR-CSF are swapped? Will the heightened pathogenicity of LAI be found to be a function of its CXCR4 tropism?
- What is the lowest number of tissue culture infectious units required in an inoculum to get at least 50% transmission? 5% transmission?
- What if the mice are given an STD (*Chlamydia* sp.) then inoculated with a very low dose of virus? Does the STD increase susceptibility to mucosal HIV-1 transmission?
- Will post-exposure prophylaxis with FTC/TDF work to prevent vaginal HIV-1 transmission? What about other drugs?

- Will pre- or post-exposure prophylaxis with FTC/TDF work to prevent rectal HIV-1 transmission? What about other drugs?
- Will a single dose of FTC/TDF given 48 hours prior to exposure prevent transmission? 24 hours? 12 hours?
- Will a single dose of FTC/TDF given 1 hour post to exposure prevent transmission? 6 hours? 12 hours? 24 hours? 48 hours?
- Do antiretrovirals, as therapy, reduce viral load and allow $CD4^+$ T cell levels to rebound?
- Do resistant mutants arise during antiretroviral therapy like in humans?
- What are the cells initially infected following vaginal inoculation?
- What are the kinetics of the GALT pathogenesis? What about other isolates or patient plasma inoculations?
- Who "wins" if LAI and JR-CSF are inoculated simultaneously into the same vaginal vault? What if the TCIU are not equal between isolates?
- What happens if human plasma from an HIV positive individual is used to mucosally inoculate BLT mice, will only CCR5 viruses be recovered?
- What outcomes are observed when HIV-1 infection is studied in the context of co-infection with another virus (EBV has been shown to infect BLT mice)? KSHV? CMV?
- What is the transmission efficiency when HIV-1 is spiked into human semen or simulated semen then inoculated mucosally?
- Can germ-free BLT mice be generated? If so, how does GALT reconstitution compare to the specific-pathogen free mice currently used?
- What effects on human immune activation does LPS have? In the context of HIV-1 infection (Brenchley, Price et al. 2006)?

- Does morphine or cocaine being present in the mouse alter its susceptibility to mucosal HIV-1 transmission? Morphine has been reported to increase CCR5 expression (Miyagi, Chuang et al. 2000).

The list can go on and on. But even then, there is no reason to limit the discussion of future experiments in the BLT model to HIV research. There has been a long standing discussion in immunology regarding the potential extrathymic development of certain gut T cells. The February 2007 issue of Immunological Reviews alone had nine separate review articles addressing this topic in mice and humans. The journal's introduction article summarize the field, "Collectively, the data from the different groups show that the CD8aa$^+$ IELs can have either a thymic or an extrathymic origin, depending on the experimental circumstances, and therefore the critical issue now is to determine which pathway is normally dominant in vivo" (Kronenberg and Havran 2007). I propose that the presence of human CD8$\alpha\alpha^+$ T cells in the small intestines of BLT mice represent and opportunity to address this issue. One approach to address this question would be to take advantage of another aspect of the BLT system: the ability to generate syngeneic cohorts of humanized mice with and without human T cells. If fetal liver CD34$^+$ cells are transplanted into NOD/SCID mice without a thy/liv implant, then SCID-hu CD34$^+$ HSC mice are produced (as described in Chapter 1). Without a thymus present, do human CD8$\alpha\alpha^+$ T cells appear in the mouse small intestine?

The benefits of making cohorts of humanized mice with and without human T cells are not limited to basic immunology questions. Cancer immunology can also benefit. Islas-Ohlmayer, et al. and Melkus, et al. have shown the experimental potential of cohorts of this nature in cancer research

(Islas-Ohlmayer, Padgett-Thomas et al. 2004; Melkus, Estes et al. 2006). In the absence of T cells, EBV induces tumor formation in the spleens of every inoculated mouse (Islas-Ohlmayer, Padgett-Thomas et al. 2004), but when the human T cells were circulating in the mice, no tumors were observed (Melkus, Estes et al. 2006).

The potential of BLT mice in many areas of research is great. The ability to transmit HIV-1 mucosally in this small animal model emphasizes the exciting potential of the BLT system to revolutionize the way HIV and research is approached. And this is just the beginning…

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Vitae

Paul Wesley Denton was born in Tyler, Texas, on June 17, 1974, the son of Shirley Curry Denton and Marvin Denton. After completing his work at North Caddo Magnet High School, Vivian, Louisiana in 1992, he entered Southern Nazarene University in Bethany, Oklahoma. During the summer of 1993, he studied at the Ausable Environmental Institute in Ausable, Michigan. During the summers of 1994 and 1995, he performed field research at the Quetzal Education Research Center in San Gerardo de Dota, Costa Rica. He received the degree of Bachelor of Science with a major in environmental studies from Southern Nazarene University in May 1997. He holds active certificates to teach all science courses, grades 7-12, in the state of Oklahoma. Paul and his new bride, Janelle, taught at the International Christian School in San Miguel, Santo Domingo de Heredia, Costa Rica during the 1997-1998 academic school year. Paul taught high school biology and physics, as well as 7^{th} grade pre-algebra. In August of 1998, he began work as a laboratory technician at the University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota. Utilizing his employee tuition waiver, he retained his technician position and he matriculated as a graduate student in the UND Department of Microbiology and Immunology in August, 1999. He was awarded the degree of Master of Science in December, 2001. In August of 2002, he entered the Ph.D. program at the University of Texas Southwestern Medical Center at Dallas, TX. Since that time he has been employed as a graduate research assistant at UTSWMC Dallas, Texas. In 1996, he married Janelle Elaine Martinson of Rolette, North Dakota. Daughter, Claire Elise, was born in 2005.

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