INVESTIGATION OF THE ROLE OF MYELOID AND T CELLS IN HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION AND PERSISTENCE \textit{IN VIVO}

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ABSTRACT

Jenna Bone Honeycutt: Role of myeloid cells and T cells in Human Immunodeficiency Virus (HIV) infection and persistence in vivo
(Under the direction of J. Victor Garcia-Martinez)

Human immunodeficiency virus (HIV) infection is the causative agent of AIDS and readily infects CD4+ T cells. I have characterized a humanized T cell only mouse (ToM) model that I have used to better understand pathogenesis of HIV infection in T cells. HIV infection is maintained over the lifetime of these animals, and viral replication is controlled using antiretroviral therapy. I have also demonstrated that latent HIV infection is readily established in ToM and that discontinuation of ART in these mice results in rapid viral rebound. These observations demonstrated that the presence of human myeloid-derived cells is not necessary for effective HIV replication or for HIV persistence in vivo.

However, T cells are not the only targets of infection, and previous studies have indicated that macrophages represent another target for infection as these cells express the cell surface receptors for HIV entry, CD4 and CCR5. Recent evidence that presence of virus in macrophages may be attributed to phagocytosis of T cells by macrophages and not to productive infection has highlighted the need for a careful re-evaluation of HIV infection of macrophages. I characterized the susceptibility of NOD/SCID mice reconstituted with human CD34+ stem cells to infection with HIV. These mice are devoid
of human (and mouse) T cells, and I established that replication in this model occurs in tissue macrophages. I have established that only macrophage-tropic HIV isolates are able to replicate in these mice, HIV is present systemically, and viral replication is sustained over time. Since the only targets for HIV infection present in these mice are of myeloid origin, they have been designated as myeloid-only mice (MoM). Treatment with antiretroviral therapy in MoM results in the rapid depletion of virus in plasma and tissues. Removal of therapy resulted in delayed viral rebound, demonstrating that macrophages are a source of viral rebound after ART-interruption.

Overall, I present herein two new and complementary models of HIV infection: ToM and MoM. These two models along with the humanized bone marrow/liver/thymus (BLT) mouse model, which is reconstituted with human T and myeloid cells, will allow us to establish definitive roles for T cells and myeloid cells in multiple areas of research including mucosal transmission, viral persistence, and new therapies for treatment or for cure.
To my husband, my parents, and my sister, I could not have done this without your love and support over these many years. Thank you for everything.
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CHAPTER 1: INTRODUCTION

HIV ORIGINS AND DISCOVERY

In 1981, the first cases of patients with acquired immunodeficiency syndrome (AIDS), presenting with Pneumocystis carinii pneumonia, in the United States (US) were reported to the Centers for Disease Control (CDC) [1]. Conclusions from the patient clinical histories were that these patients all had “cellular-immune dysfunction related to a common exposure that predisposes individuals to opportunistic infections…” [1]. The human immunodeficiency virus (HIV) epidemic began with the observation of AIDS across multiple countries that was associated with alterations in T cell populations, namely decreased numbers of helper T cells, an increased incidence of opportunistic infections and malignancies, such as Kaposi’s sarcoma [2, 3]. In 1983 two groups published that human T-cell lymphotropic virus-type III/lymphadenopathy-associated virus (HTLV-III/LAV, later renamed HIV) is the etiological agent of AIDS [2, 4].

According to the World Health Organization (WHO), there are approximately 36.9 million people living with HIV as of 2014 [5]. In 2014 alone it is estimated that an additional 2 million people acquired HIV, while 1.2 million people died from AIDS [5]. HIV is a global pandemic and is a significant financial burden worldwide as treatment is costly and required for the lifetime of the infected individual as current treatments are ineffective in eradicating the virus [6, 7]. According to the CDC, the average annual cost of treatment in the US is $23,000 (in 2010 dollars) [8] and lifetime treatment costs
$379,668 (in 2010 dollars) [9]. In the thirty years since its discovery, HIV has been the subject of intense scientific research worldwide.

HIV has a zoonotic origin, derived from simian immunodeficiency virus (SIV) in non-human primates (NHP) [10]. NHP include monkeys, chimpanzees, orangutans, gorillas, gibbons, apes, baboons, marmosets, tamarins, lemurs and lorises [11]. There are two types of HIV, HIV-1 and HIV-2. HIV-1 appears to have originated from species-crossover events in chimpanzees (SIV<sub>cpz</sub>) and gorillas (SIV<sub>gor</sub>) to humans while HIV-2 appears to have originated from crossover of sooty mangabeys (SIV<sub>sm</sub>) to humans [10, 12]. HIV-1 has crossed the species barrier at least four times, resulting in four HIV-1 groups: M, N, O and P. Group M, likely originating from chimpanzees in Cameroon, represents the vast majority (95%) of circulating viral strains worldwide and is further divided into nine subtypes (A-D, F-H, J and K), with subtype C predominating in Africa and subtype B predominating in Europe and North America. Groups N, O and P are primarily restricted to Cameroon and are considered non-pandemic [12]. HIV-2 has crossed the species barrier at least nine times [10].

**HIV PATHOLOGY & PATHOGENESIS**

**OVERALL PATHOGENESIS**

Initial infection with HIV is generally established by a single variant in an otherwise heterogeneous swarm of variants in the exposure inoculum [13]. Localized replication at the site of exposure occurs before systemic spread and established infection [14]. HIV infection is characterized by systemic depletion of CD4<sup>+</sup> T cells [15]. Acute HIV infection is characterized by high levels of plasma viremia that decline after several weeks [16]. There is a systemic inflammatory response resulting in a “cytokine
storm” that can be outwardly manifested as fatigue, fever, and diarrhea [16]. During the acute stage of infection, CD4+ T cells at mucosal sites, such as the gastrointestinal tract, are rapidly depleted [17].

Over time, peripheral and tissue CD4+ T cells are depleted, and chronic HIV infection is characterized by an asymptomatic period that lasts for years [18]. HIV infection also leads to chronic immune activation and loss of memory T cell homeostasis [18-20]. If left untreated, chronic HIV infection nearly always leads to the development of AIDS and the individual becomes susceptible to opportunistic infections [21]. According to the CDC, an AIDS diagnosis is made when an individual has a CD4 cell count less than 200 per microliter or has one or more opportunistic infections that are not generally present in healthy individuals. Progression to AIDS is associated with persistent immune activation and the nadir of the patient’s CD4 cell count [22].

The gut is a significant site of HIV replication, CD4+ T cell depletion and inflammation [23]. CD4+ T cells are rapidly depleted from the gut-associated lymphoid tissue (GALT) within four weeks of infection [17]. The breakdown of gut epithelium either due to CD8+ T cell infiltration or increases in the numbers of regulatory T cells (Treg) in the gut allows for commensal and pathogenic bacterial products to enter the bloodstream [24, 25]. During HIV and SIV infection, microbial products from the intestine, such as lipopolysaccharide (LPS), circulate systemically as a result of microbial translocation [19]. The increased levels of LPS are associated with the systemic immune activation that is seen during chronic HIV and SIV infection [19, 25].

Before widespread use of HIV therapies, neurocognitive dysfunction was another hallmark of HIV infection and was associated with presence of HIV encephalitis (HIVE),
neuronal loss, activation of microglia and the formation of microglial nodules [26-29].

The introduction of ART has greatly reduced the brain pathology that was associated with cytomegalovirus (CMV) infection and other CNS-infiltrating pathogens [26]. Even in the post-antiretroviral therapy (ART) era, nearly half of all ART-treated patients suffer some degree of cognitive impairment, although this impairment is very mild compared to the pre-treatment era [27]. Most common is asymptomatic neurocognitive impairment (ANI), diagnosed by neurological test performance that is at least one standard deviation below the mean performance of healthy controls, and mild neurocognitive disorder (MND), diagnosed by the same testing with the additional criteria that this impairment interferes with daily living [30].

CELL TYPE-SPECIFIC PATHOLOGY

Chronic immune activation during HIV and SIV infection results in increased expression of lymphocyte activation markers (such as CD38), polyclonal B cell expansion and activation, increased turnover of T cells and myeloid cells, as well as increased levels of circulating cytokines and chemokines (including interleukin-6, interferon-alpha, interleukin-10 and B cell activating factor) [31-33]. In SIV-infected rhesus macaques, increased monocyte turnover was a predictor for animals that would progress to AIDS [33]. In HIV-infected patients, monocyte apoptosis was proportional to increased levels of viremia and activation state [34]. Over the course of HIV infection, T cells lose effector functions and have a diminished proliferative capacity in response to antigen stimulation [31]. This decreased ability of T cells to respond to antigen is referred to as “immune exhaustion,” and the prolonged expression of programmed
death-1 (PD-1) molecule on the T cell surface can be used to identify these exhausted cells [35].

**OPPORTUNISTIC INFECTIONS**

The presence of opportunistic infections (OIs) is a sign of early immunodeficiency and has been extensively recognized in HIV-infected patients. One such pathogen is *Candida albicans* that can cause oropharyngeal candidiasis and is associated with reduced CD4+ T cell levels [36]. Another opportunistic pathogen is *Cryptococcus neoformans*, which is distributed worldwide and causes clinical manifestations in the lungs and/or CNS (in the form of meningitis) [37]. While these pathogens are widespread, they do not cause illness in immunocompetent individuals. Additionally, ART-treatment reduces the risk of infection with these pathogens and is recommended to prevent OIs [38].

**HIV AND CANCER**

As mentioned above, HIV infection is characterized by the presence of opportunistic pathogens that do not generally cause disease in immunocompetent individuals. Some of these pathogens are associated with the development of cancer. One such pathogen is Kaposi’s sarcoma herpesvirus (KSHV) and is the causative agent of Kaposi’s sarcoma, the most common AIDS-associated malignancy [39]. There is also a higher incidence of certain cancers associated with Epstein-Barr virus (EBV) and human papillomavirus (HPV) in HIV-infected individuals, and the incidence of disease is correlated with the level of immunosuppression in those individuals [40].

Immune dysregulation during the course of HIV infection can also lead to reduced immune surveillance and an inability to control growth of transformed cells [40,
The immune suppression resulting from HIV infection has led to the classification of HIV-1 as an indirect carcinogen [42]. HIV-infected patients are at increased risk of developing Hodgkin’s or non-Hodgkin’s lymphomas, leukemia, melanoma and anal cancer [43, 44]. The incidence and outcome of these cancers are generally dependent on the levels of immune suppression and tend to be more aggressive in severely immunocompromised patients [45].

**GENOME ORGANIZATION**

HIV is a lentivirus in the *Retroviridae* family. The HIV genome contains nine genes (*gag*, *pol*, *env*, *vif*, *vpr*, *vpu*, *rev*, *tat* and *nef*). *Gag*, *pol* and *env*, encode for the structural proteins, enzymes and envelope proteins. The remaining genes encode for regulatory and accessory proteins [46]. The provirus is flanked by two long terminal repeats (LTR) with the 5’ LTR acting as a promoter for transcription and the 3’ LTR ensuring polyadenylation [47]. There is much diversity in the HIV genome: 48.3% diversity between HIV-1 and HIV-2, 37.5% between major HIV-1 groups (M, N, O and P), 14.7% between subtypes (A, B, C, etc.), 8.2% within subtypes and even 0.6% within individual patients [46]. Envelope sequences are the most diverse, followed by the regulatory, accessory and structural proteins. The enzymatic proteins are the least diverse.

**HIV ENTRY & REPLICATION**

HIV infection requires the presence of the CD4 glycoprotein and a chemokine-receptor, either CCR5 or CXCR4, on the surface of the target cells. For this reason, CD4+ T cells, which highly express all three receptors, represent an abundant target for infection. Entry of HIV into target cells begins with binding of the HIV envelope
glycoprotein, gp120, to the target cell CD4. This binding causes a conformational change in gp120, exposing chemokine-binding domains that allow binding to co-receptors CCR5 or CXCR4 on the target cell. The gp41 peptide then inserts into the target cell membrane, causing conformational changes in gp41 and fusion of the viral and cellular membranes. At this point the viral capsid enters the target cell.

Upon entry into the target cell, the viral capsid is uncoated, and the viral enzyme reverse transcriptase copies the single-stranded RNA genome into complementary DNA (cDNA) [48]. After degradation of the RNA strand, a new DNA strand is synthesized complementary to the cDNA, and the two DNA strands form double-stranded viral DNA. This double-stranded viral DNA is then integrated into the host cell genome by the viral enzyme integrase. Integration into active genes is strongly preferred [48]. During replication, the provirus is transcribed into mRNA, and these viral transcripts are spliced into multiple mRNAs and subsequently translated into viral proteins [49]. The virus is coated in a lipid bilayer during release from the cell membrane. The viral protease then converts this immature particle into a mature virion that is capable of infecting new cells and starting the replicative cycle again [50].

**IN VIVO PLATFORMS TO STUDY HIV INFECTION**

Much has been learned about HIV infection and pathogenesis from patient samples. However, there are many limitations to human studies. First, as humans are complex autonomous beings, it is impossible to control all parameters that could potentially affect study outcomes. Using *in vivo* animal models, we can test novel therapies for potency as well as toxicity and evaluate the role that viral proteins play in the pathogenesis of HIV (or the related SIV) infection [51-53]. In animal models,
biological samples can be obtained on a regular basis, and whole organs can be harvested at necropsy for analysis at pre-determined time points. Also, the types of samples available from human patients are limited by those that are easily obtained (blood and plasma), although more invasive sampling has been performed (intestinal biopsies, bronchoalveolar lavage, cerebrospinal fluid, bone marrow aspirate or collection of peripheral lymph nodes), or those that are completely non-invasive (CT scan, MRI, questionnaire). As a result of HIV’s species restriction for replication in humans and chimpanzees, animal models have been developed and used as surrogate hosts for infection. Namely, lentiviral infection of non-human primates with SIV or SIV/HIV (SHIV) chimeras, or HIV infection of humanized mouse models.

NON-HUMAN PRIMATES

NHP represent a large animal model where SIV infection can be studied in vivo. Although chimpanzees are susceptible to HIV infection, HIV is rarely pathogenic in chimpanzees, and research utilizing chimpanzees has been largely phased out due to ethical concerns [54]. However, other NHP, such as rhesus macaques [55, 56], pig-tailed macaques [53, 57], cynomolgus macaques [58], sooty mangabeyes [59] and African green monkeys [60], have been extensively used. Indian-origin rhesus macaques are the most utilized NHP model for AIDS [61]. As HIV evolved from SIV, there are many characteristics of infection and pathogenesis in these animals that overlap with HIV infection [62]. SIV infection of NHP (with pathogenic or cross-species strains of SIV) results in systemic CD4⁺ T cell depletion and even progression to AIDS [61]. Also, SIV can be transmitted to NHP via mucosal (oral, rectal, vaginal) or
parenteral routes. As in humans, certain MHC class I alleles are associated with viral control in NHP [61].

Limitations of NHP models of infection include: 1) NHP are costly and require housing in specialized primate facilities, and 2) differences exist between SIV and HIV, including the genome, course of disease and co-receptor utilization [59]. For example, SIVmac239 exhibits approximately 50% sequence homology to HIV-1 NL4-3 [63]. Additionally, SIV contains the Vpx gene, thought to be important for counteracting SAM (Sterile Alpha Motif) domain- and HD (Histidine-Aspartic)-domain-containing protein 1 (SAMHD1) and leading to increased infection of macrophages [53, 56, 64]; but Vpx is absent in HIV-1. Similarly, HIV-1 contains the Vpu gene, which enhances virion release from the plasma membrane [47] and is absent in SIV. Genomic differences between SIV and HIV preclude the possibility of testing certain HIV-based immunogens in NHP when the corresponding viral targets are absent [61]. SIV is not sensitive to viral control with several antiretroviral drugs used to treat HIV infection, such as HIV-1 protease, reverse transcriptase and integrase inhibitors, which was an early hurdle in using these animals for treatment studies. Highly intensified ART, consisting of two non-nucleoside reverse transcriptase inhibitors (NRTIs), an integrase inhibitor, a boosted protease-inhibitor, and a CCR5 blocker, has been shown to effectively control viral replication in SIVmac251 infected rhesus macaques [65]. However, chimeric strains of HIV/SIV, called SHIVs, have been utilized to overcome the limited sensitivity of SIV to ART. For example, SHIVs containing HIV-1 reverse transcriptase (RT-SHIVs) are sensitive to treatment with non-nucleoside reverse-transcriptase inhibitors commonly used in HIV-1 [66, 67]. Additionally, simian-tropic HIV-1 (stHIV-1) isolates are being engineered and
tested in pig-tailed macaques to generate viruses that are more closely related to HIV-1 than to SIV [68]. The disease course of SIV is accelerated in NHP compared to humans (simian AIDS in 6-12 months, human AIDS in several years) [69]. Lastly, some SIV isolates are able to use alternative co-receptors for infection, and SIV can rarely utilize CXCR4 as a co-receptor [59].

**HUMANIZED MOUSE MODELS**

Humanized mice have proven to be a useful small animal model for HIV research. One of the early humanized mouse models was the SCID-hu thy/liv model in which SCID (severe combined immunodeficiency) mice are implanted with human thymus and liver tissue, resulting in the formation of a human thymic organoid [70]. Direct injection of HIV into the thymic organoid results in infection of thymocytes, but analysis of infection required direct sampling of the human thymic organoid as there was no systemic reconstitution [71-73]. The derivation of more immunodeficient mouse strains has enabled the creation of humanized mice that are systemically reconstituted with human cells. NOD/SCID and NOD/SCID/γcnull (NSG) mice lack murine T and B cells and have minimal to no NK cell activity and, as such, can be systemically reconstituted with human cells [74]. Implantation of these mice with thymus and liver tissue combined with an autologous hematopoietic stem cell transplant results in robust reconstitution of mice which are referred to as bone marrow, liver, thymus (BLT) mice. In these animals, human T cells are educated in the presence of human thymic tissue and are HLA-restricted [75, 76].

Humanized BLT mice have been extensively used by our laboratory and others to study HIV infection. These mice are susceptible to mucosal (vaginal, oral, rectal) [77-
82] and parenteral infection [51, 80] with HIV. These mice have been used to evaluate HIV prevention strategies [78-80, 83], ART treatment of HIV infection [84, 85] and viral latency and persistence during ART [86-88]. Additionally, these mice have been used to better characterize the functions of viral proteins, such as nef and vif in HIV replication and pathogenesis [20, 51, 89]. Overall, these mice offer a useful model to study full-length HIV isolates in the context of human immune cells.

Humanized mice have certain limitations. HIV-infected humanized mice do not develop an AIDS-like syndrome over the course of infection. These mice have limited humoral immune responses with regard to IgG production and B cell class switching, although there is production of antigen-specific IgM [90-92]. Additionally, cohorts of humanized BLT mice must be individually bioengineered using human tissue which is costly and requires specialized technical expertise. Once generated, due to their intrinsic immunodeficiency, they must be maintained in specialized animal facilities.

**CELLULAR TROPISM OF HIV**

HIV requires one of two chemokine co-receptors for cell entry, CCR5 or CXCR4. In the early days of HIV research, viral strains were deemed either macrophage tropic (M-tropic) or T cell tropic (T-tropic) depending on their co-receptor usage [93]. Viruses that utilized CCR5 were designated M-tropic and viruses that utilized CXCR4 were designated T-tropic [94]. However, it was later recognized that all HIV isolates were T-tropic, regardless of co-receptor usage, but few isolates were M-tropic [95, 96]. Studies have demonstrated that there is no strict correlation between macrophage tropism and utilization of CCR5, with most R5 viruses replicating only in T cells, and that some macrophage-tropic isolates even utilize CXCR4 as a co-receptor [97, 98]. The critical
factor associated with an isolate’s ability to infect T cells or macrophages has now been linked to the levels of CD4 required for viral entry on the surface of target cells [99]. Specific regions of the envelope glycoprotein 120 are thought to determine tropism for T cells and macrophages [100].

**INFECTION OF T CELLS**

The primary target for HIV infection is CD4⁺ T cells, and the levels of peripheral CD4⁺ T cells remains an important prognostic characteristic for HIV infection [101, 102]. HIV preferentially infects activated T cells, which have an abundance of host transcription factors that are necessary for viral production [103]. HIV infection leads to rapid depletion of productively infected cells via the cellular immune response (CTL-mediated) or by direct cytopathic killing during viral replication [101, 104-107]. T cells can be readily infected with CXCR4- (X4), CCR5- (R5) or dual-tropic strains of HIV. X4-tropic strains are extremely pathogenic and typically emerge late during the course of infection in some patients [108, 109]. R5-tropic isolates are almost exclusively transmitted, and the majority of transmitted-founder virus infectious molecular clones from patients are R5-tropic [110, 111].

**INFECTION OF MONOCYTES**

HIV and SIV infection of monocytes has been postulated to be a significant source of viremia after T cell depletion as well as a key player in facilitating viral transmission into the CNS [112-114]. Monocytes, which traffic to virtually all parts of the body, are highly mobile and represent an attractive candidate to traffic the virus into multiple tissues, including the brain [112, 113]. While some groups have detected virus in monocytes isolated from patient samples [114-116], others including our laboratory
(presented herein) have failed to find evidence for HIV-infection of monocytes [117-119]. Furthermore, monocytes are difficult to infect in vitro with HIV unless they are first differentiated into macrophages (monocyte-derived macrophages, MDM) [120]. Monocytes are also a short-lived cell type that undergo spontaneous apoptosis [121] and thus are unlikely to substantially contribute to long-term infection in vivo.

**INFECTION OF MACROPHAGES**

HIV infection of macrophages is determined by the expression levels of CD4 and the HIV co-receptors [99]. The viral envelope determines whether or not a viral isolate can bind to and enter into a macrophage [122, 123]. In contrast to infected T cells, infected macrophages (modeled using human MDMs) are not subject to cytopathic killing due to viral replication [124]. Production of monocyte-colony stimulating factor (M-CSF), a pro-survival cytokine, is thought to contribute to this lack of cytopathic killing as blocking M-CSF renders these cells susceptible to cytopathic death [124]. Another hallmark of HIV infection of macrophages in the pre-treatment era of HIV is the development of multi-nucleated giant cells (MNGC). These cells are formed when the membranes of several HIV-infected macrophages fuse together, and this has been most commonly documented in the brain [125, 126]

Tissue-resident macrophage populations result from both embryonic- and adult-derived hematopoietic stem cells (HSC) [127]. Specialized macrophages such as microglia in the brain as well as some Kupffer cells in the liver and Langerhans cells in the skin are derived at an even earlier time from the yolk sac [127]. Microglia are of special interest in HIV infection as these cells are extremely long-lived (many years) and express the receptors necessary for HIV infection [29, 128]. Additionally, the CNS
penetrance of HIV therapies is thought to be lower compared to other tissues and makes microglial cells attractive candidates for sustaining HIV infection over many years [129, 130].

Recent studies of HIV or SIV infection of macrophages have yielded potentially paradigm-shifting results [131, 132]. Namely, the presence of viral DNA in macrophages may not in itself be an indicator of infection, but could be the result of phagocytosis of infected T cells [131, 132]. Both studies documented evidence for phagocytosis of infected T cells, such as presence of T cell receptor (TCR) DNA [132], resulting in the presence of viral DNA in macrophages. Calatone et al. suggested that myeloid cells are not a major source of infection in vivo, and the presence of SIV DNA and RNA in these cells results from normal phagocytic clearance functions [132]. Baxter et al. reported preferential capture of HIV-infected CD4+ T cells, but concluded that this represented an alternative pathway of macrophage infection, infection by engulfment [131]. Therefore careful re-evaluation of HIV infection of macrophages is needed, especially in cases where phagocytosis of infected T cells is probable.

ANTIRETROVIRAL THERAPY

In 1985 clinical trials began for azidothymidine (AZT), a nucleoside reverse transcriptase inhibitor (NRTI) and the first successful anti-HIV medication [133]. Over time, the virus had mutated and was no longer subject to control with AZT in many patients, as is the case with virtually any antiretroviral therapy when used alone [134, 135]. The eventual failure of AZT monotherapy stems from the fact that HIV develops resistance to these and virtually any other drug used alone [134, 136]. Combinations of medications (three or more) are needed to fully control HIV replication [133, 135]. By
combining three or more drugs with distinct mechanisms of action, viremia can be suppressed for great lengths of time with infrequent emergence of drug resistance. In addition to NRTIs, non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PI), integrase inhibitors and entry (CCR5) inhibitors have been approved for use in patients [133]. The availability of new classes of drugs has been instrumental in the treatment of patients with drug-resistant viruses or those patients that are unable to tolerate the side effects of a particular regimen. In the US, the recommended ART regimen contains two NRTIs (the nucleoside/nucleotide “backbone”) and a third drug from the other classes of therapy. The introduction of ART has shifted the outcome of HIV infection from a fatal condition to management of a chronic disease [137].

HIV PERSISTENCE

LIMITATIONS OF ART

Despite the availability of regimens that effectively control HIV replication, there is no cure for HIV. Viral reservoirs are formed early on during infection, and current therapies are unable to overcome these sources of virus [138, 139]. As discontinuation of therapy results in a re-emergence of virus and continued disease progression, patients are required to remain on therapy for life [140]. There are a few exceptions, one that is the Berlin patient, who has been off therapy for seven years without viral rebound [141]. This patient received an allogeneic HSC transplant from a homozygous CCR5Δ32 donor (which confers resistance to CCR5-utilizing strains of HIV) prior to stopping ART [141]; therefore, the donor cells that reconstituted the Berlin patient were resistant to HIV infection. However, allogeneic HSC transplant are not a viable option for HIV cure strategies for the vast majority of patients, and thus, other strategies to
purge virus reservoirs must be investigated. The VISCONTI patient cohort includes 14 individuals, where control of viremia has been observed for years after ART-interruption [142]. These patients began therapy during primary HIV infection, and were treated for 2-7 years prior to ART-interruption; viral control in these patients has been hypothesized to be related to early treatment initiation [142]. However, these patients continue to be monitored for the presence of re-emerging virus, and may still harbor long-lived reservoirs of HIV.

**ACTIVE RESERVOIRS DURING ART**

There are two types of viral reservoirs during ART: residual active reservoirs that persist despite ART and latent reservoirs of transcriptionally silent virus [143, 144]. The residual active reservoir consists of cells that produce low levels of virus despite ART treatment [145]. Active viral reservoirs have been documented in PBMCs as well as in lymph nodes, tonsils and gastrointestinal samples in HIV-infected patients [146, 147]. After cessation of ART, rapid rebound of virus occurs [140, 148]. The rapid nature of this rebound suggests a persistent active source of virus.

One means of overcoming the residual active reservoir is to develop agents that are able to directly target and kill infected cells, in contrast to ART which prevents infection of new cells [149]. Strategies to kill infected cells, such as immunotoxins, have been tested *in vitro* and *in vivo* [86, 150]. Immunotoxins consist of a particular antibody or domain that targets a cell-surface antigen like HIV-1 and are combined with an effector domain of a protein toxin. The 3B3-PE38 immunotoxin targets the conserved CD4 binding site of HIV-1 gp120 with the *Pseudomonas aeruginosa* exotoxin A effector domain. This immunotoxin has been evaluated in human PBMCs and MDMs where it
effectively blocked HIV spread in culture [150]. High dose treatment with 3B3-PE38 in uninfected rhesus macaques demonstrated an absence of liver toxicity, suggesting this formulation will be well tolerated [150]. In HIV-infected humanized BLT mice on ART, treatment with immunotoxin was able to reduce both vRNA production and the number of HIV RNA+ cells in multiple tissues compared with animals receiving only ART-treatment, suggesting that the immunotoxin was effective against the residual active reservoir that persists despite ART [86].

**LATENT RESERVOIRS OF HIV IN T CELLS**

**MOLECULAR BASIS**

Latent infection of T cells occurs when the HIV provirus is integrated into the genome as the T cell enters a resting state, resulting in transcriptionally silent viral integration [143]. Specifically, as effector memory T cells are created in response to antigen stimulation, HIV can integrate into these activated cells [143, 151]. The majority of these effector cells will die, but the subset that persists can revert to a resting state as memory T cells [143]. While reversion to a resting state post-integration is the more common mechanism for establishing latency in T cells, recent evidence demonstrates that cell-to-cell interactions between human myeloid dendritic cells or monocytes with human T cells may facilitate direct infection (resulting in latency) of non-proliferating CD4+ T cells [152, 153]. HIV utilizes several host factors present in activated T cells for replication, in the resting state, these host factors are not active and therefore viral transcription cannot occur. [154].
MEASURING LATENCY

As latently infected cells are not actively replicating HIV, they are not targeted by conventional ART or the immune system. Specifically, ART treatments target specific steps in the HIV life cycle, and prevent new rounds of infection from occurring, but do not directly kill infected cells. If a cell harboring replication competent HIV is not actively replicating, ART is ineffective and the infected cell is not targeted for degradation by effector mechanisms of the immune system [149]. The frequency of latently infected cells in peripheral blood can be estimated using a quantitative viral outgrowth assay (QVOA) [155, 156], and is reported as infectious units per million (IUPM) resting CD4+ T cells [157]. During the chronic phase of infection, it is estimated that the frequency of latently infected CD4+ T cells is approximately 20 IUPM for patients not on ART and one IUPM for patients on highly-active ART [158]. The average half-life of the latent reservoir is estimated to be approximately 44 months, and eradication of this reservoir with conventional therapy is predicted to take as long as 60 years with a pool of 100,000 total latently infected cells [159]. Additionally, it has been shown that this pool of latently infected cells is established very early during infection [160, 161].

One caveat regarding the measurement of the latent reservoir in patients is that the majority of studies of have been done using peripheral blood, as this is easily accessed. However, peripheral blood may not reflect what is occurring in tissues and complementary studies in animal models of HIV have been used to evaluate latency in tissues. Specifically, the frequency of latently infected cells isolated from the tissues of ART-suppressed animals has been evaluated using HIV-infected humanized BLT mice (~9.9±2.7 IUPM resting CD4+ T cells for pooled tissues) [84, 88] and SIV-infected
macaques (1.3-2 IUPM resting CD4\(^+\) T cells for lymph nodes, spleen and PBMC) [57, 162].

**MODELS OF HIV LATENCY IN T CELLS**

Models of HIV latency include cell lines (generally Jurkat T cell-derived), primary CD4\(^+\) T cells and *in vivo* model systems [57, 84, 144, 162, 163]. Several *in vitro* models of latent infection are Jurkat-derived T cell lines that have a single integration site of HIV [144]. J-Lat and 2D10 cells are two such cell lines, each expressing GFP instead of *nef* [144, 164, 165]. J89 and THP-1 cells as well as J-LAT 6.3 cells contain the full length viral genome with a GFP gene inserted between *env* and *nef* (J89 and THP-1) or as a frameshift in *env* (J-Lat 6.3) [164, 166, 167]. The U1 model is derived from the parent U937 cell line and contains two non-replicating HIV-1 proviruses; latency in this model is associated with suboptimal levels of Tat [168]. The ACH2 model is derived from the parent A3.01 cell line, and TNF-α reactivates virus in this model [168]. The U1 and ACH2 cell lines were used to characterize how the chromatin state impacts HIV-1 transcription [169].

Patient-derived primary CD4\(^+\) T cells have also been extensively utilized to better understand latency and test latency-reversing agents (LRAs) [144]. Although all primary models begin with CD4\(^+\) T cells, there are many differences between how latency is established in the primary cell models [144, 170, 171]. From spinoculation to induction of cytoskeletal changes with chemokines, latency is established with different methods and with varying degrees of frequency [144]. Also there are a variety of viral readouts ranging from luciferase or GFP activity to limiting dilution QVOA [144]. Rather than establishing latency *ex vivo*, some groups directly measure the latent reservoir in
primary resting CD4+ T cells from infected patients [163, 172-175]. Evaluation of treatments to reverse latency in these models has yielded no consensus for potency of any particular agent, and this likely results from the varied methods by which these models were established [144].

The in vivo models of latent infection include NHP and humanized mice [57, 84, 88, 162]. In SIV-infected macaques on ART, “viral sanctuaries” of cells containing viral DNA but not expressing RNA are present in PBMC, lymph nodes, spleen and gut, which is suggestive of a latent pool of infected cells in these tissues [176]. Similar to humans, a lower IUPM was observed in resting CD4+ T cells isolated from the blood of ART-treated compared to untreated SIV-infected macaques [57]. Cells from the lymph nodes, spleen and peripheral blood of ART-suppressed SIV-infected macaques harbor latent virus at a frequency of 1.3-2 IUPM resting CD4+ T cells [57, 162]. Viral latency has also been demonstrated using pooled cells (PBMC, lymph nodes, human thymic organoid, spleen, bone marrow, liver and lungs) from ART-suppressed HIV-infected BLT mice and is present at similar IUPM (~9.9 IUPM resting CD4+ T cells) compared with patient samples (range: 1-20 IUPM resting CD4+ T cells) [87, 158].

LATENCY-REVERSING AGENTS (LRAS)

Latency is a reversible state of non-productive infection, and understanding the mechanisms by which these latently infected cells can be induced to produce virus (which would render them susceptible to ART) or finding ways to eliminate these cells will be critical for an HIV cure [177]. Global activation of T cells effectively reverses the resting state of these latently infected cells, but results in significant toxicity from increased cytokine production [178, 179]. Thus, latency-reversing agents (LRAs) that
induce expression of HIV-1 without global cell activation are needed. Once HIV expression is established, these cells could be eliminated by cytopathic killing, as a result of viral replication or by elimination of virally infected cells by the immune system ("shock and kill" or "kick and kill" strategy). LRAs under investigation include histone deacetylase inhibitors (HDACi, such as panobinostat and suberoylanilide hydroxamic acid) [163, 180], PKC activators (such as bryostatin) [181], and bromodomain inhibitors (JQ1) [182], as well as combinations thereof [183, 184]. The ability of LRAs to induce virus production in latently infected cells depends greatly on the model system used. Phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) are the only stimuli that induce latent viral activation in all cellular (primary and cell lines) models [144].

Patient studies evaluating the efficacy of several HDACi have yielded various insights as to the ability of these LRAs to purge viral reservoirs. Administration of vorinostat to patients was demonstrated to be safe and increased HIV RNA expression in T cells, but no increase in plasma viremia was observed [173, 174, 185]. Administration of panobinostat or romidepsin increased HIV-1 transcription and increased plasma viremia in some (panobinostat) or all (romidepsin) patients [172, 186]. However, none of these HDACi demonstrated a measurable impact on the size of the latent reservoir in patients. Therefore, additional agents will be required to boost the immune system and aid in viral clearance [187].

**HIV RESERVOIR IN MACROPHAGES**

While T cells are considered the major barrier to eradication of HIV, other cell types may also harbor latent virus during ART-treatment. HIV-infected macrophages possess several characteristics that support the idea that they represent a potential
reservoir for HIV [188-190]. Specifically, macrophages are long-lived cells (up to years), and HIV-infected macrophages are resistant to the cytopathic killing seen in HIV-infected T cells [124]. In addition, ART appears to be less potent in macrophages compared to T cells, with lower drug availability in macrophages [191].

The possibility of HIV persistence in macrophages during ART has been suggested by several groups [192, 193]. The presence of HIV-DNA in non-CD4+ T cells (later identified as macrophages by cell sorting) was observed in samples procured from the gastrointestinal tract of virally suppressed patients on ART [192]. However, the presence of viral DNA in macrophages may not indicate infection but rather phagocytosis of infected T cells [132], thus careful and rigorous evaluation of this potential reservoir must be carried out. Additionally, few studies have evaluated if tissue macrophages from ART-suppressed patients can produce replication competent virus \textit{ex vivo} [194], which would provide proof that these cells harbor latent replication-competent virus.

**CONTRIBUTION OF T CELLS AND MACROPHAGES TO HIV PERSISTENCE**

To better understand the role that T cells and macrophages play in facilitating long-term maintenance of infection, \textit{in vivo} models in which HIV infection of one cell type can be evaluated in the absence of other cell types are particularly useful. Namely, parsing out the roles that individual cells types can play in establishment of infection, viral replication, and establishment of latent and persistent reservoirs \textit{in vivo} is critical to developing targeted strategies for HIV eradication and cure. To this point, I have characterized two novel humanized mouse models: the T cell-only mouse (ToM) and the myeloid-only mouse (MoM). Using these two complementary model systems along
with BLT mice (having both T cells and myeloid cells), I have investigated the separate roles of T cells and myeloid cells in HIV persistence in vivo.

First, I phenotypically characterized ToM and demonstrated their capacity to replicate HIV systemically over time, and that conventional ART is able to suppress viremia in these animals (Chapter 2). Human T cells were sufficient for the establishment of latent infection, in the absence of any cytokine/chemokine contribution from human myeloid cells (which are absent). Also, ToM harbored latently infected cells at similar levels compared to HIV-infected, ART-suppressed patients (Chapter 2). Removal of ART from virally suppressed ToM resulted in the rapid rebound of virus (Chapter 2, Chapter 4).

Second, I phenotypically characterized MoM and evaluated their capacity to replicate HIV (Chapter 3). In light of the recent evidence that macrophages frequently phagocytose infected CD4+ T cells resulting in the presence of viral DNA, it was critical to determine if HIV can productively infect macrophages in the absence of human T cells [131, 132]. Using MoM, I demonstrated that macrophages can sustain HIV infection over time in the complete absence of T cells (Chapter 3). However, only macrophage-tropic HIV isolates were able to replicate in the absence of T cells. Transfer of macrophages from infected hosts into naive animals was sufficient to infect the naive animals, demonstrating that macrophages can establish de novo infection (Chapter 3).

Macrophages are not subject to the same cytopathic effects seen in infected T cells [195], are long-lived cells [190], and have lower intracellular concentrations of ART compared with PBMCs [191, 196]; therefore, these cells could be a source of
persistent HIV replication during ART or a source of viral rebound during ART-interruption [194]. However, there is a lack of evidence in primary cells that macrophages harbor latent virus. Previous attempts to characterize the “latent reservoir” in macrophages have largely depended solely on the presence of viral DNA in these cells, which could instead be the result of phagocytosis of T cells and not latent infection [131, 132]. ART effectively suppressed plasma viremia and reduced tissue viral RNA (vRNA) and viral DNA (vDNA) levels in infected MoM (Chapter 4). Removal of ART resulted in viral rebound in MoM, but this rebound was very delayed in comparison to ToM or BLT mice (Chapter 4). These results indicate that macrophages are a source of viral rebound after ART-interruption. Overall, I present herein two separate but complementary models that can be used to evaluate viral reservoirs during HIV infection.
CHAPTER 2: HIV INFECTION, RESPONSE TO TREATMENT AND
ESTABLISHMENT OF VIRAL LATENCY IN A NOVEL HUMANIZED T CELL-ONLY
MOUSE (TOM)\(^1\)

SUMMARY

The major targets of HIV infection in humans are CD4\(^+\) T cells. CD4\(^+\) T cell
depletion is a hallmark of AIDS. Previously, the SCID-hu thy/liv model was used to
study the effect of HIV on thymopoiesis \textit{in vivo}. However, these mice did not develop
high levels of peripheral T cell reconstitution and required invasive surgery for infection
and analysis. Here, we describe a novel variant of this model in which thy/liv
implantation results in systemic reconstitution with human T cells in the absence of any
other human hematopoietic lineages.

NOD/SCID-hu thy/liv and NSG-hu thy/liv mice were created by implanting human
fetal thymus and liver tissues under the kidney capsule of either NOD/SCID or NSG
mice. In contrast to NOD/SCID-hu thy/liv mice that show little or no human cells in
peripheral blood or tissues, substantial systemic human reconstitution occurs in NSG-hu
thy/liv mice. These mice are exclusively reconstituted with human T cells (hence, T cell-
only mice or ToM). Despite substantial levels of human T cells, no signs of graft-versus-
host disease (GVHD) were noted in these mice over a period of 14 months. ToM are

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response to treatment and establishment of viral latency in a novel humanized T cell-only mouse (TOM) model.
\textit{Retrovirology}. 2013 Oct 24; 10:121. PMID: 24156277. JBH, AW, NA, SC performed experiments. JBH, DM, and JVG
designed the study.
readily infected after parenteral exposure to HIV-1. HIV replication is sustained in peripheral blood at high levels and results in modest reduction of CD4$^+$ T cells. HIV-1 replication in ToM responds to daily administration of combination antiretroviral therapy (ART) resulting in strong suppression of virus replication as determined by undetectable viral load in plasma. Latently infected resting CD4$^+$ T cells can be isolated from suppressed mice which can be induced to express HIV \textit{ex vivo} upon activation demonstrating the establishment of latency \textit{in vivo}.

\textbf{INTRODUCTION}

SCID-hu thy/liv mice develop a bonafide human thymic organ and have a marginal level of systemic reconstitution with human T cells [197, 198]. The human thymic organoid present in the SCID-hu model is susceptible to HIV infection [199]. However, infection of these animals requires this tissue to be surgically exposed to virus administration via direct injection [73]. HIV injection results in infection of the human thymocytes present, but there is no detectable viremia in these mice. Thus, analysis of virus replication and its effect on thymocytes requires surgical removal of a piece of tissue [73]. Subsequent monitoring of infection over time also requires additional surgical collection of tissue for analysis. Although the use of this model is extremely labor intensive and requires large numbers of animals to make meaningful observations, the SCID-hu thy/liv model has been extensively used to evaluate HIV pathogenesis of the thymus, the effect of HIV on thymocyte development, the establishment of HIV latency in thymocytes \textit{in vivo}, the efficacy of antiviral drugs on thymocytes and the role of auxiliary genes of HIV in virus replication and CD4$^+$ thymocyte destruction [71, 72, 200, 201].
Following the development of the SCID-hu thy/liv model, several other novel strains of mice have been derived with a higher degree of immunodeficiency. These include the NOD/SCID and the NOD/SCID common gamma chain null (NSG) strains of immunodeficient mice [197]. Both of these strains have been extensively and successfully used in the derivation of a variety of humanized mouse models [202]. However, neither of these two strains has been extensively used to produce humanized thy/liv implanted mice [203].

Resting CD4+ T cells represent a well-characterized reservoir for latent HIV-1 infection, and this reservoir persists long-term despite treatment with highly active antiretroviral therapy (HAART) [87, 204, 205]. Incubating resting CD4+ T cells with CCL19, secreted by mature dendritic cells, \textit{ex vivo} increases HIV-1 integration efficiency [206]. Additionally, the chemokines CXCL9 and CXCL10, secreted by monocyte-derived cells and induced by IFN-\gamma production, seem to mediate similar effects in resting T cells [204, 206-208]. Secretion of IL-7 by dendritic cells may be important for the survival of memory T cells, and secretion of IL-15 by macrophages and other mononuclear phagocytes is important for the low level of proliferation necessary to maintain a resting memory pool over time [209]. Thus while it is known that several myeloid-derived cell types secrete cytokines and chemokines that facilitate the development of latency and maintain the resting CD4+ T cell pool, whether or not these cells are necessary for the establishment of latency \textit{in vivo} remains unknown [210].

With the long-term goal of obtaining a better understanding of HIV replication, CD4+ T cell depletion, HIV latency and persistence \textit{in vivo}, we sought to study HIV-1 in a humanized mouse model that possesses human T cells but is devoid of human
myeloid (and B) cells. To this effect, we implanted human thymus and liver into NOD/SCID and NSG mice. In this study we show that whereas NOD/SCID-hu thy/liv mice do not develop high levels of systemic reconstitution with human cells, NSG-hu thy/liv mice develop high levels of human T cells in the peripheral blood. Remarkably, flow cytometric analysis of blood and tissues demonstrate the complete absence of human B and myeloid cells in these mice. Interestingly, in contrast to mice reconstituted with human peripheral blood mononuclear cells (PBMC) and some other types of humanized mice [211, 212], these T cell-only mice (or ToM) do not develop signs of GVHD. In addition, ToM are readily susceptible to HIV infection after parenteral exposure and can sustain high levels of HIV replication. Virus replication can be efficiently suppressed by antiretroviral therapy, and HIV latency is established in resting T cells.

EXPERIMENTAL PROCEDURE

GENERATION OF HUMANIZED MICE

Humanized ToM were prepared by implanting thymus and liver tissue into 6-8 week old NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG, The Jackson Laboratory) mice. NOD/SCID-hu thy/liv (N/S-hu) mice were prepared in the same manner by implanting thymus and liver tissue into NOD.CB17-Prkdc<sup>scid</sup>/J mice (NOD/SCID, The Jackson Laboratory). Seven different tissue sets were used to generate the humanized mice presented in this manuscript. The thymus and liver implants consisted of a 1-2 mm piece of liver tissue sandwiched between two pieces of autologous thymus that were placed under the left kidney capsule (Advanced Bioscience Resources, Alameda, CA). All mice were maintained in a specific pathogen-free facility with the Division of
Laboratory Animal Medicine at the University of North Carolina at Chapel Hill (UNC-CH) according to protocols approved by the Institutional Animal Care and Use Committee. Human reconstitution of mice was monitored by flow cytometric analysis for human CD45\(^+\) cells in peripheral blood, as previously described [79, 80]. Peripheral blood samples were obtained via submandibular venipuncture and were collected in tubes containing EDTA. Whole peripheral blood was stained with antibodies, red blood cells were lysed, and the remaining cells were washed and fixed using a 1% paraformaldehyde solution. A total of 10,000-30,000 events were collected per animal at each time point as indicated below.

**TISSUE HARVESTING AND FLOW CYTOMETRIC ANALYSES OF HUMANIZED MICE**

Mononuclear cells (MNCs) were isolated from the bone marrow, spleen, lymph nodes, lung, liver, and thymic organoid as previously described [80]. Tissues were minced and/or digested and filtered through a 70 µm strainer. The liver, lung and female reproductive tract (FRT) were processed as previously described [83]. For all latency determinations, mononuclear cells, with the exception of the lymph nodes, were isolated using a Percoll gradient. Red blood cells were lysed as needed (namely for the spleen, bone marrow and liver tissues). MNCs were washed, counted via trypan blue exclusion, and flow cytometric analyses were performed for the indicated markers [79, 80, 83, 87, 213]. Live cells were distinguished by their forward and side scatter profiles as previously described [214]. Flow cytometry data was collected on either a BD FACSCanto or a BD LSRFortessa flow cytometer, and analyzed using BD FACSDiva software (v.5.0.2 or v.6.1.3).
**HIV-1 INFECTIONS**

Stocks of HIV-1\textsubscript{JR-CSF} were prepared and titered as previously described [215]. Briefly, virus supernatants were prepared via transient transfection of 293T cells, and were titered using TZM-bi cells essentially as we have previously described [77]. Parenteral exposures were performed using HIV-1\textsubscript{JR-CSF} (90,000 TCIU) administered either intravenously or intraperitoneally. A total of two intraperitoneal and six intravenous exposures were performed, yielding 2/2 and 6/6 systemically infected animals, respectively.

**ANALYSIS OF HIV-1 INFECTION**

Peripheral blood was collected via retro-orbital bleed using EDTA coated capillary tubes (approximately 100 ul total). Infection of ToM with HIV-1 was determined with a one-step reverse transcriptase real-time PCR assay (ABI custom TaqMan Assays-by-design) according to the manufacturer's instructions (with primers 5’-CATGTTTCAGCATTATCAGAAGGA-3’ and 5’-TGCTTGATGTCGCCCCACT-3’; assay sensitivity of 400 RNA copies per mL). Additionally, the percent of human CD4\textsuperscript{+} T cells in the peripheral blood of ToM pre- and post-exposure to HIV-1 were monitored by flow cytometry (using 40-60 ul of blood). Changes in the percent of CD4\textsuperscript{+} T cells present in the tissues of infected and uninfected animals were compared by two-way ANOVA, and were not significantly different. Statistical analysis was performed in Prism version 5 (GraphPad Software, Inc., San Diego, CA).

**ANTIRETROVIRAL TREATMENT OF TOM**

For HIV treatment we used a previously described triple combination of drugs that we have shown to be effective at suppressing viral load in humanized mice.
Specifically, infected ToM were administered daily intraperitoneal injections of emtricitabine (FTC; 140-200 mg/kg), tenofovir disoproxil fumarate (TDF; 146-208 mg/kg) and raltegravir (RAL; 56-80 mg/kg) for six to nine weeks, as previously described [87]. HIV-1 infection was monitored throughout ART as described above.

RESTING CELL ISOLATION AND LATENCY DETERMINATIONS OF TOM AND PATIENT SAMPLES

All MNCs from individual mice were pooled. Resting human CD4\(^+\) T cells were isolated from pooled tissues or from leukapheresis product of patient samples using negative magnetic selection (STEMCELL Technologies, Vancouver) as previously described [87, 163, 205]. Briefly, MNCs obtained from mouse tissues were incubated with a cocktail of antibodies composed of mouse anti CD45 and TER119, and anti-human CD8, CD14, CD16, CD19, CD56, CD41, CD25, CD31, CD105, HLA-DR, and glycophorin A. For the separation of cells from human samples, the mouse antibodies were not included in the isolation cocktail. Antibody-bound cells were removed using a column based-magnetic purification system and the purified resting cells were collected as flow through. This approach resulted in a >99% pure resting CD4\(^+\) T cell population. Resting CD4\(^+\) T cells were then cultured with 15 nM efavirenz and 1 \(\mu\)M raltegravir for 2 days prior to performing viral outgrowth assays to prevent any de novo infection from unintegrated virus [87]. Viral outgrowth was achieved by maximally stimulating resting cells in limiting dilution cultures containing 60 U/ml IL-2, 1\(\mu\)g/ml phytohemagglutinin (PHA) and irradiated allogeneic PBMC from an uninfected donor [87, 205]. The culture media was replaced every 3-4 days with fresh media containing 5U/ml IL-2. CD8 depleted PHA-stimulated PBMC from an uninfected donor were added twice during the
experiment to facilitate virus spread/amplification in cultures. Cultures were scored positive if p24 was detectable at day 15 and confirmed on day 19. The number of infected resting cells was estimated by a maximum likelihood method and was expressed as the infectious units per million resting CD4\(^+\) T cells (IUPM) [205].

RESULTS

CHARACTERIZATION OF THY/LIV IMPLANTED NSG AND NOD/SCID MICE

SCID-hu (thy/liv) mice have been extensively used as an \textit{in vivo} model to study HIV infection of the thymus [72]. Since the original development of the SCID-hu thy/liv model, new and improved strains of immunodeficient mice like NOD/SCID and NSG have been developed [200, 201]. We implanted human thymus and liver into NOD/SCID and NSG mice to determine whether or not these strains would be an improvement over the SCID-hu model. We then monitored the peripheral blood (PB) of these mice over time by polychromatic flow cytometry for the presence of human cells (hCD45). While the NOD/SCID implanted mice, like the original SCID-hu mice, did not have significant levels of human cells in their PB, the implanted NSG mice had substantial levels of human reconstitution as determined by presence of human CD45 in their PB (Figure 2.1A). Furthermore, human cells present in the PB of these mice were identified as T cells by their cell surface expression of human CD3 (Figure 2.1B). Interestingly, exhaustive analysis for the presence of other lymphoid or myeloid human cells did not reveal any significant levels of these cells in the PB of any animals analyzed. Specifically, we did not detect human B cells (CD19\(^+\)), human natural killer cells (CD56\(^+\)), or human myeloid cells (CD33\(^+\)) in the peripheral blood of NSG-implanted mice (Figure 2.1B). Additionally, there were no human dendritic cells present in these
mice (Lin−/HLA-DRhi, data not shown). Thy/liv implanted NSG mice showed sustained production of human T cells that reached approximately 20% in peripheral blood for up to 30 weeks (the last time point analyzed). Over this period, no signs of graft-versus-host disease (GVHD) were observed. Additionally, some animals were followed for up to 12 months post-implant (the last time point analyzed). These animals were found to sustain 20-30% human T cells in the PB even at these late time points (n=2, data not shown). From these results, we concluded that implantation of human thymus and liver into NSG mice results in sustained and exclusive production of human T cells in vivo.

In SCID-hu mice, human cells are almost exclusively found in the thymic organoid, with little reconstitution of PB or tissues [216]. To determine the systemic distribution of the human cells present in NSG mice, mononuclear cells were isolated from the bone marrow, spleen, lymph nodes, liver, lung, and the thymic organoid. Flow cytometric analyses demonstrated that the spleen, lymph nodes, liver, lung, and thymic organoid were robustly reconstituted with human cells (CD45+) (Figure 2.2A). Consistent with the lack of hematopoietic stem cell engraftment in these mice, low levels of human cells were observed in the bone marrow. To determine if the tissues were repopulated with human T cells, we used flow cytometry to assess the presence of hCD3. We observed that greater than 99% of human cells in the bone marrow, spleen, lymph nodes, liver, and lung were human T cells (CD3+) (Figure 2.2B). All tissues and the peripheral blood were reconstituted with both CD4+ and CD8+ T cells (Figure 2.2C). In the thymic organoid, we also observed a preponderance of human CD3+ cells at various stages of differentiation [217] (Figure 2.2B). We also noted an abundance of double positive (CD4+/CD8+) T cells, consistent with normal thymopoiesis (Figure 2.2C).
Additionally, the small and large intestines of ToM were analyzed by flow cytometry and immunohistochemistry. In contrast to all other tissues analyzed we found no significant levels of human cells present in the gastrointestinal tract of these animals (data not shown).

We further investigated the phenotypes of the human T cells present in PB and tissues. Both CD4$^+$ and CD8$^+$ T cells from PB and tissues of these mice had a predominantly naïve phenotype, expressing both human CD45RA and CD27 (Figure 2.3). Additionally, we found CD4$^+$ T cells with central memory (CD45RA$^-$/CD27$^+$) and effector memory (CD45RA$^-$/CD27$^-$) phenotypes (Figure 2.3A). Within the CD8$^+$ T cell population, mainly naïve and central memory phenotypes were observed, with effector memory phenotypes being extremely rare in this population (Figure 2.3B). These results demonstrate that the human CD4$^+$ T cells in these mice have a normal developmental phenotype.

**HIV INFECTION OF TOM**

Once we established the systemic reconstitution of ToM, we tested whether or not they could support HIV-1 replication. Eight ToM were infected with a single dose of cell-free HIV-1$_{JR-CSF}$, a CCR5-tropic isolate administered parenterally. We then monitored the plasma of ToM for the presence of HIV-1 RNA as previously described [83, 87]. HIV-RNA was detected in the plasma of ToM one week after exposure and high levels of viremia were maintained over time (Figure 2.4A). We also monitored CD4$^+$ T cell depletion in PB, a measure of HIV pathogenesis. The presence of HIV-RNA in PB correlated with a subsequent drop in circulating CD4$^+$ T cells (Figure 2.4A). Similar decreases in CD4$^+$ T cell levels were observed in all tissues analyzed consistent
with systemic virus spread (Fig. 2.4B). Phenotypic analysis of the remaining CD4+ T cells in the infected mice demonstrated the specific depletion of effector memory T cells (TEM) cells in PB and tissues (Figure 2.4C). These results demonstrate the susceptibility of ToM to HIV infection, their ability to sustain high levels of virus replication and the depletion of CD4+ TEM cells from PB and tissues of infected animals.

**SUPPRESSION OF HIV BY ART IN TOM**

Having established the capacity of ToM to support HIV infection, we proceeded to determine if virus replication could be suppressed by combination ART. For this purpose, HIV infected ToM were treated daily with a combination of FTC, TDF and raltegravir. This regimen has been shown by our laboratory to effectively suppress viral replication in the humanized BLT mouse model [87]. To assess the effectiveness of ART in infected ToM, levels of HIV-RNA were monitored in the plasma throughout treatment. ART administration resulted in a rapid reduction in the levels of plasma HIV-RNA in all treated animals (Figure 2.5B). Five weeks after initiation of treatment the levels of HIV-RNA in plasma were below the detection limit of our assay (750 RNA copies/ml). These results demonstrate the efficacy of ART in controlling HIV replication in humanized ToM. However, even long-term ART does not result in virus eradication and treatment interruption in patients leads to viral rebound [218]. To determine if this also occurs in ToM, one infected mouse was suppressed for four weeks on ART (treated for eight weeks total), after which treatment was ceased. One week after treatment cessation, viral RNA was detected in the plasma of this mouse demonstrating the expected virus rebound (Figure 2.5C).
QUANTIFICATION OF LATENT RESERVOIR

In humans, ART results in virus strong virus suppression, increases in CD4+ T cell levels and other significant health benefits to patients [219]. Despite this strong virus suppression seen in ART treated patients, HIV persists in resting CD4+ T cells that form a long lasting latent reservoir [220]. To determine if HIV could establish a latent reservoir in ToM, we first confirmed the presence of resting human T cells in ToM. To assess the presence of resting human T cells in ToM, we collected cells from PB, bone marrow, spleen, lymph nodes, liver, lung, and thymic organoid from HIV+ ART suppressed mice. Cells from all the tissues obtained from each individual mouse were pooled together and human CD4+ T cells in each pool of cells corresponding to one individual mouse were analyzed for expression of hCD25 and HLA-DR. This analysis demonstrated the presence of significant numbers of resting human CD4+ T cells in ToM (Figure 2.6). To determine if resting cells were latently infected with HIV, pooled cells from all tissues of each individual ART suppressed mouse were used to isolate resting CD4+ T cells by negative selection using magnetic beads [87, 205]. After magnetic selection, a highly purified population of resting CD4+ T cells, shown by a lack of CD25 and HLA-DR expression was obtained from each individual mouse (Figure 2.6).

To quantify the frequency of latently infected resting CD4+ T cells in these mice, we used a protocol validated for the same purpose for use in humans [221]. Specifically, the pooled resting cells from each individual mouse were cultured with efavirenz and raltegravir for two days to prevent any de-novo infection from unintegrated virus that may be present. Next, the resting cells were maximally stimulated in limiting dilutions
with PHA, IL-2 and irradiated PBMC from an uninfected donor, followed by co-culture with allogeneic PHA-activated CD8^+T-cell-depleted feeder cells. Fifteen days later, cultures were tested for the presence of HIV (Figure 2.7A). The number and density of cultures was then used in a maximum likelihood method to estimate the number of infectious units per million cells (IUPM). Latently infected cells were obtained from all four animals analyzed (Figure 2.7B). IUPM values from mice were compared with values obtained from outgrowth assays using resting CD4^+ T cells of HIV infected patients treated during the acute or chronic phase of infection. The levels of latently infected cells in these mice are within those observed in the chronic patients receiving suppressive ART [159, 222]. To confirm that this indeed is induction from latency, as an added control, prior to stimulation, supernatant from resting cell cultures were assayed for P24 and were all found to be negative (data not shown) suggesting that virus recovered from outgrowth experiments originated from latent provirus. These results demonstrate the establishment of HIV latency in ToM and demonstrate that in vivo human T cells alone are sufficient for establishing latency.

**DISCUSSION**

Although SCID-hu thy/liv animals have been used extensively to study thymopoiesis and HIV-1 infection of the thymus, additional applications of this model has been limited by the lack of peripheral access to the human cells [223, 224]. Specifically, in this model a lack of systemic reconstitution with human cells requires invasive surgery for infection and monitoring of virus replication [73]. In one report, low levels of human cells in PB, spleen and lymph nodes SCID-hu thy/liv implanted mice were noted [225]. However, this required implantation of twenty pieces of human thy/liv
tissue under both kidney capsules of each mouse. Using this more invasive implantation strategy combined with 20X more tissue, HIV-1 infection was achieved after IP or intra-implant injection. Using the original implantation strategy described for SCID-hu mice, the use of more immunodeficient mouse strains, like the NSG strain, has overcome the limited systemic reconstitution previously seen in SCID-hu mice. Interestingly, thy/liv implantation of NOD/SCID mice did not result in systemic reconstitution with T cells suggesting that the additional immunosuppression due to the lack of a functional common gamma chain observed in NSG mice resulting in a complete lack of natural killer cells [226] is likely contributing to the increased T cell levels in these mice.

ToM were systemically reconstituted with human T cells. This reconstitution is consistent with the continued production of human T cells from the implanted thy/liv organoid as it showed a substantial and robust population of CD3+/CD4+/CD8+ thymocytes for as long as the animals were examined (1.2 years). Consistent with the lack of cryptopatches in NSG mice [227] ToM showed essentially no significant accumulation of human T cells in the intestinal tract (data not shown). ToM show phenotypically normal CD4+ T cell development. However, we noted somewhat limited CD8+ T cell development in ToM, with few effector memory CD8+ cells. These differences in the formation of effector phenotypes in the CD4+ and CD8+ T cell populations may be due to the absence of cytokine signals from professional APCs as well as CD4+ helper T cells that limit CD8+ T cell activation/differentiation [217]. The reduction in the percentage of CD4+ T cells with an effector memory phenotype pre- and post- HIV infection cannot be attributed to differences in the source of donor tissues
since tissue from a total of 11 different donors were used to generate the mice used for these experiments.

One salient feature of ToM is the fact that despite robust levels of human T cells, they do not develop GVHD. GVHD has been observed in multiple humanized mouse models [228, 229]. Some investigators have reported a significance incidence of GVHD beginning approximately 12 weeks post-humanization and leading to death of the animals as early as 15 weeks post-humanization [228]. In contrast, we did not notice any of these effects on ToMs at these or subsequent time points. The longevity of ToM systemically reconstituted with high levels of human T cells in the absence of GVHD is an important feature of this model.

ART offers significant benefits to HIV infected patients. Our results show that combination ART is able to suppress viral replication in ToM validating this model for the evaluation of the effect of antivirals on HIV replication in vivo. As in humans, therapy interruption resulted in rapid viral rebound. Furthermore, we show that human T cells alone are sufficient for the establishment of HIV latency in resting CD4$^+$ T cells. Additionally, latently infected cells in ToM can be induced ex vivo to produce virus. The frequency of latently infected resting human CD4$^+$ T cells in ART suppressed ToM are within the range seen circulating in PB of ART suppressed patients, regardless of when therapy was initiated. T cells represent the major reservoir of latent HIV in humans. Therefore, ToM may represent a unique tool for studies of HIV eradication strategies, as they have latently infected resting CD4$^+$ T cells in the complete absence of any myeloid cells.
In summary, ToM represent a significant advance over the original SCID-hu thy/liv model because they have substantial levels of T cells in both PB and tissues. ToM are systemically but exclusively reconstituted with human T cells enhancing their utility for the study of T cell development, repopulation, function and response to stimuli \textit{in vivo}. The presence of human T cells in blood permit direct inoculations with HIV and direct monitoring of virus infection via blood plasma facilitating the longitudinal analysis of HIV infection and its effects on CD4\(^+\) T cells. ART efficiently inhibits HIV replication in ToM resulting in strong viral suppression. The ability to suppress HIV replication by ART in ToM allows the use of these mice to investigate latently infected resting human CD4\(^+\) cells \textit{in vivo}. Because these mice do not develop GVHD and systemic reconstitution with human T cells is sustained at high levels for over a year, long-term experiments are greatly facilitated in this model.
Figure 2.1: Analysis of the peripheral blood (PB) of thy/liv implanted NSG mice demonstrates long-term reconstitution with human T cells. A) Flow cytometric analysis of the PB of NOD/SCID-hu (N/S-hu) (black circles) and NSG thy/liv (gray triangles) mice demonstrates systemic reconstitution of implanted NSG mice with human cells (CD45+; solid line) and human T cells (CD3+; dashed line). Gating strategy: live cells→human CD45→human CD3. B) Flow cytometric analysis of cells from PB of a representative NSG-hu thy/liv mouse (29 weeks post-implant) demonstrates these mice are exclusively reconstituted with human T cells with greater than 99% of cells expressing human CD3. There is a lack of B cells (CD19+), natural killer cells (CD56+), and myeloid cells (CD33+) in the PB of ToM. PB CD3 expressing cells were then analyzed for CD4 and CD8 expression levels. The majority of CD3+ cells in PB expressed CD4+ (87%).
Figure 2.2: Peripheral tissues of implanted thy/liv NSG mice are extensively reconstituted with human T cells. A) Flow cytometric analysis of cells harvested from the bone marrow (BM), spleen (Spl), lymph nodes (LN), liver (Liv), lung, and the thymic organoid (TO) of implanted thy/liv NSG mice (n=15) demonstrated reconstitution with human cells (CD45⁺). B) Flow cytometric analysis of tissues harvested from an implanted thy/liv NSG mouse (29 weeks post-implant) demonstrated that all organs were reconstituted exclusively with human T cells or thymocytes. C) Flow cytometric analysis of tissues and PB harvested from an implanted thy/liv NSG mouse demonstrated that all organs were reconstituted with both CD4⁺ and CD8⁺ T cells.
Figure 2.3: Naïve/memory phenotype of T cells in the PB and tissues of ToM.

(left) The distribution of central memory, naïve and effector memory phenotypes of CD4+ (A) and CD8+ (B) T cells in the PB and tissues of ToM (n=7) was determined with flow cytometry. Animals were approximately 35 weeks post-implantation on average at time of harvest. (right) Naïve T cells were defined as CD45RA+CD27+, central memory T cells (TCM) were defined as CD45RA-CD27+, and effector memory T cells (TEM) were defined as CD45RA-CD27-. Error bars represent the Mean + SEM.
Figure 2.4: HIV-1 replication and CD4+ T cell depletion in ToM. A) ToM (n=8) were parenterally exposed to HIV-1 and the viral load monitored in PB plasma (black circles). Changes in CD4+ T cell levels in PB were measured over time using flow cytometric analysis (gray squares). Gating scheme: live cells $\rightarrow$ hCD45 $\rightarrow$ hCD3 $\rightarrow$ CD4. The limit of detection for viral load is indicated with a dashed line. B) The percentage of CD4+ T cells present in the tissues of infected (gray bars) and non-infected (black bars) ToM were analyzed using flow cytometric analysis. Gating scheme: live cells $\rightarrow$ hCD45 $\rightarrow$ hCD3 $\rightarrow$ CD4. C) HIV infection results in a reduction in the levels of effector memory cells within the CD4+ T cell population of infected mice (n=5, grey bars) versus uninfected (n=7, black bars) mice. Infected animals were approximately 48 weeks post-implantation at the time of harvest. For all graphs, error bars represent Mean $\pm$ SEM.
Figure 2.5: There is sustained HIV replication in ToM that can be efficiently suppressed by ART. A) Sustained levels of plasma HIV-RNA in infected ToM (n=5). B) The plasma viral load of infected ToM (n=4) pre- and post-initiation of ART treatment demonstrated the ability of ART to suppress viremia in these mice. ART consisted of daily injections of TDF, FTC and Raltegravir. C) Plasma viral load from an infected ToM dropped below the limit of detection and remained undetectable for the duration of ART. As seen in humans, viremia rebounded following treatment interruption. The limit of detection of the assay is indicated with a dashed line. Error bars in both graphs represent the Mean ± SEM.
Figure 2.6: Resting human CD4$^+$ T cell isolation from ToM. (Top left) Flow cytometric analysis of cells pooled from the different tissues of a ToM prior to magnetic negative selection showed the presence of both CD4$^+$ and CD4$^-$ cells. (Bottom left) Prior to negative selection CD4$^+$ T cells expressed various levels of CD25 and HLA-DR. (Top right) After isolation 99% of the cells obtained were CD4$^+$. (Bottom right) Consistent with a resting phenotype, isolated cells were CD3$^+$CD4$^+$ and did not express CD25 or HLA-DR. Gating strategy: (Top) live$\rightarrow$hCD45$^+$→hCD3$^+$. (Bottom) live$\rightarrow$hCD45$^+$→hCD3$^+$→CD4$^+$. 
Figure 2.7: Latent HIV infection of human resting CD4+ T cells in ToM and human PB. The frequency of latently infected resting CD4+ T cells was measured in resting CD4+ T cells isolated from ART-suppressed ToM and PB of suppressed patients that initiated treatment during acute or chronic phases of HIV-1 infection via co-culture of resting cells. A) Diagram depicting the resting cell co-culture assay to detect p24 expression after maxim stimulation with PHA. B) Comparison of the number of resting HIV infected cells between humanized mice and humans during the acute or chronic phase of infection. The number of infected resting cells in the individual mice was estimated using a maximum likelihood method and values reported in infectious units per million resting cells (IUPM).
CHAPTER 3: MACROPHAGES SUSTAIN HIV REPLICATION IN VIVO INDEPENDENT OF T CELLS

SUMMARY

Macrophages have been long considered as contributors to HIV infection of the CNS, a sanctuary with restricted access from the periphery. However, recent studies have contradicted early work, suggesting that macrophages are not an in vivo source of virus production. To address this question, we first analyzed monocytes isolated from viremic patients and patients undergoing antiretroviral treatment, and were unable to find viral DNA or viral outgrowth in vivo. To determine if tissue macrophages are productively infected, we used three different but complementary humanized mouse models. Two models were previously described [bone marrow/liver/thymus (BLT) and T cell-only mice (ToM)] and a third [myeloid-only mice (MoM)] was used for the purpose of investigating the role of myeloid cells in HIV replication in vivo. Using MoM we demonstrate that: 1) macrophages sustain HIV replication in vivo in the absence of T cells; 2) HIV-infected macrophages are distributed in all tissues analyzed including the brain; 3) replication-competent virus can be rescued ex vivo from infected macrophages obtained from tissues of MoM; and 4) infected macrophages can establish de novo infection. These results demonstrate that macrophages represent a bona-fide target for HIV in vivo that can sustain replication and transmit infection.
INTRODUCTION

HIV, the causative agent of AIDS, is severely species restricted and to date only humans and chimpanzee have been shown to be susceptible to infection [12, 230]. The limited species specificity of HIV represents a significant challenge for in vivo experimentation, thus there is a need for the use of animal models. Human infection by HIV (and infection by its relative SIV in non-human primates) is restricted to cells expressing the CD4 molecule [231]. In addition to CD4, productive HIV infection, meaning infection that leads to the production of viral progeny, requires one of two different G protein-coupled receptors: CCR5 or CXCR4 [232]. CD4+ T cells have been shown to harbor HIV proviruses and represent the most abundant target for HIV infection in vivo [101, 160]. Despite the prevalence of virus in CD4+ T cells, it is clear that T cells are not the only targets of HIV infection in vivo. In fact, macrophages have been shown to express CD4, CCR5 and CXCR4 and to be susceptible to HIV infection in vitro and in vivo [233-235]. Non-human primates and humanized mice have been extensively used to study HIV and SIV infection and pathogenesis in vivo. HIV/SIV infection of macrophages and microglia, the tissue-resident macrophages of the brain, is postulated to substantially contribute to the establishment and pathogenesis of HIV/SIV infection in the CNS [29, 236, 237]. The CNS is a location that has been considered to be a sanctuary for the viruses where variants of HIV can replicate and expand independently of contributions from the periphery [238, 239]. It has been suggested that the compartmentalization between the blood and CNS is associated with the ability of HIV variants in the CNS to infect cells with lower levels of CD4, such as macrophages [99]. This is especially problematic in the brain where resident
macrophages, such as microglia and perivascular macrophages, could then be susceptible to infection [29].

Whereas the ability of HIV to replicate in human macrophages in vitro has been extensively documented, evidence for HIV replication in human macrophages in vivo or ex vivo is limited and in some instances indirect [193, 240, 241]. Analysis of the gut has yielded somewhat conflicting results as human intestinal macrophages did not support HIV replication ex vivo and were found to be more monocyte-like in receptor expression patterns [241]; yet viral HIV-DNA was isolated from sorted CD13+ cells in rectal biopsies obtained from ART-suppressed patients, suggesting a non-T cell origin [242]. Analysis of monocytes from peripheral blood consistently shows very low levels or lack of infection in viremic or aviremic patients [117, 119, 243]. Evidence of both in vitro virus outgrowth from human monocytes obtained from patients and ex vivo virus outgrowth from tissue macrophages (including the brain or CNS) is largely absent. However, the presence of infected macrophages in a variety of tissues has been clearly documented via immunohistochemistry and in situ hybridization approaches [235, 244, 245].

In vivo macrophage infection is currently a topic of intense debate. Specifically, data from Calantone et al suggests that at least in SIV infected non-human primates, macrophages are not productively infected and cannot replicate SIV [132]. Rather macrophages ingest T cells and this explains the presence of HIV nucleic acids and proteins in macrophage preparations. Further evidence in support of this postulate has also been recently presented by Baxter et al [131]. In this article the authors document that human macrophages selectively capture and engulf human T cells and that detection of vDNA or viral proteins within phagocytes including macrophages may not
necessarily represent their infection but may indicate uptake of infected immune cells or their debris [131]. However, these authors indicate that uptake of virus by phagocytosis could potentially lead to infection of macrophages. Together these results strongly suggest that analysis of HIV replication in macrophages in vivo is greatly compromised by the presence of T cells. In addition to being significantly more abundant, T cells are also more susceptible to HIV infection than macrophages via cell-free or cell-associated virus [131, 132].

In order to establish the susceptibility of human myeloid cells to HIV and their ability to sustain HIV replication and productive infection in vivo, we first determined the incidence of HIV infection in peripheral blood monocytes from viremic and suppressed HIV-infected patients. Our analysis demonstrated that in contrast to the relative abundance of HIV DNA found in T cells, we found an absence (or significantly low levels) of HIV DNA in peripheral blood monocytes. Analysis of primary tissue macrophages from humans remains difficult as these samples are not easily accessible, being tissue-derived, and viability can suffer as a consequence of handling [246]. Invasive techniques such as bronchoscopy or biopsy are necessary to acquire the samples, and it is difficult to expand these cells ex vivo [247]. While blood monocytes can be differentiated in vitro into macrophages, these cells lose much of their heterogeneity, which is critical in mimicking primary tissue macrophages [247]. Because of the difficulties associated with using primary tissue macrophages from humans, we implemented a new humanized mouse model in which the only human cells present capable of supporting HIV replication are human myeloid cells [248-250]. NOD/SCID mice transplanted with human CD34+ stem cells are reconstituted with human myeloid
and B cells and are completely devoid of human T cells. Analysis of internal organs demonstrates the presence of human macrophages in all tissues analyzed including the brain. As the only cells that can support replication in these mice are of myeloid origin, we have designated these as myeloid-only mice or MoM. HIV infection of MoM with select isolates resulted in robust and sustained replication. HIV vDNA/RNA was found in virtually all tissues analyzed including the brain. Replication competent virus could be recovered from tissue macrophages and the transfer of infected macrophages into uninfected animals resulted in sustained infection. Our results also demonstrate the ability of human macrophages to fully sustain HIV infection and replication in vivo in the complete absence of human T cells confirming their role as genuine targets for HIV infection in vivo.

**EXPERIMENTAL PROCEDURE**

**PATIENT BLOOD CELL ISOLATIONS**

Approximately 8 ml of blood was obtained from de-identified viremic and ART-suppressed patients and separately into eight Sodium Citrate CPT tubes (64 ml total, BD Cat. 362761). Samples were spun down, and plasma was isolated from each tube for viral load analysis. Mononuclear cells were then collected and washed in phosphate buffered saline (PBS). Positive magnetic selection for CD3+ T cells (Miltenyi, CD3 MicroBeads, Cat. 130-050-101) was performed. Next, negative magnetic selection for monocytes (Miltenyi, Pan Monocyte Isolation Kit, Cat. 130-096-537) was performed on the non-CD3 cell population to yield a >90% pure monocyte population.
GENERATION OF HUMANIZED MICE

Humanized ToM and BLT mice were prepared by implanting allogeneic thymus and liver tissue into, 6-8 week-old NOD.Cg-Prkdc^scid Il2rg^tm1Wjl^/SzJ (NSG, The Jackson Laboratory) mice as previously described [52, 84, 251-253]. Implants consisted of a 1-2 mm piece of fetal liver tissue sandwiched between two pieces of fetal thymus. Each implant was seated under the left kidney capsule. In addition to the thymus/liver/thymus implant, BLT mice also received autologous CD34+ hematopoietic stem cells (Miltenyi CD34 Microbead Kit, Cat. 130-046-703). Humanized MoM were prepared by transplanting approximately 1x10^6 human fetal liver or cord-blood-derived CD34+ hematopoietic stem cells into NOD.CB17-Prkdc^scid/J mice (NOD/SCID, The Jackson Laboratory). Prior to humanization all mice were pre-conditioned using gamma radiation with 200 rad (ToM and BLT mice) or 250 rad (MoM).

FLOW CYTOMETRIC ANALYSIS OF PATIENT CELLS AND HUMANIZED MICE

All flow antibodies were purchased from BD Pharmingen. The antibody panel used to analyze cells isolated from HIV-infected patients included antibodies directed against hCD45 (APC, Cat. 555485), hCD3 (FITC, Cat. 555339), hCD4 (APC-H7, Cat. 560158), hCD8 (PerCP, Cat. 347314), hCD19 (PE-Cy7, Cat. 557835), and hCD11b (PE, Cat. 555388). In all samples, <0.1% T cells were present in the purified monocyte population. To monitor humanization levels in humanized mice, peripheral blood was obtained from animals via submandibular venipuncture and collected into tubes containing EDTA. Whole peripheral blood was stained with the antibody panel listed above with the exception that an antibody directed against hCD33 (PE, Cat. 340679) was substituted for the anti-hCD11b antibody. For tissue and blood analysis from
harvested mice, combinations of the above antibodies were used. Additionally, antibodies directed against hCD14 (FITC, Cat. 555397), hCD16 (PE-Cy7, Cat. 557744), hCD11b (APC, Cat. 340937), hCD45 (APC-Cy7, Cat. 557833) and HLA-DR (PerCP, Cat. 347364) were used for animals depicted in Figure 3.2D and 3.2E. Live cells were distinguished by their forward and side scatter profiles as previously described [51]. Flow cytometry data was collected on a BD FACSCanto and analyzed with BD FACSDiva software (v.6.1.3).

**Tissue Harvesting From Humanized Mice**

To prevent blood contamination of tissues (in particular brain tissues), mice were transcardially perfused with ~20 ml of room temperature PBS at necropsy. Mononuclear cells (MNCs) were isolated from the bone marrow, spleen, lung, and liver of MoM or BLT mice as previously described for ToM and BLT mice [80]. Tissues were minced and/or digested and filtered through a 70-µm strainer. The liver, lung and brain MNCs were purified with Percoll density centrifugation. MNCs were washed, counted via trypan blue exclusion, and flow cytometric analyses were performed for the indicated markers.

**Immunohistochemical Analysis of Brains**

Brains for IHC were harvested from MoM and fixed in 4% paraformaldehyde for 24 hr at 4°C, embedded in paraffin, cut into 5-µm sections and mounted onto poly-L-lysine coated glass slides. Following paraffin removal, antigen retrieval (DIVA Decloaker, Biocare Medical, Cat. DV2004.) and blocking of non-specific Ig-binding sites (Background Sniper, Biocare Medical), tissue sections were stained with primary antibodies overnight at 4°C and developed with a biotin-free horseradish peroxidase
(HRP)-polymer system (MACH3 Mouse HRP-Polymer Detection, Biocare Medical). All tissue sections were then counterstained with hematoxylin. Primary antibodies directed against CD45 LCA (2B11&PD7/26, Dako) and CD68 (KP1, Dako) were used to determine the presence of human cells and human macrophages in the brains of MoM. HIV-infected cells were detected with an antibody directed against HIV p24 Gag (Kal-1, Dako). As a control, tissue sections were stained with a mouse IgG1k (DAK-GO1, Dako) isotype control.

**HIV-1 INFECTIONS**

Stocks of HIV-1 (JRCSF, RHPA, THRO, ADA, CH058, CH040 and CH040-4013 env) and HIV-2 (7312A) were prepared and titered as previously described [215]. Briefly, virus supernatants were prepared via transient transfection of 293T cells, and were tittered using TZM-bl cells (an indicator cell line) as previously described [77]. The chimeric virus CH040-4013 env was created by replacing env in CH040 (Accession #jn944905, 6396-8863) with the corresponding restriction fragment from 4013 (Accession #jn562796). Subject 4013 had mild neurological dysfunction [238]. To test various HIV strains for macrophage-tropism, 360,000 tissue culture infectious units (TCIU) of virus was injected i.v. into MoM. BLT mice and ToM were exposed i.v. to 360,000 TCIU of ADA, CH040, or CH040-4013 env. For the non-macrophage-tropic isolates (RHPA, THRO, JRCSF, HIV-2, and CH058) ToM or BLT mice were exposed i.v. or vaginally to 90,000-360,000 TCIU of the various viruses to confirm replication competence.
ANALYSIS OF HIV-1 INFECTION

Subsequence HIV DNA analysis of patient cells and of cells isolated from MoM and BLT mice that received patient cells was performed using nested PCR analysis for HIV gag. For all patient-derived blood cells, 1x10^6 cells were analyzed. For humanized mouse samples, 1x10^5-3x10^6 cells were analyzed, depending on the cell yield from the various tissues. Genomic DNA from MNCs (5×10^5–5×10^6) from animal tissues was prepared using QIAamp DNA blood mini columns (Qiagen) according to the manufacturer’s protocol. Viral DNA was amplified by nested PCR using the Expand High Fidelity PCR System (Roche). The HIV region amplified was a 1.5-kb region in gag (gag: HIV-1JRCSF 617–2358). Amplification of gag was used to assess the presence or absence of HIV-1 gag DNA. Primer sequences were as follows: Gag outer forward primer, CTCAATAAAGCTTGCCTTGAGTGC; Gag outer reverse primer, CTTCCAATTATGTGGACAGGTGTAGG; Gag inner forward primer, GTGTGGAAAATCTCTAGCAGTGGC; Gag inner reverse primer, TAGAAGAGAAGGCTTTCAGGCC. Peripheral blood (approximately 100 ul total) was collected from mice via retro-orbital bleed using EDTA-coated capillary tubes. Infection of MoM with HIV-1 isolates was determined with a one-step reverse transcriptase real-time PCR assay (ABI custom, TaqMan Assays-by-design) according to the manufacturer’s instructions (with primers 5’-CATGTTTTCAGCATTATCAGAAGGA-3’ and 5’-TGCTTGATGTCCCCCACC-3’; assay sensitivity of ~668 RNA copies per mL). Additionally, at necropsy MNCs were isolated from tissues and analyzed for the presence of viral RNA and DNA. For viral co-culture analysis, human macrophages were plated and allowed to adhere. Within 24 hours of plating, approximately one million
allogenic, PHA-stimulated feeder cells (CD8-depleted) from healthy donors were added to the cultures as targets for viral outgrowth. After 10 days, culture supernatants were analyzed for the presence of viral RNA. Small pieces of tissue (femur, spleen, lung, and liver) from CH040 infected MoM were harvested and immersed in fixative containing 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4, with 5mM calcium chloride, 1mM magnesium chloride for several days at 4°C. Femurs were decalcified with a solution of 0.1M EDTA in 0.1M sodium cacodylate, pH 7.4, containing 1% glutaraldehyde for two weeks at 4°C. After buffer washes, the tissues were post-fixed with 1% osmium tetroxide in 0.1M sodium cacodylate, pH 7.4 for one hour at room temperature, gradually dehydrated with ethanol and propylene oxide followed by infiltration and embedment in PolyBed 812 epoxy resin (Polysciences, Inc., Warrington, PA). Ultrathin sections (70 nm) were post-stained with uranyl acetate and lead citrate and observed using a LEO EM910 transmission electron microscope operating at 80kV (Carl Zeiss SMT, Inc., Peabody, MA). Digital images were taken using an Orius SC1000 CCD camera and Digital Micrograph v2.3.1 software (Gatan, Inc., Pleasanton, CA).

**ADOPTIVE TRANSFER EXPERIMENTS**

Infected MoM or BLT mice were harvested and processed as above. For the first two adoptive transfer experiments, bone marrow cells from two separate infected MoM were injected i.v. into two separate BLT mice. In the third transfer experiment, macrophages were purified from an infected BLT mouse using the magnetic isolation strategy as above from patient cells, and injected i.v. into a MoM. For the last two adoptive transfer experiments, macrophages were purified from the pooled cells of three
infected BLT mice and injected i.v. into a MoM and BLT mouse. For the purified macrophage adoptive transfers, MNC were pooled from the spleen, liver, lung, and bone marrow of infected animals prior to macrophage isolation. Mice that received cells from infected animals were monitored for HIV-1 infection over time as above.

STATISTICS

All data was graphed and analyzed using GraphPad Prism (version 5.04). A comparison of the absolute numbers of cells in the brains of infected and uninfected MoM was performed using a two-tailed Mann Whitney analysis.

STUDY APPROVAL

HIV infected individuals were recruited at University of North Carolina at Chapel Hill (UNC) Hospital in the Infectious Diseases Department. Samples were collected under the UNC IRB #08-0047 and patient samples were de-identified prior to cell isolation in the lab (UNC IRB #14-0368). The UNC Office of Human Research Ethics determined that this study did not constitute human subjects research under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(l)] and required no additional approval. All humanized mice were maintained in a specific pathogen-free facility according to protocols approved by the Institutional Animal Care and Use Committee at UNC.

RESULTS

ABSENCE OF HIV IN PATIENT MONOCYTE SAMPLES

To determine the frequency of HIV infection in peripheral blood monocytes in humans we collected samples from eight patients. Please see Table 3.1 for patient demographic information. Four of these patients were receiving antiretroviral therapy
and four were not. We then used a multi-step protocol for the purification and enrichment of monocytes and T cells using magnetic beads (Figure 3.1A). Specifically, mononuclear cells were first isolated using Ficoll gradient centrifugation. Human T cells were removed from the mononuclear cell preparations using a CD3-specific reagent via positive magnetic selection. After T cell removal, blood monocytes were isolated using a negative selection approach that again removed any residual T cells. This procedure yielded highly purified preparations of monocytes, confirmed with flow cytometric analysis, with little to no T cell contamination (Figure 3.1B). Whole blood cells, purified monocytes and enriched T cells were analyzed for the presence of HIV-gag DNA using nested PCR analysis. Clear evidence of HIV DNA was detected in purified T cell preparations in samples obtained from infected patients regardless of their treatment status (Figure 3.1C). No evidence of viral DNA was obtained in any of the purified monocyte preparations (one million cells) analyzed from these same patients.

To begin to address the frequency at which replication competent, HIV isolates exist in blood monocytes from HIV-infected patients, we evaluated virus outgrowth from peripheral blood-derived monocytes and T cells isolated from non-suppressed HIV-infected patients injected i.v. into BLT mice. Purified patient (Pt 01-03 from Table 3.1) monocytes and T cells were injected into BLT mice. After 2-8 weeks mice were harvested and cells from the different tissues of BLT mice were analyzed using nested PCR for HIV DNA(Figure 3.1D). Consistent with the lack of HIV DNA in monocytes (Figure 3.1C), we did not observe viral DNA in the tissues of BLT mice that received patient purified monocytes (Figure 3.1D). In contrast, viral DNA was observed in the tissues of all BLT mice that received purified T cells (Figure 3.1D). This in vivo
outgrowth assay demonstrates the absence of replication competent virus in monocytes obtained from the peripheral blood of HIV-infected patients. These results underscore the need to study HIV infection in tissue macrophages rather than monocytes as monocytes are not productively infected.

**CHARACTERIZATION OF CD34-TRANSPLANTED NOD/SCID MICE**

In order to establish whether monocytes and macrophages are infected *in vivo*, in tissues that are hard to access in humans, we used an *in vivo* mouse model designed for this purpose. Pre-conditioned NOD/SCID mice were administered a bone marrow transplant with human CD34$^+$ cells [248-250] and monitored up to seven months for human reconstitution as determined by the presence of human cells in peripheral blood (n=52). Over time, human hematopoietic cells (CD45$^+$) in peripheral blood increased in numbers that remained stable after about 8-12 weeks (Figure 3.2A). The percentage of human cells in peripheral blood was sustained for the duration of the experiment. Lineage analysis of the human cells present in the peripheral blood of these mice clearly indicated the presence of human B and myeloid cells (Figure 3.2B, C). No evidence of human T cells in the peripheral blood of mice was noted at any time during these experiments.

Analysis of the cells present in tissues obtained from transplanted mice confirmed their systemic reconstitution with human hematopoietic cells (Figure 3.2B, C). Lineage analysis of the cells present in bone marrow, liver, lung and spleen demonstrated the presence of human B and myeloid cells. We consistently failed to find T cells in any of the tissues analyzed (Figure 3.2B). Analysis of hCD14 and hCD16 expression on the cells present in the peripheral blood and tissues of these mice
demonstrated the presence of classical and intermediate monocyte and macrophage subsets \[254, 255\], with the classical phenotype (CD14^+/CD16^-) representing the majority of the monocytes and macrophages present in these mice (Figure 3.3D), as is also observed in humans \[256\]. Additionally, the majority of these cells expressed MHC Class II, as denoted by expression of HLA-DR (Figure 3.2D). These results demonstrate the systemic repopulation of these mice with human myeloid and B cells in the complete absence of human T cells.

HIV infection of the brain and its sequelae are hallmarks of AIDS. Macrophages have been shown to be important targets of HIV infection in the brain \[236\]. Therefore we investigated the presence of human cells in the brains of reconstituted mice. To minimize potential contamination from cells in the blood in the brain, animals were transcardially perfused with phosphate buffered saline (PBS) at necropsy. Brain tissue was used to isolate mononuclear cells via Percoll density centrifugation and isolated cells were analyzed via flow cytometry. Human B cells as well as monocytes and macrophages were found in the brains of all mice and there was no evidence of human T cells in any of the brains analyzed (Figure 3.2E). Similar to the rest of the tissues analyzed in the previous experiment, macrophages isolated from the brains of these mice had both classical (CD14^+/CD16^-) and intermediate phenotypes (CD14^+/CD16^+) as determined by CD14 and CD16 expression levels \[256\]. These results demonstrate the reconstitution of the brains of these NOD/SCID mice with human hematopoietic cells.
HIV INFECTION OF NOD/SCID MICE RECONSTITUTED WITH HUMAN CD34+ STEM CELLS

In order to establish the fitness of each HIV isolate to be evaluated for its ability to replicate in macrophages in vivo, each was first evaluated for their replication competency in bone marrow/liver/thymus (BLT) mice and T-cell only mice (or ToM). BLT mice are reconstituted with T cells, B cells and myeloid cells [75, 257], while ToM are devoid of human monocytes and B cells but have a full complement of human T cells [251]. Both BLT mice and ToM have been shown to support HIV replication and persistent infection as determined by the presence of latently infected human T cells [84, 86, 251]. BLT mice and ToM were infected with eight different viruses: 1 primary HIV-2 isolate (7312A) [258, 259], 1 HIV-1 early passage isolate (JRCSF) [260, 261], 4 HIV-1 transmitted founder viruses (THRO, RHPA, CH058 and CH040) [110], and 1 HIV-1 macrophage-tropic virus (ADA) [262]. All viruses require CCR5 as a co-receptor for infection [110, 263, 264]. In addition, we also evaluated a chimeric virus derived from CH040 in which we replaced its envelope gene with that from a previously characterized macrophage-tropic envelope gene obtained from the CSF of a patient with moderate CNS disease (Subject #4013, Envelope #C7, denoted as CH040-4013 env) [238]. ToM and BLT mice were infected via multiple routes and viral replication was monitored weekly or biweekly in plasma via viral load analysis as previously described [51, 52, 84, 251-253]. All viruses tested without exception efficiently replicated in vivo in both ToM and BLT mouse models (Figure 3.3A, B). Each of these viruses maintained detectable levels of viral replication in the plasma of all exposed animals. These results confirm the in vivo replication competence of all the viruses being evaluated in this study, and
confirm that myeloid cells are not necessary for replication of these isolates (as these cells are absent in ToM).

To investigate HIV infection of myeloid cells *in vivo*, we utilized NOD/SCID mice reconstituted with CD34\(^+\) cells, thereby lacking human T cells [248-250]. Mice were exposed to a single i.v. dose of the HIV isolates evaluated above in BLT mice and ToM. Virus replication was monitored weekly for up to 15 weeks as a function of plasma viral load as previously described [51, 52, 84, 252, 253]. We found no evidence of HIV-1 or HIV-2 DNA in any of the NOD/SCID mice reconstituted with CD34\(^+\) cells exposed to JRCSF, THRO, RHPA, CH058 or 7312A (Figure 4.3C). Evidence of HIV replication, as determined by the presence of viral RNA in plasma, was observed in CD34\(^+\) cell reconstituted NOD/SCID mice one week after exposure to ADA, CH040 or CH040-4013 env (Figure 3C). All of the viruses that were capable of replicating in mice devoid of T cells did so efficiently, resulting in sustained levels of virus replication in the plasma for the duration of the experiment (up to 15 weeks, the last time point evaluated) (Figure 3.3C). HIV infection of NOD/SCID mice transplanted with CD34\(^+\) stem cells led to transiently increased numbers of intermediate phenotype monocytes in the peripheral blood (Figure 3.3D), similar to what is seen in humans [265].

Together, the results presented above demonstrate that all of the isolates tested can replicate efficiently in humanized mice containing human T cells. These results also demonstrate that myeloid cells can support *in vivo* replication of only a select number of virus isolates (3 out of 8 tested, Figure 3.3E). However, the viruses that were capable of replicating in the absence of T cells were able to sustain robust levels of infection that were maintained for the entire period of the experiment. Since in NOD/SCID mice
transplanted with human CD34+ cells, the only human cells that are susceptible to HIV infection and that support HIV replication are myeloid cells, we have designated them as myeloid-only mice (or MoM).

**SYSTEMIC REPLICATION OF HIV IN MOM**

Having established the replication competence of macrophage-tropic viruses in MoM in the complete absence of T cells in peripheral blood, we next determined the presence of infected cells in tissues. For this purpose, cells from the spleen, liver, lung, and bone marrow from infected MoM were analyzed for the presence of viral DNA. Consistent with systemic infection, viral DNA was readily found in all tissues examined (Figure 3.4A). To determine if systemic viral replication was occurring, tissues from MoM were analyzed for the presence of viral RNA (Figure 3.4B). Viral RNA was readily detected in all tissues analyzed. To confirm the identity of the infected cells in tissues, we used immunohistochemical staining combined with in situ hybridization analysis. The presence of viral RNA in human CD68 expressing cells in these tissues confirmed that the virus detected in the tissues of HIV-infected MoM was indeed being produced by human macrophages (Figure 3.4C). Furthermore, electron microscopic analysis of the bone marrow from HIV-infected MoM demonstrated the presence of virus budding as well as free virions in this tissue (Figure 3.4D). Together these results demonstrate systemic replication of macrophage-tropic viruses in MoM.

To confirm the presence of replication competent virus in tissue macrophages, we implemented an in vitro outgrowth assay. Cells obtained from different tissues of MoM (bone marrow, spleen, liver, and lung) were co-cultured ex vivo with CD8-depleted, HIV-negative, PHA-activated allogeneic PBMC. Culture supernatants were
analyzed by PCR after 10 days for the presence of viral RNA. We were able to
demonstrate the presence of replication competent virus from all the tissue types
analyzed (Figure 4E). These results demonstrate that human macrophages in the
tissues of MoM are productively infected with replication competent HIV.

**HIV INFECTION IS ESTABLISHED IN THE BRAINS OF MOM**

As indicated above, it has been postulated that HIV infection of the CNS is
established and maintained in human myeloid cells [29, 266]. Having established that
myeloid cells are present in the brains of MoM we investigated whether HIV infection is
established in the brains of these animals. Mice were transcardially perfused with PBS
at necropsy to minimize contamination with blood-derived cells as well as purge any
free virus that might be circulating in the brain. We isolated mononuclear cells from the
brains of MoM infected with the three macrophage-tropic viruses (ADA, CH040, CH040-
4013 env) and used HIV RNA as a surrogate for HIV replication. Viral RNA was
detected, in cells isolated from 7 of 12 infected animals, representing all three
macrophage-tropic isolates (Figure 3.5A). Immunohistochemistry demonstrated the
presence of human cells (hCD45⁺), human macrophages (CD68⁺), and HIV infected
cells (p24⁺) in the brain of an infected MoM (Figure 3.5B).

SIV infection of macaques leads to accumulation of monocytes and
macrophages in the CNS [237]; therefore, we wanted to determine the effect of HIV
infection on the number of human cells in this tissue. Compared to uninfected controls,
the total number of human cells and human macrophages in the brains of infected
animals increased significantly from 1,947 to 3,949 (p=0.0023) for total human cells and
from 635 to 1,168 for total human monocytes and macrophages (p=0.0009) (Figure
These findings show that HIV infection results in an establishment of infection in the brain of MoM as well as an increase in the absolute numbers of human cells, specifically myeloid cells.

**ADOPITIVE TRANSFER OF HIV INFECTED MACROPHAGES ESTABLISHES DE NOVO INFECTION**

To determine if the infected myeloid cells present in MoM could establish de novo infection in an uninfected host, we isolated cells from infected MoM and injected them i.v. into uninfected animals. First, bone marrow cells from two MoM (infected with CH040 or CH040-4013 env) were injected into BLT mice. This resulted in the development of rapid plasma viremia in both animals, confirming the replication competence of the viruses present in MoM (Table 3.2). To determine if macrophages in BLT mice are productively infected, myeloid cells were isolated from a BLT mouse (infected with CH040) by removing T cells from the preparation by positive magnetic bead selection. Myeloid cells were further enriched in the T cell-depleted preparation by negative magnetic bead selection, using the methodology outlined above for patient peripheral blood cells (Figure 3.1A).

Purified myeloid cells were then injected i.v. into a MoM. This resulted in sustained virus replication in the recipient animal, confirming macrophages present in this infected BLT mouse were also productively infected. The presence of productively infected macrophages in HIV-infected BLT mice was evaluated by injecting pooled macrophages purified from three BLT mice (infected with CH040) into a MoM and a BLT mouse (Table 3.2). The purified macrophages were able to establish de novo infection in both of the recipient animals confirming our initial observation (Table 3.2). These
results demonstrate that the virus replicating in macrophages of BLT mice and MoM can efficiently transmit \textit{de novo} infection into a new host and replicate \textit{in vivo} in the presence or in the complete absence of human T cells.

**DISCUSSION**

The study of HIV and SIV replication in macrophages has been confounded as presence of viral nucleic acids in macrophages \textit{in vivo} and \textit{in vitro} has been attributed to phagocytosis of infected T cells [131, 132]. Despite the postulated importance of macrophages in HIV infection and AIDS-associated neurological complications, infection of monocytes and macrophages \textit{in vivo} has been questioned [117, 119, 131, 132, 243, 267, 268]. Several groups, including ours, have failed to find significant amounts of HIV DNA in human peripheral blood monocytes from viremic and aviremic patients [117, 119, 243]. These outcomes have been ascribed to differences in the susceptibility of monocytes and macrophages to HIV infection during differentiation [267, 269] and highlight the need to study infection of tissue macrophages rather than monocytes.

To investigate \textit{in vivo} HIV infection of macrophages, we took advantage of two humanized mouse models that we had previously developed (ToM and BLT mice) and implemented a new model in which only human myeloid cells can serve as targets for HIV infection (MoM). Three of the viruses tested (ADA, CH040, and CH040-4013 env) were able to sustain infection in the absence of human T cells, although all viruses utilize the co-receptor CCR5 [110, 263, 264]. It should be noted that the HIV-infected MoM in this study represent animals reconstituted with cells from 24 different human donors, with no donor to donor differences in susceptibility to infection; this contrasts
from an *in vitro* study of monocyte-derived macrophages where donor variation in susceptibility to infection was identified as a confounding factor [234]. CH040 infection of MoM demonstrated that transmitted/founder viruses can efficiently infect and replicate in macrophages [270-272]. In addition, a chimeric virus in which the envelope gene of CH040 was replaced by one obtained from the CNS of a patient [238], also replicated well in MoM.

Previous experiments to better understand HIV infection of macrophages *in vivo* have been conducted using SIV infection [273]. Using rhesus macaques, it has been shown that CD4 depletion (pre-infection) leads to robust SIV infection of macrophages [274, 275], and there is no post-peak drop in viremia, resulting from the infection of macrophages that maintain a constant level of viral production [274]. In CD8-depleted (post-infection) rhesus macaques, increased numbers of monocytes and macrophages entered into the CNS of SIV infected compared with uninfected animals [237]. Similar to studies above, we noted no post-peak drop in viremia and found higher numbers of human macrophages in the brains of infected compared to uninfected MoM. One advantage of MoM is the complete absence of T cells, thus the aforementioned attributes can now be defined as occurring during HIV infection of macrophages without contribution from T cells. HIV replication in MoM cannot be ascribed to the phagocytosis of T cells by macrophages and replication occurs only in the human myeloid cells present. Replication in macrophages was verified by sustained plasma viremia, immunohistochemical and *in situ* hybridization analyses, electron microscopy, bulk DNA and RNA detection, and *in vitro* outgrowths. Given the difficulties of procuring of tissue
samples that invariably will also contain T cells, the availability of an *in vivo* model such as MoM represent a significant advance in the field.

Monocyte and macrophage transmigration into the brain has been considered to play a central role in establishing HIV infection of the CNS [276]. Antiretroviral therapy has resulted in significant overall benefits to HIV-infected patients, but does not always result in neuropsychological improvements [277-279] and which may stem from differences in the tropism of viruses found in the periphery compared to the CNS [29, 266, 280, 281]. Here we demonstrate that T cells are not necessary to traffic the virus from the periphery to the brain and macrophages can sustain infection of the CNS. However, several issues remain to be addressed in future work. Based on our analysis, we cannot ascertain whether the appearance of HIV+ cells in the CNS represents *de novo* infection or migration of infected cells from other tissues into the brain. If it represents *de novo* infection, it will be important to determine whether it was initiated via cell-free or cell-associated virus. Numbers of infected myeloid cells in the periphery of MoM is very low compared to the relatively high viral load, suggesting that cell-free virus is seeding the infection in the brain of MoM. Our results demonstrating the lack of HIV infection of blood monocytes from humans suggest that infected monocytes might not be directly responsible for the seeding of HIV in the CNS of humans, but rather cell-free virus or infected T cells in peripheral blood are responsible for introducing HIV into the CNS.

Despite the lack of infected monocytes in peripheral blood, we found strong evidence of productive macrophage infection in tissues. In addition, our data demonstrating that macrophages isolated from BLT mice infected with the macrophage-
tropic strains are capable of establishing HIV infection when injected into naïve recipients indicates that macrophages can be productively infected with HIV in the absence or presence of human T cells.

Our studies have significant implications for the field. They establish tissue macrophages as true targets of productive HIV infection. The fact that no infected monocytes are present (or are present at very low levels) in peripheral blood of patients suggests that future analysis of HIV latency in myeloid cells in humans will be complicated by the need to directly sample tissues in sufficient amounts to perform persistence, latency and induction analyses. MoM can be used to directly access the role of tissue-derived macrophages in persistence of HIV infection in vivo during ART, and this will represent an important future direction for this model. Specifically, we will be able to address the effectiveness of ART to control viral replication in macrophages as well as whether or not viral rebound occurs in these animals after removal of ART. Overall, this model is a unique tool to better understand HIV replication in macrophages in vivo.
Figure 3.1: Absence of HIV DNA in monocytes but not in T cells isolated from peripheral blood of infected patients. A) Schematic of monocyte and T cell purification strategy. Total mononuclear cells (MNCs) were isolated from the peripheral blood of eight HIV-infected patients (from Table 1). T cells were depleted using positive magnetic selection and monocytes were enriched using negative magnetic selection. B) Flow cytometric analysis of the total MNCs, T cells, non-T cells, non-monocytes and monocytes demonstrated very pure populations of both T cells and monocytes isolated using this protocol (Patient 04 shown). C) One million purified T cells or monocytes were then analyzed for the presence or absence of HIV-gag DNA using nested PCR analysis in untreated or treated patients. The lower limit of detection for these analyses is 2 copies of HIV DNA. Purified T cells or monocytes from untreated Pt01, Pt02, and Pt03 were injected into BLT mice. D) vDNA analysis demonstrated systemic infection of BLT mice injected with purified patient T cells but not monocytes.
Figure 3.2: NOD/SCID mice transplanted with human CD34+ hematopoietic stem cells are reconstituted with human B cells and myeloid cells but lack T cells. A) Flow cytometric analysis of the peripheral blood of mice demonstrates a sustained presence of 20%-30% human CD45+ cells over time (28 weeks shown, n=52). B) Flow cytometric analysis of the bone marrow, spleen, liver, lung and peripheral blood of a representative mouse demonstrates the presence of human B cells (CD19+) and human myeloid cells (CD33+) with a complete absence of human T cells (CD3+). C) Average levels of human cell reconstitution in bone marrow (n=36), spleen (n=36), liver (n=35), lung (n=35) and peripheral blood (n=18) and the distribution between myeloid and B cells of the human cells. D) Phenotypic characterization of the human monocytes and macrophages in the tissues of a mouse reconstituted with human CD34+ cells (gating scheme: live → hCD45+ → hCD33+/hCD11b+). Most cells demonstrated a classical phenotype with CD14+/CD16- expression, but intermediate phenotype monocytes and macrophages were also present. Almost all monocytes and macrophages expressed HLA-DR. E) Flow cytometric analysis of the brains of MoM demonstrated the presence of human B cells and macrophages with a lack of human T cells. The majority of macrophages in the brain also expressed a classical phenotype.
Figure 3.3: HIV replication can be sustained over time in human macrophages in vivo, but it is limited to very few HIV strains. A) Efficient replication of all HIV strains tested in BLT mice as determined by viral load analysis. B) Efficient replication of all HIV strains tested in ToM mice as determined by viral load analysis ToM. C) Plasma viral load of infected myeloid-only mice (MoM). Only HIV-1 ADA, CH040, and CH040-4013 env (a patient-envelope-derived chimeric virus) were detectable in the plasma over time. D) Increase in numbers of CD14+/CD16+ cells (intermediated phenotype monocytes) in blood of during the first few weeks of infection. E) Summary of viruses that were evaluated for replication in MoM, ToM and BLT mice with regard to plasma viremia and tissue vDNA analysis. For viruses in MoM marked with a minus sign ("-"), at least one animal was evaluated for presence of vDNA in the spleen, liver, lung, and bone marrow and was determined to be uninfected.
Figure 3.4: Systemic replication of HIV-1 in the tissues of MoM. A) Cell-associated viral DNA copies/100,000 total cells and B) cell-associated viral RNA copies/100,000 total cells were measured from cells isolated from bone marrow (BM), spleen (Spln), liver (Liv), and lung of MoM infected with ADA (n=2), CH040 (n=10) or CH040-4013 env (n=9). C) Electron micrograph of infected MoM bone marrow shows the presence of viral budding (black arrows) as well as free virions (green and red arrows) in the tissue. Virions where the viral core was visible are denoted with red arrows. D) *In situ* hybridization analysis of the tissues of a MoM infected with CH040 demonstrates the presence of human macrophages (expressing CD68, brown staining) producing HIV RNA transcripts (black granules). E) Plated macrophages from infected MoM tissues [bone marrow (n=20), spleen (n=11), liver (n=11) or lung (n=8)] were cultured with allogenic donor CD8-depleted feeder cells. Viral outgrowth was as determined by presence (black squares) or absence (white squares) of viral RNA in the culture supernatants after 10 days. Number of human macrophages plated in each culture is graphed on the y-axis. We were able to measure outgrowth in 41/50 total samples (All).
Figure 3.5: Human cells are present in the brains of MoM and increase in numbers during HIV infection. A) Cell-associated viral RNA copies/100,000 total cells were measured from the brains of infected MoM. We were able to detect virus in the brains of 7 of 12 animals (lower limit of detection 30 copies per $10^5$ cells). B) Immunohistochemical analysis of the brain was used to confirm the presence of human cells, human macrophages (CD68) and HIV p24 in the brain of an infected MoM. C) Mononuclear cells from the brains of infected (n=24) and uninfected MoM (n=35) were analyzed via flow cytometry and the absolute numbers of human cells (CD45+), human B cells (CD19+) and human monocytes and macrophages (CD33+/CD11b+) were calculated per brain. There was a significant increase in the overall numbers of human cells (p=0.0023) and human monocytes and macrophages (p=0.0009) in the whole brains of infected mice compared to uninfected mice.
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**Table 3.1: HIV-1 infected patient characteristics.** No, not currently on any antiretroviral therapy (no exclusion if previously treated); Yes, currently suppressed on antiretroviral therapy; M, male; F, Female; CD4:CD8 ratio, percentage of CD4+ T cells in peripheral blood divided by percentage of CD8+ T cells in peripheral blood by flow cytometry.
Table 3.2: Human macrophages can establish *de novo* infection of humanized mice. Mononuclear cells were isolated from the bone marrow of infected MoM and injected into humanized BLT mice resulting in systemic infection of the BLT mice. Total mononuclear cells were harvested from the liver, lung, spleen and bone marrow of BLT mice infected with CH040. Two sequential steps purified tissue macrophages. First, positive magnetic selection was used to deplete T cells. Second, negative magnetic selection was used to further purify the pool of human macrophages. Macrophages from infected BLT mice were analyzed for vDNA levels and then injected into MoM. MoM and BLT mice were evaluated for HIV infection over time in the blood and in tissues at necropsy. Results indicated by a plus sign are samples that tested positive for HIV-1 RNA (plasma viral load) or DNA (for tissue cells).
CHAPTER 4: HUMAN MACROPHAGES ARE SOURCE OF HIV REBOUND DURING ART-INTERRUPTION

SUMMARY

HIV infection results in a life-long condition that, when treated, resembles a chronic disease. Despite years of fully suppressive therapy, HIV persists in the host and is never eradicated. One major barrier to eradication is that multiple cell types are infected and may contribute to persistence. Macrophages have been long recognized as contributors to HIV infection and as likely candidates for harboring HIV during suppressive antiretroviral therapy. Using a novel myeloid-only mouse (MoM) model, we demonstrate that HIV infection of macrophages (in the complete absence of T cells) is rapidly suppressed using antiretroviral therapy (ART). Treatment results in a decline of viral RNA/DNA levels in tissues compared to untreated mice. After ART-interruption in suppressed HIV-infected MoM, viral rebound was observed. In animals that experienced viral rebound, it occurred 49 days after therapy discontinuation. These results identify macrophages as a source of viral rebound and suggest that macrophages may represent a barrier to HIV eradication.

INTRODUCTION

While antiretroviral therapy (ART) has significantly improved the outcome for HIV infected patients, there is no cure for HIV [156, 282]. Despite effective ART, viral reservoirs are established early on during infection and result in viral rebound after treatment interruption [282-284]. The viral reservoir consists of a heterogeneous
population of cells at various anatomical sites that contain HIV despite treatment with highly active ART [285]. The latent reservoir, defined as infected cells that can be activated to produce replication-competent virus, consists mostly of resting CD4+ T cells, but other cellular reservoirs have been suggested [194, 286]. A viral reservoir in T cells is established in HIV-infected humans [222], SIV-infected rhesus macaques [162] and HIV-infected humanized mice [87, 287, 288]. While the HIV reservoir in T cells is well characterized, other cell types may contribute to persistence as reservoirs as well. Historically, the long-lived properties of HIV infected macrophages have made them a candidate for being a viral reservoir [188-190].

As the latent reservoir exists in the context of suppressive ART, it is important to first understand the efficacy of ART in HIV infected macrophages. Specifically, the effectiveness of ART in suppressing viral replication in macrophages in vivo and whether or not ART can eradicate virus from macrophages. In an in vitro model using MDMs, Gavenano et al demonstrate that the intracellular levels of nucleoside analogs after treatment are lower in macrophages compared to PBMCs [289]. Specifically, TDF and FTC treated macrophages had EC50s below the intracellular concentration of drug levels achieved in these cells, highlighting the possibility that current treatments may be ineffective against macrophages in patients with macrophage-tropic viruses. For virtually all drugs evaluated, the EC50 of the nucleoside analogs differ greatly between activated macrophages (cultured with M-CSF) and resting macrophages, with greater potency in resting compared to activated macrophages [289]. Macrophages are generally non-dividing cells with smaller nucleotide pools compared to other PBMCs; this can lead to decreased competition for cellular kinases, more rapid phosphorylation
of NRTIs, and greater efficacy of ART despite lower intracellular concentrations of NRTIs [196]. However, the in vivo efficacy of ART in macrophages has yet to be directly addressed.

The possibility of persistence of HIV in macrophages during ART has been suggested by several groups [192, 193]. In Yukl et al, authors demonstrated the presence of HIV-DNA in non-CD4+ T cells (later identified as macrophages by cell sorting) isolated from the gut of virologically suppressed patients on ART [192]. However, the presence of viral DNA in macrophages may not indicate infection but rather phagocytosis of infected T cells [132]. Using humanized myeloid-only mice (MoM), we are able to investigate ART treatment of macrophages and evaluate whether or not macrophages are a source of virus during ART-interruption in the complete absence of T cells in vivo.

We have previously demonstrated that macrophages can sustain HIV replication independently of human T cells using the MoM model, which is devoid of human T cells (Chapter 3). Using macrophage-tropic HIV isolates, viral replication is sustained in the plasma and tissues of MoM over time. Most importantly, since there is an absence of human T cells in MoM, the presence of viral RNA and DNA in this system cannot be attributed to phagocytosis of infected T cells, but rather indicates productive infection of macrophages. Using this model system allows us to directly assess the macrophage reservoir in vivo without the possible contamination from the known T cell reservoir.

Using MoM, we directly investigate the effects of ART-treatment on HIV-1 infected macrophages. Specifically, is viral replication in tissue macrophages controlled by ART, does viral replication persist in these cells during treatment, and are
macrophages a source of latent HIV? For this study we used two NRTIs, emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) in combination with an integrase inhibitor (Raltegravir, RAL). ART was effective in reducing plasma viremia below the limit of detection of our assay within two weeks of treatment initiation, and reduced the cell-associated vDNA and vRNA levels in tissues of HIV-infected MoM compared to uninfected controls. As a cure to HIV remains elusive, efforts towards defining and understanding the viral reservoirs that exist during ART has come to the forefront of HIV research [188, 282, 290]. We demonstrate that macrophages can be a source of rebound after ART treatment is interrupted, as virus was observed in the plasma of three MoM seven weeks post-ART interruption. This suggests that strategies to eradicate HIV from patients should consider macrophages as a potential viral reservoir.

EXPERIMENTAL PROCEDURE

HUMANIZED MOUSE GENERATION

Humanized MoM were created by transplanting sub-lethally irradiated NOD.CB17-Prkdc\textsuperscript{scid}/J mice (NOD/SCID, The Jackson Laboratory) with approximately 1x10\textsuperscript{6} cord blood or fetal liver-derived CD34+ hematopoietic stem cells. Humanized ToM and BLT mice were created as previously described [86, 87, 288]. Briefly, human thymus and liver tissues were implanted under the kidney capsule of sub-lethally irradiated NOD.Cg-Prkdc\textsuperscript{scid} Il2rg\textsuperscript{tm1Wjl}/SzJ (NSG, The Jackson Laboratory) mice. BLT mice also received 3.5x10\textsuperscript{5} autologous CD34+ hematopoietic stem cells. All animals were pre-conditioned using gamma radiation with either 200 rad (ToM and BLT mice) or 250 rad (MoM). Mice were maintained in a specific pathogen-free facility with the Division of Laboratory Animal Medicine at the University of North Carolina at Chapel Hill.
(UNC-CH) according to protocols approved by the Institutional Animal Care and Use Committee.

**VIRAL STOCK PREPARATION**

Stocks of CH040 were prepared and titered as previously described [215]. Briefly, virus supernatants were prepared via transient transfection of 293T cells, and were titered using TZM-bl cells (an indicator cell line). We also prepared stocks of a chimeric virus, CH040-4013 env (4013-env). The chimeric virus was created by replacing *env* in CH040 (Accession #jn944905, 6396-8863) with the corresponding restriction fragment from 4013 (Accession #jn562796). Humanized MoM, ToM or BLT mice were injected intravenously with $3.6 \times 10^5$ TCID of HIV. Non-suppressed ToM and BLT mice in Figure 2A and 2B were exposed vaginally with $3.6 \times 10^5$ TCID of HIV.

**TISSUE HARVESTING OF HUMANIZED MICE**

To minimize the numbers of blood-derived monocytes in tissues of infected MoM, mice were transcardially perfused with ~20 ml of room temperature PBS before harvest. Mononuclear cells (MNCs) were isolated from the bone marrow, spleen, lung, and liver as previously described. Tissues were minced and/or digested and filtered through a 70 µm strainer. Liver and lung samples were purified with Percoll density centrifugation. Red blood cells were lysed as needed (namely for the spleen, bone marrow and liver tissues). MNCs were washed and counted via trypan blue exclusion.

**ANALYSIS OF HIV-1 INFECTION**

Peripheral blood was collected via retro-orbital bleed using EDTA coated capillary tubes (approximately 100 ul total). Infection of MoM, ToM and BLT mice with HIV-1 was determined with a one-step reverse transcriptase real-time PCR assay (ABI
custom TaqMan Assays-by-design) according to the manufacturer’s instructions (with primers 5’-CATGTTTTTCAGCATTATCAGAAGGA-3’ and 5’-TGCTTGATGTCCCCCACC-3’; assay sensitivity of 668 RNA copies per mL). Additionally, at necropsy MNCs were isolated from tissues and analyzed for the presence of viral RNA and DNA. For viral co-culture analysis (Table 1), human macrophages were plated and allowed to adhere. Within 24 hours of plating, approximately one million allogenic, PHA-stimulated feeder cells (CD8-depleted) from healthy donors were added to the cultures as targets for viral outgrowth. After 10 days, culture supernatants were analyzed for the presence of viral RNA.

**ANTIRETROVIRAL TREATMENT OF MICE**

For HIV treatment we used a previously described triple combination of drugs that we have shown to be effective at suppressing viral load in ToM and BLT mice [87, 288]. Mice were administered daily intraperitoneal injections of emtricitabine (FTC; 211 mg/kg), tenofovir disoproxil fumarate (TDF; 205 mg/kg) and raltegravir (RAL; 56 mg/kg) for three to eleven weeks as indicated on individual graphs. HIV-1 infection was monitored throughout ART duration via plasma viral load analysis as described above.

**VIRAL OUTGROWTH EXPERIMENTS**

For viral co-culture analysis (Table 4.1), human macrophages were plated and allowed to adhere. Within 24 hours of plating, approximately one million allogenic, PHA-stimulated feeder cells (CD8-depleted) from healthy donors were added to the cultures as targets for viral outgrowth (kindly provided by Nancie Archin at UNC-CH). After 10 days, culture supernatants were analyzed for the presence of viral RNA. For the adoptive transfer experiments (Table 4.1), MNCs were isolated from ART-suppressed
MoM and ~3 million cells were injected i.v. into naïve BLT mice. Plasma viral load was monitored for 4-8 weeks post-injection, and tissues were analyzed for the presence of vDNA at necropsy.

**T CELL AND MACROPHAGE SORTING**

Total MNCs were isolated from the spleen, liver, lung, and bone marrow of HIV-infected BLT mice. Mouse cells were depleted using the EasySep Mouse Human Chimera Isolation kit followed by positive magnetic selection for human CD3⁺ T cells (Miltenyi, CD3 MicroBeads, Cat. 130-050-101). Next, negative magnetic selection for human monocytes and macrophages (Miltenyi, Pan Monocyte Isolation Kit, Cat. 130-096-537) was performed on the non-CD3 cell population to yield a >90% pure macrophage population.

**STATISTICS**

All data was graphed and analyzed using GraphPad Prism (version 5.04). Comparisons between models in Figure 4.1 and Figure 4.5 were carried out using a Kruskal-Wallis test with Dunn’s post-test. Bonferroni multiple comparisons test was used to compare groups of mice in Figure 4.3. Mann Whitney test was used to calculate differences between cell types in Figure 4.6. P-values are indicated by asterisk(s) according to Prism guidelines: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

**RESULTS**

**ANTIRETROVIRAL THERAPY EFFECTIVELY SUPPRESSES HIV-1 PLASMA VIRAL LOAD IN INFECTED MYELOID-ONLY MICE (MOM)**

Using MoM, described in Chapter 3, in which the only cells susceptible to HIV infection are macrophages, we determined if virus replication in these cells is
suppressed by combination ART. For this purpose MoM were infected with HIV-1\textsubscript{CH040} or HIV-1\textsubscript{CH040-4013env}, two macrophage-tropic viruses that replicate well in presence or absence of T cells (Chapter 3). HIV replication after exposure was monitored using plasma viral load analysis as previously described [81, 87, 288]. Once infection was well established (for 5-8 weeks) mice were treated once daily, via intraperitoneal injection, with emtricitabine (FTC; 211 mg/kg), tenofovir disoproxil fumarate (TDF; 205 mg/kg) and raltegravir (RAL; 56 mg/kg). We have previously demonstrated the effectiveness of this particular regimen in ToM and BLT mice to suppress HIV-1 viremia [87, 288]. Using MoM, ToM and BLT mice, we have the unique ability to compare viral suppression kinetics in T cells, macrophages, and T cells plus macrophages specifically. With all three models we can determine cell type specific characteristics of infection and their response to therapy.

MoM, ToM and BLT mice were infected for several weeks prior to ART initiation (top three panels, Figure 4.1A). The pre-ART plasma viral load was significantly lower in MoM compared with ToM and BLT mice (\(p\leq0.05\) and \(p<0.001\), Figure 4.1B), and this may result from either differences in viral output of T cells compared with macrophages or from differences in levels of human reconstitution in these models. The rate of reduction in plasma viral load [denoted as change in Log(Plasma VL)] was compared between models after one week of ART (Figure 4.1C). An approximate 2-log decrease in plasma viral load was observed in MoM and ToM after one week of ART (Figure 4.1C). The initial decline in plasma viremia was significantly less in BLT mice (~1 log) compared with MoM (\(p<0.01\)). The modest decline in viremia of BLT mice compared with MoM may result from the higher viral burden in BLT mice. Consistent with the
higher viral burden and slower viral decline in BLT mice compared with MoM, viral suppression was achieved later in BLT mice compared with MoM (average of 30 days compared to 11 days post-ART initiation, respectively, p<0.001, Figure 4.1D).

The *in vivo* lifespan of productively infected cells was estimated for MoM, ToM and BLT mice by modeling the slope of viral decay (reflecting the death rate of virus producing cells) after ART-initiation [formula: ln(2)/decay rate], as reported previously for SIV-infected rhesus macaques [275]. The decay rate was estimated by taking the linear slope of the natural log-transformed data of viral load from day 0 to day 7 of treatment. The average lifespan of productively infected cells was calculated to be an average of 1.35 days in MoM, 1.23 days in ToM, and 1.95 days in BLT mice (Figure 4.1E). This difference was statistically significant between MoM and BLT mice (Kruskal-Wallis test with Dunn’s post-test, p≤0.05). The lifespan of infected cells in ToM and MoM were not statistically different, suggesting that the lifespan of productively infected macrophages or T cells is similar.

We noted several differences between BLT mice and MoM including: 1) pre-ART plasma viral load, 2) rate of plasma viral decline, 3) time to viral suppression and 4) infected cell lifespan. In an effort to minimize these differences and facilitate a direct comparison, we infected BLT mice for only one week prior to ART-initiation (BLT short infection, bottom panel, Figure 4.1A). Infecting BLT mice for one week, rather than five weeks, resulted in similar plasma viral suppression kinetics between MoM or ToM (Figure 4.1B-E. This suggests that the rate of HIV suppression in BLT mice is in proportion to the viral burden.
A viral “blip” is defined as a transient increase in viral load from a previous time point during ART, after initial suppression was achieved; “blips” are occasionally observed in patients on durable ART [291, 292]. Viral “blips” were observed in infected ToM (4/8 mice, Figure 4.2A) and BLT mice (3/13 mice, Figure 4.2B) receiving ART. However, no viral “blips” were observed in MoM or in short-infection BLT mice. This may result from the lower pre-ART viral load in MoM and short-infection BLT mice compared with ToM and BLT mice infected for five weeks.

**HIV-RNA AND DNA LEVELS ARE DECREASED IN TISSUE MACROPHAGES DURING ART**

After demonstrating ART-mediated control of plasma viremia we next determined the ability of ART to reduce HIV RNA and DNA levels in the tissues. HIV-infected MoM, ToM and BLT mice were harvested and cells were isolated from the liver, lung, spleen, and bone marrow of suppressed (MoM n=5, ToM n=3, BLT mice n=8) and non-suppressed (MoM n=8, ToM n=3, BLT mice n=4) animals. All mice receiving ART were suppressed below the limit of detection in plasma at time of necropsy. Within each model, vDNA levels (Figure 4.3A) and vRNA levels (Figure 4.3B) were reduced compared to non-suppressed animals.

Compared to untreated BLT mice, vDNA levels were significantly reduced in the liver and spleen of ART-treated BLT mice. Although we have previously demonstrated there is a reduction in vRNA levels in ART-suppressed BLT mice [86, 87], this was the first demonstration that the overall levels of vDNA are also reduced. The magnitude of vDNA reduction was calculated for each model (Figure 4.3C). Compared to untreated MoM, vDNA levels were reduced 2-log in the liver and spleen and 3-log in the lung and
bone marrow of ART-treated MoM (Figure 4.3C). In treated ToM, compared to untreated ToM, vDNA levels were lower by 2-log in the liver and lung and by 2.5-log in the spleen and bone marrow (Figure 4.3C). Compared to non-suppressed BLT mice, vDNA levels were reduced by 1.5-log in the bone marrow and lung and by 2-log in the liver and spleen of treated BLT mice (Figure 4.3C).

Similar to the reduction in vDNA levels in ART-treated mice compared with untreated animals, vRNA levels were also reduced. There was a significant decrease of vRNA in the bone marrow of ART-treated MoM compared to untreated MoM (p<0.01, Figure 4.3B). Compared to untreated BLT mice, there was significantly less vRNA in the liver, lung, and spleen of ART-treated BLT mice (p<0.0001 for all three tissues, Figure 4.3B). The magnitude of vRNA reduction was calculated for each model (Figure 4.3D). Compared to untreated MoM, vRNA levels were reduced 2-log in the spleen, 3-log in the liver and 4-log in the lung and bone marrow in ART-treated MoM (Figure 4.3D). In ART-treated ToM, compared to untreated ToM, vRNA levels were reduced 3-log in the liver and lung and 3.5-log in the spleen and bone marrow (Figure 4.3D). Compared to non-suppressed BLT mice, vRNA levels in treated BLT mice were lower by 1.5-log in the liver and 2-log in the lung, spleen and bone marrow (Figure 4.3D).

**VIRAL REBOUND OCCURS RAPIDLY IN THE PRESENCE OF T CELLS DURING ART-INTERRUPTION**

To determine the time to viral rebound after ART-interruption, therapy was removed from suppressed ToM (n=5, Figure 4.4A) and BLT mice (n=5, Figure 4.4B). Plasma viral load was measured weekly and all animals had at least two consecutive plasma viral loads below the limit of detection prior to ART-interruption. In all ToM and
BLT mice, virus rebounded in the plasma within two weeks of ART-interruption. ToM were treated for 7-11 weeks and rebounded in an average of 1.6 weeks. BLT mice were treated for 5-10 weeks and rebounded in an average of 1.4 weeks. This demonstrated the reproducible and rapid rebound of virus in the presence of T cells.

**HIV REBOUND FROM MACROPHAGES AFTER ART-INTERRUPTION**

In ToM and BLT mice, we saw rapid rebound of virus in the plasma during ART-interruption (1-2 weeks after interruption). For rebound experiments, a total of 14 MoM were infected and treated with ART for different periods of time (3-5 weeks). All animals were monitored at least 2 weeks post-ART interruption as this was the maximum time required to observe viral rebound in ToM and BLT mice. In eight MoM treated for five weeks, no viral rebound was observed in the plasma (Figure 4.5A, top panel). Mice were harvested at various intervals as denoted by arrows in the viral load plot (from 3-9 weeks post-ART interruption) and tissues (liver, lung, spleen, and bone marrow) were assayed for the presence of viral DNA and/or RNA. Cell-associated vDNA and vRNA levels were below our limit of detection (~3 copies) in these samples (Table 4.1). In three MoM treated for three weeks, and observed a similar pattern whereby no viral rebound was observed up to nine weeks post-ART interruption (Figure 4.5A, middle panel) and vRNA and vDNA levels were below our limit of detection (Table 4.1); demonstrating the effectiveness of this ART regimen in macrophages.

To further evaluate the presence of HIV in the tissues of ART-treated MoM, we attempted two viral outgrowth techniques: *in vivo* outgrowth of MoM cells in uninfected BLT mice and *in vitro* co-culture of MoM cells with activated allogenic CD8-depleted, CD4+ T cells. Cells from four of the MoM were viral rebound was not observed (MoM
#5, 7, 10 and 11, listed by number in Table 4.1) were injected into uninfected BLT mice. Plasma viral load was monitored over time. No viral replication was observed in these animals (no vRNA in plasma over time and no vDNA in cells from BLT tissues at necropsy, limit of detection was 668 RNA copies/ml plasma and ~3 DNA copies per 100,000 cells). Additionally, cells from three of these non-rebounding MoM (MoM #5, 6 and 7, listed by number in Table 4.1) were plated and PHA-activated, CD8-depleted allogenic PBMCs from healthy donors were added to the cultures to determine if virus could be rescued from the cells isolated from these MoM. After 10 days the culture supernatants were collected and analyzed for HIV-RNA, but all samples were below our level of detection by this technique (<668 RNA copies/mL of culture supernatant). This outgrowth technique was validated in Chapter 3. We observed no viral replication in any of the BLT mice that received isolated MNCs or in any of the in vitro cultures, suggesting that the levels of replication competent virus in MoM are minimal after ART-treatment.

While we were unable to recover replication competent HIV from many of the ART-treated MoM, we did observe viral rebound seven weeks after therapy interruption in three animals (bottom panel, Figure 4.5A). To our knowledge, these MoM with viral rebound after ART-interruption represent the first in vivo evidence that macrophages can be a source of viral rebound after ART-interruption.

BLT mice infected for one week had similar suppression kinetics compared to MoM (Figure 4.1); therefore, only the short-infection BLT mice were used for direct comparison to MoM. In short-infection BLT mice treated with ART for five weeks (Figure 4.5B, top panel) or eight weeks (Figure 4.5B, bottom panel), viral rebound was
observed 1-2 weeks post-ART interruption, significantly earlier than in MoM (7 weeks) 
(p=0.036). It should be noted that in MoM where viral rebound was observed, the pre-
ART plasma viral load was significantly higher when compared to MoM where viral 
rebound was not observed (218,746 versus 55,773 RNA copies per ml plasma, p≤0.05) 
(Figure 4.5C, Table 4.1). The pre-ART viral load of MoM with or without rebound was 
not different from that observed in short-infection BLT mice.

**LACK OF DETECTABLE HIV RNA/DNA FROM INFECTED MACROPHAGES BUT NOT T CELLS IN ART-TREATED BLT MICE**

In ToM, the only source for viral rebound after ART-interruption is T cells [288]; however, BLT mice contain both T cells and macrophages that are susceptible to infection. After noting an absence of viral rebound and undetectable levels of vRNA and vDNA in some ART-treated MoM and a significant delay of rebound in others, we proceeded to investigate the presence of infected macrophage in infected BLT mice receiving ART.

To investigate the possible source of virus rebound in BLT mice (i.e. T cells or macrophages), we employed the cell-sorting strategy illustrated in Figure 4.6A. We used four BLT mice treated for 5-8 weeks, with plasma viral load suppressed for 2-4 weeks (Figure 4.6B), and non-suppressed animals infected for 4-6 weeks prior to harvest. MNCs from the spleen, liver, lung, and bone marrow were collected and the cells from the different tissues combined into a single pool of cells for each individual mouse. Then mouse cells were depleted from the pooled MNCs. After removal of the mouse cells, human T cells were isolated via positive magnetic selection. The remaining
non-T cell fraction was then subjected to negative magnetic selection yielding a highly purified population of human macrophages (<1% T cell contamination).

Our results show the presence of readily detectable levels of viral DNA (Figure 4.6C) and viral RNA (Figure 4.6D) in macrophages and T cells purified from untreated BLT mice. Viral DNA and RNA was also readily detectable in T cells purified from ART treated BLT mice. In contrast, we were unable to detect HIV-DNA (Figure 4.6C) or RNA (Figure 4.6D) in purified macrophages from ART-suppressed BLT mice (limit of detection ~25 copies). Viral DNA was reduced by approximately 2-log in T cells (p=0.029) and 1-log in macrophages (p=0.021) of ART-suppressed mice compared with non-suppressed animals. Viral RNA was reduced by almost 3-log in macrophages (p=0.046) of ART-suppressed mice compared with non-suppressed animals. These results are consistent with those obtained in MoM and reflect a dramatic decrease in infected macrophages as a result of antiretroviral treatment.

DISCUSSION

Macrophages are long-lived cells (half-life of months to years) that have been postulated as a potential reservoir of HIV [188, 191, 194, 286]. However, there has been a lack of direct evidence for the role of macrophages as a viral reservoir in vivo. The critical questions we sought to answer in this study were: 1) is viral replication in tissue macrophages controlled by ART? 2) Does viral replication persist in these cells during treatment? 3) Are macrophages a source of viral rebound after ART-interruption? To address these questions we used a novel in vivo model that we recently developed (Chapter 3). We treated HIV-infected MoM with ART consisting of FTC/TDF/RAL and showed that this combination therapy effectively suppressed plasma viral load and
reduced vDNA and vRNA levels in the tissues of treated mice. In addition, ART administration resulted in a lack of viral rebound in the majority of HIV-infected MoM after analytical therapy interruption. In our study we utilized an InSTI-based ART regimen containing two NRTIs, FTC and TDF, and an integrase-inhibitor, RAL. Despite *in vitro* evidence that NRTIs are present at much lower concentration in macrophages compared to T cells [289], we found this regimen to be highly effective for reducing plasma viremia as well as vDNA and vRNA levels in the tissues of MoM.

Differences were observed in the viral suppression kinetics in macrophages and T cells. First, viral suppression is achieved rapidly in the plasma of MoM (1.6 ± 0.1 weeks). Comparatively, ToM and BLT mice required approximately 2-3X longer to achieve initial suppression. However, ToM and BLT mice had significantly higher pre-ART plasma viral loads compared to MoM, and the log-reduction in plasma viral load was significantly less in BLT mice compared to MoM after one week of therapy. These results suggest that ART may be less potent in T cells than in macrophages or that initial viral burden impacts the efficacy of ART, as has been demonstrated in humans [293]. Notably, BLT mice infected for only one week had a similar viral load to MoM, and in these BLT mice, ART was able to suppress plasma viremia in a similar time frame to MoM (2.2 ± 0.2 weeks) (Figure 4.1D).

We observed that ART effectively suppressed viral replication in tissue-macrophages within 1-3 weeks of treatment-initiation (Figure 4.3). We noted a 2-4 log reduction in tissue vRNA levels of treated compared to untreated MoM. This suggests that viral production from macrophages during ART is effectively suppressed to undetectable or near undetectable levels. Consistent with the low to undetectable vRNA
levels, we noted a 2-3 log reduction in tissue vDNA levels of treated compared to untreated MoM, suggesting that most infected cells were eliminated. Although we were only able to detect minimal levels of vDNA or vRNA in ART-treated MoM, this does not preclude the possibility that small numbers of macrophages harbor HIV during ART.

We observed viral rebound in the plasma of MoM several weeks after ART-interruption. We therefore demonstrate that macrophages can be a source of virus rebound after ART-interruption. Viral rebound in patients generally occurs within two weeks of ART-interruption [148]. In MoM, viral rebound was not observed until seven weeks after ART-interruption. This would suggest that viral rebound in most patients during ART-interruption is likely the result of virus in T cells. However, macrophages cannot be ruled out.

We were able to detect some vDNA and some vRNA in the tissues of ART-suppressed MoM harvested on therapy (Figure 4.3A,B). This seemed in contrast to our data in BLT mice on therapy where we were unable to detect any vDNA or vRNA in the purified macrophages. However, this might be explained by the total length of ART-treatment in the different groups of mice. MoM were treated for 1-3 weeks (average 2 weeks) and BLT mice were treated for 5-8 weeks (average 6.2 weeks). The BLT mice were treated longer because it takes longer to suppress viremia in these animals compared to MoM, and our intent was to analyze the macrophages once viral suppression was achieved in plasma. Overall, we demonstrate that this ART regimen, consisting of FTC/TDF/RAL, is extremely effective in reducing viral levels in infected macrophages in vivo.
There are several caveats to our study in MoM. First, interactions between T cells and macrophages may be critical for persistent HIV infection in macrophages. To address this, we purified macrophages from ART-suppressed BLT mice, and the levels of virus in these cells were below our limit of detection. Second, we could not evaluate the possible contribution of microglia to viral rebound. Microglia derive from early precursor cells in the yolk sac [294] and the human CD34+ stem cells used to reconstitute MoM have not been shown to generate microglial cells in the brain of transplanted mice. Therefore we could not assess the efficacy of ART to suppress viral replication in microglia or their contribution to viral persistence and rebound. A third caveat is that NOD/SCID mice develop spontaneous thymic lymphomas resulting in a relatively short life (9-14 months) [295]. This limitation prevented us from extending our observations of infected MoM after ART-interruption for periods of time greater than 63 days which means we could be underestimating the frequency of viral rebound in macrophages.

Humanized MoM represent a unique small animal model for investigating the role of macrophages in viral persistence during ART and viral rebound during ART-interruption in the complete absence of human T cells. We demonstrate that HIV replication in macrophages in MoM is rapidly controlled with conventional ART, and there is a robust reduction in the levels of vRNA and vDNA in the tissues of these mice. However, delayed viral rebound was observed in MoM after removal of therapy, demonstrating that macrophages can be a reservoir for HIV.
Figure 4.1: HIV replication in myeloid cells is effectively suppressed by ART. A) Plasma viral load of mice after ART-initiation in MoM (n=19, magenta triangles), ToM (n=8, green diamonds), BLT mice (n=13, light blue circles) and BLT mice infected for one week (also called short-infection, n=5, blue squares). Dashed vertical line denotes the start of ART. Dashed horizontal line represents the limit of detection for the assay (~668 RNA copies per ml of plasma). B) Comparison of plasma viral load at the time of ART-initiation between groups of mice. C) Comparison of the individual log reduction in plasma viral load one week after ART-initiation between groups of mice. D) Comparison of the time to viral suppression (defined as the time point of the first viral load below the limit of detection) between groups of mice. Kruskal-Wallis test with Dunn’s Multiple Comparison post-test was used to compare groups of mice in B-D. E) The half-life of infected cells was estimated as the time to a two-fold reduction in plasma viremia in mice after starting ART.
Figure 4.2: Viral “blips” observed during ART-treatment of ToM and BLT mice. Viral “blips” were observed in A) ToM (in 4 out of 8 mice) and in B) BLT mice (in 3 out of 13 mice). Individual plasma viral load plots are shown for animals where “blips” were detected after initial viral suppression was achieved. Black arrows indicate viral “blips.”
Figure 4.3: Reduction of HIV-DNA and RNA levels after ART-suppression from MoM tissues. Cell-associated viral A) DNA and B) RNA (copies per 100,000 total cells) was measured for MoM suppressed on ART (n=5, light purple circles), MoM not on ART (n=8, dark purple squares), ToM suppressed on ART (n=3, light green circles), ToM not on ART (n=3, dark green squares), BLT mice suppressed on ART (n=7, light blue circles), and BLT mice not on ART (n=4, dark blue squares). Comparisons between suppressed and non-suppressed animals for each model were performed using Bonferroni’s multiple comparisons test. Solid gray line in A and B denotes the limit of detection for the assay (~3 DNA or RNA copies). C) The fold-difference in HIV-DNA levels in non-suppressed animals compared to suppressed animals was calculated as: [vDNA(non-suppressed)] / [vDNA(suppressed)]. This represents the magnitude of the reduction in the number of HIV-infected (vDNA+) cells in ART-treated animals. D) The fold-difference in HIV-RNA levels in non-suppressed animals compared to suppressed animals was calculated as: [vRNA(non-suppressed)] / [vRNA(suppressed)].
Figure 4.4: Viral rebound is rapid in the presence of T cells *in vivo*. Therapy was interrupted from suppressed ToM (n= 5) and BLT mice (n=5) at different times and plasma samples were collected to determine the time to viral rebound. Viral rebound after ART discontinuation was observed in both A) ToM and B) BLT mice. Duration of ART is indicated with a gray box. All mice rebound in two weeks or less.
Figure 4.5: Viral rebound after ART-cessation observed in 3/14 of HIV-infected MoM. Plasma viral load was measured over time in A) infected MoM and B) “short-infection” BLT mice. Only mice observed for greater than two weeks after cessation of ART were considered for viral rebound (this is the maximum time needed to observe viral rebound in ToM and BLT mice (Figure 4.4). Duration of ART is indicated by gray box. Infected MoM are grouped by animals treated for five weeks with no detectable rebound (top), MoM treated for three weeks (short-ART) with no detectable rebound (middle), and MoM treated for five weeks that rebounded seven weeks after ART-cessation (bottom). Infected BLT mice are grouped by length of ART, either five weeks of therapy (top) or eight weeks of therapy (bottom). For all plots, arrows indicate time points where mice were harvested and vDNA and/or vRNA levels were assessed in tissues of mice where rebound was not observed (Table 1). C) The pre-ART plasma viral load of MoM where rebound was not observed was compared with the pre-ART viral load of MoM and BLT mice where rebound was observed after ART-cessation. MoM where rebound was observed had a significantly higher viral load than MoM where rebound was not observed. Each symbol represents an individual mouse and is color-coded for the harvest time point in either A (MoM) or B (BLT mice). Kruskal-Wallis test with Dunn’s Multiple Comparison post-test was used to compare groups of mice in C.
Figure 4.6: No infection of macrophages in ART-suppressed BLT mice. ART-suppressed and non-suppressed BLT mice were harvested and total MNCs (from the spleen, liver, lung, and bone marrow) were isolated from individual animals, and mouse cells were depleted. Purified T cells and macrophages were isolated from individual mice using magnetic beads. A) Schematic of cell isolation from pooled human MNCs of BLT tissues. B) Viral suppression kinetics for treated BLT mice used for macrophage and T cell isolation. Arrows indicate time points where BLT mice were harvested and cells isolated/purified. C) DNA levels from T cells (circles) and macrophages (Macs, triangles) isolated from untreated (No ART) or ART-suppressed (+ ART) BLT mice. D) RNA levels from T cells and macrophages isolated from untreated (No ART) or ART-suppressed (+ ART) BLT mice. In C-D, each point represents an individual BLT mouse, and the ART-suppressed animals are color-coded with the lines and arrows in B. Mann-Whitney test was used to compared DNA and RNA levels between T cells (with or without ART) or between macrophages (with or without ART). Dashed lines in C-D represent the limit of detection for each assay (~25 DNA or RNA copies).

Table 4.1: No vDNA or vRNA in tissue macrophages isolated from MoM where viral rebound was not observed. Cell-associated vDNA and vRNA analysis was performed on cells isolated from MoM tissues (liver, lung, spleen and bone marrow) at necropsy. Additionally, cells from MoM #5, 7, 11 and 12 were injected into BLT mice, but no viral outgrowth was observed in any of the BLT mice. Cells from MoM #5, 6 and 7 were cultured with activated-feeder cells for 10 days, but no viral outgrowth was observed in any of the cultures. “nd” denotes not done.
Table 4.1: No vDNA or vRNA in tissue macrophages isolated from MoM where viral rebound was not observed. Cell-associated vDNA and vRNA analysis was performed on cells isolated from MoM tissues (liver, lung, spleen and bone marrow) at necropsy. Additionally, cells from MoM #5, 7, 11 and 12 were injected into BLT mice, but no viral outgrowth was observed in any of the BLT mice. Cells from MoM #5, 6 and 7 were cultured with activated-feeder cells for 10 days, but no viral outgrowth was observed in any of the cultures. “nd” denotes not done.
CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

STUDY SUMMARY

While T cells are the more abundant targets of HIV infection, we have demonstrated herein that macrophages are also capable of sustaining HIV replication over time in the complete absence of T cells. HIV infection of human T cells and macrophages differs in many aspects: overt toxicity during infection [124, 296], the number of isolates that can actively replicate in macrophages compared with T cells (Chapter 3) and the effectiveness of ART in macrophages compared with T cells [191, 196] (Chapter 4). Specifically, fewer HIV isolates are able to replicate in macrophages compared with T cells (Chapter 3). ART treatment of MoM, ToM and BLT mice effectively controls viral replication (Chapter 4). Also, while viral latency is consistently established in human T cells and rebound occurs rapidly (7-14 days) after the cessation of ART (Chapter 2 and Chapter 4), this was not always the case with macrophages (Chapter 4). In MoM viral rebound was not always observed; and when it was observed, it was 49 days post ART-interruption. However, we have demonstrated that macrophages can represent a source of viral rebound during ART-interruption and cannot be excluded as a source of persistent virus (Chapter 4).

SUMMARY OF STUDIES ON HIV INFECTION OF T CELLS

With the long-term goal of obtaining a better understanding of HIV replication, CD4+ T cell depletion, HIV latency and persistence in vivo, we sought to study HIV-1 in a humanized mouse model that possesses human T cells (ToM) but is devoid of human
We implanted human thymus and liver tissues under the kidney capsule of NSG mice, resulting in sustained human T cell levels in the spleen, liver, lung, lymph nodes and bone marrow (Figure 2.2). Without a bone marrow transplant, ToM do not support human myeloid or B cell development (Chapter 2). ToM have much higher levels of human reconstitution than thy/liv-implanted SCID [70] or NOD/SCID mice (Figure 2.1), demonstrating the critical nature of the IL-2 gamma chain knockout in NSG mice to support high levels of systemic human engraftment [226]. ToM are primarily reconstituted with naïve (CD45RA+/CD27+) and central memory (CD45RA-/CD27+) CD4+ and CD8+ T cells as well as effector memory (CD45RA-/CD27-) CD4+ T cells (Figure 2.3).

ToM support HIV infection with multiple isolates including JRCSF, RHPA, THRO, CH058, CH040, CH040-4013 env, ADA and 7312A (HIV-2) (Figure 2.4, Figure 3.3). Although infection with JRCSF results in a modest (10-20%) reduction in the overall levels of CD4+ T cells in tissues and blood, nearly all of the effector memory CD4+ T cells are depleted (Figure 2.4). ART-treatment of ToM infected with JRCSF, CH040 or CH040-4013 env resulted in a decline in plasma viremia (Figure 2.5, Figure 4.1). Tissue vDNA and vRNA levels were also decreased below the limit of detection in CH040 and CH040-4013 env-infected ToM (Figure 4.3). Additionally, removal of ART in suppressed ToM resulted in the rapid rebound of virus in plasma (1-2 weeks) (Figure 2.5, Figure 4.4). These results demonstrate that ART effectively controls HIV replication in T cells in vivo. Additionally, we demonstrated that human myeloid cells are not required for the establishment of a latently infected T cells, and that virus rebounds rapidly after removal of ART in the presence of T cells.
Evaluation of latency in resting (CD25−/HLA-DR−) CD4+ T cells isolated from ART-suppressed ToM using a quantitative viral outgrowth assay (QVOA) [175] demonstrated the presence of inducible virus at a frequency of ~3.9 infectious units per million (IUPM) resting CD4+ T cells (Figure 2.7). These results are comparable to those observed in the peripheral blood of ART-suppressed human patients that initiated therapy during acute or chronic HIV infection (~1.4 or ~2.4 IUPM, respectively) (Figure 2.7). These results also confirm that T cells alone are sufficient for the establishment of viral latency, and latency establishment does not require cytokine or chemokine input from human myeloid-derived cells. As T cells are thought to be the major reservoir for viral latency, ToM represent a unique tool for the in vivo evaluation of T cell-targeted HIV eradication strategies.

SUMMARY OF STUDIES ON HIV INFECTION OF MYELOID CELLS

Although CD4+ T cells are a well-characterized and abundant target of HIV infection, other target cells exist. Specifically, human macrophages which express CD4 and CCR5, the cell surface receptors necessary for viral entry. As these cells are considered to be long-lived and not subject to cytopathic killing by HIV after infection, there is interest in establishing whether or not macrophages represent a persistent reservoir during ART-treatment or a source of viral rebound during ART-interruption. I have used a humanized mouse model that is reconstituted with human myeloid cells and a complete absence of human T cells (Figure 3.2). We prepared MoM by injecting human CD34+ hematopoietic stem cells into pre-conditioned NOD/SCID mice (Chapter 3, Chapter 4). MoM are systemically reconstituted with human myeloid cells (including blood monocytes, tissue macrophages and dendritic cells) and B cells. Most
importantly, MoM completely lack human T cells. Monocytes present in the blood and macrophages present in the tissues (bone marrow, spleen, liver and lung) of MoM express a classical phenotype (CD14+/CD16-) and MHC Class II (HLA-DR+).

There is a lack of HIV DNA in patient monocytes (Figure 3.1); therefore, monocytes cannot serve as a surrogate for understanding HIV infection of myeloid cells as these cells are refractory to infection, and only become susceptible upon differentiation into macrophages [297]. Thus, in vivo evaluation of HIV infection in myeloid cells must be performed using tissue macrophages, not blood monocytes. MoM were susceptible to HIV infection with three different R5-tropic HIV clones (ADA, CH040 or CH040-4013 env) as determined by plasma viral load (Figure 3.3). Infection was evident one week after intravenous exposure and was maintained over several weeks (up to 15 weeks, last time point evaluated). However, MoM were not susceptible to infection with other R5-tropic HIV isolates including JRCSF, THRO, RHPA, CH058 and 7312A (HIV-2) (summarized in Figure 3.3E).

Viral replication occurs systemically in MoM as HIV DNA and HIV RNA are detectable in all tissues (Figure 3.4). Furthermore, in situ hybridization demonstrated that CD68-positive macrophages produce HIV RNA transcripts in these mice (Figure 3.4). Analysis of the brains of infected MoM demonstrated that T cells are not required for HIV trafficking into the CNS (Figure 3.5). HIV RNA was detected in over half (7/12) of the infected MoM brains analyzed, and HIV p24-positive cells in the brain were detectable by IHC (Figure 3.5). There were an increased number of human cells and human macrophages present in the brains of infected MoM compared to uninfected animals.
To determine the frequency at which replication-competent virus exists in macrophages, we cultured macrophages isolated from the tissues of infected MoM and performed viral outgrowth assays (Figure 3.4). Co-culture of cells from infected MoM with activated allogeneic CD4\(^+\) cells resulted in viral outgrowth in the majority of wells from all tissues, with as few as 135 human macrophages leading to infection of the cultures. This demonstrates that infected macrophages are found in all tissues, and even a small number of macrophages can lead to productive infection of T cells in culture. Expanding this to an *in vivo* model, adoptive transfer of bone marrow cells from infected MoM into BLT mice resulted in the rapid (within one week) infection of the BLT mice (Table 3.2). To determine if macrophages are productively infected in the presence of T cells, macrophages were purified from BLT mice infected with CH040 or CH040-4013 env and injected in MoM. These MoM became infected, further demonstrating that macrophages are capable of establishing *de novo* infection; and the evidence from BLT mice demonstrates that macrophages are infected in the presence of T cells (Table 3.2).

HIV replication in MoM is rapidly controlled by ART (Figure 4.1). Within two weeks of ART-initiation, HIV RNA levels in plasma were below the limit of detection in all MoM. The first week of ART was characterized by a near 2-log reduction of virus in the plasma of MoM. Using a method demonstrated in Micci *et al.*, the half-life of HIV-infected cells during ART of MoM, ToM and BLT mice was estimated [298]. The half-life of the infected cells during ART was estimated to be 1.35 days in MoM, 1.23 days in ToM, and 1.76-1.95 days in BLT mice (Figure 4.1). HIV DNA levels are greatly reduced (by 2-3 log) in ART-suppressed MoM compared with untreated mice, suggesting an
elimination of many infected cells during therapy (Figure 4.2). HIV RNA levels were similarly reduced (by 2-4 log) in ART-suppressed MoM tissues compared with untreated mice, showing that RNA production was minimal in the presence of therapy (Figure 4.2).

The kinetics of viral rebound during ART-interruption was distinct for MoM (Figure 4.5) compared to ToM and BLT mice (Figure 4.4). While ToM and BLT mice rebounded in 1-2 weeks after removal of therapy, no MoM rebounded within this same timeframe. Rebound was observed in 3/14 MoM, and this occurred seven weeks after removal of ART (Figure 4.5). Analysis of the tissues of MoM that did not rebound after ART-interruption demonstrated a lack of virus DNA and/or RNA, suggesting that near-eradication of virus-infected cells was achieved in these mice (Table 4.1). Viral rebound in MoM was associated with animals that had a higher pre-ART viral load in comparison to MoM where rebound was not observed (Figure 4.5).

In order to determine if these observations of MoM would translate into models where both T cells and macrophages are present, we analyzed macrophages from infected BLT mice (Figure 4.6). BLT mice infected with CH040 harbor HIV DNA and HIV RNA in their purified T cells and macrophages. After suppression with ART, there are decreased HIV DNA and HIV RNA levels in the T cells, but a further reduction of these levels to below the limit of detection are seen in the purified macrophages. This would suggest that HIV rebound during ART-interruption in BLT mice results from virus in the T cells, not virus in the macrophages.

**IMPLICATIONS OF CURRENT STUDIES**

Our results have significant implications for HIV latency and cure research with regard to T cells and myeloid cells as viral reservoirs. The establishment of latency in T
cells results from reversion of an activated HIV-infected T cell to a resting state [143], but direct infection of resting CD4\(^+\) T cells, although ineffective, has also been demonstrated [299, 300]. However, recent studies have demonstrated that culture of myeloid dendritic cells (mDCs) or monocytes along with resting CD4\(^+\) T cells induces post-integration latency in non-proliferating CD4\(^+\) T cells, and suggests that direct cell contact is important in this induction of latency [152, 153]. Using ToM, we demonstrate that latency is established in resting CD4\(^+\) T cells in the complete absence of human myeloid-derived cells (Chapter 2). Our results suggest that T cells, with no induction of post-integration latency by human myeloid cells, are sufficient for the establishment of HIV latency \textit{in vivo}.

Using the complementary MoM model of HIV infection, with a complete absence of human T cells, we have demonstrated sustained and systemic HIV replication \textit{in vivo} (Chapter 3). We also demonstrate that HIV infected macrophages are susceptible to viral control with ART (Chapter 4). The potential contribution of macrophages to HIV and SIV persistence has been suggested for many years [188, 301], but there has been a lack of direct \textit{in vivo} evidence [194]. We present the first direct evidence that tissue macrophages can be a source of viral rebound during ART-interruption, in the absence of human T cells. More striking was the delay in viral rebound from macrophages (49 days) compared with T cells (7-14 days), suggesting that the mechanisms controlling viral persistence may be distinct in T cells and macrophages. Human MDM harbor unintegrated viral DNA in a circular form (extrachromosomal 2-LTR circles or cc2LTR) for up to two months in culture [302]. Gillim-Ross \textit{et al}, suggest that these circular forms may contribute to viral persistence in macrophages [303], although these unintegrated
circular products are considered as “dead end products” that cannot sustain replication [304]. Future studies in MoM will useful in elucidating the mechanisms of viral persistence in macrophages in vivo.

MOVING FORWARD (FUTURE STUDIES)

MECHANISM OF VIRAL PERSISTANCE IN MACROPHAGES

One critical question remaining is whether or not macrophages could serve as an active or latent reservoir during ART. This question has been the subject of a major controversy within the HIV research community that has been further confounded by recent evidence that the presence of virus in macrophages can be the result of phagocytosis of virally infected T cells and therefore does not represent a true infection [132]. We have shown that HIV latency in T cells can be established in the complete absence of human myeloid cells in ToM, and have now also demonstrated that persistent macrophage infection can be established in the complete absence of T cells using the MoM. The fact that we observed viral rebound in ART-treated MoM after treatment cessation suggests that HIV latency can occur in macrophages that latency is a real phenomenon in macrophages and is established in a similar way as in T cells, because we observed viral rebound in ART-treated MoM after treatment cessation. It will be critical in future experiments to be more discerning about the ways that we define latency, the ways that we investigate latency, and the ways that we address latency therapeutically in T cells and macrophages. While we have performed pivotal experiments to determine the role of macrophages in viral persistence and rebound after ART-interruption, many additional questions remain to be addressed.
The most important remaining question is whether or not the viral rebound in MoM is the result of true viral latency (as integrated, transcriptionally silent virus that can be induced to produce infectious virus) or if the duration of ART (five weeks) was too short to purge a small number of cells actively replicating virus from the tissues of MoM, capable of leading to viral rebound after ART-interruption. Either outcome would have important implications for HIV cure research. If macrophages indeed serve as a latent reservoir, the large numbers of macrophages throughout the body could represent a formidable barrier to HIV eradication. If macrophages are a cellular source of viral replication with a short half-life they might eventually be eliminated during treatment and not contribute to HIV persistence.

To address these questions, we must establish whether HIV rebound in MoM results from latently infected cells or cells actively replication virus. There are several possible strategies to address if rebound results from active viral production in macrophages. One could be to extend the length of treatment. Another could be to intensify the ART regimen with additional classes of drugs. One last alternative could be to use the 3B3-PE38 immunotoxin [86]. Treatment with 3B3-PE38 has been demonstrated to significantly decrease the active reservoir that persists in BLT mice during ART [86] and evaluation of this immunotoxin in tissue culture experiments with MDM demonstrated great potency with minimal toxicity [150]. Perhaps the best way to address if rebound in MoM results from a latent reservoir would be to evaluate ex vivo induction of viral production from cells isolated from infected, ART-suppressed MoM. Using these cells, a quantitative viral outgrowth assay could be performed in a manner similar to what has been done for CD4+ T cells. If infected macrophages represent a
true long-lived latent reservoir, it will be critical to determine how latency is established and maintained in these cells, and where these latently infected cells reside. However, it is possible that macrophages could represent both active and latent reservoirs of virus, as is observed in T cells.

With regard to these future experiments, it will also be important to consider the combinations of ART that are used for HIV suppression in macrophages. My results show that HIV replication in macrophages is effectively controlled by a regimen consisting of TDF, FTC and RAL. As not all patients are on the same ART regimen, it will be critical to determine if other regimens are as potent in macrophages. However, there are several additional treatment regimens that have yet to be evaluated specifically for HIV suppression in macrophages in vivo. Additionally, newly formulated long-acting ART tailored for macrophages [305, 306] could also be evaluated using MoM. We can then determine whether or not viral rebound is observed in MoM suppressed with other combinations of ART, or more importantly, if rebound occurs more rapidly after ART-interruption. If rebound in more rapid, this would suggest that certain ART regimens may be preferred for patients that harbor macrophage-tropic isolates of HIV.

**FUTURE UTILIZATION OF ART IN MOM**

Pre-exposure prophylaxis (PrEP) against HIV has included systemic and topical administration of ART to prevent HIV transmission. We have demonstrated that systemic PrEP with TDF/FTC is effective in preventing vaginal and rectal as well as intravenous transmission in BLT mice [79, 80]. Using MoM, we can now determine if these drugs are capable of preventing HIV infection of macrophages when administered
systemically prior to exposure. We could also evaluate post-exposure prophylaxis (PEP) in MoM, which would help determine the time it takes to establish infection in macrophages in vivo.

**VIRAL FACTORS INVOLVED IN HIV INFECTION OF MACROPHAGES AND T CELLS**

The viral protein vpx, found in HIV-2 and SIV, may facilitate viral replication in macrophages by counteracting SAM domain- and HD-domain-containing protein 1 (SAMHD1) \[^{53, 56, 64}\]. Insertion of HIV-2\_rod vpx into HIV-1 allowed a previously non-macrophage tropic isolate to replicate in MDM \[^{64}\]. However, as recent evidence that vpx was unable to facilitate SIV replication in macrophages \[^{132}\], we can specifically address the role that vpx might play in facilitating viral replication in macrophages in vivo with MoM.

Much that is known about the function of viral proteins is in the context of T cells. However, MoM represent a unique model that can be used to answer important questions in the field with regard to viral replication in tissue macrophages in vivo in the absence of T cells. For example, the Vpr protein has been shown to enhance macrophage infection in MDM \[^{307}\]. Using a Vpr-deficient isolate in MoM, we could determine if replication is significantly decreased or is unaffected by the absence of Vpr. Another viral protein of interest in MoM could be Nef. Nef is a moderate enhancer of viral infectivity that plays a major role in CD4 and MHC downregulation in T cells \[^{20, 47, 52}\]. Also, exogenous Nef can lead to production of transcriptional factors such as AP-1 and NF-κB and phosphorylation of MAP kinases by MDMs \[^{301}\]. By modifying CH040 Nef, either by deletion or other mutations causing a frameshift in the protein, we
would be able to directly access the requirements for Nef in HIV replication in macrophages.

**VIRAL TRAFFICKING IN THE CNS**

I have demonstrated that human cells populate the brain of uninfected and infected MoM and that virus is detectable in the brains of the majority (58%) of infected MoM. However, careful evaluation of the brains of ToM is needed to determine 1) Are human T cells present in the brains of these mice? If so, how many? and 2) Is virus able to traffic to the brain of ToM in the complete absence of myeloid cells? In a study of SIV-infected rhesus macaques, animals were given natalizumab (Tysabri), an anti-alpha4 blocking antibody [308]. This antibody is able to minimize lymphocyte trafficking into the brain. We could evaluate this antibody in ToM, MoM and BLT mice to determine if this treatment at various points of infection can prevent virus from getting to the brain. This would help to determine if cell-free infection of the distinct cell types is possible in the CNS.

**MECHANISMS OF VIRAL TRANSMISSION**

Even though the bulk of my work has dealt with different aspects of HIV persistence, both ToM and MoM models can contribute to other important areas of HIV research. For example, ToM and MoM represent unique models where the cellular requirements for HIV transmission via mucosal routes (oral, vaginal and rectal) can be studied. Recent mapping of HIV target cells, including IHC of CD4, CD16, CD3 and CD1b, in human oral, rectal/sigmoid and cervical mucosal samples of healthy donors, was performed [309]. As the luminal surfaces of the mucosa are those which are in direct contact with HIV, careful evaluation of the peri-luminal HIV target cells was
performed. This demonstrated that peri-luminal target cells of HIV were most prevalent at areas containing simple columnar epithelium (endocervix and rectal/sigmoid surfaces). Also, this demonstrated that CD4\(^+\) T cells and macrophages are present near the lumen of mucosal sites and could potentially come into contact with HIV.

While mucosal CD4\(^+\) T cells represent the primary target for vaginal HIV transmission, many other target cells are present in the vaginal cavity such as Langerhans cells, dendritic cells and macrophages [310]. Langerhans cells have been shown \textit{ex vivo} and \textit{in vitro} to endocytose HIV-1 virions and transfer them to CD4\(^+\) T cells, and this is attributed to long dendrites from Langerhans cells that are able to traverse through the epithelium [311, 312]. Of the mononuclear cells present in the vaginal cavity, dendritic cells are one of the first to take up HIV-1 virions \textit{ex vivo}, so they may help facilitate early establishment of HIV-1 infection [313]. Analysis of vaginal mucosa explants demonstrated that vaginal macrophages express higher levels of CD4, CCR5 and CXCR4 than intestinal macrophages, and vaginal macrophages were susceptible to HIV-1 infection [241]. However, models for vaginal transmission also include myeloid-cell independent mechanisms. Using various epithelial cell types and various cell lines, it has been shown that transcytosis may occur whereby infectious virions enter an epithelial cell and then migrate from the apical to basal surface, leading to subsequent infection of target cells [314]. However, there is limited \textit{in vivo} evidence as to which mechanisms are most important in facilitating mucosal transmission.

Humanized ToM and MoM (alongside the complementary BLT mouse model) could be useful tools in understanding the cellular requirements for mucosal transmission. I have some preliminary data demonstrating that the female reproductive
tract of MoM and ToM and the gut of MoM are reconstituted with human cells, and that vaginal transmission is effective in ToM. Strategies to prevent vaginal HIV transmission, such as topical formulation of existing ART, can be evaluated using ToM. Evaluation of rectal transmission in MoM could also be important to determine if macrophages are capable of establishing systemic infection after rectal exposure to HIV. No evaluation of the oral mucosa of MoM and ToM has been performed yet, but if these tissues (nasal-associated lymphoid tissue or NALT, esophagus, stomach) are reconstituted with human cells, these models could be useful in determining if macrophages or T cells are sufficient to facilitate oral HIV transmission in vivo. By understanding if mucosal transmission can occur in the presence of T cells or macrophages alone, targeted strategies against these cells can be evaluated.

**FINAL SUMMARY**

Herein I describe the characterization and validation of two complementary humanized mouse models for HIV infection in vivo. Using humanized ToM and MoM, we have demonstrated that HIV replication can occur in the presence of either T cells or macrophages, but macrophage infection is limited to macrophage-tropic HIV isolates. ART suppression of virus in the plasma is rapid in ToM or MoM, but kinetics of plasma rebound differed greatly between the two models. Specifically, viral rebound is rapid in ToM (1-2 weeks) but is significantly delayed in MoM. This is the first demonstration that macrophages can be a source of viral rebound in the absence of T cells after ART-interruption. We also demonstrate that a latent HIV reservoir is established in T cells in the complete absence of myeloid-derived cells. Using these models we demonstrate that myeloid-derived cells and T cells can independently contribute to re-emergence of
HIV infection when ART is interrupted and further delineation between the mechanisms contributing to viral rebound from T cells or from myeloid cells can be achieved using ToM and MoM.
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