Elucidating the roles of αβ and γδ T cells in HIV infection

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BA, Indian Academy Degree College, 2013

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Submitted to the Graduate Faculty of the

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2021

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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It was defended on

February 26, 2021

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Abstract

Untreated HIV infection leads to acquired immunodeficiency syndrome (AIDS) in nearly all cases [1]. However, a small proportion of HIV-infected individuals, known as long-term non-progressors (LTNPs), suppress viremia to undetectable levels without antiretroviral treatment [2, 3]. Additionally, while most HIV-infected individuals display defects in T cell frequency and function, including the γδ T cell subpopulations [4, 5], these parameters are maintained in LTNPs [6-8]. Although their role in HIV infection is not well-defined, γδ T cells display anti-HIV properties *in vitro* [9], and their maintained numbers in LTNPs implies that they may contribute to HIV control *in vivo*. An alternate hypothesis for the limited disease progression noted in LTNPs is based on the infecting HIV strain itself [10, 11], as defects in HIV Nef have been shown to impact the pathogenic properties of HIV *in vitro* [12, 13] and are associated with nonprogressive infection [14, 15]. However, it remains unclear if these reported modifications in HIV Nef and the preservation of γδ T cell frequency demonstrated in LTNPs are mechanistically interrelated and associated with *in vivo* outcomes. Thus, we optimized a robust bone marrow-liver-thymus (BLTS)-huMouse (huMouse) model to elucidate the role of Nef and γδ T cells in chronic HIV infection. In doing so, we demonstrated that preventing Nef dimerization abrogates HIV viremia and associated immune dysregulation *in vivo*, suggesting that Nef dimerization may serve as an effective HIV therapeutic target. To our knowledge, we also demonstrate for the first time that human γδ T cell

subsets can be successfully reconstituted in a huMouse model, and we show that their *in vivo* dynamics during infection recapitulate those observations described in humans during natural HIV infection, with a particular loss in the Vδ2 subset occurring with wild-type but not Nef-defective HIV infection. Additionally, we found that replenishing the Vδ2 T cell numbers through adoptive transfer failed to control HIV infection, but instead exacerbated viremia, with the transferred cells serving as targets for HIV infection. Importantly, this small animal model provides an effective and useful platform for *in vivo* mechanistic studies to explore HIV pathogenesis and HIV therapeutic strategies.

Table of Contents

List of Tables

List of Figures

Preface

I would like to sincerely thank all of my committee members for their guidance and aid over the past 5 years during the completion of this dissertation work. Their time and consideration have contributed significantly to the shape and quality of my work. I would like to thank Dr. Robbie Mailliard, Dr. Moses Bility and Dr. Charles Rinaldo for the opportunity to finish my PhD in their laboratories, as well as Dr. Michael Lotze, and Tatiana Garcia Bates for their support and encouragement during that transition. I am grateful to Dr. Mailliard and to my committee for their expertise, advice, and constructive criticism, which have been instrumental in the maturation of my project and in my development as a scientist.

I would like to thank the participants of the Multicenter AIDS Cohort Study for their tireless contribution to this study and to the fight for an HIV cure. I express my utmost appreciation to Dr. Michael Lotze and Dr. Moses Bility, whose thought-provoking discussions, scientific and otherwise, have been invaluable to me personally and as a researcher. In addition, many thanks to Dr. Tatiana Garcia-Bates, Dr. Mariana Palma, and Jan Kristoff for their mentorship and for always making the time to help me solve any problem I encountered. I also thank Holly Bilben, Yash Agarwal, Priyanka Talukdar, Kathy Hurtle, Cole Betty and Sara Ho for their technical assistance and flexibility. This accomplishment was a group effort, and without all of you it would not have been possible.

1.0 Introduction

1.1 Human immunodeficiency virus (HIV-1)

1.1.1 History and global burden of HIV/AIDS

Human immunodeficiency virus (HIV) was discovered in the early 1980s. The disease was named acquired immunodeficiency syndrome (AIDS) by the Center for Disease Control and Prevention. In the early 1980s, two leading scientists Robert Gallo (USA) and Luc Montagnier (France) separately discovered that AIDS patients may have been infected by a new retrovirus [16]. Gallo's group isolated the virus from peripheral blood mononuclear cells (PBMC) of an AIDS patient. This virus was similar in structure to human T-lymphotropic viruses (HTLVs), which is why they initially named it HTLV-III [17]. However, Montagnier's group demonstrated that viral structural proteins p19 and p24 isolated from a lymphadenopathy patient were indeed antigenically different to those of HTLV and named it as lymphadenopathy-associated virus [16]. Initially each group gave the virus different name based on the symptoms observed in the infected patients or similarities to known viruses, however in 1986, a group of scientists suggested the name HIV-1 and this name was globally accepted.

HIV belongs to Retroviridae family (genus Lentiviridae) and is transmitted by blood transfusion, vertically (in utero, breast milk), and sexually via the vaginal tract or rectal mucosal tissues [18]. HIV predominantly infects cells expressing the CD4 receptor, these include CD4+ T cells, dendritic cells (DC), and macrophages. One of the hallmarks of HIV infection is dramatic depletion of CD4+ T lymphocytes leading to asymptomatic chronic phase and opportunistic

infections in those who are untreated, which ultimately leads to AIDS and death [19-23]. Despite several decades of research, the development of a successful prophylactic vaccine or cure for HIV infection remains elusive. The discovery of antiretroviral drugs in the 1990s and implementation of cART (combined anti-retroviral therapy) in the mid-1990s, has increased the life expectancy and quality of life of many HIV infected individuals. Despite successful suppression of virus by cART, HIV remains one of the most fatal infectious diseases worldwide. Currently over 37 million people living with HIV globally, approximately 1.8 million new infections occur each year, and one million people die due to HIV/AIDS annually [24].

Cross-species transmission of simian immunodeficiency viruses (SIV) from chimpanzees and gorillas in West Central Africa resulted in the establishment of HIV-1, the virus strain that initiated the HIV/AIDS epidemic in humans. Whereas multiple transmissions of SIVs in sooty mangabeys in West Africa resulted in HIV-2 [25]. Within these HIV types multiple groups of genetically different HIV have been identified and categorized. Groups A – H have been assigned for HIV-2 strains, and HIV-1 is divided into groups M, N, O, and P, with some of these groups being even further categorized into additional subtypes [25]. These groups vary in many ways including pathogenicity, infectivity, and geographical prevalence [26]. In North America, South America, Europe, and Australia, HIV-1 group M subtype B is the most common, whereas subtype C is the most common type identified in Africa and Asia [27]. Although HIV-1 and HIV-2 share similar mechanisms of infection and pathogenicity, HIV-1 is more infectious and causes more severe and rapid disease progression compared to HIV-2 strains [28, 29].

1.1.2 HIV-1 genome

The HIV-1 genome is composed of 9kb single-stranded positive sense RNA. It encodes fifteen protein flanked on either side by the 5' and 3' long terminal repeats (LTR), which help in viral transcription and insertion of the HIV-1 proviral DNA into the infected host cell genome [30, 31]. Three fundamental genes of HIV-1 are Gag, Pol, and Env, which are translated into structural and enzymatic proteins. The Gag polyprotein is processed into single protein products such as the matrix protein (MA), capsid (CA or p24), and nucleocapsid (NC) [32]. Pol gene encodes enzymes such as viral protease (PR), reverse transcriptase (RT), and integrase (IN), which helps in processing and maturation of viral proteins, reverse transcription of single-stranded viral RNA into double-stranded cDNA and insertion of viral cDNA into the host cell genome respectively [33-35]. The Env gene expresses the gp160 polyprotein, which is cleaved by the cellular protease Furin to produce the transmembrane glycoprotein 41 (gp41) and the surface glycoprotein 120 (gp120), which binds with host cell receptors such as CD4 and CCR5 or CXCR4 and mediates viral binding and fusion with the host cell membrane [36]. HIV-1 also encodes regulatory proteins such as transactivator protein (Tat) and the regulator of virion expression protein (Rev). Tat helps in transcription of integrated viral DNA and enhance the viral replication within infected cells [37]. Rev hijacks the host nuclear export machinery to transport unspliced viral RNA out of the nucleus and into the cytoplasm [38]. HIV also encodes accessory proteins such as Vif, Vpr, Vpu, and Nef, which are not essential for viral replication but play and important role in viral pathogenesis, viral spread and immune escape [39]. Nef protein plays an important role in immune dysregulation and disease progression and is discussed in the next section.

1.1.3 HIV-1 Nef

HIV-1 nef gene encodes a 27 kDa myristoylated Nef non-enzymatic protein, which is one of the first proteins expressed during the viral life cycle. Nef plays a critical role in HIV pathogenesis by promoting viral replication and disease progression to AIDS by enabling immune escape of HIV-infected cells. Individuals infected with Nef-deleted or -defective HIV display slower progression to AIDS [40, 41], while Nef-deleted SIV has similarly been shown to impair SIV replication and progression to AIDS in rhesus macaques [42]. Moreover, expression of Nef alone in CD4+ T cells induce AIDS-like disease in a transgenic mouse model, which suggests that Nef has a direct role in disease progression [43].

1.1.4 Key functions of Nef

In 1994, it was demonstrated that deletion or disruption of Nef gene severely impairs the infectivity of HIV [44]. Subsequent studies demonstrated the Nef-mediated enhancement in viral replication and infectivity across various viral subtypes [45]. Later it was confirmed that enhancement of infectivity required the myristoylation of Nef, AP-2 association and clathrin-mediated endocytosis [46]. However, the underlying mechanism(s) was not still clear. In 2015, the host cell restriction factor SERINC5 was identified as a critical factor involved in Nef-mediated infectivity of HIV. SERINC5 is a multipass transmembrane protein expressed on the surface of HIV-1-infected cells that gets incorporated into the newly produced virions [47-49]. SERINC5 disrupts the fusion of viral particle and host cell membrane through and Env-dependent mechanism. However, Nef downregulates SERINC5 from the cell membrane through AP-2-

depedent pathway and thereby prevents its incorporation in the budding virion and aids in enhancing the infectivity [48].

Another critical function mediated by HIV Nef is the downregulation of MHC-1. MHC-1 is required for loading digested viral peptide antigens for presentation to the effector $CD8^+$ T cells needed for clearing the infected cells. By downregulating MHC-1 from infected cell surfaces, HIV escapes the immune recognition and thereby continues to replicate in the host cell. Two different models have been reported for Nef-mediated MHC-1 downregulation. In the first model, Nef is recruited by the phosphofurin acidic cluster 2 (PACS-2) adaptor protein to the trans-Golgi network, where it activates the Src-family kinases such as Hck in macrophages or Lyn in T cells. Subsequent downstream signaling cascade by the Src-family kinases leads to increase in levels of membrane phosphatidylinositol (3,4,5)-trisphosphate via phosphoinositide 3-kinase, which causes activation of the small GTPases Arf1 and Arf6 and endocytosis of cell-surface MHC-I [50, 51]. MHC-1 is further prevented from recycling back to the plasma membrane. In the second model, Nef associates with endocytic adaptor protein complex 1 (AP-1) and Arf1 and traps newly synthesized MHC-I within Trans Golgi Network, thereby prevents anterograde trafficking to the plasma membrane [52]. Proposed sequences of these two models suggest that the first model occurs earlier during the infection cycle [50, 53].

Nef also downregulate CD4 because as a means to protect the cell from cytotoxic superinfection. Moreover, it has been shown that interactions between CD4 and Env triggers antibody-dependent cell-mediated cytotoxicity [50, 54]. Nef engages the S2 subunit of endocytic adaptor protein complex 2 (AP-2) and cytosolic tail of CD4 and initiates clathrin-mediated endocytosis of CD4 molecule of HIV infected cells [55, 56]. CD4 downregulation is also induced

by the HIV-1 accessory protein Vpu, which is expressed later in the viral infection and downregulates CD4 through a ubiquitination-dependent process [57].

Nef interacts with range of host cell proteins such as Src-family kinases to modulate the host immune system. Nef preferentially binds to Src-family kinase-Hck and Lyn via SH3 domain, [58, 59], which displaces the SH-3 domain from its regulatory position resulting in constitutive activation of these kinases [60, 61]. Studies have demonstrated that knockdown of Hck or dominant negative Hck mutant reduces the HIV-1 transcription and viral replication in macrophages [62, 63]. Nef also induces intracellular relocalization of Lck, another Src-family kinase that plays an important role in T cell activation [64]. However, it is not clear if Nef binds directly or indirectly to Lck for mediating immune dysregulation.

1.2 HIV-1 replication cycle

HIV-1 begins its infection cycle by binding glycosylated envelope protein (gp120 + gp 41) to CD4 molecule on the host T cells, monocytes, macrophages, and dendritic cells [65, 66]. Interaction of gp120 and CD4 results in change in configuration of variable loops of gp120 and that allows binding to chemokine receptors C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) [67, 68]. This conformational change exposes gp41, which facilitates fusion of the virus and host cell membrane and thereby allows entry of the virus into the cells [69]. Upon entry, the viral core is destabilized, and uncoating of capsid proteins and reverse transcription of HIV genome begins simultaneously [70, 71]. Reverse transcriptase converts the viral single-stranded RNA genome into a double-stranded DNA genome using host cell deoxynucleoside triphosphates (dNTPs) [72, 73]. Reverse-transcribed viral genetic material is

detected within 4 hours of infection, and maximum double stranded viral DNA is detected by 12 hours post infection [74]. Next, newly synthesized viral DNA assembles with the capsid proteins, integrase, and host cell nuclear import factors to form the pre-integration complex (PIC) that crosses the nuclear membrane [75, 76]. Next, integrase performs 3' endonuclease processing of the viral DNA and nicks the host cell DNA and ligates with the with host DNA [77]. Host DNA repair enzymes repair the gaps and stabilize the integration of proviral DNA into host genome for further replication by host cell machinery [78]. Next, host cell transcription factors bind to the 5' long terminal repeat of HIV-1 to begin transcription of the proviral genome [79]. Tat and Rev are produced early in the replication cycle. Tat protein binds with transactivation-response (TAR) region and recruit transcription elongation factor to enhance the transcription of HIV-1 RNA products [80]. Rev binds to the Rev response element in Env coding region of unspliced viral RNA and helps in the nuclear export of unspliced products such as gag, gag-pol, env by interacting with nuclear pore proteins [81, 82]. After translation of viral mRNA, matrix protein, capsid, nucleocapsid, protease, RT, integrase and envelope proteins assemble with the viral RNA, and then the assembled viral particles are transported to the cell membrane [83, 84]. Next, viral structural proteins multimerize in lipid rafts of host cell membrane, and the virions finally release from the cell by the host endosomal sorting complexes [85].

1.3 HIV-1 pathogenesis

$CD4^+$ T cells are the primary targets of HIV-1, which play an essential role in regulating the adaptive immune system by interacting with other immune cells and cytokine production. HIV-1 binds to receptor CD4 and co-receptor CCR5 and CXCR4 molecule expressed on various immune

cells. Although CD4+ T cells are the main targets of HIV-1, it can also productively infect monocytes/macrophages and dendritic cells by binding to CD4 and DC-SIGN [86-88]. HIV-1 transmission occurs through various routes such as sexual contact, blood transfusion, blood exposure, vertical transmission from mother to baby. It is observed that <1% of HIV-1 exposure events in the female or male genital tract led to infection [89], whereas 5-20% of transmission occurs through direct blood contact (needles or transfusion) [89]. Approximately three weeks post infection, plasma viremia reaches a peak and then dropped to a viral set-point as the cells of adaptive immune system responds to limit the virus replication [90]. The period when virus replication is steady and the individual is asymptomatic is called "clinical latency," which can last for years. Early signs of HIV-1 infection can be asymptomatic or manifest as a flu-like symptoms, rashes, and unexplained weight loss within 4 weeks of infection. However, the systemic depletion of $CD4_+$ T cells is a pathogenic hallmark of HIV-1 infection [91]. Moreover, immune homeostasis is disrupted as a result of cytotoxic HIV-1 production, pyroptosis/apoptosis of infected and uninfected cells, and killing of infected cells by CTLs and NK cells [91-93]. Disruption of gut epithelial barrier caused by HIV-1 infection results in microbial translocation [94] from gut to other body parts and into the circulation, which further perturbs immune function and contributes to pathogenicity [95]. HIV-1 infection is also associated with progressive neurocognitive decline and brain atrophy resulting from inflammation and damage to glial cells [96, 97]. Finally subsequent opportunistic infections due to a weakening immune system and viral reactivation ultimately leads to the development of AIDS [98].

1.4 HIV-1 and therapeutics

HIV-1 infection is dynamic, and it displays varying rate of disease progression in infected patients. Based on the rate of progression, people with untreated HIV infection can be divided into

three major groups: (1) rapid progressors (AIDS developed within 3 years of infection), (2) intermediate progressors, (AIDS developed within a span of 3-10 years after seroconversion), and (3) long-term non-progressors (LTNP), where normal $CD4^+$, $CD8^+$ T-cell and γδ T cell counts and function are maintained for decades in in the absence of anti-retroviral therapy [99]. LTNP comprises less than 5% of total HIV-1 infected individuals. With an advent of new viral load testing technology LTNP can be further classified in two sub-groups, with those having detectable plasma viremia <5000 HIV-RNA copies/ml defined as LTNP, and individuals with persistent viral copies below 50 copies/ml being termed elite controllers [100].

In rapid progressors during acute infection, robust viral replication results in peak plasma viremia mirrored by a dramatic decrease in blood $CD4^+$ T cell and Vδ2 T cell counts [101, 102]. With the advent of anti-retroviral drugs (ARD), viremia is reduced significantly in those infected undergoing therapy. Currently, there are more than 25 FDA approved ARDs, which target various viral components such as reverse transcriptase (RT), integrase, and protease to inhibit their function. The first class of ARD, such as Enfuvirtide and maraviroc, block viral fusion with host cell by interacting with gp41 and CCR5 respectively [103, 104]. The second class of ARD inhibit reverse transcriptase, of which there two types that nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). NRTIs are competitive inhibitors and they compete with natural dNTPs to be incorporated by RT but they lack 3' hydroxyl group necessary for DNA elongation and thereby terminate the viral DNA replication [105]. On the other hand, NNRTIs are allosteric inhibitors that bind directly to hydrophobic region of RT known as NNRTI-binding pocket and inhibit its function [106, 107]. A third class of ARD are integrase inhibitors, which bind directly to the enzyme and prevent viral DNA strand transfer to host genome [108]. The fourth class of ARD are the protease inhibitors, which bind to the catalytic

domain of protease to prevent its polyprotein cleavage activity necessary for viral maturation and infectivity [109]. All of these ARDs can be successfully used in various combinations as an anti-retroviral therapy (ART) for people living with HIV. Early ART suppresses viremia, reduces transmission and mortality [110, 111], and also prevents immune cell depletion and dysregulation. However, there is a subset of T cells known as γδ T cells, whose frequency and functions are severely altered in HIV infection, and they fail to recover to normal levels even after years of ART. In the following section impact of HIV on γδ T cells is discussed in detail.

1.5 γδ T cells

The Unknown Unknowns: Recovering Gamma-Delta T Cells for Control of Human Immunodeficiency Virus (HIV).

Shivkumar Biradar , Michael T Lotze , Robbie B Mailliard

Viruses. 2020; 12(12):1455.

Although alpha beta T cells have been studied intensively as a current 'known' with the basis of their recognition of processed peptides in the context of MHC molecules understood, far less attention has been paid to gamma delta T cells, in part because of their relative limited numbers in the blood and spleen but in particular because they reside primarily in tissues which are less readily accessible for study. They clearly play a role in infectious diseases, the second leading cause of death worldwide. The development of vaccines or treatments targeting viral pathogens with a high mutation rate has been ineffective because of their immune evasion properties. Conversely, focusing on host factors that regulate disease progression could be very useful. Hence,

utilizing underappreciated host immune cells such as gamma-delta (γδ) T cells could enable developing therapies, surmounting the difficulties associated with escape from αβ T cells. They recognize relatively conserved molecules on the pathogen, recognize stress ligands expressed on infected cells, and can mount a rapid and effective immune response, including mobilizing other immune effectors such as myeloid cells. Unconventional T cell, including those bearing γδ TCR, make up a small proportion of T cells as compared to conventional αβ T cells in humans. They are widely distributed throughout the body [112] and recognize a variety of self and non-self-antigens in an MHC independent fashion, producing cytokines and inducing cytotoxicity in target cells [113]. Their frequency and function are severely altered in the setting of several diseases, including HIV.

1.6 Development of γδ T cells

γδ T cells are important components of both innate and adaptive immunity. Mechanisms underlying their expansion and regulation following development and selection in the thymus remain largely unknown. Both αβ and γδ T cells originate during fetal development from a common CD4- CD8- (double negative; DN) progenitor cell in the thymus [114]. Based on their stage of development and expression of CD44 and CD25, DN cells can be further divided into four subsets: DN1 (CD44+ CD25-), DN2 (CD44+ CD25+), DN3 (CD44- CD25+), and DN4 (CD44- CD25-) [115]. At the DN2 stage TCR δ, TCR γ, and TCR β loci undergo rearrangement [116] and at DN3 stage cells those undergoing productive β selection rearrange with a pre-TCR α chain to mature into double-positive cells (CD4+ and CD8+), giving rise to conventional αβ T cells following secondary rearrangement of the TCR α chain. γδ TCR rearrangement precedes β

selection. β selection does not operate in γδ T-cell development [117]. Since TCR α and TCR δ are interleaved within the same locus, their expression is mutually exclusive [118]. Hence, at the stage of β selection, DN thymocytes must express either pre-TCR α or γδ TCR to mature into a distinct T-cell lineage. Furthermore, the downregulation of expression of CD73 and CD24 on DN T cells determines the final fate of the γδ lineage [119]. TCR signaling is stronger downstream of the γδ TCR than the pre-TCR, driving γδ TCR progenitors into γδ T-cell lineage [120]. γδ T cell development from $CD34^{+}$ hematopoietic progenitor is depicted in figure 1. Unlike αβ T cells, which are dependent on the thymus for their development, γδ T cells can be developed in

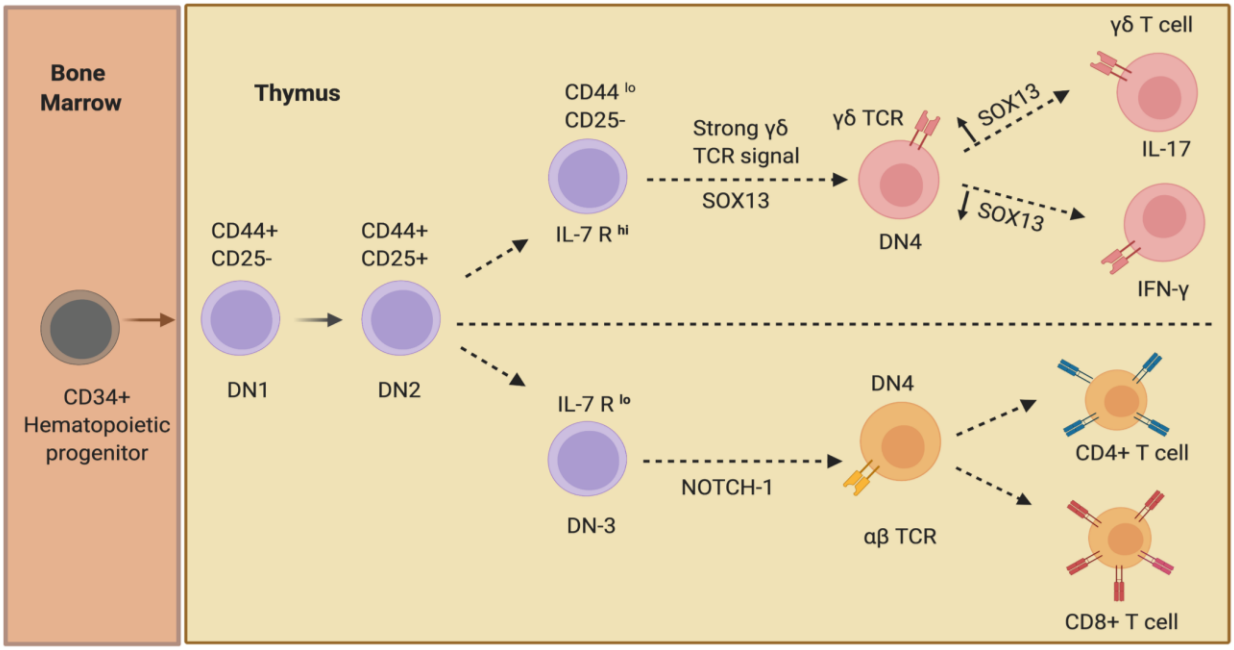

Figure 1. An overview of γδ T cell development.

The diagram illustrates the differentiation and development of mouse γδ T cells from thymic progenitor cells. First, (CD4neg and CD8neg) double-negative 1 (DN1) progenitor cells with CD44posCD25neg phenotype differentiate to the CD44posCD25pos DN2 stage. At the DN2 stage the β-, γ-and δ-chains of the T cell receptor (TCR) are rearranged. Expression of the γδ -TCR and strong γδ TCR downstream signaling drives cells into the γδ -T cell lineage, supported by the expression of SRY-box 13 (SOX13), an HMG family protein, binding to TCF7/TCF1. Rearrangement of γδ TCR precedes β selection, whereas DN3 cells which fail to produce functional γδ _TCR undergo β-selection and rearrange with pre-TCR α chain to mature into double positive cells (CD4+ and CD8+) and give rise to αβ T cells. Furthermore, in the presence of strong γδ TCR activating signals they differentiate into IFN-γ (SOX13 is downregulated) producing cells whereas in weaker γδ TCR activating signals, SOX13 expression is maintained, and cells differentiate into IL-17 producing γδ cells.

extrathymic sites such as the fetal liver and gut epithelium during the 6–8-week period of gestation [121]. Although there is a partial overlap of thymic and extrathymic repertoires early in development, they diverge and become non-overlapping during the second trimester [122]. In humans, there are only a few Vγ and Vδ germline genes (Vγ 2,3,4,5,8,9 and Vδ 1,2,3,4,6,7,8) among which rearrangement takes place [123]. It is believed that pairing of Vγ and Vδ genes is not random because in neonates and adults, a majority of Vδ2 variable genes rearrange with the Vγ9 gene and are pre-programmed to recognize phosphoantigens (pAgs) from birth, providing a level of innate immunity. Rearrangement of Vγ9 and δ2 variable (V) gene segments proceeds in the fetal liver as early as five weeks of gestation, and by 20-30 weeks of gestation they dominate the γδ repertoire. Later, Vδ1+ T cells increase within the cord blood and thymus [124, 125]. Postnatal exposure to microbes causes a dramatic increase in Vγ9Vδ2 T cells, which become the predominant γδ T-cell population in the peripheral blood [126]. Interestingly, the Vδ2 chain can also rearrange with other γ chains besides Vγ9 and circulate in the blood as naïve T cells and differentiate into effector T cells upon antigen specific encounter[127]. Although the distinct antigens recognized by the Vγ9negVδ2 T cells have yet to be characterized, they do respond to non-pAgs such as CMV and contribute to adaptive immunity [128, 129].

1.7 Structural and functional subsets of γδ T cells

Based on TCR δ chain usage, human γδ T cells can be categorized into three major subsets: Vδ1, Vδ2, and Vδ3 T cells. Vδ1 T cells constitute less than 30% of total γδ T cells in the peripheral blood, but they are the predominant population in tissues, including the dermis, spleen, liver, gut

epithelia, lung, and other mucosal sites [130]. Vδ1 T cells maintain epithelial tissue integrity, and function through recognition of the stress-inducible ligands MICA and MICB expressed by transformed and virus-infected cells, and self-glycolipids presented by CD1c/d molecules [131]. Vγ9Vδ2 T cells circulating in the peripheral blood and lymphatics recognize phosphoantigens produced by various microbes and transformed host cells in an MHC non-dependent fashion [113]. The third subtype of γδ T-cells, the Vδ3 T cells, comprise approximately 0.6% of peripheral blood T lymphocytes and are also found in the liver and gut [132, 133]. Although there are no known specific antigens identified for Vδ3 T cells, they can function through cognate interactions with HLA-A2 and CD1d [134], another β2 macroglobulin containing MHC-like molecule, primarily presenting lipid moieties. Vδ3 T cells are expanded in peripheral blood of renal and stem cell transplant recipients with cytomegalovirus reactivation [135], and B-cell chronic lymphocytic leukemia [136]. Similar to Vδ2 T cells, Vδ3 T cells can also act as a bridge linking innate and adaptive immunity through their capacity to modulate B-cell and dendritic cell (DC) maturation [137, 138]. Furthermore, Vδ4, Vδ6, Vδ7, and Vδ8 T cells have been detected in the peripheral blood of lymphoma patients, but their chain pairing and activation remains unclear [139], serving as distinct minorities of γδ T cells.

γδ T cells can also be categorized into different subgroups based on their functional characteristics, which include the Th1-like IFN-γ producing subset, the IL-17 producing cells, and those which function as regulatory γδ T cells. Human peripheral blood γδ T cells activated with IPP can be differentiated into Th1-like cells by culturing them in the presence of recombinant IL-12 and anti-IL-4 blocking antibody [140]. Whereas culturing γδ T cells in the presence of recombinant IL-4 and anti-IL-12 blocking antibody differentiates them into Th2-like cells, mediating immune responses that are particularly effective against extracellular pathogens [140].

1.8 Ligand recognition by γδ T cells

Although γδ TCRs are less diverse than αβ TCRs, they recognize a plethora of molecules such as non-peptidic metabolites of isoprenoid biosynthesis, stress molecules (MICA and MICB), heat-shock proteins, etc. [141]. Recognition of these molecules allows them to respond to many microbial components as well as transformed and infected host cells by inducing direct cytotoxicity and rapid secretion of inflammatory chemokines and cytokines [142]. Each of these ligands recognized by γδ T cells is explored in detail below.

1.8.1 Phosphoantigens

The breadth and variety of antigens γδ T cells are specifically capable of recognizing have yet to be completely defined. However, their capacity to recognize non-peptidic antigens has been studied in great depth. The predominant circulating Vγ9δ2 T cells, which contribute to 90-95% of the total γδ T cells in human peripheral blood, recognize phosphorylated isoprenoid precursors, collectively called phosphoantigens (pAg) [143]. The most studied pAg in prokaryotes is HMBPP, an intermediate of the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway, and in eukaryotes isopentenyl pyrophosphate (IPP), intermediate of the mevalonate pathway [143]. Besides microbial activation of γδ T cells, pAg that accumulates in transformed cells can also trigger γδ T cell activation [144]. In contrast to αβ T cells, which recognize peptide antigens presented on MHC molecule, γδ T cells recognize pAg in the context of members of the immunoglobulin superfamily members butyrophilin 2A1 and 3A1 (BTN2A1 and BTN3A1). Thus, butyrophilin mediated interaction activates the signaling of the γ9δ2 TCR. Specifically, BTN2A1 binds the Vγ9+ domain of the TCR, whereas BTN3A1, binds the Vδ2 and γ-chain regions on the opposite side of the TCR

to mediate signaling [145]. This new understanding of BTN and the γδ TCR signaling pathway may facilitate development of future γδ T-cell–based immunotherapies. Interestingly, the pAg is thought to bind within the cytosolic portion of the butyrophilins, promoting allosteric changes [146].

1.8.2 MHC-like ligands

γδ T cells recognize cellular stress proteins and pathogen-associated molecules **(Fig. 2)** through their expression of several common natural killer (NK) cell receptors, including NKp30, NKp44,

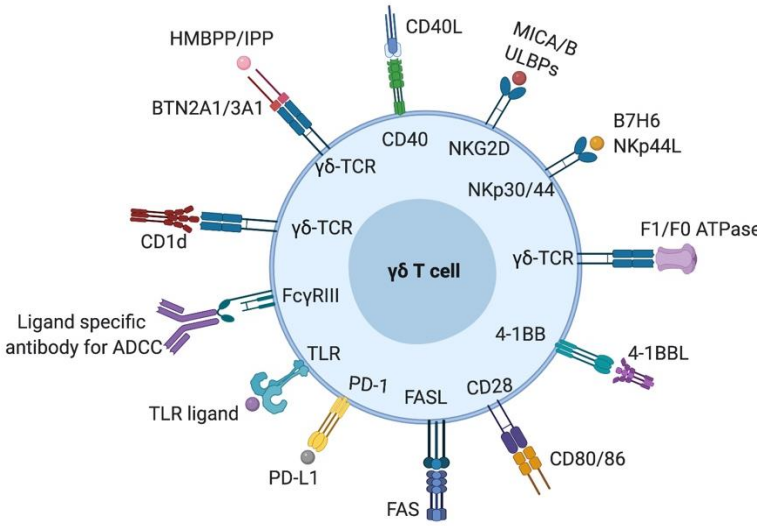

Figure 2. Ligand recognition by gamma delta (γδ) T cells.

γδ T cells provide a wide range of immunologic functions, recognizing a diverse array of self- and non-self-ligands through their expression of various signaling receptors (depicted in the figure). Abbreviations: HMBPP: (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate, IPP: Isopentenyl pyrophosphate, BTN2A1: Butyrophilin 2A1, ADCC: Antibody-dependent cell-mediated cytotoxicity, TLR: Toll-like receptor, PD1: Programmed cell death protein 1, MICA/B: MHC class I chain-related protein A/B, ULBP- UL16 binding protein, NKG2D: Natural killer group 2D.

NKp46, and the C-type lectin-like receptors NKG2D, NKG2A, and NKG2C. Specifically, Vδ1 T cells recognize the MHC-like molecules CD1a, b, c, and d that present lipid Ags (glycolipids and

certain microbial lipids) and are primarily expressed on professional Ag presenting cells [147]. Tissue resident Vδ1 T cells demonstrate reactivity to these ligands by producing several effector proteins, including IFN-γ and granulysin. γδ T cells also maintain epithelial tissue integrity by recognizing stress-induced MHC class I-related molecules MICA and MICB in an MHC independent fashion [148].

1.8.3 Other cell surface and soluble proteins

Besides phosphoantigens, γδ T cells also recognize the mitochondrial F1/F0-ATPase-related structure expressed on the Burkitt's lymphoma cell line Daudi [149]. Several bacterial proteins can elicit γδ T cells responses, including staphylococcal enterotoxin A [150], toxin listeriosis O [151], tetanus toxoid from Clostridium tetani [152], and ESAT-6, the highly virulent and immunogenic protein of Mycobacterium tuberculosis (Mtb) [153].

γδ T cells can also express co-stimulatory molecules such as tumor necrosis factor (TNF) receptor family molecules including CD27, CD30, CD137, and inhibitory factors such as CD279 (PD-1), regulate innate and adaptive immune responses [154, 155]. Moreover, Vδ2 T cells express CD16 (Fcγ receptor), which facilitates their antibody-mediated cellular cytotoxicity (ADCC) activity [156, 157].

1.9 γδ T cell interaction with other immune cells

In addition to γδ T cells ability to recognize microbial and cellular stress ligands to carry out their direct effector functions, these cells play an integral role in the immune system in part

through their extensive crosstalk and intercellular communication activities with other immune cells **(Fig. 3)**.

1.9.1 γδ and B cells

The ability of γδ T cells to produce a wide range of cytokines and chemokines aids in their interaction with a wide variety of immune cells. One such chemokine produced by activated γδ T cells is the CXC-chemokine ligand 13 (CXCL13), which facilitates B-cell arrangement in the germinal centers of lymphoid tissue [158]. While αβ T cells are important for germinal center formation and B cell production of immunoglobulins (Ig's), mice deficient in TCRα efficiently develop normal lymph node germinal centers and maintain the capacity to produce immunoglobulins (Ig's) [159]. This study suggests that in the absence of αβ T cells, γδ T cells may play a role in providing help to support B cell function [159]. In vitro studies have demonstrated that γδ T-cells in the presence of IL-4 can induce B-cell activation, Ig isotype switching, and secretion of IgE [160]. Co-culturing Vδ3 T cells and B cells results in B-cell maturation, characterized by upregulation of CD40, CD86, and HLA-DR surface expression on both cell types [137]. This suggests that similar to Vδ2 T cells, Vδ3 T cells can induce B cell maturation and differentiation into functional APC capable of activating conventional T cells [161]. Thus, γδ T cells play a critical role in promoting B-cell maturation antigen presentation, and antibody production, thus contributing to humoral immunity [162].

1.9.2 γδ and dendritic cells

γδ T cells interact with DC to induce maturation in vitro [163], characterized by the upregulation of MHC molecule and co-stimulatory molecules such as HLA-DR, CD86, and CD83 on DCs. DC maturation is contact independent and predominantly driven by TNF-α secreted from activated γδ T cells. Phosphoantigen mediated activation of γδ T cells induces IL-12p70 production by DC, which in turn is critical for driving the differentiation of naïve αβ T cells into IFN-γ producing effector cells [163, 164]. Besides Vδ2 T cells, Vδ1 T cells can also induce DC maturation. Tissue resident Vδ1 T cells, interacting with CD1a, b, and c molecules expressed on immature DCs, promotes DC maturation [165]. Early during microbial infection, when there is no apparent microbe specific CD8 T cells, these Vδ1 T cells can induce maturation of DCs and enhance their ability to present antigen to naïve CD4 T cells. γδ T cells produce a large quantity of IFN- γ early during TB infection, which help DCs prime CD8 T cells, generating protection against TB infection [166]. Recent studies suggest that human Vδ3 T cells are also capable of inducing maturation of DC into cytokine-producing APC [161]. Thus, γδ T cells are a bridge between innate and adaptive immunity, and their unique properties can be potentially used for immunotherapeutic purposes.

1.9.3 γδ and monocyte/macrophages

Monocytes and γδ T cells, are rapidly recruited to the sites of infection or inflammation, and they affect each other's ability to effectively eliminate infected cells [167] [168]. Microbe activated Vγ9Vδ2 T cells can differentiate monocytes into inflammatory DCs, which in turn display effector function such as antigen presentation and inflammatory cytokine production [167].

Increased cytotoxic activity of γδ T cells is noted when they are incubated with M. tuberculosis (Mtb)-infected monocytes [168]. Besides monocytes, macrophages and γδ T cells also regulate each other's function. Macrophages infected with Mtb release chemokines to efficiently recruit γδ T cells and regulate their function [169]. PAg-expanded γδ T cells kill human or avian influenza virus-infected macrophages, promoting viral clearance [170]. The terminal effector memory cell following malaria infection is an M-CSF producing γδ T cell [171]. Thus, γδ T cells play a crucial role in regulating macrophage function to promote control and elimination of microbes.

1.9.4 γδ and NK cells

NK cells crosstalk with γδ T cells to modulate and regulate the adaptive immune response [172-174]. Provision of Zoledronic acid (ZA) activates γδ T cells that in turn interact with NK cells via CD137, resulting in the upregulation of the cytotoxic receptor NKG2D on NK cells, enhancing their capacity to recognize and kill tumors that are usually resistant to NK cytolysis [175]. In Listeria monocytogenes-infected mice, γδ T cells produce IFN-γ early during the infection, aiding in the NK cell's capacity to mount effective innate immune responses against the intracellular pathogen [176]. Thus, it is now accepted that γδ T cells play an essential role in regulating NK cell-mediated immunity and that the absence of or dysfunction of γδ T cells may impair NK cell activation and function [177].

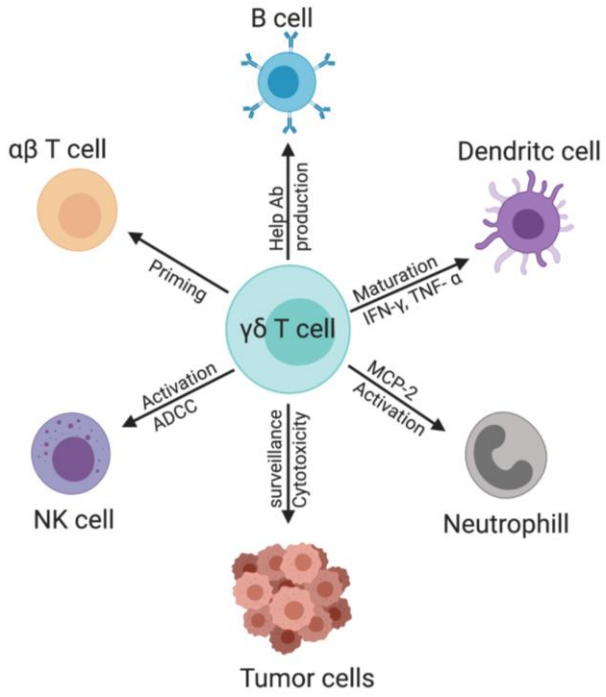

Figure 3. Crosstalk of γδ T cells with innate and adaptive immune cells.

DC: Activated γδ T cells secrete IFN-γ and TNF-α, which promotes upregulation of CD86 and MHC class I molecules on DC and provide help to increase IL-12 secretion by DC. B cell: γδ T cells secretes IL-4, IL-10 and CXCL13, and provides help to B cell for antibody production. Neutrophil: γδ T cells secretes IL-17A and CXCL8, which recruit neutrophils to the site of inflammation. NK cell: γδ T cells provide costimulatory signal (CD137-CD137L interaction) to NK cells and promotes upregulation of activation markers on NK cells which facilitates antitumor activity of NK cells. αβ T cell: activated γδ T cells can process soluble antigens and present them in the context of both MHC class I and Class II to naïve CD8+ and CD4+ αβ T cells respectively, to drive their maturation, proliferation, and differentiation. Tumor cell: γδ T cells can provide immunosurveillance and induce cytotoxicity in tumor cells.

1.10 γδ T cells as antigen presenting cells

Besides producing cytokines and having cytotoxic potential, human γδ T cells can also function as professional antigen-presenting cells [178]. While Vγ9Vδ2 T cells predominantly circulate in peripheral blood, following their activation, they can express MHC class I and class II molecules, the co-stimulatory molecules CD80 and CD86, and the lymph node homing CC-chemokine receptor 7 (CCR7) [178]. Furthermore, activated human γδ T cells can take up and process soluble antigens [179] [180], and present them in the context of both MHC class I and

Class II to naïve CD8+ and CD4+ αβ T cells respectively, to drive their maturation, proliferation, and differentiation [178]. Although precise mechanisms of antigen uptake is not very well studied, Seino et al have demonstrated that activated γδ T cells phagocytose apoptotic or live cancer cells and tumor antigens possibly through the scavenger receptor CD36 in a C/EBPα (CCAAT/enhancer-binding protein α)-dependent mechanism and mount a tumor antigen-specific CD8+ T-cell response [181]. This fact highlights their therapeutic potential to initiate antigen-specific adaptive responses against various pathogens.

1.11 Impact of HIV on γδ T cells

1.11.1 HIV-specific destruction of phosphoantigen responsive γδ2 T cells

In healthy individuals, Vγ9Vδ2 T cells contribute to 90-95% of the total γδ population in the peripheral blood, and the remaining 5-10% are Vδ2neg such as Vδ1 and Vδ3s [182]. In HIV-infected individuals, Vγ9Vδ2 T cells are drastically depleted and Vδ2: Vδ1 ratio is inverted in peripheral blood [182]. Interestingly Vδ2 cell depletion occurs very early during the infection and correlates with viral load and CD4 depletion [183]. HIV preferentially depletes phosphoantigen responsive Vδ2 cells with Vγ9-Jγ1.2 TCR rearrangement [184]. Although the precise mechanism of depletion of Vδ2 cells in HIV-infected individual is not understood, some studies indicate that α4β7 and CCR5 receptors form a complex on Vδ2 cell which facilitate the V3 loop of HIV gp120 to bind to CCR5 in the absence of expression of the CD4 co-receptor. This interaction leads to p38 kinase activation and induces apoptosis in these cells [185]. Also, HIV infection leads to loss of Th17 CD4 cells, which regulate epithelial barrier integrity in the gut [186]. Depletion results in

dysregulation of mucosal immunity and allows microbial translocation into the circulation, resulting in systemic immune activation [94] and expansion of Vδ1 cell in viremic patients [187]. Moreover, the Vδ2 subset from HIV-infected individuals fail to proliferate or produce cytokine in response to mycobacterial infection in vitro, suggesting that Vδ2 cells are functionally inactive [4]. This functional anergy in residual Vδ2 cells mediates their less responsive phenotype to phosphoantigens as well as recognition and lysis of Daudi cells [188].

1.11.2 Impact of γδ T-cell perturbations on immune control of HIV

Much of the impact of γδ T cells on immune function in health and disease is mediated through their bi-directional cross-talk with other immune cells [177]. In healthy individuals activated γδ T cells induce the maturation and differentiation of DCs and B cells into functional APC, but in HIV-infected individuals, this immunomodulatory capacity of γδ T cells is compromised [189]. Typically, upon activation Vγ9Vδ2 T cells can drive the upregulation of CD80, CD86, HLA-DR, and CD40 surface expression on APCs, enhancing their capacity to induce primary αβ T-cell responses [178]. HIV infection alters the ability of APC to process and present antigen, inhibiting the ability of Vγ9Vδ2 T cells to effectively interact with and positively impact the phenotype and function of APC [189].

Activated γδ T cells can also act as APC. Impairment of γδ T cells in HIV infection may impact their ability to present antigen and induce a primary response in αβ T-cell [190]. Activated γδ T cells produce large quantities of chemokines including macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, and CCL5/RANTES which bind to CCR5, a coreceptor for HIV, blocking HIV infection of target cells. Again, alteration of γδ T cells in HIV infection may lead to reduced protection against new infection of target cells. Vδ2 T cells cultured in the presence of

HMBPP and IL-21 express B-cell-attracting chemokine CXCL13, and the CXCL13 receptor CXCR5. This promotes B-cell somatic mutation, productive rearrangement, and maturation in the germinal center thereby promoting antibody production by B cells [191-193]. Due to the loss of γδ T cells in HIV infected individuals, it is likely that these crucial helper functions will also be compromised.

Numerous studies demonstrate the protective role of γδ T cells in controlling HIV replication and inducing cytotoxicity in HIV infected cells [170, 194, 195]. However, γδ T cells can also contribute to negative outcomes in HIV infection. γδ T cells express high levels of the inhibitory receptor TIGIT in aviremic HIV-infected individuals. They are the primary inflammatory driver in ART-suppressed HIV infection and contribute to age-associated morbidity and mortality [196]. Alveolar immune cell homeostasis is disrupted in HIV-infected adults by increasing infiltration of γδ T cells along with other immune cells in broncho-alveolar lavage fluid, enhancing the susceptibility of these individuals to lower respiratory tract infections [197].

1.12 Impact of ART on γδ T cells in HIV infection

Although ART is very effective in restoring CD4 counts and suppressing the virus below detectable levels, it fails to fully restore standard frequencies of Vδ2 T cells in HIV-infected individuals [182]. Long term ART treatment partially restored the Jγ1.2 repertoire of the Vδ2 subset [184]. These Vδ2 cells are highly activated [198] but are functionally compromised, including diminished cytokine production, cytotoxicity, and proliferation [199]. At the same time, expanded Vδ1 levels remain stable and produce proinflammatory cytokine during ART treatment in peripheral blood and mucosal sites and express the exhaustion molecule, CD279 (PD-1) [200].

Table 1. HIV immunotherapy studies using Vδ2 T cells

Treatment	Participant status	Result	Reference
Adoptive transfer of zol + IL-2 expanded PBMCs	HIV infected huMice	No impact of Vδ2 T cell on CD4+ depletion or plasma viremia	[201]
HMBPP + IL-2 injected in Macaque	SHIV- infected Macaque	Expansion and activation of Vδ2 T cell. Increase in Env specific antibody but no impact on viral load during chronic infection.	[202]
Pamidronate + IL-2 expanded PBMCs	Human HIV+ ART	Inhibition of HIV replication in vitro. PAM expanded Vδ2 T cell suppress p24 production by CD4+ T cells in the presence of vorinostat.	[203]
Ex vivo Vδ2 T cells	Human HIV+ ART	CD16 activation on Vδ2 T cells from HIV-ART treated individuals induced ADCC in target cells in vitro.	[204]
Ex vivo IPP + IL-18	Human HIV+ ART	IL-18 stimulation improves IPP induced Vδ2 activation, proliferation and degranulation in HIV infected individuals	[205]

Whether ART has an impact on reducing the Vδ1 number, and activation is not clearly understood. Studies suggest that loss of Vδ2 cells in viral controllers is significantly lower than untreated or ART-treated individuals, and γδ17 cells are highly preserved. This preserved γδ population may be responsible for preventing the microbial translocation and controlling the chronic systemic immune activation [6, 206].

1.13 HIV reservoirs

Even though ART suppresses HIV replication significantly, the viral genome remains transcriptionally silent in host chromatin, representing a major challenge towards efforts to

eradicate infection [207]. To eradicate the virus, it is essential to identify and understand various host cells that can harbor the integrated HIV genome for prolonged periods. Resting memory CD4+ T lymphocytes are considered the major reservoir site for HIV [208]. Recent studies demonstrate that other cell types can also harbor latent HIV including γδ T cells [209]. Replication-competent HIV can be obtained from highly purified Vδ2 T cells of ART treated HIV patients. Interestingly, this study has identified a previously unrecognized Vδ2 reservoir in which latent HIV infection is unexpectedly frequent [210]. Although precise mechanism is not known, γδ T cells could be infected by the CXCR4-tropic laboratory clone HIVLAI. [188]. Possible mechanisms of infection includes binding of HIV envelope glycoprotein to CCR5/α4β7 receptor on Vδ2 T cells and establishes infection in the absence of expression of the CD4 receptor, the natural receptor for HIV infection [185]. HIV induced immune activation causes CD4 receptor upregulation on γδ T cells, making them susceptible to HIV infection [210].

B-cell follicles are the other major source of latent HIV reservoirs. Specifically, in B-cell follicles, T-follicular helper and T-follicular regulatory cells are significantly more permissive to HIV infection and persistence. Furthermore, extracellular HIV virions accumulated on the surface of follicular dendritic cells in germinal centers and thus are also a major source of virus. Although activated γδ T cells with cytotoxic properties can traffic to secondary lymphoid organs, their role in clearing HIV reservoirs in B-cell follicles is not clearly understood [211]. There are contrasting views about the integration of HIV DNA in monocytes and macrophages. A few studies suggest that macrophages are even more susceptible to HIV infection [212, 213]. Detection of HIV DNA and HIV p24 in CD68+ macrophages present in duodenal tissue from gut biopsies of virologically suppressed HIV+ individuals can be found [212]. Similarly, replication-competent HIV in urethral macrophages from virologically suppressed donors [213] is also observed. Active replication of

HIV within CD68+ macrophages in brain tissue of ART treated patients may contribute to the development of HIV associated neurocognitive disorders [214, 215].

1.14 Use of γδ T cell for immunotherapy in HIV

γδ T cells are the first line of defense against many pathogens, but their number and functions are severely altered in many infectious diseases, including HIV. Despite the long-term ART treatment, γδ T cells are not reconstituted to the original frequency in HIV-infected individuals. However, in viral suppressors, Vγ9Vδ2 T cell number is maintained at normal levels, implying that immunotherapy using Vγ9Vδ2 T cells might recreate the immune function as viral suppressors and control HIV infection [6]. Several efforts were made to develop suitable methods for expanding γδ T cells in vitro and *in vivo*. In vitro method involves stimulating PBMCs with bisphosphonates such as Isopentenyl pyrophosphate (IPP), HMBPP and FDA approved drug zoledronic acid (ZA) [216]. ZA blocks the metabolic conversion of isopentenyl pyrophosphate (IPP), allowing this phosphoantigen to accumulate until stimulatory levels are reached, resulting in Vγ9Vδ2 T cell activation and expansion [216]. Bisphosphonate mediated expansion of γδ T cell is a rapid means to generate large quantities of γδ T cells for adoptive cell therapy. On the other hand, delta one T cells (DOT) can be expanded using OKT-3 monoclonal antibody and cytokine cocktail (rIL4, rIFNγ, rIL21 and rIL1β) [217]. Siegers et al achieved high yield of DOT cells by treating PBMCs with Con-A and recombinant human IL-2 and IL-4 [218]. Alternative strategies for expanding γδ T cells that do not respond to pAgs or N-BP involves use of monoclonal antibodies (mAb). Using γδ TCR specific antibodies low level of expansion of Vδ1 and Vδ2 T cells has been achieved but it's not very successful to date. However, 20.1 an agonistic Ab specific

for CD277 (a member of BTN3 subfamily), mimics pAg-induced Vγ9Vδ2 cell activation. This antibody may simulate a conformational change in the CD277 molecule to activate and expand Vγ9Vδ2 T cells [219].

In vivo approach includes ZA and IL-2 combination treatment to induce Vγ9Vδ2 T-cell expansion and maturation [220]. Expanded Vγ9Vδ2 T cell belong mainly to the central memory and effector memory subgroups. Increased DC maturation and HIV-specific CD8 T-cell responses were observed in ZA treated patients [220]. Very limited adverse events of ZA are found, highlighting the safety of the ZA treatment. Moreover, the expansion of Vγ9Vδ2 T cells in HIV-infected patients could also improve tumor immunity and enable better control of opportunistic pathogens. However, impact of ZA treatment on viral RNA and CD4 levels is lacking in this study thus it needs to be addressed in future clinical trials [220].

Specifically in case of HIV infected individuals, phosphoantigen responsive Vγ9Vδ2 T cells can be recovered by IL-18 supplementation, promoting inflammasome formation and triggering more robust activation and expansion of Vγ9Vδ2 cells in vitro [205]. Differences in the capacity of γδ T cell to recover and expand from early vs. late ART treated individuals might be an obstacle for immunotherapy. On the other hand, γδ T cells from healthy individual can be grown efficiently, which could be used in the setting of allogeneic transfer in HIV-infected individuals, hopefully without significant toxicity to the host. Transplantation of γδ-T cells from an allogenic donor promotes engraftment and are incapable of mediating GVHD since they recognize antigen in an MHC independent fashion [221]. Adoptive transfer of ex vivo expanded γδ T cells in HIV infected individuals is a promising strategy to increase host control over HIV disease **(Table 1).** An immune reconstruction syndrome [222], associated with immune reconstitution and production of proinflammatory cytokines by activated γδ T cells could produce undesirable toxicity. Some

studies reported relatively minor adverse effects, including fever, injection site soreness, nausea, and diarrhea for bisphosphonate/IL-2 therapy for cancer [223]. Other cancer studies reported decreased responses during extended bisphosphonate/IL-2 therapy dosing schedules due to the development of anergy [223]. Since HIV is a chronic disease, repeated administration of bisphosphonate/IL-2 therapy may be needed. The development of functional anergy in γδ T cells needs to be evaluated carefully. Treating γδ T cells with rapamycin (targeting both MTORC1 and MTORC2) reduces the anergy induced in γδ T cells and this could be an effective strategy for overcoming some of the potential problems in γδ T cell-based immunotherapy [224].

1.15 Animal models for hiv-1 infection

Moving beyond the mousetrap: current and emerging huMouse and rat models for investigating prevention and cure strategies against HIV infection and associated pathologies.

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Retrovirology 17, 8 (2020). https://doi.org/10.1186/s12977-020-00515-3.

Cell culture-based *in vitro* studies cannot accurately reflect the complexities of pathogen infection in a host. For example, a novel therapeutic compound/ cell therapy that targets a pathogen may be highly effective *in vitro* but host factors such as bioavailability, and tissue penetration/ tissue trafficking may affect clinical efficacy. Thus, robust *in vivo* models should be used to study such intricate interactions.

To truly understand host and pathogen interactions, it is ideal to study disease pathogenesis, and therapeutic strategies in the host species. However, HIV-1 is a human-specific pathogen, and

it does not infect any other species. Currently, the two most common animals used for HIV-1 modeling are macaques and huMice. Use of these models help in translating *in vitro* discoveries to *in vivo* studies and mitigates the high costs associated with long-term human trials that require hundreds to thousands of participants. Animal models allows researchers to manipulate experimental variables for hypothesis-driven investigation and provide *in vivo* proof-of-concept data that can guide clinical trial studies in human.

1.16 Non-human primate models

HIV-1 evolved from multiple cross-species transmission of simian immunodeficiency virus (SIV) from African non-human primates (NHP) to humans [225-228]. However, HIV-1 does not readily infect NHPs nor recapitulate AIDS disease progression [71]. Multiple NHP restriction factors such as Macaque variations of tripartite-motif-containing protein 5α, APOBEC3, tetherin, and SAM domain- and HD-domain-containing protein 1 inhibit HIV-1 replication in macaques [229-233]. Moreover, SIV infection does not progress to AIDS in NHP and their immune system abrogate the pathogenesis and control the chronic immune activation which is not observed in humans [234, 235]. Furthermore, SIV contains only about 50 percent of the genetic code of HIV and there are substantial differences in γδ subset composition and phenotype in monkeys and humans [236]. Alike humans, alteration in the peripheral Vδ1/Vδ2 ratio in SIV-infected macaques was not observed [237]. Hence, studying γδ T cells' role in SIV-infected animals provides limited information about whether these cells will respond in a similar fashion against the human immunodeficiency virus. Besides physiological limitations, macaques' studies can be limited by

availability and high costs associated with purchase and maintenance. Hence, an ideal model would be less expensive small rodent model with easy maintenance and permit the use of HIV-1.

1.17 HuMouse (huMouse) models

A second animal model for studying HIV-1 infection is the huMouse. HuMice are generated by engraftment of human cells and/or tissue into an immunodeficient mouse strain such as NSG for reconstitution of a human immune system in a small animal [238]. HuMice provide the platform to study the *in vivo* system in which actual HIV-1 and human cell response can be understood. There are multiple advantages of using mice over NHPs for studying HIV infection. Mice are readily available or can be bred quickly in lab, less expensive than NHPs and they require very less space and maintenance, and they are very easy to handle. Because of all these advantages larger cohorts of mice can be studied. HuMice can be created by various methods and each one of them has pros and cons which are described below **(Table 2)**.

1.18 Peripheral blood lymphocytes (PBL)- huMouse model

Human $CD4^+$ T cells are the major target for HIV infection; thus, a mouse model with human $CD4^+$ T cells provides a platform for modeling HIV/AIDS. Various immunodeficient mouse models lacking mature T, B, [239] and NK cells, along with defects in macrophage phagocytic function [240] support robust reconstitution of human CD4+ T cells and other lymphocytes (*e.g.* CD8+ T cells) following transplantation of human peripheral blood

mononuclear cells (PBMCs) or CD4+ T cells. Such cells can be transplanted via intravenous (IV) or intraperitoneal (IP) injection into myoablated, immunodeficient juvenile mice (6-8 weeks old), to generate peripheral blood lymphocyte (PBL)-huMice. Human CD4+ T cells are readily detectable in the blood at 4 weeks post-transplantation [240, 241], providing a huMouse model that can be generated in a relatively short period of time. The PBL-huMouse model supports HIV replication and provides a means of evaluating the efficacy of direct-acting therapeutics (*e.g.* antivirals drugs, antibodies) geared towards preventing HIV transmission [242] and controlling HIV replication [240]. Additionally, PBL-huMouse models constructed using PBMCs from HIV-infected individuals with undetectable viral load can be employed as an *in vivo* assay (mouse-quantitative Viral Outgrowth Assay, mouse-qVOA) for evaluating the eradication of the HIV reservoir in said individuals [243, 244]. A major limitation of this model is the rapid development of graft-versus-host (GvHD) disease within 6-7 weeks following transplantation of lymphocytes, thus significantly restricting the experimental window [245]. Additionally, the PBL-huMouse model does not incorporate human macrophages, which are a major HIV reservoir in various organs, including the brain [240].

1.18.1 Hematopoietic stem cells (HSC)-huMouse model

In order to reconstitute a broader spectrum of human immune cells in HIS-huMice, myoablated, immunodeficient mice are transplanted with CD34+ HSCs via intrahepatic injection (neonatal mice) [241] or intravenous injection (juvenile/adult mice) [246]. These HSCs can be obtained from a myriad of sources including fetal liver tissue [247], mobilized stem cells in the blood, and neonatal cord-blood stem cells [246, 248, 249]. Human immune reconstitution in the HSC-huMouse model requires 10-12 weeks to develop [250]. Various hematopoietic lineages,

including T cells, monocytes/macrophages, B cells, and dendritic cells, are developed in the blood and other tissues (e.g., spleen, liver, brain) [250]. Moreover, the HSC-huMouse model generates a naïve human immune system, which negates confounding factors associated with prior pathogen exposure [246, 251]. The HSC-huMouse model supports HIV infection, CD4+ T cell depletion, chronic immune activation and limited anti-HIV T and B cell immune responses [248]. A major advantage of the HSC-huMouse model over the PBL-huMouse model is the delayed and reduced incidence of GvHD, which provides the opportunity for long-term modeling of HIV infection and replication [240]. The HSC-huMouse model provides a means of evaluating the efficacy and safety of direct-acting therapeutics (*e.g.* antivirals drugs, antibodies) and immune-modulatory agents (*e.g.* pDC modulators [252]) geared towards preventing HIV transmission, controlling HIV replication, and ameliorating CD4+ T cell depletion and chronic immune activation [248]. The HSC-huMouse model supports HIV transmission via the intravenous (along with intraperitoneal) route [247]; however, conflicting reports exist for mucosal route of transmission [253, 254]. Additionally, the reconstituted human T cells are educated in the mouse thymic epithelium, thus limiting antigen-specific responses [255]. This limitation of T cell education in the murine thymic epithelium has been partially overcome by the construction human leukocyte antigen (HLA) class I transgenic-immunodeficient mice to support robust T cell development of HLA-matched HSC transplants [256].

Moreover, lymph nodes and spleen are poorly reconstituted, including a limited development of human B cells and myeloid cells in the white and red pulps of the spleen [257]. Several modifications have been made to the HSC model to address these limitations. Li *et. al.* constructed an immunodeficient mouse model that incorporated a lymphoid tissue-stromal cytokine transgene (i.e. thymic-stromal-cell-derived lymphopoietin) and demonstrated improved

lymph node development in HSC-huMice [257]. Additionally, studies have demonstrated enhanced human B and myeloid cell development in murine secondary lymphoid tissues via transgenic expression of critical cytokines (i.e. IL6; IL3, GM-CSF and SCF) for B and myeloid cell maturation [258-260]. Although incorporation of requisite human transgenes in HIS-huMice has been successful in demonstrating improved development of immune cells, often the resultant lineage is skewed, as the transgene expression is not synchronized for physiological expression, and supporting stromal cells and other essential cytokines are absent [258-260].

1.18.2 Bone marrow-liver-thymus (BLT)-huMouse model

Another strategy for improving human immune cell development in HIS-huMouse models is to implant human lymphoid tissues containing the requisite microenvironment for supporting robust immune cell development. To facilitate human T cell education and associated function, human thymic tissues are incorporated in HIS-huMice, and termed, Bone Marrow-Liver-Thymus (BLT)-huMice [261, 262]. BLT-huMice have served as a major animal model for HIV research for over a decade and are a cost-effective alternative to the surrogate, simian immunodeficiency virus (SIV)-non-human primate (NHP) models. The BLT-huMouse model is generated via surgically transplanting myoablated, immunodeficient mice with autologous fetal human liver and thymus tissues, followed by IV injection of autologous CD34+ HSC [263-265]. Transplanted mice require 10-12 weeks for systemic reconstitution of human cells post-transplantation [263-265]. The most widely utilized strain for constructing BLT huMice is the NOD-Prkdcscid IL2rgTm1Wjl (NSG) [251, 262], which is readily available from Jackson Laboratory. BLT-huMice can also be constructed using comparable immunodeficient mouse strains, such as, C57BL/6 Rag2-/-γc-/-CD47-/- (TKO) [251, 266]. The key benefit of the BLT-huMouse model over PBL- and HSC-

huMouse models is the presence of human thymic microenvironment, which facilitates T cell education in an autologous human tissue that contains the requisite stromal cells (as well as cytokines and factors, presumably at physiological levels) [251]. BLT-huMice have systemic tissue reconstitution with human immune cells, including in mucosal tissues, which enables mucosal transmission [267-274] and recapitulates the main route of HIV transmission in humans [89, 267-274]. Other hallmarks of HIV infection and replication in BLT-huMice include robust T cell depletion [267, 273], CNS infiltration [275, 276], immune response [266, 277-280], and latency [281-283]. The BLT-huMouse model is a robust platform for evaluating antiretroviral therapy, pre-exposure prophylaxis (PrEP), latency reversing agents (LRA), vaccination, proviral excision, and T cell engineering. Despite scientific advances gained from the BLT model, the system does have disadvantages. The generation of BLT-huMice requires surgical expertise and access to human fetal tissues, which may not be readily available. Recently, a novel BLT-like huMouse model has been developed using non-autologous cord blood-derived hematopoietic stem cells and neonatal thymus, which enable investigators to construct >1000 BLT-like huMice using cryopreserved thymus and readily available cord-blood stem cells [284]. Recent studies demonstrate that these BLT-like huMice develop a myriad of human immune cells and supports HIV infection and replication (unpublished data from Elie Haddad, Chloé Colas, et al, at the CanCure 5th Annual General Meeting – 2019, Poster Session, in Montreal, Canada). BLT-huMice are prone to GvHD, which limits the experimental window these animals can be utilized to approximately 6 months post-engraftment [285, 286]. However, BLT-huMice constructed with a C57BL/6 immunodeficient background are resistant to GvHD [266, 283]. Despite systemic immune cell reconstitution and HIV-specific immune responses, BLT huMice do not develop a complete human immune system. The current widely used immunodeficient mouse models

possess an IL-2 receptor γ chain deletion [287-290]. As a result, mouse lymphoid organs do not fully develop in those models, [251] and the loss of lymphoid tissue microenvironment impairs the ability of BLT-huMice to develop a robust humoral immune response, as immunoglobulins are skewed towards IgM or weak IgG response [266, 279, 289, 291-293]. Constructing a BLT-huMouse model using immunodeficient mice with requisite human transgenic factors/cytokines may optimize human B cell development and overcome limitations of humoral immune response in the model [294-296]. An alternative strategy, which is consistent with the BLT-model strategy, is to incorporate the requisite secondary human lymphoid tissue (i.e. spleen) microenvironment for robust human immune cell (e.g. B cells, macrophages) development and response [297].

1.18.3 Bone marrow-liver-thymus-spleen (BLTS)-huMouse model

To address the limitations of the BLT-huMouse model, namely, poor development of secondary lymphoid tissue and modest macrophage reconstitution, we incorporated human spleen into the BLT-huMouse model, and termed those animals, Bone Marrow-Liver-Thymus-Spleen (BLTS)-huMice [297]. The BLTS-huMouse model exhibits significant improvement over the BLT-huMouse model by addressing several limitations [297]. Successful spleen growth dramatically lowers the incidence of GVHD in BLTS-huMice, thus allowing for experimental studies that extend up to 9 months post-transplantation [297]. Decreased interference from the effects of GVHD and a longer experimental window may improve insights gained from long-term studies of HIV infection, such as investigation of therapeutics once chronic infection is established. The human spleen in the BLTS-huMouse model recapitulates human adult spleen architecture and facilitates better reconstitution of immune cells, including human red pulp macrophages, which are poorly reconstituted in the BLT-huMouse model [297]. It is well established that macrophages

can serve as a reservoir for HIV [213, 298, 299]; thus, the BLTS-huMouse model provides a system for investigating human splenic macrophage-HIV interactions [297]. Additionally, the spleen is a major lymphoid tissue reservoir, with B cell follicles in the white-pulp serving as an immune privilege site for anti-HIV T-cells [211]. The human spleen in BLTS-huMice provides a model for investigating anti-HIV immune response within the white-pulp and the role of B cell follicle in mediating HIV persistence. The BLTS-huMouse model supports cART-mediated HIV load suppression to undetectable levels, and replication competent HIV reservoirs can be detected in human spleen tissues [297]. Lymphoid tissue fibrosis is an immuno-pathogenic feature associated with HIV infection and plays a major role in mediating chronic inflammation and abrogating the development of a robust immune response [300]. A major advantage of the BLTS-huMouse model is that HIV infection results in lymphoid tissue fibrosis; this disease manifestation is absent in HIV-infected BLT-huMice [297]. Although the BLTS-huMouse model exhibits more robust immune reconstitution compared to its BLT counterpart, the two models share some limitations. The transplantation of the human tissues under the renal capsule requires an individual with advance surgical skills to successfully and safely perform the procedure.

Table 2. HuMice models used for HIV studies

Model	Approach	Characteristics	Application
Hu-PBL-SCID	Engraftment of human PBMCs or PBLs	Rapid human immune cell reconstitution, predominantly engrafts T cells, Early GVHD	HIV infection, Mature T cell response, mouse-quantitative viral outgrowth assay
Hu-HSC-SCID	Engraftment of human (CD34+) hematopoietic stem cells	Robust development of lymphoid and myeloid lineage immune cells, and naïve immune system, delayed GVHD	Long term HIV infection, ART suppression, anti-HIV T and B-cell immune responses in chronic HIV infection,
Hu-BLT (Bone Marrow-Liver-Thymus)	Engraftment of human hematopoietic stem cells, fetal liver and thymus	Robust development of hematopoietic and naïve immune system. Improved T cell education in human thymus.	Mucosal HIV infection, sustained viremia, cART-mediated suppression of the virus, and cellular immune response, therapeutic testing of drugs/ vaccines, latent HIV infection.
Hu-BLTS (Bone Marrow-Liver-Thymus-Spleen)	Engraftment of human hematopoietic stem cells, fetal liver, thymus and spleen	Robust development of hematopoietic and naïve immune system. Improved B cell follicle and macrophage development.	HIV reservoir studies, therapeutic drug screening for HIV eradication.

The BLTS-huMouse model uses human fetal tissues, which introduces logistical and operational constraints. The use of frozen fetal tissues and hematopoietic stem cells can alleviate some of those constraints (unpublished data). Demonstrating robust anti-HIV immunity in HIS-huMouse models has been a long-term goal in the field; said system would allow robust evaluation of HIV vaccine candidates against circulating viral strains. The incorporation of human primary and secondary lymphoid tissues in HIS-huMice brings us one step closer to this goal. At present, we are actively investigating the anti-HIV human immunity in the BLTS-huMouse model to determine if this system provides a means for evaluating HIV vaccines.

2.0 Specific Aims

2.1 Aim 1

Optimize the huMouse model for studying HIV (Nef) and αβ/γδ T cells interaction *in vivo*.

Hypothesis - Transplantation of immunodeficient mice with human lymphoid tissues and hematopoietic stem cells provides the requisite microenvironment for robust human immune system development and HIV infection.

Rationale - Although in vitro studies can be used to understand direct interactions and effects of HIV on αβ/γδ T cells, it does not reflect complex interactions occurring in an *in vivo* microenvironment, especially in immunological studies where multiple cell types work in coordination with each other to produce an effective immune response. Thus, it is necessary to study the function of αβ/γδ T cells using an *in vivo* model. Rhesus and cynomolgus macaques infected with simian immunodeficiency virus (SIV) have been commonly used as *in vivo* models for SIV studies [301]. However, SIV contains only about 50 percent of the genetic code of HIV and there are substantial differences in γδ subset composition and phenotype in monkeys and humans [236]. The information we can extrapolate from non-human primate models of SIV becomes limited by the unaltered peripheral Vδ1/Vδ2 T cell ratio in SIV-infected macaques [237] and the genetic differences between SIV and HIV [236]. Therefore, an alternate approach is needed to understand the *in vivo* dynamics of γδ T cells in HIV infection. Among the widely used small mammal platforms for investigating HIV pathogenesis and therapeutics is the huMouse model

[302], generated via retro-orbital injection of CD34+ hematopoietic stem cells and autologous transplantation of fetal thymus and spleen organoids into immunodeficient mice, this huMouse provides both the peripheral immune circulation and human lymphoid microenvironment to study HIV in blood and human lymphoid tissues. We and others have previously shown that human CD4+/CD8+ T cell ratios before and after HIV infection of huMice are comparable to clinical values, but γδ T cells have yet to be characterized in this model.

2.2 Aim 2

Determine the impact of WT and Nef defective HIV infection on αβ and γδ T cells in huMice.

Hypothesis- Nef defective HIV but not WT HIV will maintain the normal frequency, phenotype, and function of αβ and γδ T cells in huMice.

Rationale- HIV Nef plays an important role in optimal infection, efficient viral replication, and persistence and accelerated disease progression to AIDS in HIV-1 infected humans [14, 15]. Individuals infected with Nef deleted HIV exhibit reduced viral load, immunopathogenesis, and slower disease progression [303, 304]. Nef-deleted HIV exhibit reduced infectivity and replication as compared to the wild type HIV [4, 5]. However, whether Nef has any direct impact on peripheral blood and lymphoid tissue-resident γδ T cells remains unresolved. Moreover, *in vivo*, mechanistic studies confirming the impact of Nef-dimerization defective virus infection on T cells are lacking. Thus, we have studied the interactions between wild type/Nef defective HIV and immune cells αβ and γδ T cells in huMice.

2.3 Aim 3

Elucidate the role of γδ T cells in control of HIV infection in huMouse.

Hypothesis- Adoptive transfer of Vδ2 T cells in huMice will control HIV infection.

Rationale- Although in vitro studies have shown that γδ T cells interact with various immune cells and play important role in regulating the innate and adaptive immune system, their role in controlling HIV infection *in vivo* remains unresolved [9, 305, 306]. We designed an adoptive transfer experiment to test the protective function of allogenic Vδ2 cells against HIV infection in an *in vivo* setting by injecting ex vivo expanded Vδ2 T cells, into huMice.

3.0 Materials and Methods

3.1 Construction of huMice

Non-Obese Diabetic. Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were obtained from the Jackson Laboratory and bred in the Division of Laboratory Animal Resources facility at the University of Pittsburgh. The mice were bred and housed under biosafety level 1, pathogen-free conditions according to the guidelines approved by the Institutional Animal Care and Use Committee and were fed irradiated chow (Prolab Isopro RHM 3000 Irradiated, catalog 5P75-RHI-W 22, PMI Nutrition International) and autoclaved water. Human fetal tissues were obtained from the Health Sciences Tissue Bank at the University of Pittsburgh Advanced Bioscience Resources Inc and processed under biosafety level 2 conditions. Within 12 hours of receipt of fetal human liver and thymus, CD34+ hematopoietic stem cells (HSCs) were isolated from the fetal liver as previously described [307] and cryopreserved at -170° C until transplantation. Portions of the fetal liver and thymus sample were cryopreserved at -170 C until transplantation. 4 to 6-week-old NSG mice received a radiation dose of 1.50 Gray before transplantation to suppress any residual immune functions and were immediately transferred to biosafety level 2+ animal housing. On the day of operation, the cryopreserved CD34+ HSCs and tissues were thawed, and the tissues were morcellated into ~1 mm^3 fragments, and the irradiated mice were anesthetized using 1.5-3% isoflurane. Autologous human fetal thymus and spleen tissue sections were implanted under the kidney capsule, and 150,000 CD34+ HSCs were engrafted via retroorbital injection in 100 uL of PBS. Immediately following the procedure, the mice received 150uL injections of carprofen (1 mg/mL) and ceftiofur (1 mg/mL) as a painkiller and antibiotic, respectively. These injections

continued once a day for 2 days for a total of three sets of injections. Successful engraftment was determined by flow cytometric analysis of human CD45 expression on blood cells of mice, now termed huMice. Mice harboring > 30% of human $CD45^+$ cells were used for further experiments.

3.2 Study participants

Participants of the Pittsburgh clinical site of the Multicenter AIDS Cohort Study (MACS) were included in this study. These participants were HIV-1 infected men who were on ART for a median duration of 12.08 years, who had a median $CD4^+$ T cell count of 620 cell/μl and a viral load of <50 copies/ml. Wherever mentioned blood products from age-matched HIV-negative MACS participants were used in the study. Whole blood products from HIV-1-seronegative blood donors were purchased from the Central Blood Bank of Pittsburgh. Written informed consent was obtained from participants before inclusion in the study, which was approved by The University of Pittsburgh Institutional Review Board.

3.3 Isolation of monocytes and peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) obtained from a buffy coat or whole blood were isolated by standard density gradient separation using Lymphocyte Separation Medium (Corning). Monocytes were isolated from PBMC by positive magnetic bead selection (Miltenyi Biotec), and CD4+ T cells and γδ T cell subsets were isolated by negative selection (EasySep™

CD4 T cell, Cat #-17952 and γδ T cell isolation kit, Cat #- 19255) according to the manufacturer's specifications, and the differentially isolated cells were cultured or cryopreserved until use.

3.4 Flow cytometry

Single-cell suspensions prepared from peripheral blood, splenocytes and thymocytes of huMice were stained with a live/dead fixable aqua dead cell stain kit (Thermo Fisher Scientific) and fluorochrome-conjugated antibodies [anti-human CD45, anti-human CD4, anti-human Vδ2, (BioLegend); anti-human CD8, CD3, CD-19, CD56, PD1, HLA-DR, CD25, CD69, CD45RA, and CD27, IFN-γ (Becton Dickenson); and anti-human Vδ1, (Thermo Fisher Scientific)], CD16-PerCP-Cy5.5 (Beckman Coulter), and HIV-p24 (KC57, Beckman Coulter). Cells were fixed using 2% paraformaldehyde, and data were acquired using an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software. Gating was done based on fluorescence minus one (FMO).

3.5 Virus production

The dimerization mutant constructs used in this study are based on the HIV NL4-3 backbone in which part of the NL4-3 Nef ORF is replaced with that of Nef from the closely related HIV-1 B-clade isolate, SF2. HIV-1 stocks were produced in 293T cells (ATCC; CRL-3216) after transfection and amplified in the T-cell line MT2 (AIDS Reagent Program) [12, 308]. Viral titers

were quantified by HIV-1 p24 AlphaLISA assay (PerkinElmer; AL291F) according to the manufacturer's protocol.

3.6 Generation of monocyte-derived DC

Bone marrow cells were harvested from huMice and performed ACK lysis to get rid of RBCs. Then 1 X 10^6 cells were cultured for 7 days in the presence of 100ng/ml of recombinant human stem cell factor (cat# 255-SC-010),100 ng/mL Flt3-ligand (Cat #-308-FK-005), 50 ng/mL of recombinant human GM-CSF (Sanofi-aventis Cat# NAC2004–5843-01) and 2.5 ng/mL of tumor necrosis factor (TNF)-α (25 ng/mL; R&D Systems Cat# 210-TA). Next, immature DCs were generated from bone marrow cells and cultured for 7 days in Iscove's Modified Dulbecco's Media (IMDM; Gibco Cat# 12440-053) containing 10% fetal bovine serum Atlanta biologicals Cat# S12450H) and 0.5% gentamicin (Gibco Cat# 15710-064) in the presence of granulocyte-monocyte colony-stimulating factor (GM-CSF; 1000 IU/mL; Sanofi-aventis Cat# NAC2004–5843-01) and interleukin-4 (IL-4; 1000 IU/mL; R&D Systems Cat# 204-1 L). Mature, high IL-12p70-producing Type 1 DC and IL-12p70 deficient, prostaglandin E2-treated DC (Type 2-DC) were generated as previously described (29) by exposure of immature DC cultures at day 5 for 48 h to a cocktail of maturation factors containing either interferon (IFN)-α (1000 U/mL; Schering Corporation Cat# NDC:0085–1110-01), IFN-γ (1000 U/mL; R&D Systems Cat# 285-1F), IL-1β (10 ng/mL; R&D Systems #201-LB), tumor necrosis factor (TNF)-α (25 ng/mL; R&D Systems Cat# 210-TA), and polyinosinic:polycytidylic acid (20 ng/mL; Sigma-Aldrich Cat# P9582-5MG), or IL-1β (10 ng/mL), TNF-α (25 ng/mL), IL-6 (1000 U/mL; R&D Systems Cat# 206–1 L), and PGE2 (2 μM; Sigma-Aldrich Cat# P6532-1MG), respectively. Differentiation of DC was

confirmed by assessing the DC-specific markers such as CD83, CD86, Sig 1, OX40L, CCR-5 by flow cytometry.

3.7 Functional characterization of differentially matured DCs

To test the IL12p70-producing capacity of DC, they were harvested, washed, and plated in flat-bottom 96-well plates at 2×10^4 cells/well. To mimic the interaction with CD40L-expressing Th cells, recombinant human CD40 ligand was added to the culture. Supernatants were collected after 24 h and tested for the presence of IL-12p70 by ELISA.

3.8 Functional characterization of T cells

Immunomagnetic selected human splenic T cells from BLTS-huMice and human T cells from PBMCs were seeded at 8x10^4 cells per well (96-well plate) and stimulated with Dynabeads™ Human T-Activator CD3/CD28 (Life Technologies Cat# 11131D) and PBS was used as vehicle control. The cells were stimulated with beads at a ratio of 1:1. T cells were harvested after 24 hr and used directly for flow cytometry analysis of the human IFNg levels (T cell activation marker).

3.9 Functional characterization of macrophages

HuMice were euthanized and human splenocytes were isolated from spleen tissue by mechanical grinding. CD163+ splenic macrophages were isolated using Milteny biotech human CD163 MicroBead Kit (cat #- 130-124-420). 100,000 macrophages were plated in 96 well plate and incubated in a humidified CO2 incubator for 1 hr. After the cells have adhered culture medium was replaced with 100 µL of the prepared pHrodo™ Red . coli BioParticles (Cat #- P35361). Next, microplate was transferred to an incubator warmed to 37°C for 1–2 hours to allow phagocytosis and acidification to reach their maximum. Fluorescence emitted by the pHrodo BioParticle phagocytosed macrophages was analyzed by the flow cytometry.

3.10 Tissue culture assay

TZM-bl cells and Dynabeads™ Human T-Activator CD3/CD28 (Life Technologies Cat# 11131D) treated human PBMCs-derived CD4+ T cells (via immunomagnetic selection) from an HIV negative donor (10,000 cells per cell) were added to separate 96-well plates in RPMI medium 1640-based growth medium (100 ul volume) and cultured overnight. The cells were inoculated with 0.1 ng p24 inoculum (100 ul) of wild-type, nef-deleted, or dimerization defective HIV-1 (NL4-3 strain) and incubated for the indicated time (2, 4 and 6 days). For measuring HIV infectivity and replication in TZM-bl cells, culture supernatant was removed from each well and Beta-Glo® reagent (Promega, Madison, WI) was added and the chemiluminescence activity (Relative Light Units-RLU) was subsequently (after 1 hour) measured using a luminometer

following manufacturer's recommended protocol. For measuring HIV infectivity and replication in human CD4+ T cells, culture supernatant was removed and measured using qPCR.

3.11 In vitro expansion of γδ T cells

HuMice were sacrificed and fully developed lymphoid tissues were collected and single cells were isolated following mechanical dissociation. Cells isolated from the lymphoid tissue were cocultured with allogeneic monocytes (4:1 ratio) from HIV-seronegative human blood bank donors in the presence of nitrogen-containing bisphosphonate zoledronate (ZOL, 5uM) (Zoledronic Acid, Selleckchem, S1314) and recombinant human (rh)IL-2 (Proleukin®, 100 IU/mL; Prometheus Laboratories) for 10 days as previously described [309]. rhIL-2 (100 IU/ml) was subsequently added every 3 days. The 10 day-cultured γδ T cells were characterized by flow cytometry analysis.

3.12 HIV infection of huMice

The CCR5-tropic strain of HIV-1 (NL4-3) [310] was generated by transfection of 293T cells (ATCC; ATCC CRL-3216) with a plasmid containing a full-length HIV genome and collecting the HIV containing culture supernatant. The viral titer was determined using GHOST cells (NIH AIDS Reagent Program; catalog 3942) as previously described [311]. Supernatant from uninfected 293T cells was used as a mock control. huMice were anesthetized and inoculated with

mock control supernatant or HIV-1 (~1 × 10^5 infectious units) by i.v. injection via retroorbital delivery.

3.13 HIV-1 genomic RNA detection

Total RNA was purified from plasma using RNA-Bee (AMSBIO). The RNA was then reverse-transcribed using TaqMan Reverse Transcription Reagents (Invitrogen) and quantitatively detected by real-time PCR using the TaqMan Universal PCR Master Mix (Invitrogen) with primers (forward primer, 5′ - CCCATGTTTTCAGCATTATCAGAA - 3′, and reverse primer, 5′ - CCACTGTGTTTAGCATGGTGTTTAA - 3′) and detection probe targeting HIV Gag gene (5′ - AGCCACCCCACAAGA - 3′) [312]. The assay sensitivity/cutoff was 10 copies/ml.

3.14 Adoptive transfer of T cells to huMice

PBMC derived $CD4^+$ T cells were isolated from HIV-infected MACS participants using EasySep™ Human $CD4^+$ T Cell Isolation Kit and activated overnight with Human T-Activator CD3/CD28 Dynabeads® (Life Technologies). The next day Dynabeads were separated from the $CD4^+$ T cells by manual dissociation followed by magnet isolation. The activated $CD4^+$ T cells were, washed, resuspended in PBS, and adoptively transferred into huMice via intraperitoneal injection (5 million cells/100µl/mouse). PBMC from the allogenic HIV non-infected donor were cultured in the presence of ZOL and rhIL-2 for 10 days to expand the Vδ2 cells. Activated and expanded Vδ2 cells were adoptively transferred to huMice via intraperitoneal injection (10

million/100µl/mouse). The huMice were divided into two treatment cohorts; one that received only activated $CD4^+$ T cells from HIV-infected donor, and the other that received the activated HIV-infected $CD4^+$ T cells as well as *in vitro* expanded allogenic Vδ2 cells.

3.15 Gene expression profiling using nCounter analysis

HIV infected, and Mock-inoculated huMice were sacrificed at the end of the study and total RNA was isolated from engrafted spleen tissue of huMice using Qiagen RNA isolation kit Cat# 74104. Nanostring profiling of host response was performed at the University of Pittsburgh Genomic Core facility using the nCounter Human Immunology v2 Panel, Cat #-XT-CSO-HIM2-12. Total RNA (50ng) was hybridized to reporter and capture probe sets at 65°C for 24 h. Hybridized samples were loaded on the nCounter cartridge and post-hybridization steps and scanning was performed on the nCounter Profiler. RCC files were analyzed using nSolver analysis software (Version 4.0) as per the manufacturer's protocols. Negative and positive controls included in probe sets were used for background thresholding, and normalizing samples for differences in hybridization or sample input respectively.

3.16 Statistics

Differences between HIV-infected/uninfected humans and huMice were compared using the two-tailed unpaired Student t-test. Differences among the human or huMice groups were compared using the two-tailed paired students t-test. The normality of the samples was tested using

the Shapiro-Wilk normality test. Statistical analyses were performed using the Prism8 (GraphPad Software) and p values <0.05 were considered statistically significant. The sample numbers and statistical analyses used are specified in each figure legend.

3.17 Approval for acquisition and use of human fetal tissue and biological agents

Human fetal liver and thymus (gestational age of 18–20 weeks) were obtained from medically or elective indicated termination of pregnancy through Magee-Women's Hospital of UPMC via the University of Pittsburgh, Health Sciences Tissue Bank. Written informed consent of the maternal donors was obtained in all cases, under IRB of the University of Pittsburgh guidelines and federal/state regulations. The use of human fetal organs/cells to construct huMice was reviewed by the University of Pittsburgh's IRB office, which has determined that this submission does not constitute human subject research as defined under federal regulations (45 CFR 46.102 [d or f] and 21 CFR 56.102[c], [e], and [l]). The use of human hematopoietic stem cells was reviewed and approved by the Human Stem Cell Research Oversight (hSCRO) at the University of Pittsburgh. The use of biological agents (e.g., HIV), recombinant DNA, and transgenic animals was reviewed and approved by the Institutional Biosafety Committee (IBC) at the University of Pittsburgh. All animal studies were approved by the IACUC at the University of Pittsburgh and were conducted following NIH guidelines for housing and care of laboratory animals.

3.18 Human ethical approval and informed consent:

The study was performed in accordance with the guidelines of "Ethical Principles for Medical Research Involving Human Subjects" provided by the World Medical Association Declaration of Helsinki (1964), and its subsequent amendments [41]. Written informed consents were obtained from the human study participants from Pittsburgh clinical site of the Multicenter AIDS Cohort Study (MACS), as well as from the maternal donors of fetal tissues used in the study following the University of Pittsburgh IRB guidelines as well as federal/state regulations. The ethical use of human fetal organs/cells to perform the studies was reviewed prior to study initiation by the University of Pittsburgh IRB, which determined that the submitted study does not constitute human subject research as defined under federal regulations (45 CFR 46.102 [d or f] and 21 CFR 56.102[c], [e], and [l]). The ethical use of human hematopoietic stem cells was reviewed and approved by the University of Pittsburgh Human Stem Cell Research Oversight (hSCRO) committee.

4.0 Results

4.1 The BLTS-huMouse model supports functional human innate and adaptive immune cells in the blood and the human lymphoid tissues.

We demonstrate here the reconstitution of γδ T cells, NK cells, CD4+ and CD8+ αβ T cells, monocytes, memory B cells, and plasma blasts in peripheral blood mononuclear cells (PBMCs) and the primary (thymus) and secondary (spleen) lymphoid tissue grafts **(Fig. 4 & 5)**. Additionally, we demonstrate the human antigen presenting cells' functionality in the BLTS-huMouse model **(Fig. 6)** Bone marrow-derived hematopoietic stem cells in the BLTS-huMice can differentiate into type 1 and type 2-polarized dendritic cells (DCs). Type-1 DCs secrete type-1 cytokine (IL12 p70) upon stimulation with physiological ligand (CD40L) **(Fig. 6A).** Human CD163+ splenic macrophages from BLTS-huMice phagocytized bacteria (pHrodo™ Red E. coli BioParticles) upon co-culture **(Fig. 6B).** Furthermore, we demonstrate the functionality of T cells in the BLTS-huMouse model. We show robust splenic T cell response (type-1 cytokine, IFNγ) to physiological stimulation (CD3/CD28) that was comparable to the cytokine (IFNγ) response by T cells from human PBMCs that was comparable to the cytokine (IFNγ) response by T cells from human PBMCs **(Fig. 6C).**

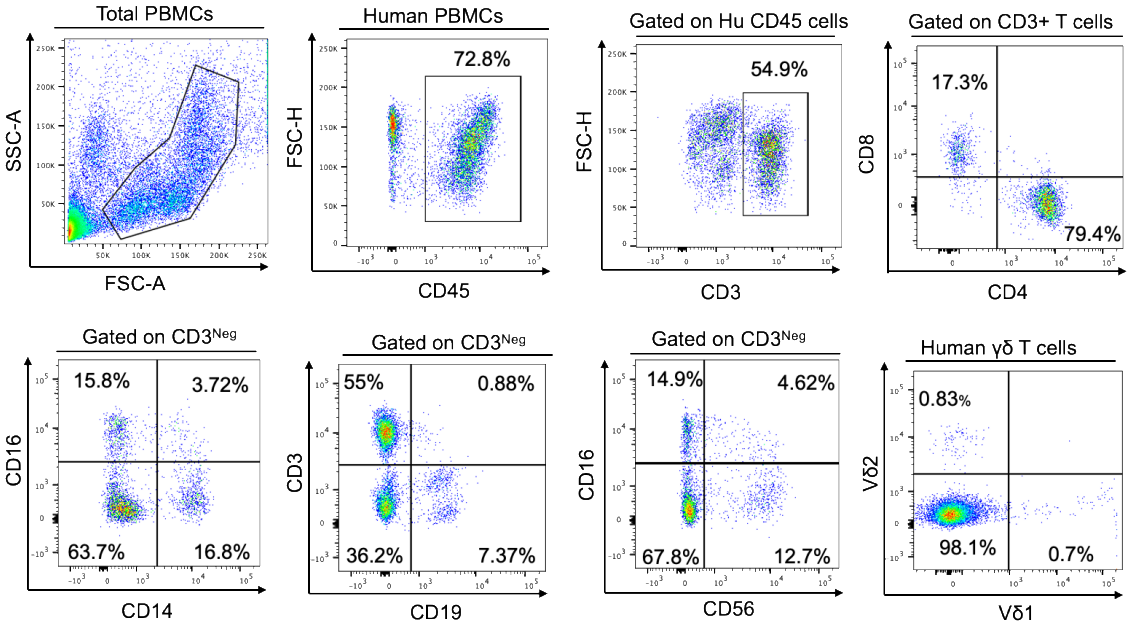


Figure 4. Human peripheral blood cell reconstitution in BLTS-huMouse model.

Flow cytometry analysis of the blood from representative BLTS-huMouse demonstrate the presence of human leukocytes (CD45+ cells), T cells (CD4+ and CD8+ cells), γδ T cells (Vδ1 and Vδ2 T cells), monocytes (CD14+ CD16+ cells), B cells and NK cells.

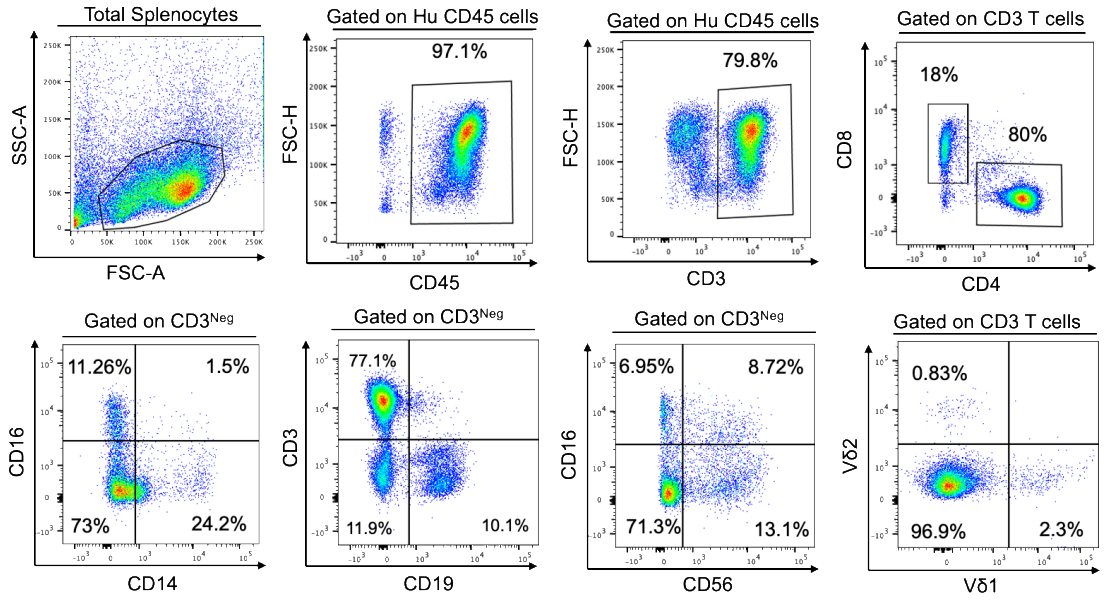


Figure 5. Human immune cell reconstitution in the human spleen in the BLTS-huMouse model.

Flow cytometry analysis of the human splenocytes from a representative BLTS-huMouse demonstrate the presence of human leukocytes (CD45+ cells), T cells (CD4+ and CD8+ cells), monocytes (CD14+ CD16+ cells), NK cells and B cells and γδ T cells (Vδ1 and Vδ2 T cells).

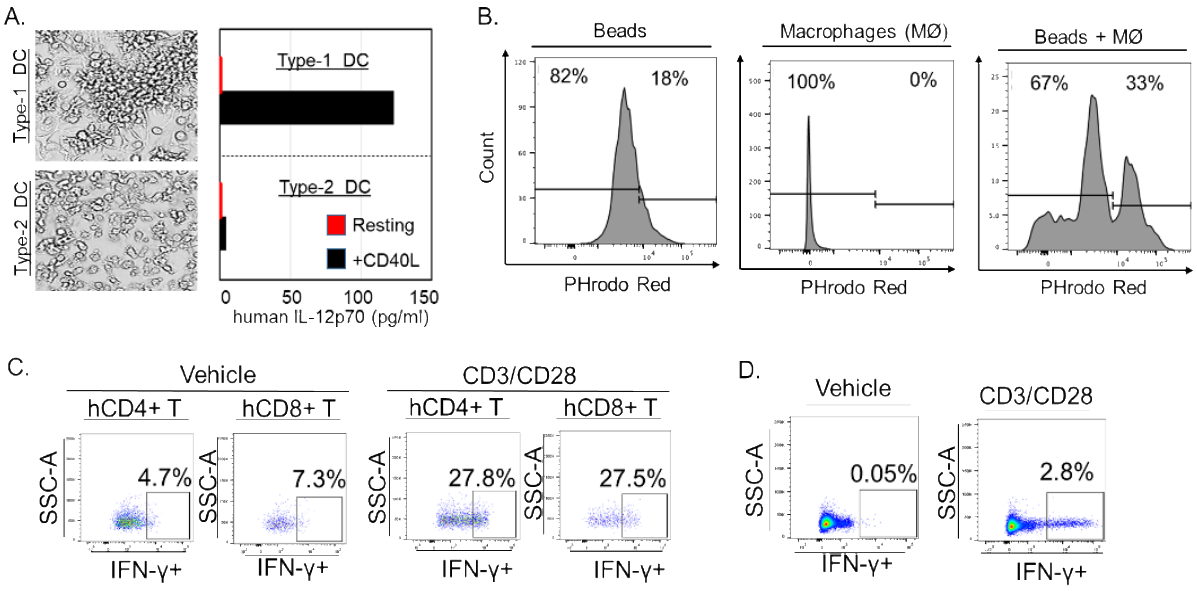


Figure 6. The functionality of human antigen presenting cells and T cells in the BLTS-huMouse model.

(A) Human CD34+ cells were differentiated into Type-1 and Type-2 dendritic cells (DC), and subsequently stimulated with CD40 ligand or vehicle, and human IL12p70 was measured. (B) Human CD163+ macrophages were immunoselected from human splenocytes and cultured with or without pHrodo™ Red E. coli BioParticles™ (ThernoFisher), and phagocytosis was measured using flow cytometry. (C-D) Human CD3+ T cells (immunomagnetic selected) were stimulated with CD3/28 beads and human IFN-g secretion.

The human immune system in the BLTS huMouse model abrogates nef-deleted HIV viremia.

We previously reported that BLTS-huMice support HIV replication and associated immunopathogenesis [297]. We demonstrated that human macrophages (CD163+ splenic cells) and CD4+ T cells facilitate HIV replication [297]. Therefore, BLTS-huMice provide an *in vivo* model for investigating Nef-immune system interactions in HIV infection. Nef enhances HIV infectivity and replication in some cell lines, although other studies have reported that Nef enhancement of HIV replication is variable in human PBL-derived activated-primary CD4+ T cells [314-316]. Here we show that nef-deleted HIV (NL4-3 strain) exhibits reduced replication compared to wild-type HIV in the TZM-bl cell line **(Fig. 7A)**.

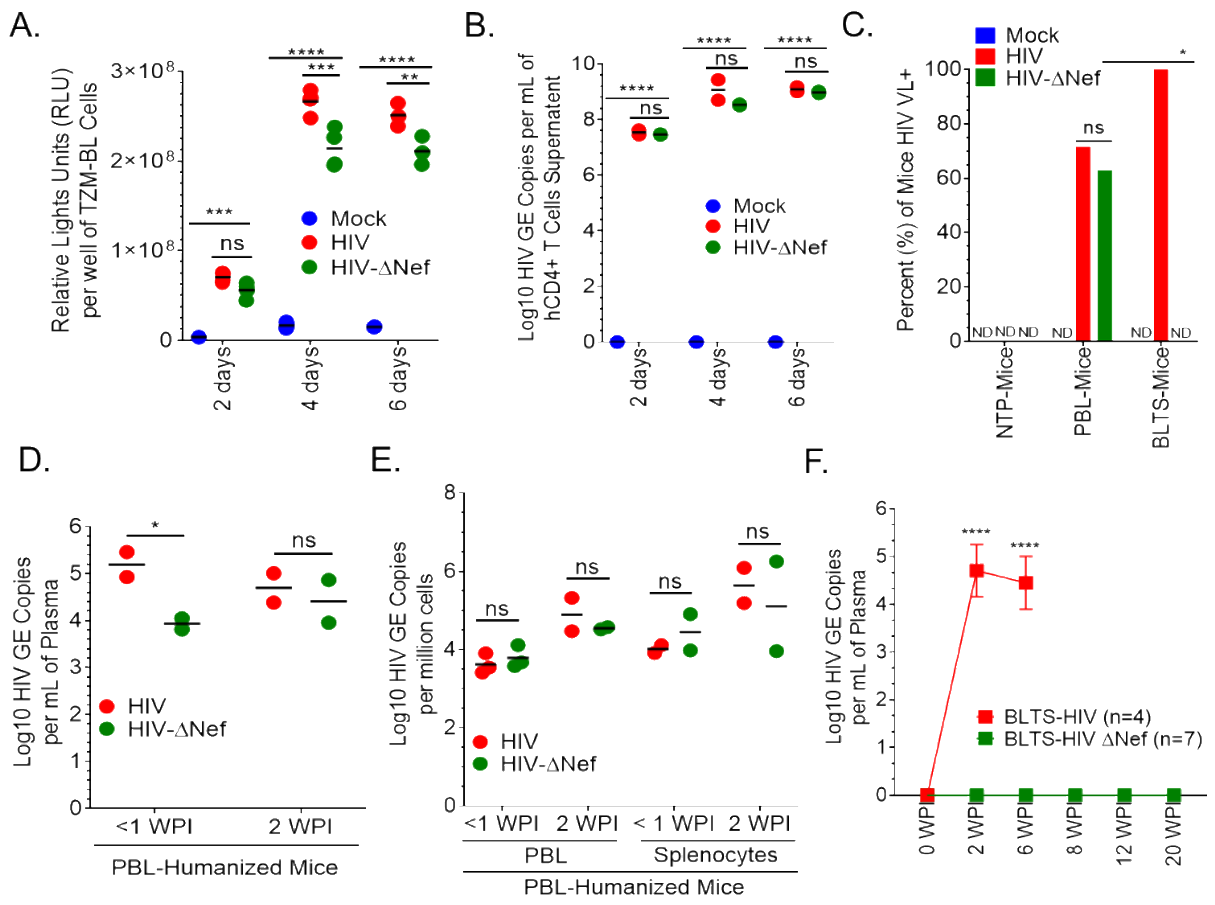


Figure 7. The human immune system in the BLTS huMouse model abrogates *nef*-deleted HIV viremia.

Analysis of the infectivity and replication of wild-type HIV and *nef*-deleted HIV (0.1 ng HIV p24 per 1x10^4 cells) demonstrate (A) reduced infectivity and replication kinetics in TZM-BL cells (N=3-4 wells per group); on the contrary, (B) similar infectivity and replication kinetics were recorded in activated (CD3/CD28)-primary CD4+ T cells (N=2 wells per group). (C-E) The infectivity and replication of wild-type and *nef*-deleted HIV (10 ng HIV p24 inoculum per mouse) in peripheral blood lymphocytes (PBL; reconstituted with predominately human CD4+ T cells) huMice (PBL-huMice; N=7-8 mice per group, and 3-5 per timepoint) is comparable as measured in the blood (plasma and PBL) and splenocytes at indicated weeks post-infection (WPI). (C) For additional controls, non-transplanted NSG mice (NTP) were inoculated with the different HIV strains and mock to demonstrate that HIV replication (i.e., viral load) requires the presence of human cells. (C) For negative controls, the absence of viral load was demonstrated in the blood and splenocytes of mock-inoculated PBL-huMice. (F) On the contrary, BLTS-huMice (N=4-7 mice per group; 10 ng HIVp24 inoculum per mouse) exhibit complete inhibition of *nef*-deleted HIV viremia. For BLTS-huMice inoculated with *nef*-deleted HIV, 7 mice were evaluated up to 6 weeks post-infection (WPI), and only 2 huMice and 1 huMouse remained in the study at 8-12 WPI and at 20 WPI, respectively. Mock inoculated BLTS-huMice (n=4) were used for controls, with those mice showing no detectable HIV genome (C; *timepoints data not shown*). Not significant (ns) = P>0.05, * = P≤ 0.05, ** = P≤ 0.01, *** = P≤ 0.001, **** = P≤ 0.0001.

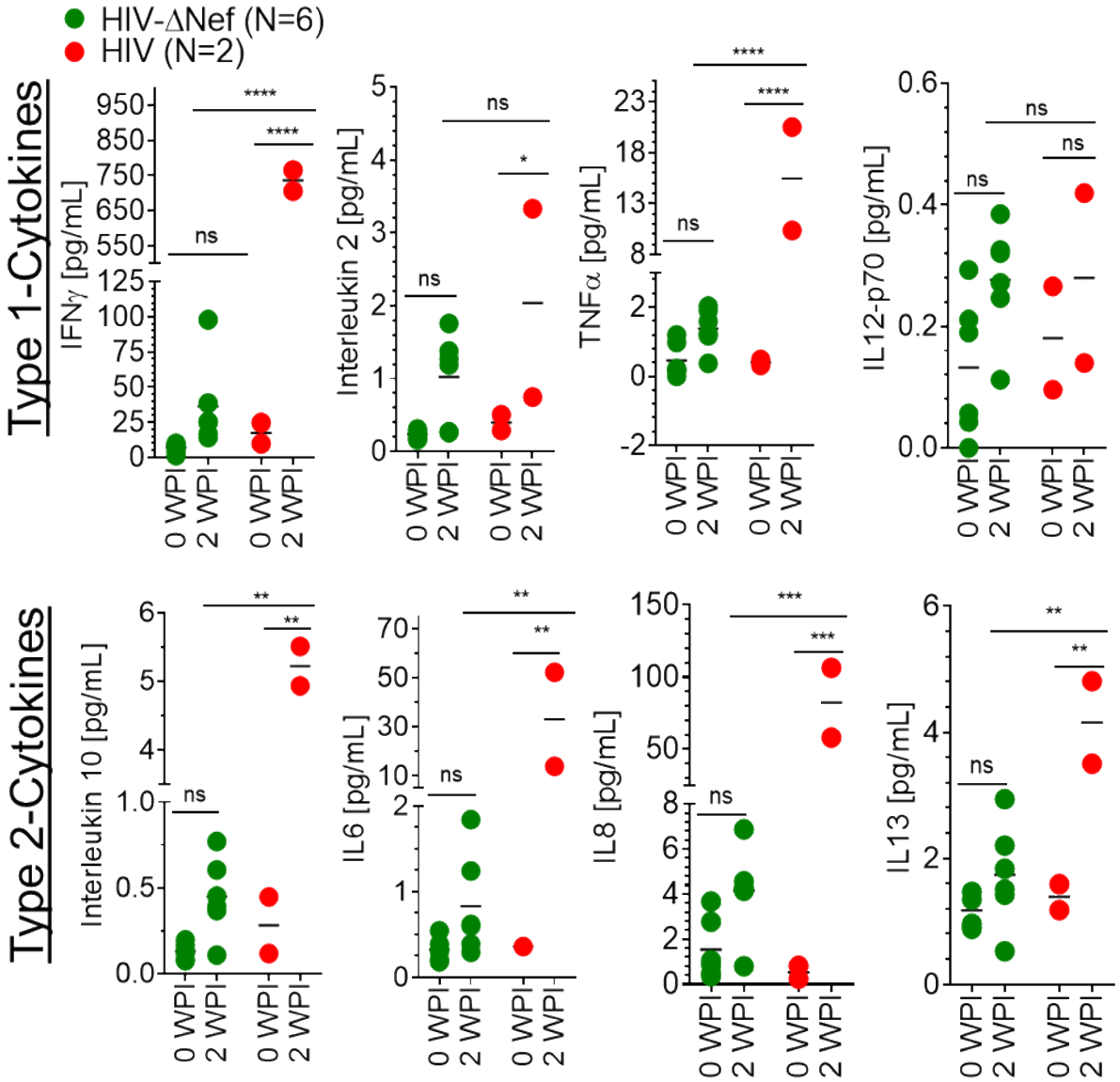


Figure 8. The human-immune system in BLTS-huMice mediate a cytokine response to *nef*-deleted HIV and wildtype HIV transmission.

Analysis of the human cytokine levels before and after inoculation with wild-type HIV and *nef*-deleted HIV (10 ng HIVp24 ≈ 1x10^6 infectious units per mouse) in BLTS-huMice (N=2-6 mice per group) demonstrate that both viruses induce Th1 and Th2 cytokines albeit, relatively higher levels with wild-type HIV replication. N=2-3 mice per group. ns = P>0.05, * = P≤ 0.05, ** = P≤ 0.01, *** = P≤ 0.001.

However, nef-deleted HIV and wild-type HIV exhibit negligible differences in viral replication in the activated-primary CD4+ T cell culture model **(Fig. 7B)**. The *in vivo* equivalent of the activated-primary CD4+ T cell culture model is the PBL-huMouse model [317, 318]. Using

the PBL-huMouse model, we demonstrate that nef-deleted HIV and wild-type HIV exhibit similar infectivity and replication in the blood and lymphoid tissues **(Fig. 7D-E)**, which is consistent with previous studies [319]. On the contrary, inoculation of BLTS-huMice with nef-deleted HIV and wild-type HIV resulted in divergent outcomes, with viremia in wild-type HIV inoculated mice and no viremia in nef-deleted HIV-inoculated mice **(Fig. 7F)**. We inoculated BLTS-huMice with the same high dose of nef-deleted HIV and wild-type HIV stocks used to infect PBL-huMice; therefore, the nef deleted HIV inoculum is infectious and replication-competent. These observations demonstrate that Nef overrides host immune control of HIV-1 replication in the BLT-S huMouse model.

HIV transmission in humans results in a cytokine burst (includes IFNγ, IL10, and TNFα) within two weeks of exposure [320], which stimulates immune cells to initiate an anti-viral immune response [321]. Inoculation of BLTS-huMice with nef-deleted HIV induces a modest increase in the secretion of Type 1 cytokines (IFNγ, IL-2, TNFα, IL12p70) and Type 2 cytokines (IL10, IL6, IL8) as compared to the highly viremic wild-type HIV **(Fig. 8)**. The cytokine burst in wild-type HIV infection in humans does not eliminate the virus or reduce viremia to undetectable levels; instead, it may fuel immune dysregulation [320]. The resulting chronic HIV infection induces progressive T cell-immune activation and [320, 322, 323] inadequate anti-viral T cell responses [320, 322, 323], and general immune dysregulation (including B cell hyperactivation) [324, 325].

4.2 Nef dimerization defect abrogates HIV viremia and associated immunodeficiency in the BLTS-huMouse model.

Previous X-ray crystallography studies of HIV-1 Nef proteins either alone or in complexes with host cell kinase regulatory domains revealed that Nef forms homodimers [326]. Comparison of these structures identified several residues common to these Nef dimer interfaces, including L112, Y115, and F121. Mutagenesis of these residues prevents homodimerization of recombinant Nef in vitro and reduces Nef dimer formation in a cell-based bimolecular fluorescence complementation (BiFC) assay. [12, 327].

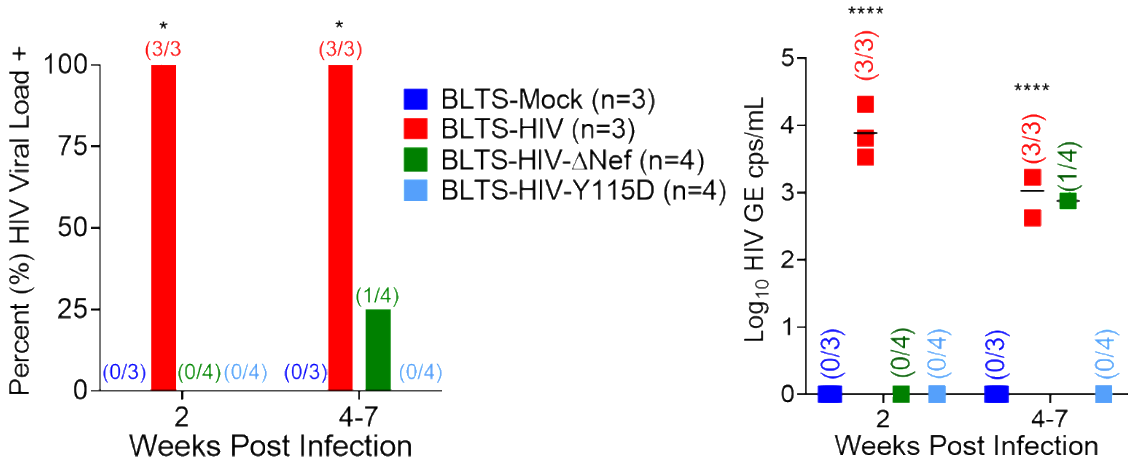


Figure 9. Nef-dimerization defect abrogates HIV viremia in in BLTS-huMice.

BLTS-huMice (BLTS-Mice; N=3-4) exhibit viremic control of *nef*-dimerization defective-HIV. N=3-4 mice per group, with mock (2 mice at 7 weeks post-inoculation and 1 huMouse at 4 weeks post-inoculation), wild-type HIV (3 mice at 7 weeks post-infection), Nef-deletion-ΔNef (2 mice at 7 weeks post-inoculation and 2 mice at 4 weeks post-inoculation) and Nef dimerization-defective-Y115D (2 mice at 7 weeks post-inoculation and 1 huMouse at 6 weeks post-inoculation) for indicated 4-7 weeks timepoints. Not significant (ns) = P>0.05, * = P≤ 0.05, ** = P≤ 0.01, *** = P≤ 0.001, **** = P≤ 0.0001.

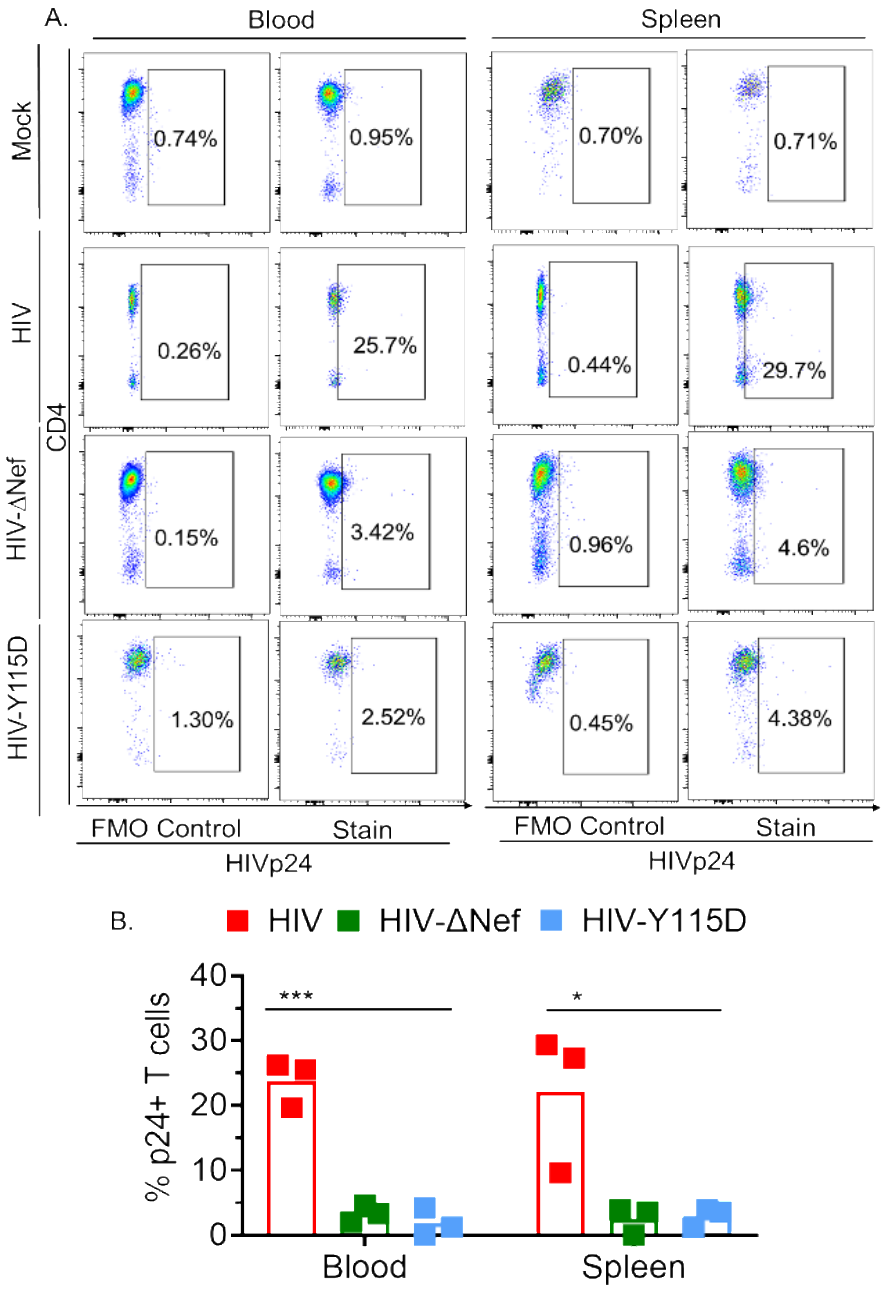


Figure 10. Nef-dimerization defect reduces HIV infected-CD4+ T cells in BLTS-huMice.

(A-B) Inoculation of BLTS-huMice with a *nef*-dimerization defective HIV (Y115D; 10 ng HIVp24) results in reduced HIVp24+ CD4+ T cells in the human spleen, as compared to wild-type HIV infection, as measured using flow cytometry. Mock inoculated huMouse was used as a control in flow cytometry analysis. N=3 mice per group, with wild-type (3 mice at 7 weeks post-infection), Nef-deletion-ΔNef (2 mice at 7 weeks post-inoculation and 1 huMouse at 4 weeks post-inoculation) and Nef dimerization-defective-Y115D (2 mice at 7 weeks post-inoculation and 1 huMouse at 6 weeks post-inoculation) for the 4-7 weeks sacrifice-timepoints. Not significant (ns) = P>0.05, * = P≤ 0.05, ** = P≤ 0.01, *** = P≤ 0.001, **** = P≤ 0.0001.

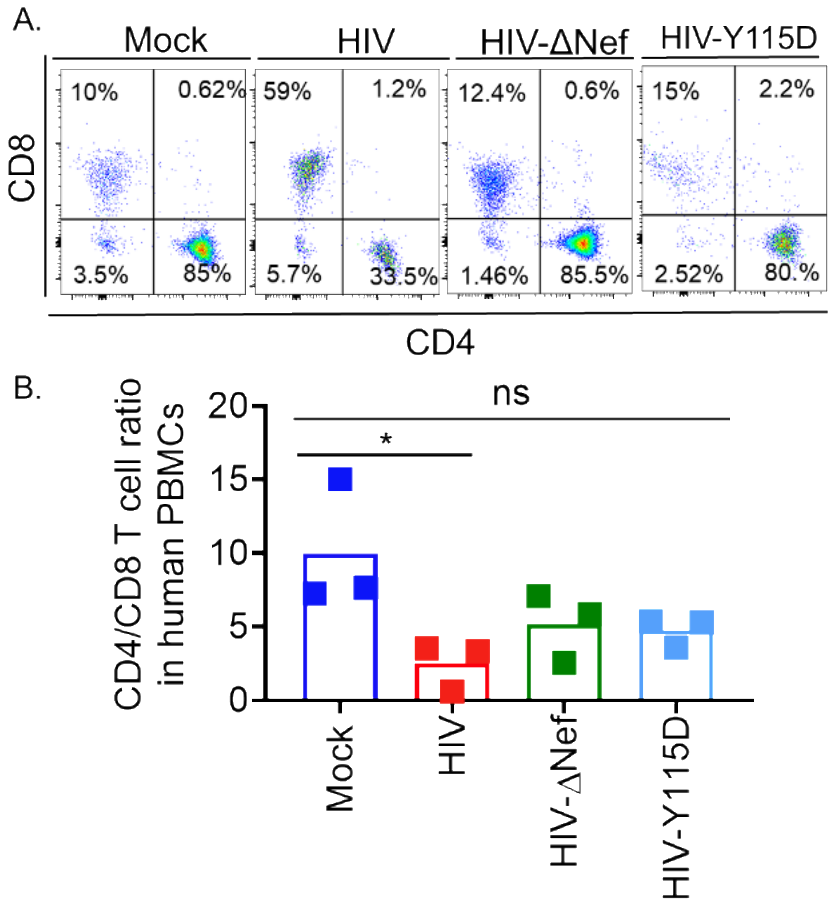


Figure 11. HIV-*nef* dimerization defect abrogates HIV-induced CD4+ T cell depletion in the BLTS-huMouse model.

Flow cytometry analysis of human CD4+ and CD8+ T cells (CD4+/CD8+ T cell ratio) in mock, wild-type HIV, and *nef*-defective HIV infection (*nef*-deleted-ΔNef, and *n*ef-dimerization defective-Y115D; 10 ng HIVp24) in BLTS-huMice. N=3 mice per group, with mock (2 mice at 7 weeks post-inoculation and 1 huMouse at 4 weeks post-inoculation), wild-type HIV (3 mice at 7 weeks post-infection), *nef*-deleted-ΔNef (2 mice at 7 weeks post-inoculation and 2 mice at 4 weeks post-inoculation) and *nef*-dimerization defective-Y115D (2 mice at 7 weeks post-inoculation and 1 huMouse at 6 weeks post-inoculation) for indicated timepoints. ns = $P>0.05$, * = $P\leq 0.05$.

Based on the BiFC assay, the relative disruption of dimerization in the Nef mutants is: L112D+Y115D double mutant (87%) > Y115D (59%) > F121A (33%) [12, 308].Previous studies showed that these dimerization-defective Nef mutants also reduce HIV replication [12] and abrogate HIV-induced impairment of host defense [328] in tissue culture models, implicating Nef dimers in signaling mechanisms by which HIV-Nef promotes viremia and immune dysregulation

[12]. Here, we showed that BLTS-huMice inoculated with the *nef*-dimerization defective HIV strains (Y115D) abrogate HIV viral load in the blood (aviremic) **(Fig. 9)** and results in reduced HIV-infected CD4+ T cells in human spleen xenografts when compared to wild-type HIV infection **(Fig. 10)**. BLTS-huMice inoculated with *nef*-deleted HIV (NL4-3 strain) were predominately aviremic (75% of the mice) and had low levels of HIV-infected CD4+ T cells in the human spleen xenografts **(Fig. 10)**. Additionally, *nef*-defective HIV (ΔNef and the dimerization-defective mutant, Y115D) and mock-inoculated BLTS-huMice exhibited similar CD4+/CD8+ T cell ratios, whereas wild-type HIV-infected BLTS-huMice resulted in immunodeficiency (reduced CD4+/CD8+ T cell ratio; CD4+ T cell loss) **(Fig. 11)**.

4.3 Nef dimerization defect attenuates HIV-induced immune dysregulation in the BLTS-huMouse model.

Many studies have shown that Nef impairs the anti-viral immune response to HIV infection [329]. Furthermore, several lines of evidence demonstrate that Nef-dimerization defective mutants and Nef dimerization inhibitors abrogate HIV-induced immune dysregulation [330, 331]. We show that Nef dimerization defect (Y115D mutant) attenuates HIV-induced T cell-immune activation (elevated CD25 and HLA-DR levels) **(Fig. 12)** and (elevated PD1 levels) **(Fig. 13)** in the blood and human spleen. Consistent with the role of Nef in promoting HIV-induced immune dysregulation, complete deletion of the nef gene in the HIV genome attenuates HIV-induced T cell-immune activation (elevated CD25, HLA-DR levels and PD1 levels) in the blood and the human spleen in BLTS-huMice **(Fig. 12)**.

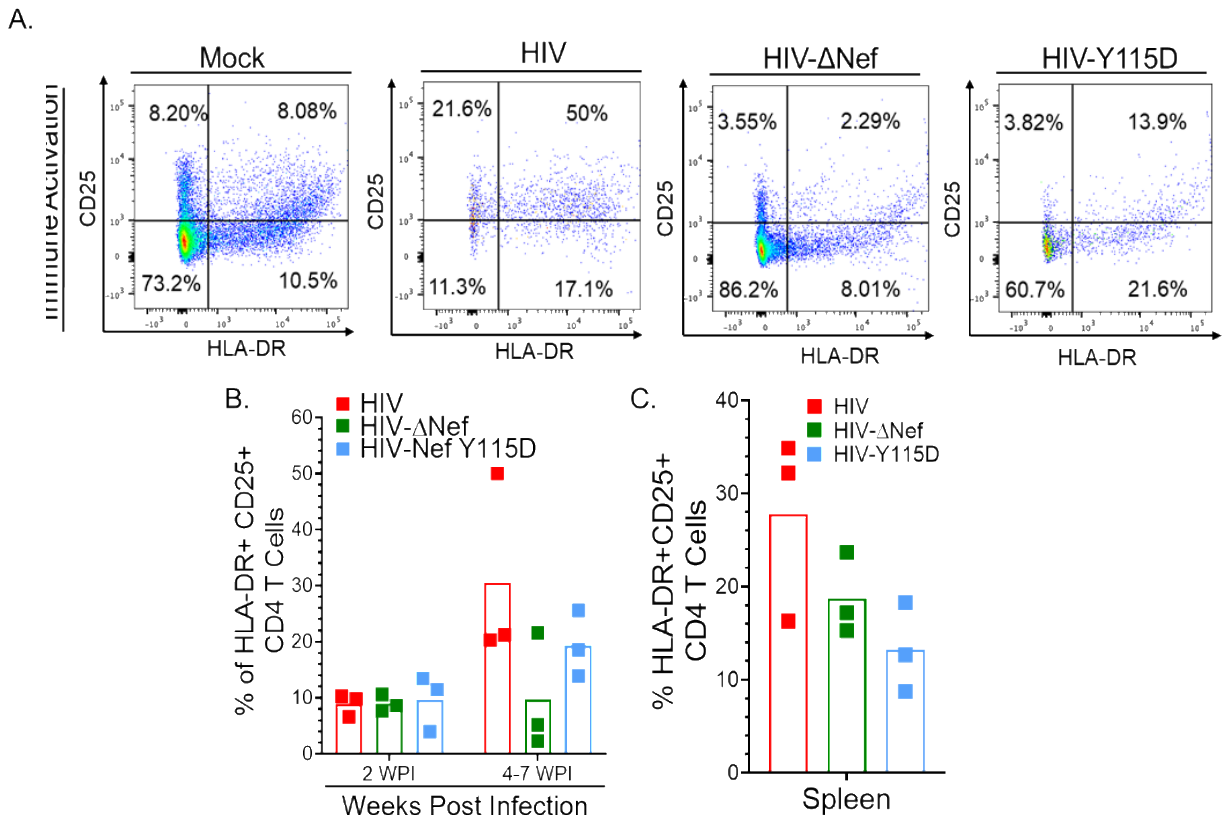


Figure 12. Nef dimerization defect abrogates HIV-induced elevated HLA-DR+ and CD25+ CD4+ T cells in the blood and human spleen in BLTS-huMice.

(A, B) Flow cytometry analysis of human CD4+ T cells in the blood in BLTS-huMice demonstrates HIV-induced HLA-DR+ and CD25+ CD4+ T cells are reduced in *nef*-defective HIV infection (*nef*-deleted-ΔNef, and *nef*-dimerization defective-Y115D; 10 ng HIVp24 per huMouse). Human CD4+ T cells from Mock-BLTS-huMouse served as a control. (C) Flow cytometry analysis of human CD4+ T cells in the human spleen xenografts in BLTS-huMice demonstrates reduced HLADR+ and CD25+ CD4+ T cells in *nef*-defective HIV infection (*nef*-deleted-ΔNef, and *nef*-dimerization defective-Y115D) compared to wild-type HIV. N=3 per group, with wild-type HIV (3 mice at 7 weeks post-infection), *nef*-deleted-ΔNef (2 mice at 7 weeks post-inoculation and 1 huMouse at 4 weeks post-inoculation) and *nef*-dimerization defective-Y115D (2 mice at 7 weeks post-inoculation and 1 huMouse at 6 weeks post-inoculation) at indicated 4-7 weeks sacrifice-timepoints.

The current consensus posits that chronic HIV infection in humans results from an inadequate antiviral-Th1 immune response, which is associated with T cell immune exhaustion [322], systemic immune inflammation (i.e., elevated levels of CXCL13) [332], B cell hyper-activation and dysregulation [324, 333], and reduced levels of anti-viral factors (i.e., CXCL12) [334].

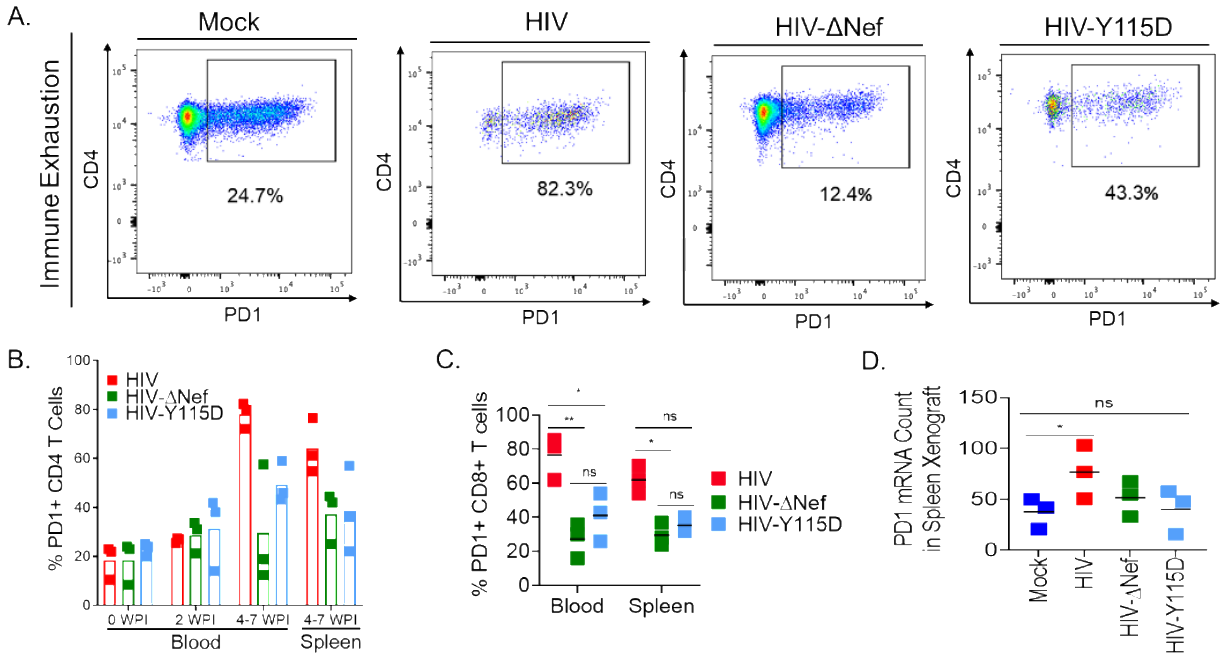


Figure 13. Nef dimerization defect abrogates HIV-induced T cell-checkpoint inhibitor expression in the blood and human spleen in BLTS-huMice.

(A, B, C) Flow cytometry analysis of human CD4+ and CD8+ T cells in the blood and human spleen in BLTS-huMice demonstrates elevated CD4+ and CD8+ T cell-checkpoint inhibitor expression (PD1) in the wild-type HIV group compared to *nef*-defective HIV groups (*nef*-deleted-ΔNef, and *nef*-dimerization defective-Y115D; 10 ng HIVp24 per mouse). (A) Human CD4+ T cells from Mock-BLTS-huMouse served as a control. (D) Additionally, gene expression analysis of the human secondary lymphoid tissue xenograft (Spleen) in BLTS-huMice demonstrates elevated expression of PD1 in the wild-type HIV group compared *nef*-defective HIV groups (*nef*-deleted-ΔNef, and *nef*-dimerization defective-Y115D) and mock. Not significant (ns) = P>0.05, * = P≤ 0.05, ** = P≤ 0.01. N=3 per group, with wild-type HIV (3 mice at 7 weeks post-infection), *nef*-deleted-ΔNef (2 mice at 7 weeks post-inoculation and 1 huMouse at 4 weeks post-inoculation) and *nef*-dimerization defective-Y115D (2 mice at 7 weeks post-inoculation and 1 huMouse at 6 weeks post-inoculation) at indicated 4-7 weeks sacrifice-timepoints.

Previous studies also show that HIV Nef dimers are required to activate non-receptor tyrosine kinases of the Src and Tec families to enhance viral replication (Staudt et al. JBC 2020) and are also linked to downregulation of MHC-I and CD4 receptor on T cells [12]. Additionally, Nef promotes B cell hyper-activation and dysregulation; this pathogenic effect has been associated with T cell interaction [324, 333]. Dysregulated immune activation and signaling by Nef are presumed to drive chronic HIV infection and progression to AIDS [335]. Gene expression analysis demonstrates that wild-type HIV infection in BLTS-huMice induces systemic inflammation

(elevated CXCL13), B cell hyper-activation (elevated CD19, CD79, PAX5, and BLNK), and reduced levels of anti-viral factors (CXCL12, PML/TRIM19) in the human spleen xenograft **(Fig. 14A)**. Wild-type HIV infection also induced the expression of the non-receptor tyrosine kinase, ABL1, in the human spleen xenografts of BLTS-huMice **(Fig. 14A)**. ABL1 plays a critical role in cytoskeletal rearrangement and facilitate HIV entry into the host cell. ABL1 also enhances HIV-1

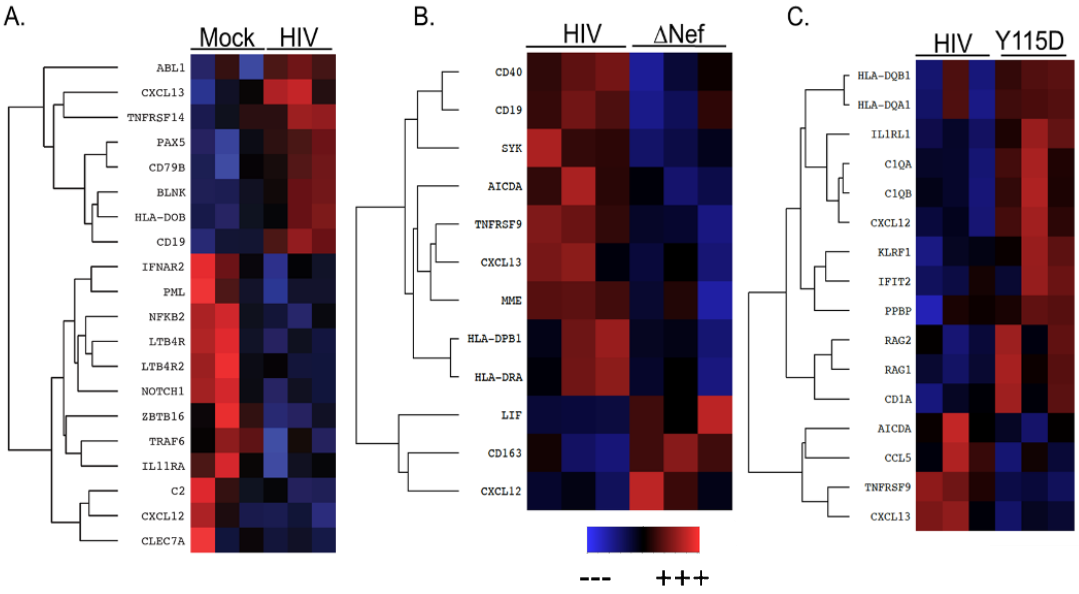


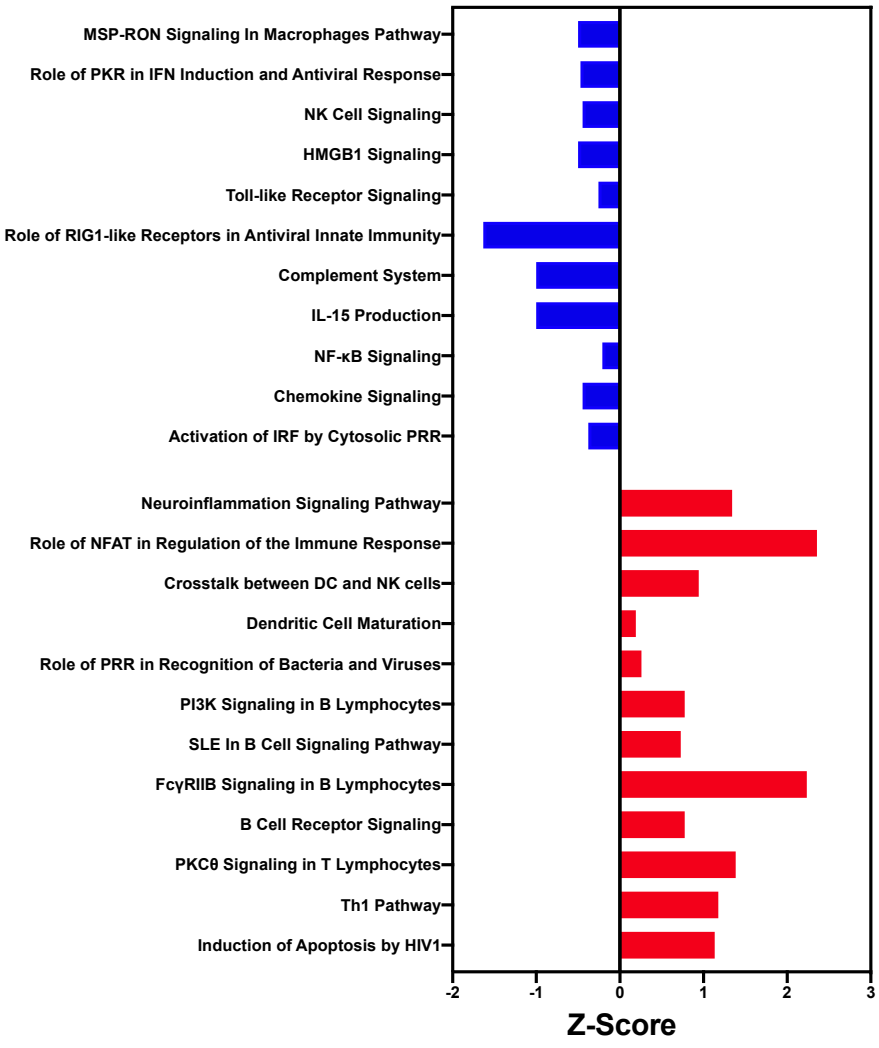
Figure 14. Nef dimerization defect abrogates HIV-induced immune dysregulation in the human spleen in BLTS-huMice.

(A-C) Nanostring analysis of the human spleen in BLTS-huMice demonstrates elevated expression of systemic immune activation, B cell hyper-activation and non-receptor tyrosine kinase genes and reduced anti-viral inhibitors genes in Wildtype HIV infection compared to (A) mock and (B-C) *nef*-defective HIV infection (*nef*-deleted-ΔNef, and *nef*-dimerization defective-Y115D; 10 ng HIVp24 per mouse) and mock inoculation. Note: blue denotes downregulation and red denote upregulation. N=3 per group, with wild-type HIV (3 mice at 7 weeks post-infection), ΔNef (2 mice at 7 weeks post-inoculation and 1 mouse at 4 weeks post-inoculation) and *nef*-dimerization defective-Y115D (2 mice at 7 weeks post-inoculation and 1 mouse at 6 weeks post-inoculation) at 4-7 weeks sacrifice-timepoints.

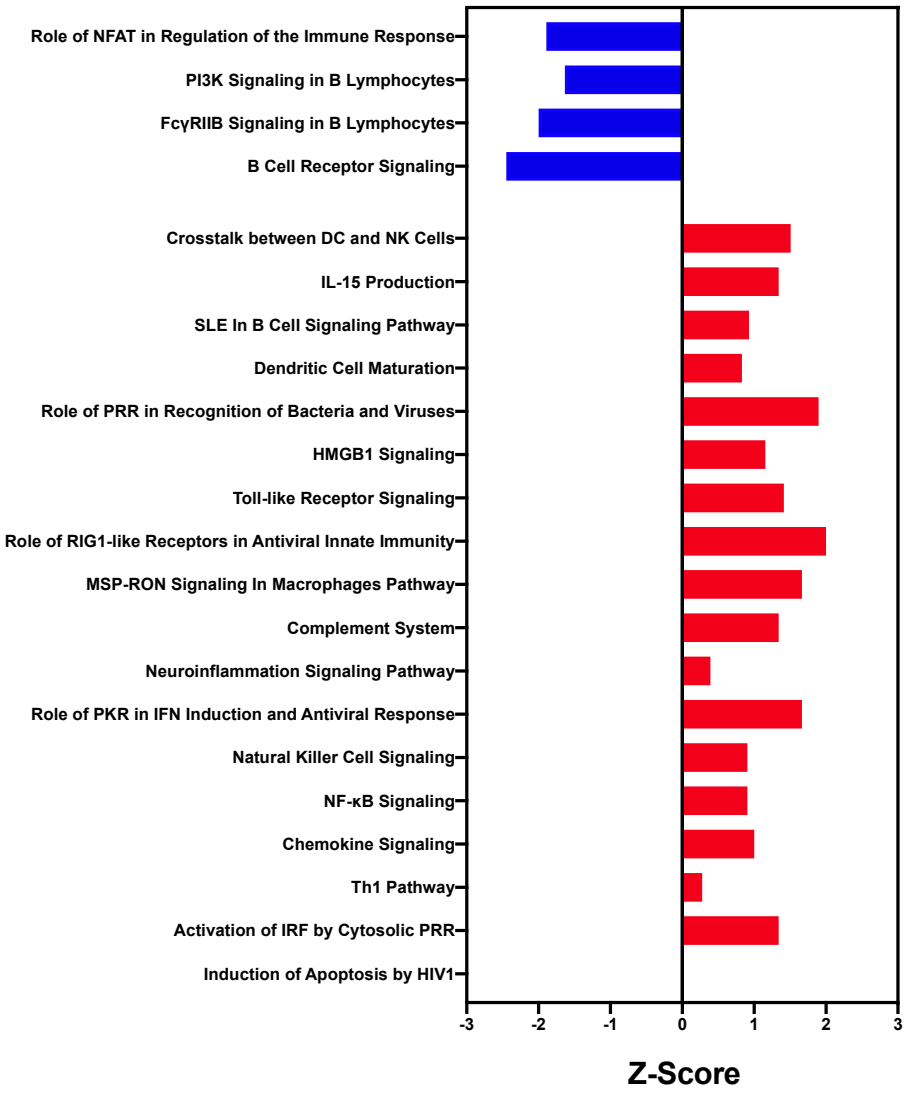
replication by activating RNA pol-II [336]. Comparative analysis of gene expression in BLTS-huMice inoculated with wild-type HIV and nef-deleted HIV showed reduced expression of the non-receptor tyrosine kinase, SYK, reduced systemic inflammation (CXCL13, HLA-DR), decreased B cell hyper-activation (CD19, AICDA), and elevated levels of anti-viral factors

(CXCL12, LIF) in the human spleen xenografts in nef-deleted HIV infection compared to wild-type HIV infection **(Fig. 14B)**. A similar analysis of the human spleen xenograft revealed reduced systemic inflammation (CXCL13); elevated levels of anti-viral factors (CXCL12, IFIT2); upregulation of genes involved in complement activation (C1Q); increased expression of RAG1/2 for the development of diverse repertoire of immunoglobulins; and elevated levels of MHC-related

A. HIV-WT Vs Mock



B. dNef Vs HIV-WT



C. HIV-Y115D Vs HIV-WT

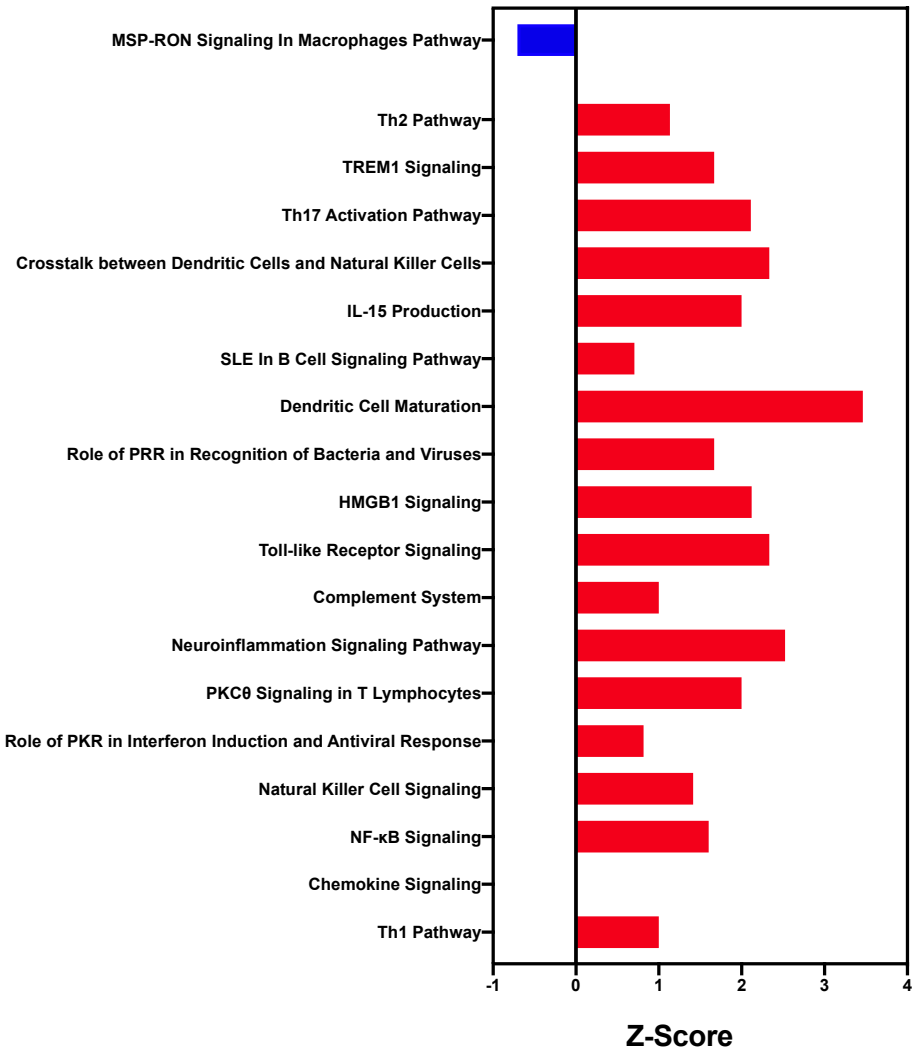


Figure 15. Nef dimerization defect abrogates HIV-induced immune dysregulatory signaling in human spleen in BLTS-huMice.

(A-C) Ingenuity Pathway Analysis of the Nanostring gene expression data demonstrates elevated systemic immune activation signaling and B cell signaling and reduced anti-viral immune signaling in wildtype HIV infection compared to mock (A). Ingenuity Pathway Analysis also demonstrates elevated anti-viral immune signaling and Th1 signaling in *nef*-defective HIV infection (*nef*-deleted-ΔNef, and *nef*-dimerization defective-Y115D; 10 ng HIVp24 per mouse) compared to wild-type HIV (B, C). Note: blue denotes downregulation and red denote upregulation. N=3 per group, with wild-type HIV (3 mice at 7 weeks post-infection), ΔNef (2 mice at 7 weeks post-inoculation and 1 mouse at 4 weeks post-inoculation) and *nef*-dimerization defective-Y115D (2 mice at 7 weeks post-inoculation and 1 mouse at 6 weeks post-inoculation) at 4-7 weeks sacrifice-timepoints.

molecule CD1A in BLTS-huMice challenged with the nef-dimerization mutant (Y115D) as compared to huMice challenged with wild-type HIV **(Fig. 14C)**. Consistent with the gene

expression profile in the human spleen in BLTS-huMice, Ingenuity pathway analysis (IPA) demonstrates elevated systemic immune activation signaling (Th1 pathway, Neuroinflammatory pathway, Induction of apoptosis by HIV) and B cell signaling (PI3K signaling, FcγRIIB signaling, B cell receptor signaling) in HIV infection **(Fig. 15A)**. It also revealed reduced anti-viral immune signaling, specifically Toll-like receptor signaling, HMGB1 signaling, NK cell signaling, RIG1-like receptors and pattern recognition receptors (PRR) signaling) and enhanced Th1 signaling and decreased B cell signaling (PI3K signaling, FcγRIIB signaling, B cell receptor signaling) in nef-defective HIV infection cohorts (ΔNef and dimerization-defective Nef-Y115D) compared to wild-type HIV **(Fig. 15B&C)**. This suggests that components of the innate immune system such as complement system, PRRs, HMGB1, NK cells, dendritic cells, and macrophages are playing an essential role in controlling viremia in nef-defective HIV infected huMice. Furthermore, deletion or mutation of nef abrogates its ability to interact with host proteins to downregulate MHC-I, which allows CTLs to recognize infected cells in the context of MHC-I and induce cytotoxicity to control viremia in BLTS-huMice.

4.4 HIV infection alters γδ T cell populations in huMice and humans

To investigate the impact of HIV infection on γδ and αβ T cell populations, we infected huMice reconstituted with a CCR5-tropic laboratory strain of HIV-1 (NL4-3). We have previously shown HIV replication kinetics in huMice [297], which is similar to HIV replication kinetics in adult humans [320]. Consistent with the previous studies, HIV RNA copies were detected in the peripheral blood of the HIV-infected huMice as early as 2 weeks post-infection **(Fig. 16A)** [267, 297]. PBMC from mock-inoculated and HIV-infected huMice were collected before and after HIV

infection for further viral load analysis, and these huMice were sacrificed for tissue collection 4 weeks after infection. We first determined the proportion of γδ T cells present in PBMC of HIV-infected and non-infected huMice before and after HIV infection. Representative flow cytometry analysis plots displaying the percentage of γδ T cells present at pre-and post-infection time points are shown in Fig. 16B. We noted an approximate 3-fold increase in total γδ T cell population in peripheral blood of both HIV-infected (p=0.03) and mock-infected huMice (p=0.001) at the post-infection time point as compared to pre-infection (Fig. 16C). Interestingly we found that post-infection peripheral blood γδ T cell frequency was approximately 2.3-fold higher HIV-infected

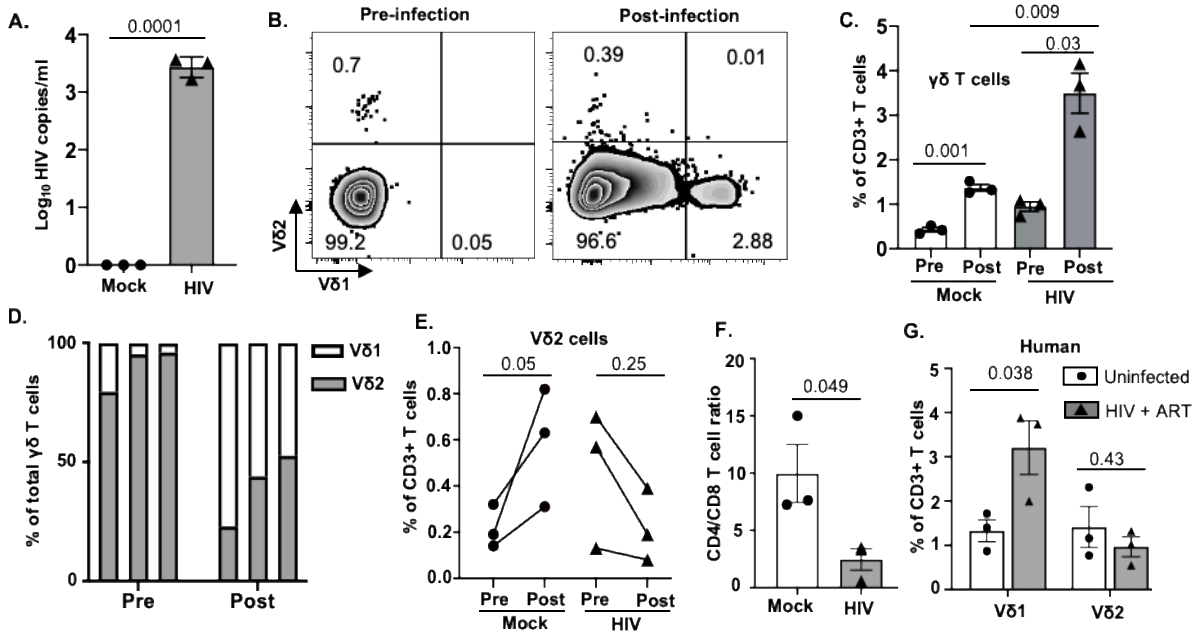


Figure 16. Peripheral blood γδ T cell number is altered in HIV-infected humans and huMice.

(A) HIV-1 replication (HIV RNA genome copies per ml) in the blood following HIV_{NL4} inoculation at 1X 10^5 IU per mouse measured by qPCR (n = 3 per group). (B) Frequency of total γδ T cells before and after HIV infection in mock and HIV-infected huMice analyzed by flow cytometry (n = 3 per group). (C) Representative flow plot showing the change in frequency of γδ T cell subsets before and after HIV infection. (D) Graphical representation of the change in frequencies of Vδ1 and Vδ2 cells within γδ population pre- and post-HIV infection. (E) Quantitation of changes in Vδ2 T cell frequency pre- and post-HIV infection in peripheral blood of HIV-infected and non-infected huMice. (F) Comparison of changes in $CD4^+/CD8^+$ T cell ratio in peripheral blood of HIV-infected and non-infected huMice analyzed by flow cytometry. (G) Frequency of Vδ1 and Vδ2 T cell subsets in peripheral blood of ART-treated HIV-infected and non-infected MACS participants analyzed by flow cytometry. Data are presented as a mean value ±

SEM. p values <0.05 were considered statistically significant. P values were determined using paired 2-tailed Student's t-test for comparing changes in γδ T cells population within the same cohort at two different time points, whereas an unpaired, 2-tailed Student's t-test was used to compare differences between 2 groups.

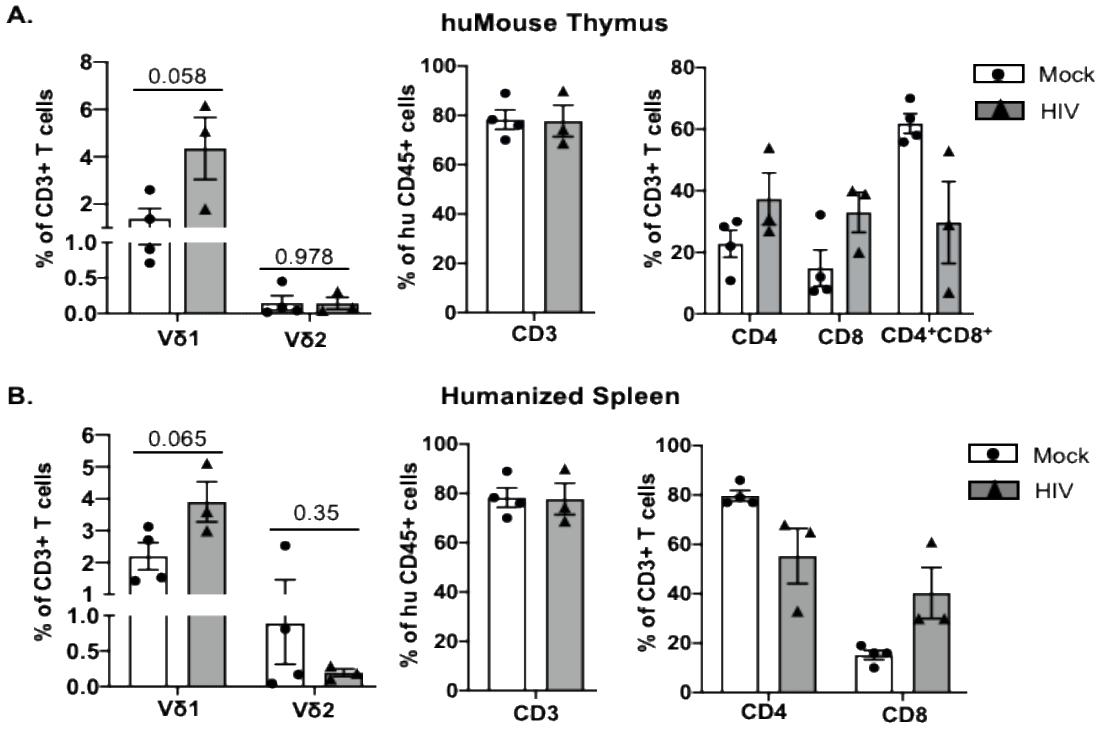


Figure 17. T cell number is altered in lymphoid tissue of HIV-infected huMice.

(A-B) Quantification of human T cell subsets, γδ T cells and αβ T cells in human thymus and humanized spleen tissue of HIV-infected (n=3) and non-infected (n = 4) huMice at 22 weeks post-transplantation. Data are presented as mean values ± SEM. P values <0.05 were considered statistically significant as determined using an unpaired, 2-tailed Student's t-test.

in huMice than non-infected huMice [p=0.009] (**Fig. 16C**). This suggests that HIV infection elevates the total γδ T cell population in huMice.

Next, we determined the impact of HIV infection on the frequency of γδ T cell subsets. We found that HIV infection leads to an approximate 2-fold depletion of peripheral blood Vδ2 T cells of huMice during early infection, while the Vδ1 T cell frequency increased approximately 3-fold (**Fig 16D**). The observed decrease of Vδ2 T cells in huMice with HIV-infection was not seen in the mock-infected mice, which actually showed an increase in Vδ2 T cell frequency (**Fig. 16E**),

suggesting that HIV infection causes the selective elimination of the Vδ2 cells. Furthermore, depletion of peripheral blood $CD4^+$ T cells of the HIV-infected huMice results in a dramatic decrease in the $CD4^+/CD8^+$ T cell ratio (**Fig. 16F**). These results are consistent with what has been previously reported in human γδ T cell studies [337, 338], and similar to the trend we find among HIV-infected MACS participants on ART who display higher frequencies of Vδ1 cells and lower percentages of Vδ2 cells (**Fig. 16G**).

In HIV-infected humans, lymphoid tissues are known to be sanctuaries for the latent HIV reservoir during ART [339]. Therefore, we assessed the impact of HIV infection on the lymphocytes derived from lymphoid tissues of huMice by flow cytometry analysis. Although not statistically significant, we observed an approximately 3-fold increase in the frequency of Vδ1 T cells in the human thymus ($p = 0.058$), and approximately 2-fold increase in the humanized spleen of HIV-infected huMice ($p = 0.065$) when compared to respective tissues from non-infected huMice (**Fig. 17A**). This suggests that the frequency of Vδ1 T cells is increased in lymphoid tissue of huMice during HIV infection. We did not find a significant difference between the Vδ2 T cell population frequencies derived from the lymphoid tissues of HIV-infected or mock-infected huMice. Besides γδ T cells, we found approximately a 2-fold increase in the proportion of cytotoxic CD8+ T cells derived from thymus and humanized spleen tissue of HIV-infected huMice as compared to the mock-inoculated huMice, suggesting a rapid proliferation of cytotoxic T cells in response to HIV infection (**Fig. 17B**).

4.5 Selective *in vitro* expansion of Vδ2 T cells from HIV-infected and non-infected huMice

We cultured leukocytes derived from lymphoid tissue of huMice (n = 6), peripheral blood of ART-suppressed HIV-infected (n = 4), and age-matched uninfected MACS participants (n = 5)

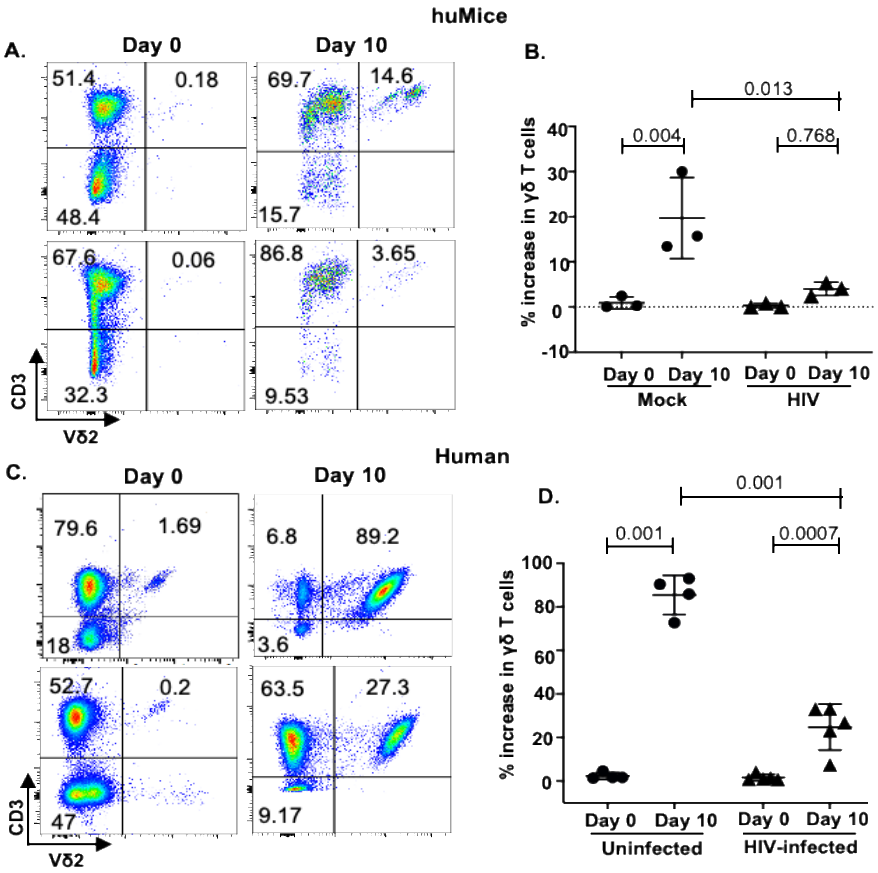


Figure 18. HIV infection impairs the *in vitro* expansion of Vδ2 T cells.

(A-B) Splenocytes from HIV-infected/non-infected huMice were cultured in the presence of zoledronate, IL-2, and allogenic uninfected monocytes (n= 3 mice per group). (C-D) Flow plots representing *in vitro* expansion of Vδ2 cells from HIV-infected and non-infected individuals in the presence of zoledronate and IL-2. Expansion of Vδ2 cell frequency was significantly higher in non-infected donors (n=4) compared to HIV-infected donors (n=5). Data are presented as mean values ± SEM. P values <0.05 were considered statistically significant as determined using a 2-way ANOVA test.

and stimulated them with the combination of ZOL and rhIL-2 to selectively promote the in vitro expansion of Vδ2 cells. The basal percentage of Vδ2 cells within the $CD3^+$ population of lymphocytes were analyzed by flow cytometry, which revealed a range of inter-individual

differences among uninfected donors (1.2% - 2.2%), ART-suppressed HIV-infected individuals (0.5% - 1.2%), and huMice (0.2% - 1%). Initially, when we cultured Vδ2 T cells from the peripheral blood or the lymphoid tissues of huMice in the presence of ZOL and rhIL-2, we observed minimal expansion of Vδ2 T cells, but it was not optimal. Next, we supplemented the cultures with allogenic monocytes from healthy individuals and obtained higher expansion of Vδ2 T cells. Our results show that Vδ2 T cell expansion from lymphoid tissues of non-infected huMice after 10 days was approximately 4-fold higher than HIV-infected huMice (p=0.013) (**Fig. 18B**). Similarly, we expanded Vδ2 T cells from HIV-infected and non-infected MACS participants and found that Vδ2 T cell expansion was approximately 3-fold higher in non-infected individuals than those with HIV-infection (p=0.001) (**Fig. 18D**). This suggests that HIV infection not only depletes the frequency of Vδ2 T cells *in vivo* but it also severely impacts the ability of these cells to expand *in vitro* in response to ZOL and rhIL-2 treatment.

4.6 The phenotype of *ex-vivo* expanded Vδ2 T cells

The phenotype of expanded Vδ2 cells after 10 days of exposure to ZOL and rhIL-2 was analyzed in a subgroup of HIV-infected and uninfected MACS participants and HIV-infected/uninfected huMice by measuring the expression of markers of activation and differentiation by flow cytometry **(Fig. 19A)**. Surface expression of the inhibitory receptor PD-1 was observed in a mean of 78% and 45% on the cultured Vδ2 cells derived from HIV-infected and uninfected huMice, respectively (p=0.04). Similarly, the mean percentage of Vδ2 cells expressing PD-1 from HIV-infected and uninfected human donors was respectively 40% and 20% (p=0.001) **(Fig. 19B)**. The activation markers CD69 and CD25 were co-expressed on a mean of 80% and

65% of the Vδ2 cells cultured from HIV-infected and uninfected huMice, respectively. Similarly, CD69 and CD25 co-expression was observed in a mean of 50% and 25% of the Vδ2 T cells from HIV-infected and uninfected humans, respectively **(Fig. 19C)**. Together, these results suggest that

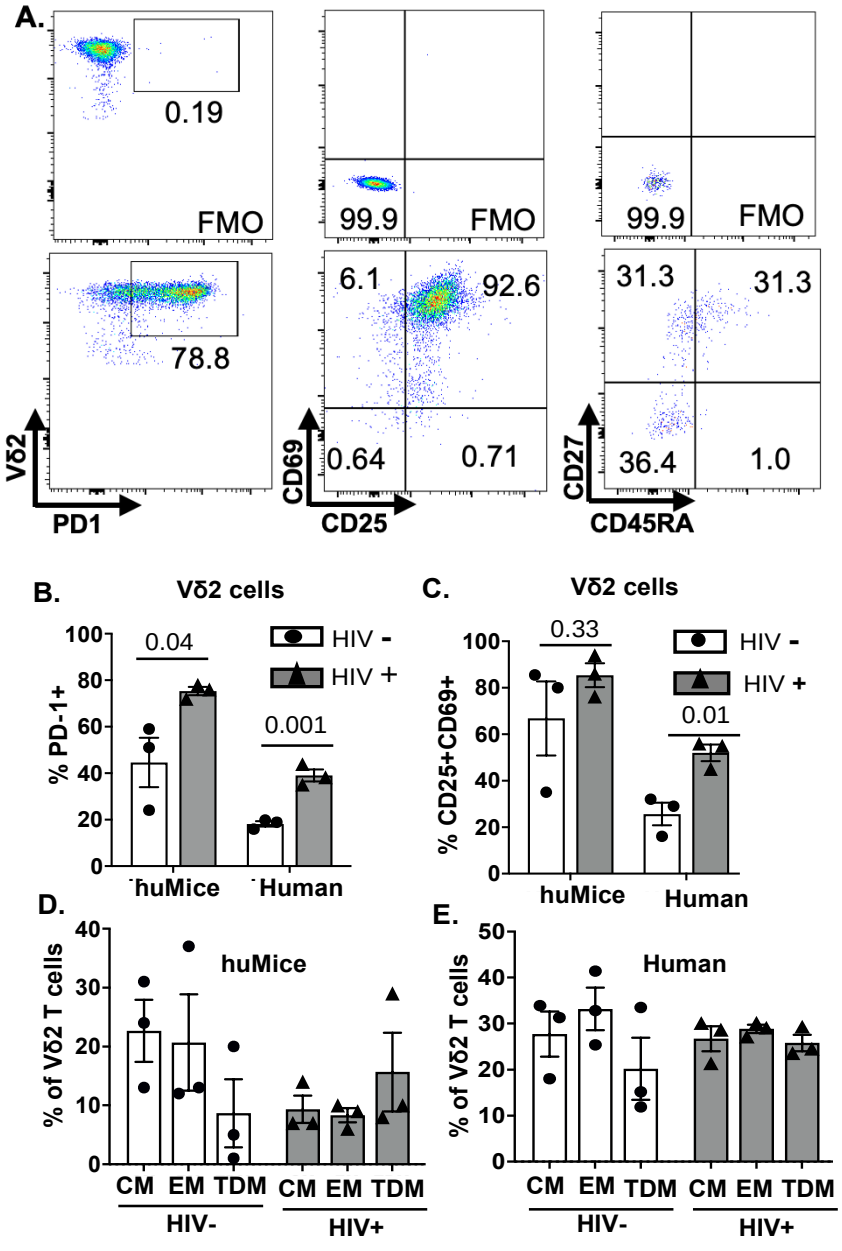


Figure 19. Phenotype characterization of cultured Vδ2 cells.

The phenotype of Vδ2 cells from 6 huMice and 6 HIV-infected/non-infected donors after the expansion was analyzed by flow cytometry. (A) Representative flow cytometry analysis of expanded Vδ2 cells from lymphoid tissue of huMice expressing activation, inhibitory, and differentiation markers. (B) Expression of the checkpoint inhibitory marker PD-1 on Vδ2 cells expanded from HIV-infected and non-infected huMice and humans. (C) Dual expression of activation

markers CD69 and CD25 on Vδ2 cells expanded from HIV-infected and non-infected huMice and humans. (D) Percentage of Vδ2 cells defined as central memory (CM) ($CD45^{-}CD27^{+}$), terminally differentiated (TDM) ($CD45^{+}CD27^{-}$) and effector memory (EM) ($CD45^{-}CD27^{-}$) derived from HIV-infected and non-infected huMice. (E) Percentage of Vδ2 cells derived from HIV-infected and non-infected human MACS participants defined as having EM, CM, TDM phenotypes. Data are presented as mean values ± SEM. P values were determined using 2 tailed unpaired t-tests between the 2 groups.

the expression of activation markers on Vδ2 cells expanded in vitro are slightly higher in those derived from HIV-infected humans and huMice than from their uninfected counterparts. We also evaluated the differentiation status of the cultured Vδ2 cells based on memory cell phenotypes defined as follows: (CM) central memory (CD45–CD27+), (TDM) terminally differentiated (CD45+CD27–-) and (EM) effector memory (CD45–CD27–). Although not statistically significant, we noted an increase in the TDM phenotype and a decrease in the CM and EM phenotypes in the in vitro expanded Vδ2 T cells derived from HIV-infected huMice compared to the Vδ2 cells cultured from uninfected huMice **(Fig. 19D)**. However, in humans, we found an approximately equal distribution (20-30%) of EM, CM, TDM phenotypes between HIV-infected and non-infected individuals **(Fig. 19E)**.

4.7 Adoptive transfer of Vδ2 T cells enhances HIV infection in huMice

Many in vitro studies have demonstrated a protective role of γδ T cells against HIV infection [9, 305, 306]. Therefore, we tested the impact of Vδ2 cells on HIV infection in the in vivo huMice model through the adoptive transfer of ex vivo expanded allogeneic Vδ2 cells. As discussed above, in vitro expansion of Vδ2 T cells from HIV-infected individuals was not optimal. We overcame this barrier in our adoptive transfer experiment by utilizing Vδ2 T cells expanded from allogeneic non-infected individuals. A similar strategy was previously demonstrated to be

safe and effective in humans [340]. Moreover, it is therapeutically relevant because Vδ2 T cells lack functional MHC-restriction and therefore pose a minimal risk for developing graft-versus-host complications [341]. They may however serve as targets for an allogeneic response by the engrafted immune cells. HuMice were grouped into 2 different cohorts: one cohort received only

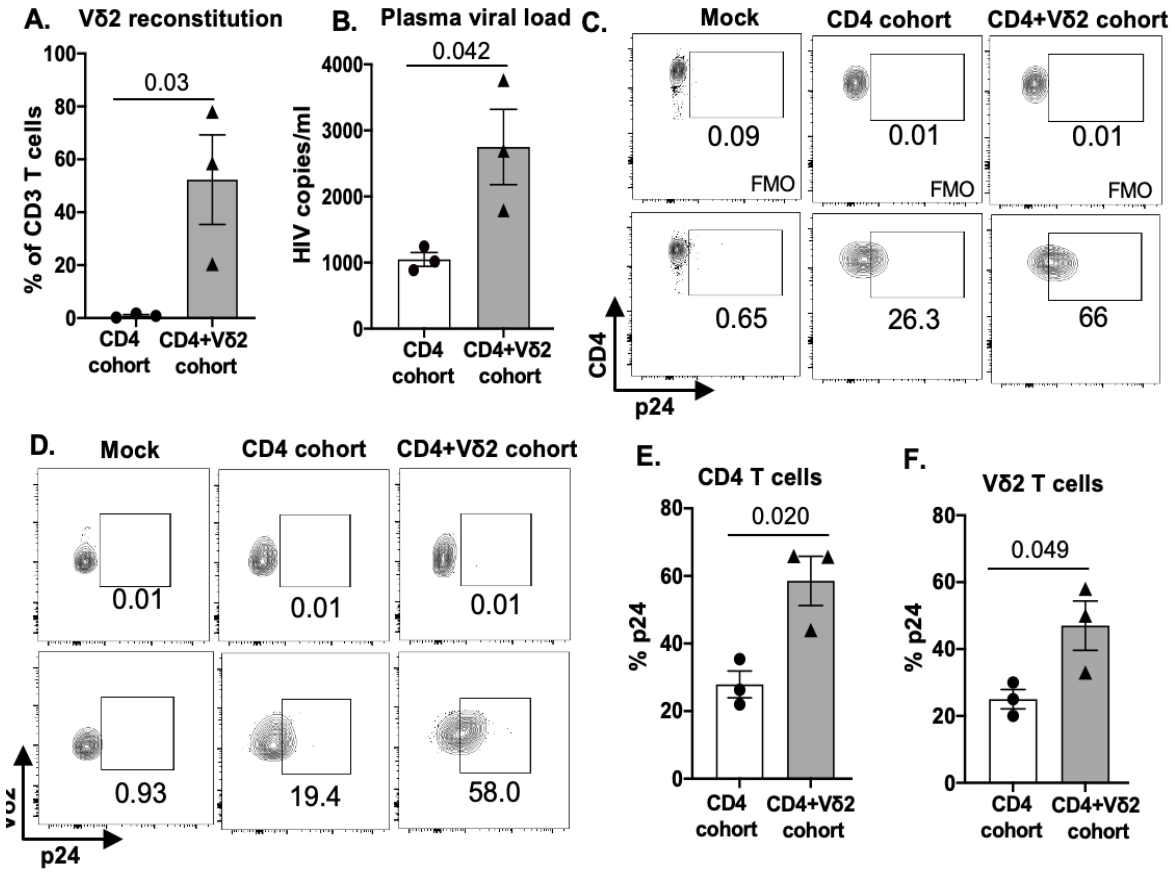


Figure 20. Adoptive transfer of Vδ2 T cells exacerbates HIV infection in huMice.

A) Vδ2 cell number significantly increased post-adoptive transfer in peripheral blood of huMice (n=3 per group); analyzed by flow cytometry. (B) HIV viral load increased significantly in plasma of Vδ2+CD4-treated huMice as compared to CD4-treated huMice; measured 2 weeks post-adoptive transfer by qPCR (n=3 per group). (C & E) Representative flow cytometry analysis of peripheral blood $CD4^+$ T cells and Vδ2 cells that are expressing HIV p24 respectively. (D & F) HIV p24 is significantly higher in peripheral blood $CD4^+$ and Vδ2 T cells of huMice that received CD4+Vδ2 treatment as compared to the huMice that received only $CD4^+$ T cells treatment respectively (n=3 per group). Data are presented as mean values ± SEM. P values were determined using 2 tailed paired t-test within the treatment groups.

activated CD4+T cells from HIV-infected human donor (CD4-only cohort), while the other cohort received activated CD4+ T cells from HIV-infected human donor as well as the cultured activated allogenic Vδ2 cells from an HIV non-infected human donor (CD4+Vδ2 cohort). Reconstitution of human Vδ2 and CD4+ T cells in the peripheral blood of huMice was examined via flow cytometry two weeks after the adoptive transfer procedure. We found that a mean of 50% of all T cells were Vδ2 T cells in the peripheral blood of CD4+Vδ2 cohort, whereas less than 1% of T cells were Vδ2 T cells in CD4 only cohort ($p=0.03$) **(Fig. 20A)**, which indicated successful engraftment of human Vδ2 T cells in the huMice. Next, we confirmed HIV replication in the plasma of huMice by qPCR. Surprisingly, we observed a viral load in the CD4+Vδ2 cohort was approximately 2-fold higher than the CD4-only cohort **(Fig. 20B)** ($p=0.042$). Hypothesizing that this increase in viral load could be due to HIV-infection of the adoptively transferred Vδ2 T cells, we decided to analyze the CD4+ and Vδ2 T cell subsets in the peripheral blood of both cohorts at 2 weeks post-adoptive transfer. Representative flow cytometric plots of HIV p24 from both cohorts are shown in **Figure 20C &D**. Again, we observed an approximate 2-fold higher presence of HIV p24 in CD4+ T cells ($p=0.020$) and Vδ2 T cell ($p=0.049$) in the CD4+Vδ2 cohort of huMice compared to the reference CD4-only cohort **(Fig. 20E&F)**. Therefore, our results indicate that the adoptive transfer of Vδ2 T cells exacerbated HIV infection.

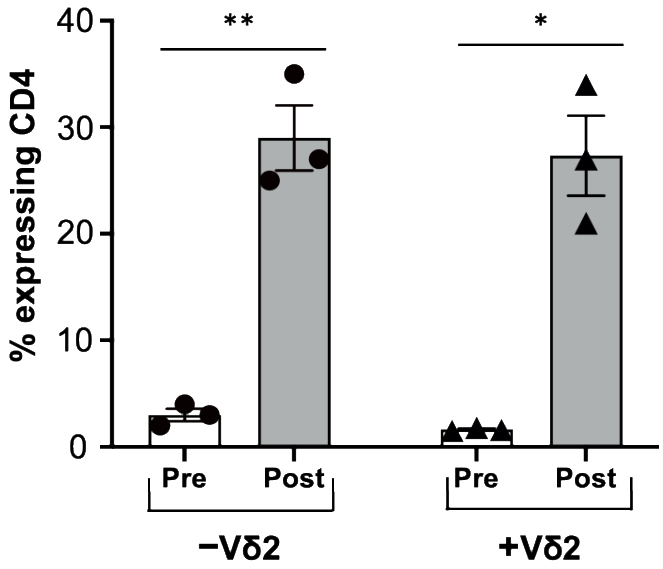


Figure 21. Induction of CD4 expression on Vδ2 T cells *in vivo* during HIV infection.

$CD4^+$ T cells from an HIV-infected MACS participant were administered to huMice with or without co-transfer of *in vitro* activated Vδ2 T cells. CD4 expression on human Vδ2 T cells from the huMice was measured by flow cytometry analysis pre-and post- (2 weeks) cell transplant. Data are presented as mean values ± SEM. P values were determined using 2 tailed paired t-test within the treatment groups.

Despite low or lack of CD4 receptor expression on Vδ2 T cells, our in vivo data suggest that these cells can indeed be targets of HIV infection. This is in accordance with a previous study from Sarabia et al, which reported that resting Vδ2 cells act as a reservoir for latent HIV infection [342]. We posited that HIV infection could impact the phenotype of Vδ2 T cells to make them more susceptible to direct infection. Since Vδ2 T cells already express high levels of the CCR5 co-receptor, we examined whether the expression of the CD4 receptor on Vδ2 T cells was induced on this cell type during HIV infection. Prior to adoptive transfer, less than 5% of endogenous and in-vitro cultured Vδ2 T cells expressed the CD4 receptor, but at 2 weeks after adoptive transfer, we indeed detected a mean of 30% of Vδ2 T cells expressing the CD4 receptor in both the cohorts **(Fig. 21)**. Contrary to the previous reports [9, 343] highlighting the protective function of Vδ2 T cells in controlling HIV infection *in vitro,* our result suggests that HIV infection can drive CD4

expression on Vδ2 T cells *in vivo*, priming them to become targets for HIV infection and contributors to viral dissemination.

5.0 Discussion

The role of HIV accessory protein Nef has been implicated in HIV replication, immune dysregulation, and immunodeficiency [12, 324, 325, 344-346]. Although humans and monkeys infected with nef-defective HIV and SIV, respectively, have provided insights into the pathogenic and immune inhibitory role of Nef [303, 304, 347], humanized rodents provide the ideal small animal model for *in vivo* mechanistic studies of HIV-immune system interactions [297, 317, 318]. Previous studies in the BLT-huMouse model confirmed the role of HIV Nef in mediating HIV-induced CD4+ T cell depletion [348]. However, nef-defective HIV viremia in the BLT-huMouse model was only delayed for a few weeks (at low doses of inoculum) or reduced (at high doses of inoculum) [348], suggesting that an improved human immune system in huMice could completely mitigate nef-defective HIV viremia.

We recently incorporated a human secondary lymphoid tissue (the spleen xenograft) in the BLT-huMouse model to create the BLTS-huMouse model. The BLTS-huMouse model enabled T cell development and education in the human thymic microenvironment [349] and antigen-specific T cell expansion in the human splenic microenvironment [350]. Furthermore, the BLTS-huMouse model is reconstituted with various human innate and adaptive immune cells, including macrophages, T, and B cells [297]. Here, we demonstrate the functionality of the antigen-presenting cells and T lymphocytes cells in BLTS-huMice, suggesting that those cells can enable an effective antiviral T cell immune response. We previously demonstrated that the BLTS-huMouse model supports HIV replication [297]. HIV viremia kinetics in BLTS-huMice mimic patterns in HIV-infected adult humans, such as the partial control of viremia after peak viremia 2-

weeks post-infection [297]. Here, we employed the BLTS-huMouse model to determine the role of Nef expression and homodimerization in HIV viremia and associated immune dysregulation.

First, we demonstrated that nef-defective HIV and wild-type HIV exhibit similar infectivity and replication at high inocula in a tissue culture model (activated CD4+ T cells) and an *in vivo* model (PBL-huMice containing activated CD4+ T cells) of HIV infection and replication. In contrast, nef-deleted HIV and wild-type HIV exhibit divergent viremia outcomes at high inocula in BLTS-huMice; the nef-deleted HIV group remains aviremic for 20-weeks post-inoculation, while the wild-type HIV group exhibits high viremia by 2 weeks post-infection followed by a plateau in viral load up to 6 weeks. Furthermore, HIV viremia was associated with CD4+ T cell depletion in the blood. Both nef-defective HIV and wild-type HIV elicited an initial burst of human cytokine responses in the BLTS-huMice that was higher in the wild-type HIV infection. This elevated cytokine response in wild-type HIV infection is likely due to the high viremia, compared to the aviremic, nef-defective HIV. Notably, the elevation of human cytokine levels suggests a human immune response to both nef-defective HIV and wild-type HIV, with an adequate response elicited by nef-defective HIV and an ineffective response elicited by wild-type HIV.

Previous studies have demonstrated that homodimerization is essential for Nef-mediated enhancement of HIV infectivity, replication, and immune dysregulation in tissue culture models [12, 346]. Here, we show that Nef dimerization enhances HIV viremia and associated T cell activation (i.e., elevated CD25 and HLA-DR expression and PD1 expression) and general immune dysregulation in a small animal model with a robust human-immune system. The level of disruption in Nef dimerization activity was consistent with the viremia outcomes. Additionally, gene expression analysis suggests that functional Nef promotes the expression of nRTK such as

Syk, general immune dysregulation (including B cell dysregulation), and impairs the expression of anti-viral factors *in vivo*.

In summary, we report for the first time that Nef dimerization mediates enhancement of HIV viremia and promotes T cell activation and general immune dysregulation *in vivo* in the BLTS-huMouse model. T cell activation, and widespread immune dysregulation are believed to be significant drivers in maintaining the HIV reservoir. Furthermore, recent evidence demonstrates that Nef is expressed in HIV-infected individuals receiving highly active-ART, suggesting Nef could play a significant role in maintaining the HIV reservoir by supporting low-level infection and replication and dysregulating the anti-viral immune response[351]. The BLTS-huMouse model supports an ART-mediated HIV reservoir [297], thus providing an ideal model for investigating the impact of Nef-inhibitors (including inhibitors targeting Nef dimerization) in HIV cure strategies.

γδ T cells are the first line of defense against many pathogens, but their frequency and functions are severely altered in the setting of many infectious diseases, including HIV. Despite long-term ART and viral control, γδ T cells do not reconstitute in HIV-infected individuals to their levels set prior to infection [338]. However, in HIV elite controllers, Vδ2 T cell numbers are maintained at normal levels throughout infection, implying that Vδ2 T cells play an important role in HIV infection and control. Therefore, a better understanding of their function during HIV infection will be necessary in order for them to be effectively utilized or targeted for therapeutic benefit. While prior studies have demonstrated the protective effect of γδ T cells against HIV infection in vitro [9, 305, 306], there is a paucity of information available and a gap in knowledge regarding their therapeutic potential *in vivo*.

In this study, we offer the first evidence that clinical trends of γδ T cell subpopulations before and after HIV infection can be modeled in huMice. Immunodeficient NSG mice exhibited robust reconstitution of human immune cells, including γδ T cells, by 12 weeks post-engraftment of CD34+ human fetal liver cells and thymic tissues. Flow cytometric analysis of human T cell subsets revealed that Vδ1/Vδ2 T cell ratios and CD4+/CD8+ T cell ratios in both the blood and lymphoid tissues of healthy huMice were comparable to that seen in healthy humans. Furthermore, we observed high levels of viremia two weeks following infection, an associated depletion of Vδ2 T cells, and an expansion of Vδ1 T cells in the peripheral blood of these animals. These features suggest that huMice can overcome some of the translational limitations that exist in non-human primate models of SIV, which include unremarkable changes in Vδ1/Vδ2 T cell ratios otherwise common to human HIV infection. Our study demonstrating the *in vivo* reconstitution of Vδ2 T cells in the huMouse model also provides a proof-of-concept and basis for the design of future in vivo studies for further evaluating the role of human γδ T cells in the setting of HIV infection as well as other chronic diseases such as cancer.

Current HIV cure strategies center on utilizing the effector functions of conventional CD8+ cytotoxic T cell lymphocytes (CTL) to kill the HIV-infected cellular reservoir following the induction of latency reversal. Unfortunately, the need to specifically stimulate or target the activation of autologous HIV-antigen specific autologous CD8+ T cells ex vivo or in vitro on an individual MHC/peptide-specific level, and the existence of HIV CTL escape variants within the latent reservoir has challenged the progress of this approach [352, 353]. γδ T cells offer an attractive alternative to CTL as a potential therapeutic tool to mediate anti-HIV effector functions. Their lack of MHC-restriction may provide added benefits by raising the threshold for HIV to achieve immune escape. Moreover, since they pose a reduced risk of inducing allogeneic graft

rejection, they may be considered for application in allogeneic immunotherapy settings. A previous study has shown that γδ T cells mediate inhibition of HIV replication [190], but the natural scarcity of γδ T cells in tissues and circulation indicates that these cells would likely need to be expanded ex-vivo in order for them to have the intended therapeutic effect. Although there are numerous in vitro protocols for expanding γδ T cells from bulk PBMC, two major approaches can be considered for targeting γδ T cells for clinical translation. First, both ZOL and rhIL-2 can be administered to directly increase the proliferation of endogenous Vδ2 T cells [220]. The other approach would be ex-vivo activation and expansion of Vδ2 T cells for adoptive therapy. In the HIV setting, this approach is limited by the substantial loss of Vδ2 T cells that occurs during the early stages of the infection cycle, which fail to fully recover after initiation of ART. An alternative would be to harvest Vδ2 T cells from healthy donors and expand them in vitro using ZOL and rhIL-2 for allogeneic delivery, as has been previously reported in human cancer clinical trials [340, 354] and non-human primate models [355]. One of these cancer trials demonstrated that the adoptive transfer of haploidentical expanded Vδ2 T cells from relatives of cancer patients was safe and effective for achieving meaningful responses [340]. We attempted to culture and expand Vδ2 T cells derived from PBMC as well as lymphoid tissue of huMice using ZOL and rhIL-2. Unfortunately, while we were able to expand these huMouse derived cells in vitro, we were not able to collect and generate an adequate number to carry out *in vivo* studies using this method. However, when we supplemented the cultures with allogeneic monocytes from healthy individuals to enhance ZOL-induced phosphoantigen presentation, we achieved a 20-fold increase in Vδ2 T cell expansion. Importantly, this was the first reported evidence that Vδ2 T cells derived from the lymphoid tissue of huMice can indeed be expanded in vitro.

Our study examined the therapeutic potential of adoptively transferred Vδ2 T cells in HIV infection of BLT huMice. Although previous *in vitro* studies described the protective effect of γδ T cells against HIV infection [9, 305, 306], we did not see a therapeutic benefit with the delivery of Vδ2 T cells in huMice. In fact, treatment with the activated γδ cells resulted in a significant enhancement of HIV infection as compared to the HIV-infected huMice that were not treated with the Vδ2 T cells. Our results demonstrate that during HIV infection the Vδ2 T cells can transiently upregulate surface expression of CD4 and served as viable targets for active HIV infection and dissemination. These findings raise more questions about the role of γδ T cells in the initial sequelae of HIV infection and their potential contribution to the HIV cellular reservoir as has been previously reported [342].

To our knowledge, this is the first report demonstrating that functional human γδ T cells can be robustly reconstituted in a huMice model. This small animal model provides a platform for future mechanistic studies to explore interactions between HIV and T cell subsets and, more broadly, for *in vivo* evaluation of γδ T cells and γδ T cell-based therapies in the setting of various human diseases.

6.0 Implications to Public Health

The development of antiretroviral therapy (ART) has played a significant role in controlling the human immunodeficiency virus (HIV) epidemic. ART inhibits viral replication and prevents progression to acquired immunodeficiency syndrome (AIDS), resulting in decreased overall mortality [356, 357]. Unfortunately, despite highly active-ART that reduces the viral genome load to undetectable levels in the blood, HIV persists in cellular reservoirs, abrogates the anti-HIV immune response, and promotes immune dysregulation [356-358]. Indeed, clinical data reveals that the HIV-accessory protein negative factor (Nef) remains detectable in the blood and blood cells of individuals living with ART-controlled HIV, suggesting that Nef promotes immune dysregulation during ART [351, 359].

Individuals infected with HIV containing defects in the *nef* gene exhibit reduced viral load and delayed AIDS progression [304]. Some individuals infected with *nef*-defective HIV exhibit a negligible viral load without needing ART [304]. However, a few individuals infected with *nef*-defective HIV develop AIDS after years of naturally controlling the virus [304]. Data from this subset of HIV-infected individuals established Nef as a pathogenic factor in HIV infection [303, 304]. However, the mechanisms by which Nef promotes viremia and immune dysregulation *in vivo* remain unresolved [331, 345].

In this study, we reported here for the first time that Nef dimerization mediates enhancement of HIV viremia and promotes T cell activation and general immune dysregulation *in vivo* in the BLTS-huMouse model. T cell activation, and widespread immune dysregulation are believed to be significant drivers in maintaining the HIV reservoir. Furthermore, recent evidence demonstrates that Nef is expressed in HIV-infected individuals receiving highly active-ART,

suggesting Nef could play a significant role in maintaining the HIV reservoir by supporting low-level infection and replication and dysregulating the anti-viral immune response [351]. Therefore, Nef dimerization domain may represent a novel and effective target for ART development. We have optimized the BLTS-huMouse model which supports an ART-mediated HIV reservoir [297], thus providing an ideal model for investigating the impact of Nef-inhibitors (including inhibitors targeting Nef dimerization) in HIV cure strategies.

In the present study, we also provide the first reported phenotypic and functional characterization of human γδ T cells in a huMouse and evaluated how they are impacted by HIV infection *in vivo*, and we assess their therapeutic potential following adoptive cell transfer. We demonstrate that the huMouse model recapitulates the clinical changes in Vδ1 and Vδ2 T cell frequencies in the peripheral blood reported during natural HIV infection, providing for the first time an *in vivo* model relevant for studying human γδ T cell biology. When tested therapeutic impact on HIV infection, the adoptive transfer of cultured allogenic Vδ2 T cells into huMouse implanted with $CD4^+$ T cells from HIV-infected individuals on ART surprisingly resulted in enhancement rather than control of HIV replication. This escalation in viral production was accompanied with a marked increase in detection of HIV p24 positive Vδ2 T cells isolated from the huMice following adoptive transfer, suggesting that the Vδ2 T cells served as targets for HIV infection. These findings raise more questions about the role of γδ T cells in the initial sequelae of HIV infection and their potential contribution to the HIV cellular reservoir as has been previously reported [342]. This small animal model provides a platform for future mechanistic studies to explore interactions between HIV and T cell subsets and, more broadly, for *in vivo* evaluation of γδ T cells and γδ T cell-based therapies in the setting of various human diseases.

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