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HTLV-1 infection and neuropathogenesis in the context of Rag1^{-/-}γc^{-/-} (RAG1-hu) and BLT mice

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Abstract

To date, the lack of a suitable small animal model has hindered our understanding of Human T-cell lymphotropic virus (HTLV)-1 chronic infection and associated neuropathogenesis defined as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The host immune response plays a critical role in the outcome of HTLV-1 infection, which could be better tested in the context of humanized (hu) mice. Thus, we employ here the Balb/c-Rag1^{-/-}γc^{-/-} or Rag1 as well as Bone marrow-Liver-Thymic (BLT) mouse models for engraftment of human CD34⁺ hematopoietic stem cells. Flow cytometry and histological analyses confirmed reconstitution of Rag1 and BLT mice with human immune cells. Following HTLV-1 infection, proviral load (PVL) was detected in the blood of Rag-1 and BLT hu-mice as early as 2 weeks post-infection (wpi) with sustained elevation in the subsequent weeks followed by Tax expression. Additionally, infection was compared between adult and neonatal Rag1 mice with both PVL and Tax expression considerably higher in the adult Rag1 mice as compared to the neonates. Establishment of peripheral infection led to lymphocytic infiltration with concomitant Tax expression and resulting myelin disruption within the central nervous system of infected mice. In addition, up-regulation in the expression of several immune checkpoint mediators such as programmed cell death-1 (PD-1), T-cell Ig and ITIM domain (TIGIT), and T cell Ig and mucin domain-3 protein (Tim-3) were observed on CD8⁺ T cells in various organs including the CNS of infected hu-mice. Collectively, these studies represent the first attempt to establish HTLV-1 neuropathogenesis in the context of Rag-1 and BLT hu-mice as potential novel tools for understanding HTLV-1 neuropathogenesis and testing of novel therapies such as immune checkpoint blockade in the amelioration of chronic HTLV-1 infection.

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Keywords

HTLV-1; chronic viral infection; humanized mice; RAG1; BLT

Introduction

Human T-cell lymphotropic virus (HTLV)-1 belongs to the delta-retroviruses family of retroviruses and was the first human retrovirus to be discovered (Hajj et al. 2012; Lairmore et al. 2005; Yoshida 2005). HTLV-1 is known to be the causative agent of diseases like adult T-cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) amongst other clinical outcomes (Banerjee et al. 2010; Hajj et al. 2012; Lairmore et al. 2005; Nagai and Osame 2003). While ATL is a monoclonal malignancy of mature activated CD4⁺ T cells, HAM/TSP is an inflammatory neurological disease characterized by lesions in the central nervous system (CNS) (Banerjee et al. 2010; Fuzii et al. 2014; Hajj et al. 2012; Nagai and Osame 2003). The characteristic feature of HAM/TSP is spastic paresis along with urinary disturbances and high antibody titers to HTLV-1 in serum and CSF. In most patients, the progression of the disease is gradual. Several inflammatory lesions in both the brain and spinal cord as well as spinal cord atrophy have been observed during biopsies of HAM/TSP patients (Izumo 2010). HTLV-1 infects approximately 15–20 million people worldwide with endemic areas in Brazil, the Caribbean, Japan, the Middle East, Eastern Europe and inter-tropical Africa (Amorim et al. 2014; Cabral et al. 2012; Hajj et al. 2012; Morgan 2011; Oliere et al. 2011). While most of the individuals remain asymptomatic throughout life, about 2–6% will develop ATL after a prolonged latency period of 20–40 years (Banerjee et al. 2010; Hajj et al. 2012). There is no effective treatment or vaccine for either ATL or HAM/TSP.

HTLV-1 pathogenicity is mainly a result of infection of CD4⁺ T cells but CD8⁺ T cells, monocytes/macrophages, and dendritic cells (DCs) may also be infected (Amorim et al. 2014). The infection is characterized by abnormal growth and proliferation of T cells brought about by the viral regulatory protein Tax that is encoded by the HTLV-1 genome in addition to other genes that control retroviral replication (Hajj et al. 2012; Hyun et al. 2015). Evidence suggests that high proviral load (PVL), constant expression of Tax, and an associated cell-mediated immune response play a critical role in disease development. Through activation of transcription factors such as NF- κ B, Tax brings about the increased expression and secretion of inflammatory cytokines namely tumor necrosis factor (TNF), Interferon (IFN)- γ , interleukin (IL)-2, granulocyte macrophage colony-stimulating factor (GM-CSF), chemokine (C-X-C motif) ligand 9 (CXCL9) and CXCL10 (Hajj et al. 2012; Yari et al. 2014). In addition to discrepancies of the adaptive branch of the immune system, dysfunction of innate mediators such as monocytes/macrophages and DCs has also been reported (Amorim et al. 2014; Manuel et al. 2013b). In previous studies with Jamaican cohort, HTLV-1 infected subjects present a decreased amount of plasmacytoid DCs (pDCs) in ATL and HAM/TSP patients as compared to asymptomatic carriers (ACs). Additionally, myeloid DCs (mDCs) showed a decreased expression of HLA-DR in HAM/TSP patients but an increased expression of CD86 was seen in both pDCs and mDCs compared to other groups (Manuel et al. 2013a; Manuel et al. 2013b).

HTLV-1 replication is limited in vivo by anti-HTLV-1 cytotoxic T-cell (CTL) responses, which in turn are governed by human leucocyte antigen (HLA) haplotypes (Bangham 2008, 2009; Bangham et al. 2009; Jacobson et al. 1990; Saito and Bangham 2012). Protection against development of HAM/TSP in ACs is determined by the presence of oligoclonal anti-Tax CTLs (Traggiai et al. 2004). The recognition of cognate antigenic peptides presented by MHC molecules triggers T-cell receptor (TCR) signaling, while co-stimulatory and co-inhibitory (collectively named co-signaling) receptors direct T cells' function and fate. The majority of the co-inhibitory receptors belong to the immunoglobulin and tumor necrosis factor receptor superfamily (IgSF and TNFRSF) (Chen and Flies 2013). In earlier studies, we observed a direct correlation between programmed cell death-1 (PD-1), its ligand Programmed death-ligand 1 (PD-L1) and CTL dysfunctions in ATL and HAM/TSP patients (Manuel et al. 2013a; Manuel et al. 2013b). In our earlier studies with 10 HTLV-1 negative and 10 HTLV-1 positive subjects, we observed a 3-fold increase in co-expression of PD-1 and T-cell Ig and ITIM domain (TIGIT) on both CD4⁺ and CD8⁺ T cells from infected samples compared to control with the expression being more pronounced in Tax positive T cells. Similarly in a separate study using samples from asymptomatic carriers and HAM/TSP patients we observed that CD8⁺ T cells from a HAM/TSP patient overexpressed T cell Ig and mucin domain-3 protein (Tim-3) and 2B4 along with PD-1.

Animal models have been widely used to study HTLV-1 viral transmission, disease pathogenesis, and treatment. These models range from naturally infected hosts such as rabbits, some non-human primates and to a lesser extent rats to engineered animal models (Hajj et al. 2012; Lairmore et al. 2005). Studies with rabbit, rat and simian models have successfully led to the understanding of routes of transmission of the HTLV-1 virus, anti-viral immune responses, and the means of preventing its transmission (Lairmore et al. 2005). However, these models remain expensive and difficult to maintain in addition to unreliable disease induction and inconsistent disease development after a prolonged duration of time (Hajj et al. 2012; Lairmore et al. 2005). Mouse models have traditionally been cost-effective and easy to develop and maintain: however, HTLV-1 transmission in mice using usual methods of infection does not produce reliable infection and virus expression due to the inefficient fusion of HTLV-1 envelope with murine cells (Hajj et al. 2012; Lairmore et al. 2005). Hence, mice have been manipulated to establish HTLV-1 infection through the generation of transgenic and humanized mice models (Dodon 2014; Panfil et al. 2013). The first humanized mouse model was generated by introducing the *SCID* mutation that impaired the development of mature B and T cells thus improving engraftment efficiency. Further modifications resulted in better engraftment efficiencies by introducing the non-obese diabetic (NOD) mutation that reduces NK cell activity in addition to loss of B cells, T cells and complement activity. The generation of NOG (NOD/Shi-scid IL2r γ ^{-/-}) mice includes the IL-2R γ gene mutation that disrupts the γ chain that is common to receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. The generation of the Rag2^{-/-} γ c^{-/-} model was targeted to reduce the innate immune response in the mice in order to ease the engraftment of human CD34⁺ hematopoietic stem cells, which are shown to be infected by HTLV-1. These mice were a result of crossing homozygous recombinaise activating gene 2 (Rag2) knockout mice with homozygous common cytokine receptor γ chain (γ c) knockouts. These mutations result in loss of thymus-derived mature T cells and peripheral B cells along with

loss of functional NK cells and lymphocytes. Inoculation of immunodeficient mice with ATL cells or HTLV-1 transformed cell lines resulted in the replication of ATL features and a suitable model to test treatments aimed at tumor suppression. However, there is no suitable small animal model to better understand the immuno- and neuropathogenesis of HTLV-1 leading to the development of HAM/TSP in an in-vivo system. Here, we employed the Balb/c-Rag1-hu^{-/-}γc^{-/-} (Rag1) and Bone marrow Liver Thymic or BLT mouse models for engraftment of human CD34⁺ hematopoietic stem cells (HSCs). Both Rag1 and BLT humanized mice (hu-mice) have not been used in HTLV-1 research yet and their role in CNS-related diseases is just beginning to be explored. Our studies as presented here revealed reconstitution of Rag1 and BLT hu-mice with human immune cells, including macrophages, dendritic cells, T cells and B cells. Both the Rag1 and BLT hu-mice demonstrate susceptibility to HTLV-1 infection with the presence of Tax in spleen and the CNS. Taken together, this is the first attempt to establish HTLV-1 neuropathogenesis in the context of Rag1 and BLT hu-mice.

Materials and Methods

Generation of humanized mice and infection with HTLV-1

CD34⁺ HSCs ($3-5 \times 10^5$) were injected into sub-lethally irradiated (350 rads) 2–5 day old neonatal Rag1 mice (Jackson Laboratories, Bar Harbor, ME) intra-hepatically, as previously described (Akkina et al. 2011; Berges et al. 2006; Veselinovic et al. 2012) to generate Rag1 humice. BLT mice were generated from 6–10 week old NOD/SCID mice as described previously (Lan et al. 2006; Melkus et al. 2006). Briefly, human fetal thymus and liver tissues (gestational age of 17–20 weeks) were surgically implanted under the kidney capsule of irradiated immunodeficient mice followed by intravenous transfer of $3-5 \times 10^5$ CD34⁺ autologous HSCs. At 12 weeks post-engraftment, transplanted Rag1 and BLT-hu mice were screened for human pan-leukocyte marker, CD45, and T cell marker, CD3, levels by flow cytometry in blood collected from a tail bleed. Upon achieving 60% engraftment, Rag1 and BLT hu-mice were injected with 100μl of PBS containing 2×10^6 irradiated MT-2 cells (Yari et al. 2014) or PBS alone intraperitoneally. The neonatal Rag1 HTLV-1 infected hu-mice model was generated by injecting 50μl of MT-2/CD34⁺ co-culture containing 5×10^5 CD34⁺ cells and 4×10^5 lethally irradiated MT-2 cells or CD34⁺ cells alone intra-hepatically. All mice were observed daily, and blood samples were drawn weekly. At different timepoints during the study, mice were sacrificed after the administration of isoflurane and tissue collected under aseptic condition for flow cytometric, droplet digital polymerase chain reaction (ddPCR), real time PCR, and histopathological analysis.

Flow cytometry

For the confirmation of engraftment, 100μl of blood was collected from both Rag1 and BLT mice via tail vein bleed and treated with heparin. Blood was blocked with 7μl mouse blocking buffer [Normal mouse serum (Jackson ImmunoResearch Labs, West Grove, PA)), Rat anti-mouse CD16/CD32 (Mouse FC Receptor Monoclonal), Human gamma globulin (Jackson ImmunoResearch Labs)] for 5–8 minutes. 3μl each of the following monoclonal antibodies: FITC-conjugated anti CD45, PE-conjugated anti-CD3, and PE-Cy5-conjugated CD4 were added to each sample, vortexed, and incubated for 30 minutes at RT in the dark.

Samples were lysed and washed following the Human Erythrocyte lysing kit and cells were fixed in 1% paraformaldehyde/DPBS for flow analysis. Samples were acquired using BD Acurri C6 flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo V.X. software (Tree Star, Ashland, OR).

ddPCR detection of proviral load and confirmation of infectivity of hu-mice

Blood samples for analyses of proviral load and Tax expression were collected via tail vein bleed. About 100µl of blood was collected from each animal at four different time points two weeks apart followed by a terminal bleed. For Rag1 hu-mice, DNA was then extracted from the blood and other organs using Qiagen QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in 200µL TE buffer, and Zymo Research Quick-gDNA Blood Kit (Zymo Research, Irvine, CA) in 70µL TE buffer. For BLT hu-mice DNA was extracted using Zymo Quick-gDNA extraction kit according to manufacturer's protocol and eluted in 50µl elution buffer. Samples were stored at -80°C. PVL was then determined from the DNA samples using a newly developed HTLV-1-specific PCR (ddPCR) assay as described below (Pinheiro et al. 2012). Briefly, DNA was fragmented using a restriction enzyme. The digested DNA was diluted and mixed with the HTLV-1 Tax and RPP30 primers (HTLV-1 tax, Fwd: CTT ATT TGG ACA TTT ACC GAT G and Rev: TGA GGC CGT GTG AGA GTA GA; RPP30, Fwd: GAT TTG GAC CTG CGA GCG and Rev: GCG GCT GTC TCC ACA AGT) and probes (HTLV-1 tax, 6FAM TGATTTCGGGGCCCTGC MGBNFQ; RPP30, VIC CTGACCTGAAGGCTCT MGBNFQ) and Bio-Rad 2X Supermix. The PCR samples were then partitioned into thousands of nanoliter-sized droplets that were subsequently amplified on a thermocycler. Droplets were queried for a fluorescent signal. Each independent event (droplet) was defined as either positive or negative for the target probe by the amplitude of its recorded fluorescence signal (Hindson et al. 2011). Due to the random segregation of DNA fragments into droplets, Poisson algorithm was used to determine absolute copy numbers independently of a standard curve. The PVL was calculated as the percentage of infected cells using the following formula as described earlier (Brunetto et al. 2014):

$$PVL = \left[\frac{\text{Quantity of HTLV-1 Tax}}{\frac{\text{Quantity of housekeeping gene}}{2}} \right] \times 100$$

RNA isolation and quantitative PCR (qPCR)

5 wpi a terminal bleed was performed on 2 adult RAG1 mice and blood was collected in an EDTA tube and stored at RT until all were ready for processing. The blood was centrifuged at 3000rpm for 3 minutes and plasma was collected and stored at -80°C. RNA was extracted from one of the aliquots of plasma using an E.Z.N.A.® Viral RNA Kit (Omega Bio-Tek, Norcross, GA) following manufacturer's protocol eluting out in DEPC treated water. cDNA was then synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. cDNA was stored at -20°C until further use in qPCR. The amplification of HTLV-1 tax (Fwd: 5'-CGT GTT TGG AGA CTG TGT AC -3' and Rev: 5'-CTG TTT CGC GGA AAT GTT TT -3') and human GAPDH (Fwd: 5'-CAA TGA CCC CTT CAT TGA CC -3' and Rev: 5'-TTG ATT TTG GAG GGA TCT CG -3')

was then set-up using Power SYBR® Green PCR Master Mix in the 7500 Real-Time PCR Systems (Applied Biosystems®, Foster City, CA).

Tissue Processing

Hu-mice were euthanized by CO₂ asphyxiation using approved IACUC protocol. Spleen, lymph nodes, human thymus graft, brain, and spinal cord were collected and placed in vinyl specimen molds, encased in O.C.T. compound and stored at –80°C. The spinal cord was cut in half due to specimen mold size; spinal cord molds were labeled with the spinal cord region, i.e. cervical to thorax or thorax to lumbar. O.C.T. compound tissue blocks were sectioned on a cryostat in to 5µm sections and stored at –20. Paraffin embedded sections were cut at 4µm thick and stored at –20°C until used. Sections were stained with antibodies using standard immunohistochemistry and immunofluorescence protocol as explained below. Spinal cord sections were also subjected to Luxol Fast Blue/Cresyl Violet staining (Nova ultra, Woodstock, MD) for assessment of demyelination. The stained slides were scanned at high resolution using (Nikon 80i microscope), viewed using the associated proprietary viewing software (Spot 2.0) and 20×, 40×, and 100× photomicrographs were generated. NIH Image J software was used to score the extent of demyelination in the cervical to thoracic region of the spinal cord. Three different areas of interest were selected per section to obtain an average score. Ratio of the area of demyelinated region to the entire area of interest was calculated and converted in to percentage. Percentage demyelination was then subjectively scored as follows: 0 – 10% demyelination represented a score of 0; 11 – 30% was a score of 1; 31 – 50% was a score of 2; 51 – 70% was a score of 3 and 71 – 100% was a score 4. Demyelination scores from at least 2 different sections per animal were averaged.

Immunohistochemistry/Immunofluorescence

Paraffin-embedded hu-mice spleen, brain, and spinal cord sections were baked overnight at 55°C to firmly affix and dehydrate the tissue. The sections were then deparaffinized in xylene and rehydrated in a graded series of alcohol following which they were boiled in 10mM sodium citrate pH 6.0 for 20 minutes for antigen retrieval. Frozen sections were rinsed with PBS and fixed with 1% PFA for 15 minutes. This was followed by permeabilization for 30 minutes in 0.1% triton X-100 where required. Slides were blocked in 10% normal serum for 2 hours at RT depending on the animal in which the secondary antibody was raised. After blocking, the slides were stained with primary antibodies at carefully titrated concentrations overnight at 4°C followed by probing with secondary antibody for 1 hour. Where indicated, slides were counter-stained with 1µg/ml of nuclear marker DAPI. The sections were then treated with chromogen- substrate solution depending on the enzyme tag (horseradish peroxidase (HRP) or alkaline phosphatase (AP)) of the secondary antibody and counter stained with hematoxylin. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide or for 20 minutes at RT before probing with secondary antibody. Endogenase phosphatase activity was quenched by adding levamisole (240µg/ml) to the chromogen-substrate solution. Following staining, slides were dried, mounted and a series of 20x, 40x and 100x images were taken using the Nikon Eclipse 80i (Nikon, Tokyo, Japan). NIH Image J software was used for quantification of immune cells and Tax speckles.

Antibodies

Mouse monoclonal anti-Tax antibody, rabbit monoclonal anti-human CD4 antibody (Abcam, Cambridge, MA), rabbit polyclonal pre-diluted anti-human CD3 (Invitrogen, Carlsbad, CA) and mouse anti-human CD3 monoclonal antibodies (mAbs) (Novus Biologicals, Littleton CO), goat polyclonal anti-human PD-1 antibody (Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal anti-human CD20 antibody (Thermo Scientific, Grand Island, NY), mouse monoclonal anti-human CD11b (eBioscience), mouse anti-human TIGIT mAb (ebioscience, San Diego, CA), mouse anti-human Tim-3 mAb (Biolegend, San Diego, CA) and rabbit monoclonal anti-human CD8 (Thermo Fisher Scientific, Waltham, MA) were used for fluorescent immunohistochemistry analyses. Secondary antibodies donkey anti-mouse TRITC (Jackson ImmunoResearch), goat anti-rabbit IgG FITC (Santa Cruz, Dallas, TX), anti-mouse rhodamine red (Thermo Fisher Scientific), anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific) were used for the detection of immune cell infiltration and Tax expression. DAPI (Sigma-Aldrich, St. Louis, MO) and Hematoxylin (Polyscientific, Bayshore, NY) were used for counterstaining.

Results

Rag1 and BLT hu-mice demonstrated successful human immune cell engraftment

Previous work by Akkina et al. has demonstrated Rag1 mice to support multilineage human hematopoiesis and population of different lymphoid organs with human cells (Akkina et al. 2011). At 12 weeks post-engraftment, transplanted Rag1 and BLT hu-mice were screened for human cell engraftment by staining for human pan-leukocyte marker CD45 and CD3 in peripheral blood by flow cytometry (Fig. 1A). The percent expression of human CD45 in each mouse has been listed in Table 1. The mean percentage of human CD45⁺ cells was 41.71% and about 42% of the mice demonstrate at least 50% of human immune cell engraftment. The neonatal Rag1 mice that received the virus together with CD34⁺ HSC engraftment showed greater reconstitution with human immune cells (56% v 29% for adult Rag1 and 40% for BLT). We next evaluated the presence of different lymphoid and myeloid cell subsets in the spleen by immunohistochemistry staining. Rag1 (Fig. 1B) and BLT hu-mice (data not shown) demonstrated significant reconstitution by human immune cells, including CD11b⁺ monocytes/dendritic cells, CD3⁺ T cells and CD20⁺B cells.

Adult and neonatal Rag1 and BLT hu-mice are susceptible to HTLV-1 infection with persistent viremia

Adult and neonatal Rag1 hu-mice were infected intraperitoneally and intra-hepatically with HTLV-1 producing MT-2 cells and MT-2/CD34⁺ HSC co-culture respectively. Proviral load (PVL) was detected in the peripheral blood of Rag1 hu-mice as early as 2 weeks post infection (wpi) (Fig. 2A, left). At 5 wpi 2 adult Rag1 mice were euthanized to analyze viral loads in the different organs. 193% PVL was detected in the peripheral blood of Rag1 hu-mice which was high compared to the PVL levels in the spleen (40%) (Fig. 2A, right). At 5 wpi Tax expression was found to be significantly higher in infected as compared to control (Fig 2A, below). The viral protein Tax showed peak expression at 14 wpi in Rag1-hu mice with continued expression until 16 weeks (data not shown). The blood PVL in the neonatal animals was 17% at week 11 post-infection, followed by a drop in viremia and subsequent

increase at week 17. Tax expression in these animals rose from 6% at week 11 to 18% at week 17. The PVL was considerably lower in the neonates as compared to the adult Rag1 hu-mice with less than 20% PVL observed in the peripheral blood, brain, and liver of these animals. Conversely, 60% PVL was seen in the spleen (Fig. 2B). We then tested whether the BLT hu-mice were similarly susceptible to HTLV-1 infection and observed persistent viremia in these animals. Adult BLT hu-mice were infected intraperitoneally with 100 μ L of PBS containing 2 million irradiated MT-2 cells or PBS alone. These mice were observed daily and blood samples were drawn every two weeks. PVL was determined using ddPCR in the peripheral blood, spleen and thymus. At 2 wpi PVL was detected in the blood, spleen and thymus of infected BLT hu-mice compared to that of the control (Fig. 2B lower panel) indicating that the BLT hu-mice are also susceptible to HTLV-1 infection. Moreover, the PVL at five different time points showed persistent viremia (Fig. 2C upper panel) indicating that the mice could be used as a good model to study HTLV-1 infection. We also assessed Tax expression and presence of CD4⁺ T cells in spleen sections of HTLV-1-infected BLT mice and found an increased expression of Tax within CD4 T cells compared to non-infected control (data not shown) by immunofluorescence.

Tax presence and the resultant immune infiltration in brain and spinal cords of infected Rag1 and BLT mice

Neurodegeneration in HAM/TSP occurs mainly due to the bystander tissue damage inflicted by HTLV-1 specific CTL cells through their ability to destroy Tax-expressing cells and by the production of proinflammatory cytokines (Hanon et al. 2000). In our study, the infected mice demonstrated considerable amount of immune cell infiltration in the spinal cord and brain sections (data not shown) as detected by immunohistochemistry and immunofluorescence. CD45⁺ cells were detected in spinal cord sections of both Rag1 (Fig. 3A) and BLT hu-mice (data not shown). Also Tax speckles were observed and quantified within the CD3⁺ T cell population in the brain sections from the infected mice (Fig. 3B and C). Sections from brain and spinal cord of the control and HTLV-1 infected BLT hu-mice were stained with Luxol Fast Blue and MOG with the infected mice showing considerable areas of demyelination (Fig. 4). This data suggests that hu-mice models can be infected with HTLV-1 virion producing cells resulting in viable infection with considerable immune cell infiltration into the CNS and resultant demyelination.

T cells in infected hu-mice show expression of co-inhibitory receptors

Preferential recruitment or expansion of HTLV-1-specific CTLs in the CNS is central to HAM/TSP pathogenesis. We measured the intensity of CD8⁺ CTLs in the brain and spinal cord of infected hu-mice. Intensity of CTL staining was higher in the spinal cord of the infected animals (Fig. 5C) Progressive loss of T cell function, a signature feature of T cell exhaustion during chronic viral infection, is phenotypically characterized by the expression of co-inhibitory receptors (e.g. PD-1, Tim-3, TIGIT, LAG-3, CTLA-4 and 2B4). Thus, we analyzed sections from the periphery and CNS of control and HTLV-1 infected hu-mice to assess the difference in T cell functionality between the animals. Figure 5 revealed slightly increased expressions of PD-1 in spleen, thymus, and CNS (brain and spinal cord) in infected as compared to control, while the expression of PD-1 in lymph nodes of infected mice seemed to be downregulated. This was further confirmed by quantifying PD-1

expression using the ImageJ software (Fig. 5C). The expression of TIGIT in the peripheral organs was mostly higher in the infected groups (Fig. 6C). We observed a slightly increased expression of TIGIT in the brain of the infected group as compared to control, while the expression in the spinal cord was elevated almost 2.5 fold in the infected animals compared to control (Fig. 6). The expression of Tim-3 was downregulated in all the CNS organs of the infected group but up-regulated in the periphery (Fig. 7)

Discussion

HTLV-1 infection leads to a large spectrum of diseases whose biology and possible preventive and curative measures against them can be tested using suitable animal models. In addition to ATL and HAM/TSP, more than 40% of the infected individuals may present with other diseases such as infectious dermatitis, polymyositis, sicca syndrome, overactive bladder and/or erectile dysfunction, chronic periodontitis, sjogern's syndrome, polymyositis, inflammatory arthritis and uveitis (Amorim et al. 2014; Yari et al. 2014). HTLV-1 is generally transmitted vertically from mother to child by breastfeeding, horizontally through sexual transmission and parentally through infected blood and sharing of needles among intravenous drug users (Banerjee et al. 2010; Hajj et al. 2012; Lairmore 2014).

The advances in the generation of mice models are still plagued with shortcomings and hence the quest for a superior strain of mice still continues. Features such as litter size, robustness, and general health problems may interfere with experimental procedures using mouse models. In order to comprehend the immune- and neuropathogenesis of HTLV-1 in an in vivo system we chose to utilize both neonatal and adult Rag1 and the BLT mouse models for engraftment of human CD34⁺ hematopoietic stem cells and subsequent infection with HTLV-1 infected cell line MT-2. Both these mouse models have not yet been previously employed in the study of HTLV-1 infection although they have been successfully utilized to study human immune cell responses to HIV infection. These mice show generation of B and T cells, macrophages, NK cells and DCs along with appropriate human MHC restriction over at least 6 months homing to secondary lymphoid organs without being massively activated (Traggiai et al. 2004). HIV-1-infected BLT mice accurately mirror human cellular immune responses to infection (Dudek and Allen 2013; Dudek et al. 2012) and PD-1 blockade suppress HIV-1 viral load in these mice (Seung et al. 2013). Recently, human macrophages were shown to populate the perivascular spaces and meninges of the CNS of HIV-1 infected hu-NSG mice (Gorantla et al.). Additionally, hepatitis B infection follows a different course in adults v children, with 95% adults clearing the virus spontaneously whereas 90% neonates develop chronic infection (Publicover et al. 2011). This is suggestive of age-related differences within the immunological makeup, which have not been elucidated so far. Thus, the development of both adult and neonatal hu-mice models may serve to test similar differences in the course of HTLV-1 infection. Here we observed reconstitution of Rag1 and BLT hu-mice with human immune cells, including macrophages, dendritic cells, T cells and B cells (Fig. 1). Rag1 hu-mice have been successfully reconstructed with human immune cells since the early 2000s paving way for the generation of humanized mice for the study of underlying mechanisms of human disease (Ito et al. 2012; Legrand et al. 2006; Li et al. 2014). In a previous study, 41% of Rag1-hu mice demonstrated at least 70% engraftment with a mean peripheral blood human cell

engraftment of $59.3 \pm 2.5\%$ (Akkina et al. 2011), which is comparable to the results achieved in our study with 34% of Rag1-hu mice showing at least 60% engraftment. Generally, engraftment data from other studies with humanized mice indicate more than 40–50% of cells to be huCD45⁺ (Akkina et al. 2011; Dudek et al. 2012; Seung et al. 2013). Furthermore, HTLV-1 infection was confirmed in the infected mice through the detection of PVL determined in the peripheral blood and spleen of Rag1 and BLT hu-mice by ddPCR (Fig. 2). Peripheral blood PVL was detected as early as 2 wpi, which was high compared to PVL levels in the spleen at its peak. A high blood PVL is usually associated with oligoclonal proliferation of HTLV-1⁺ T cells in individuals with HAM/TSP or ACs (Cook et al. 2012; Coutinho et al. 2014). Thus these animals may develop neurological complications due to the infection thus potentially paving a path towards generation of a suitable animal model for studying HAM/TSP. PVL and Tax levels in the mice were quantified by using a recently established precise method of quantification known as ddPCR (Brunetto et al. 2014; Hindson et al. 2011; Pinheiro et al. 2012). The viral protein Tax showed peak expression at 14 wpi in Rag1hu-mice with continued expression until 16 weeks. Interestingly, PVL and Tax expression was considerably higher in the adult Rag1 hu-mice as compared to the neonates with the latter showing less than 20% PVL in the peripheral blood, brain, and liver. Successful reconstitution of spleen with human CD4⁺ T cells and their subsequent infection with HTLV-1 is evident in Fig. 2(C). The presence of Tax in the brain and spinal cord of infected mice was confirmed by immunofluorescence imaging.

The progressive neurodegeneration in HAM/TSP has been attributed to the host immune response to the virus, especially the response of HTLV-1 tax specific CTLs (Barmak et al. 2003; Kubota et al. 2002; Levin and Jacobson 1997). Several mechanisms have been postulated to be responsible for the demyelination and cellular death observed in the CNS (Barmak et al. 2003; Lepoutre et al. 2009). For example, according to the bystander mechanism, proinflammatory cytokines and cytotoxins released by activated lymphocytes lead to destruction of neighboring uninfected cells (Biddison et al. 1997) while autoimmune molecular mimicry causes destruction of CNS-resident cells that express cross-reactive antigens (Levin et al. 2002). In the present study, CD45⁺, CD3⁺ (Fig. 3) and CD11b⁺ (data not shown) cells were also seen infiltrating in regions of the brain and spinal cord. The amount of infiltrating CD45⁺ cells in the infected Rag1 mice was higher compared to the BLT mice but the difference was not significant (Fig. 3C). Subsequently, the infiltrating immune cells may be responsible for the demyelination observed in the spinal cord sections of the Rag1 and BLT hu-mice (Fig. 3 and 4). Based on the demyelination scores, there is no significant change in the amount of myelin degeneration between the two mouse models, albeit the Rag1 mice show marginally higher scores. This correlates with other studies that have shown axonal loss and demyelination as clinical manifestations of HAM/TSP seen on histopathology (Liu et al. 2014). In this study the data from the hu-mice has also corroborated previous findings that have reported stable PVL despite disease progression due to neurodegeneration (Liu et al. 2014; Olavarria et al. 2012). Thus far, both the mouse models show comparable level of infection and neuropathogenesis. Although the neonatal Rag1 and BLT mice show better engraftment efficiency, higher PVL in adult Rag1 mice suggest stronger susceptibility to HTLV-1 infection.

Finally, since T cell function in chronic viral infections like HTLV-1 is tightly regulated via both co-stimulatory and co-inhibitory signaling molecules (Abdelbary et al. 2011; Manuel et al. 2013b), we looked at the expression of some of the members of the immunoglobulin superfamily of receptors. Increased CD8⁺ CTLs were seen in the spinal cord of the infected humice. Interestingly, expression of PD-1 and TIGIT was increased upon HTLV-1 infection in the brain and spinal cord of infected animals while that of Tim-3 was downregulated. All three co-inhibitory receptors showed increased expression in the thymus. PD-1 also showed increased expression in the lymph nodes while TIGIT and Tim-3 showed elevated expression in the spleen of the infected animals. Conflicting reports have emerged regarding the expression of PD-1 and Tim-3 on HTLV-1 specific CTLs with increased PD-1 expression linked to higher proviral loads and impaired T cell functionality in HAM/TSP patients (Fuentes Marraco et al. 2015; Kozako et al. 2011). At the same time downregulation of systemic Tim-3 levels in HAM/TSP have been associated with sustained inflammatory response of T cells leading to pathogenic events (Ndhlovu et al. 2011). Our results show that HTLV-1 infection and its associated pathologies can be well studied using Rag1 and BLT hu-mice for the targeted development of therapeutic alternatives.

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References

- Abdelbary NH, Abdullah HM, Matsuzaki T, Hayashi D, Tanaka Y, Takashima H, Izumo S, Kubota R. Reduced Tim-3 expression on human T-lymphotropic virus type I (HTLV-I) Tax-specific cytotoxic T lymphocytes in HTLV-I infection. *J Infect Dis.* 2011; 203:948–959. [PubMed: 21402546]
- Akkina R, Berges BK, Palmer BE, Remling L, Neff CP, Kuruvilla J, Connick E, Folkvord J, Gagliardi K, Kassu A, Akkina SR. Humanized Rag1^{-/-} gammac^{-/-} mice support multilineage hematopoiesis and are susceptible to HIV-1 infection via systemic and vaginal routes. *PLoS One.* 2011; 6:e20169. [PubMed: 21695116]
- Amorim CF, Souza AS, Diniz AG, Carvalho NB, Santos SB, Carvalho EM. Functional Activity of Monocytes and Macrophages in HTLV-1 Infected Subjects. *PLoS Negl Trop Dis.* 2014; 8:e3399. [PubMed: 25521499]
- Banerjee P, Tripp A, Lairmore MD, Crawford L, Sieburg M, Ramos JC, Harrington W Jr, Beilke MA, Feuer G. Adult T-cell leukemia/lymphoma development in HTLV-1-infected humanized SCID mice. *Blood.* 2010; 115:2640–2648. [PubMed: 20124219]
- Bangham CR. HTLV-1 infection: role of CTL efficiency. *Blood.* 2008; 112:2176–2177. [PubMed: 18779398]
- Bangham CR. CTL quality and the control of human retroviral infections. *Eur J Immunol.* 2009; 39:1700–1712. [PubMed: 19582737]
- Bangham CR, Meekings K, Toulza F, Nejmeddine M, Majorovits E, Asquith B, Taylor GP. The immune control of HTLV-1 infection: selection forces and dynamics. *Front Biosci (Landmark Ed).* 2009; 14:2889–2903. [PubMed: 19273242]
- Barmak K, Harhaj E, Grant C, Alefantis T, Wigdahl B. Human T cell leukemia virus type I-induced disease: pathways to cancer and neurodegeneration. *Virology.* 2003; 308:1–12. [PubMed: 12706085]

- Berges BK, Wheat WH, Palmer BE, Connick E, Akkina R. HIV-1 infection and CD4 T cell depletion in the humanized Rag2^{-/-}gamma c^{-/-} (RAG-hu) mouse model. *Retrovirology*. 2006; 3:76–89. [PubMed: 17078891]
- Biddison WE, Kubota R, Kawanishi T, Taub DD, Cruikshank WW, Center DM, Connor EW, Utz U, Jacobson S. Human T cell leukemia virus type I (HTLV-I)-specific CD8⁺ CTL clones from patients with HTLV-I-associated neurologic disease secrete proinflammatory cytokines, chemokines, and matrix metalloproteinase. *J Immunol*. 1997; 159:2018–2025. [PubMed: 9257869]
- Brunetto GS, Massoud R, Leibovitch EC, Caruso B, Johnson K, Ohayon J, Fenton K, Cortese I, Jacobson S. Digital droplet PCR (ddPCR) for the precise quantification of human T-lymphotropic virus 1 proviral loads in peripheral blood and cerebrospinal fluid of HAM/TSP patients and identification of viral mutations. *J Neurovirol*. 2014; 20:341–351. [PubMed: 24781526]
- Cabral F, Arruda LB, de Araujo ML, Montanheiro P, Smid J, de Oliveira AC, Duarte AJ, Casseb J. Detection of human T-cell lymphotropic virus type 1 in plasma samples. *Virus Res*. 2012; 163:87–90. [PubMed: 21924305]
- Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*. 2013; 13:227–242. [PubMed: 23470321]
- Cook LB, Rowan AG, Melamed A, Taylor GP, Bangham CR. HTLV-1-infected T cells contain a single integrated provirus in natural infection. *Blood*. 2012; 120:3488–3490. [PubMed: 22955925]
- Coutinho R Jr, Grassi MF, Korngold AB, Olavarria VN, Galvao-Castro B, Mascarenhas RE. Human T lymphotropic virus type 1 (HTLV-1) proviral load induces activation of T-lymphocytes in asymptomatic carriers. *BMC Infect Dis*. 2014; 14:453. [PubMed: 25148903]
- Dodon MD. Of mice, men, and HTLV-1. *Blood*. 2014; 123:303–304. [PubMed: 24434991]
- Dudek TE, Allen TM. HIV-Specific CD8⁺ T-Cell Immunity in Humanized Bone Marrow-Liver-Thymus Mice. *J Infect Dis*. 2013; 208(Suppl 2):S150–154. [PubMed: 24151322]
- Dudek TE, No DC, Seung E, Vrbanac VD, Fadda L, Bhounik P, Boutwell CL, Power KA, Gladden AD, Battis L, Mellors EF, Tivey TR, Gao X, Altfeld M, Luster AD, Tager AM, Allen TM. Rapid evolution of HIV-1 to functional CD8(+) T cell responses in humanized BLT mice. *Sci Transl Med*. 2012; 4:143ra198.
- Fuertes Marraco SA, Neubert NJ, Verdeil G, Speiser DE. Inhibitory Receptors Beyond T Cell Exhaustion. *Front Immunol*. 2015; 6:310. [PubMed: 26167163]
- Fuzii HT, da Silva Dias GA, de Barros RJ, Falcao LF, Quaresma JA. Immunopathogenesis of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). *Life Sci*. 2014; 104:9–14. [PubMed: 24704970]
- Gorantla S, Makarov E, Finke-Dwyer J, Castaneda A, Holguin A, Gebhart CL, Gendelman HE, Poluektova L. Links between progressive HIV-1 infection of humanized mice and viral neuropathogenesis. *Am J Pathol*. 177:2938–2949.
- Hajj HE, Nasr R, Kfoury Y, Dassouki Z, Nasser R, Kchour G, Hermine O, de The H, Bazarbachi A. Animal models on HTLV-1 and related viruses: what did we learn? *Front Microbiol*. 2012; 3:333. [PubMed: 23049525]
- Hanon E, Hall S, Taylor GP, Saito M, Davis R, Tanaka Y, Usuku K, Osame M, Weber JN, Bangham CR. Abundant tax protein expression in CD4⁺ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes. *Blood*. 2000; 95:1386–1392. [PubMed: 10666215]
- Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, Kitano TK, Hodel MR, Petersen JF, Wyatt PW, Steenblock ER, Shah PH, Bousse LJ, Troup CB, Mellen JC, Wittmann DK, Erndt NG, Cauley TH, Koehler RT, So AP, Dube S, Rose KA, Montesclaros L, Wang S, Stumbo DP, Hodges SP, Romine S, Milanovich FP, White HE, Regan JF, Karlin-Neumann GA, Hindson CM, Saxonov S, Colston BW. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem*. 2011; 83:8604–8610. [PubMed: 22035192]
- Hyun J, Ramos JC, Toomey N, Balachandran S, Lavorgna A, Harhaj E, Barber GN. Oncogenic HTLV-1 Tax Suppression of Primary Innate Immune Signaling Pathways. *J Virol*. 2015
- Ito R, Takahashi T, Katano I, Ito M. Current advances in humanized mouse models. *Cell Mol Immunol*. 2012; 9:208–214. [PubMed: 22327211]

- Izumo S. Neuropathology of HTLV-1-associated myelopathy (HAM/TSP): The 50th Anniversary of Japanese Society of Neuropathology. *Neuropathology*. 2010; 30:480–485. [PubMed: 20573027]
- Jacobson S, Shida H, McFarlin DE, Fauci AS, Koenig S. Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. *Nature*. 1990; 348:245–248. [PubMed: 2146511]
- Kozako T, Yoshimitsu M, Akimoto M, White Y, Matsushita K, Soeda S, Shimeno H, Kubota R, Izumo S, Arima N. Programmed death-1 (PD-1)/PD-1 ligand pathway-mediated immune responses against human T-lymphotropic virus type 1 (HTLV-1) in HTLV-1-associated myelopathy/tropical spastic paraparesis and carriers with autoimmune disorders. *Hum Immunol*. 2011; 72:1001–1006. [PubMed: 21851845]
- Kubota R, Soldan SS, Martin R, Jacobson S. Selected cytotoxic T lymphocytes with high specificity for HTLV-I in cerebrospinal fluid from a HAM/TSP patient. *J Neurovirol*. 2002; 8:53–57.
- Lairmore MD. Animal models of bovine leukemia virus and human T-lymphotropic virus type-1: insights in transmission and pathogenesis. *Annu Rev Anim Biosci*. 2014; 2:189–208. [PubMed: 25384140]
- Lairmore MD, Silverman L, Ratner L. Animal models for human T-lymphotropic virus type 1 (HTLV-1) infection and transformation. *Oncogene*. 2005; 24:6005–6015. [PubMed: 16155607]
- Lan P, Tonomura N, Shimizu A, Wang S, Yang YG. Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation. *Blood*. 2006; 108:487–492. [PubMed: 16410443]
- Legrand N, Weijer K, Spits H. Experimental models to study development and function of the human immune system in vivo. *J Immunol*. 2006; 176:2053–2058. [PubMed: 16455958]
- Lepoutre V, Jain P, Quann K, Wigdahl B, Khan ZK. Role of resident CNS cell populations in HTLV-1-associated neuroinflammatory disease. *Front Biosci (Landmark Ed)*. 2009; 14:1152–1168. [PubMed: 19273122]
- Levin MC, Jacobson S. HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP): a chronic progressive neurologic disease associated with immunologically mediated damage to the central nervous system. *J Neurovirol*. 1997; 3:126–140. [PubMed: 9111175]
- Levin MC, Lee SM, Kalume F, Morcos Y, Dohan FC Jr, Hasty KA, Callaway JC, Zunt J, Desiderio D, Stuart JM. Autoimmunity due to molecular mimicry as a cause of neurological disease. *Nat Med*. 2002; 8:509–513. [PubMed: 11984596]
- Li F, Cowley DO, Banner D, Holle E, Zhang L, Su L. Efficient genetic manipulation of the NOD-Rag1^{-/-}IL2RgammaC-null mouse by combining in vitro fertilization and CRISPR/Cas9 technology. *Sci Rep*. 2014; 4:5290. [PubMed: 24936832]
- Liu W, Nair G, Vuolo L, Bakshi A, Massoud R, Reich DS, Jacobson S. In vivo imaging of spinal cord atrophy in neuroinflammatory diseases. *Ann Neurol*. 2014; 76:370–378. [PubMed: 25042583]
- Manuel SL, Sehgal M, Connolly J, Makedonas G, Khan ZK, Gardner J, Betts MR, Jain P. Lack of recall response to Tax in ATL and HAM/TSP patients but not in asymptomatic carriers of human T-cell leukemia virus type 1. *J Clin Immunol*. 2013a; 33:1223–1239. [PubMed: 23888327]
- Manuel SL, Sehgal M, Khan ZK, Goedert JJ, Betts MR, Jain P. An altered maturation and adhesion phenotype of dendritic cells in diseased individuals compared to asymptomatic carriers of human T cell leukemia virus type 1. *AIDS Res Hum Retroviruses*. 2013b; 29:1273–1285. [PubMed: 23750452]
- Melkus MW, Estes JD, Padgett-Thomas A, Gatlin J, Denton PW, Othieno FA, Wege AK, Haase AT, Garcia JV. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nat Med*. 2006; 12:1316–1322. [PubMed: 17057712]
- Morgan O. HTLV-1 associated myelopathy/tropical spastic paraparesis: how far have we come? *West Indian Med J*. 2011; 60:505–512. [PubMed: 22519224]
- Nagai M, Osame M. Human T-cell lymphotropic virus type I and neurological diseases. *J Neurovirol*. 2003; 9:228–235. [PubMed: 12707853]
- Ndhlovu LC, Leal FE, Hasenkrug AM, Jha AR, Carvalho KI, Eccles-James IG, Bruno FR, Vieira RG, York VA, Chew GM, Jones RB, Tanaka Y, Neto WK, Sanabani SS, Ostrowski MA, Segurado AC, Nixon DF, Kallas EG. HTLV-1 tax specific CD8+ T cells express low levels of Tim-3 in HTLV-1

- infection: implications for progression to neurological complications. *PLoS Negl Trop Dis*. 2011; 5:e1030. [PubMed: 21541358]
- Olavarria VN, do Gomes AN, de Kruschewsky RA, Galvao-Castro B, Grassi MF. Evolution of HTLV-1 proviral load in patients from Salvador, Brazil. *Braz J Infect Dis*. 2012; 16:357–360. [PubMed: 22846124]
- Oliere S, Douville R, Sze A, Belgnaoui SM, Hiscott J. Modulation of innate immune responses during human T-cell leukemia virus (HTLV-1) pathogenesis. *Cytokine Growth Factor Rev*. 2011; 22:197–210. [PubMed: 21924945]
- Panfil AR, Al-Saleem JJ, Green PL. Animal Models Utilized in HTLV-1 Research. *Virology (Auckl)*. 2013; 4:49–59. [PubMed: 25512694]
- Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, Emslie KR. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Anal Chem*. 2012; 84:1003–1011. [PubMed: 22122760]
- Publicover J, Goodsell A, Nishimura S, Vilarinho S, Wang ZE, Avanesyan L, Spolski R, Leonard WJ, Cooper S, Baron JL. IL-21 is pivotal in determining age-dependent effectiveness of immune responses in a mouse model of human hepatitis B. *J Clin Invest*. 2011; 121:1154–1162. [PubMed: 21393863]
- Saito M, Bangham CR. Immunopathogenesis of human T-cell leukemia virus type-1-associated myelopathy/tropical spastic paraparesis: recent perspectives. *Leuk Res Treatment*. 2012; 2012:259045. [PubMed: 23198155]
- Seung E, Dudek TE, Allen TM, Freeman GJ, Luster AD, Tager AM. PD-1 blockade in chronically HIV-1-infected humanized mice suppresses viral loads. *PLoS One*. 2013; 8:e77780. [PubMed: 24204962]
- Traggiai E, Chicha L, Mazzucchelli L, Bronz L, Piffaretti JC, Lanzavecchia A, Manz MG. Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science*. 2004; 304:104–107. [PubMed: 15064419]
- Veselinovic M, Neff CP, Mulder LR, Akkina R. Topical gel formulation of broadly neutralizing anti-HIV-1 monoclonal antibody VRC01 confers protection against HIV-1 vaginal challenge in a humanized mouse model. *Virology*. 2012; 432:505–510. [PubMed: 22832125]
- Yari A, Rezaee SA, Valizadeh N, Rajaei T, Jazayeri SM, Soltani M, Norouzi M. Evaluation of HTLV-1 activity in HAM/TSP patients using proviral load and Tax mRNA expression after In Vitro lymphocyte activation. *Iran J Basic Med Sci*. 2014; 17:531–536. [PubMed: 25429345]
- Yoshida M. Discovery of HTLV-1, the first human retrovirus, its unique regulatory mechanisms, and insights into pathogenesis. *Oncogene*. 2005; 24:5931–5937. [PubMed: 16155600]

B)

Figure 1. Human immune cell engraftment in peripheral blood and spleen

(A) CD45⁺ CD3⁺ CD4⁺ human leukocyte expression data in peripheral blood of Rag1 and BLT hu-mice analyzed by flow cytometry. (B) Tissue sections of spleen from Rag1 hu-mice were stained for the presence of human leukocytes lightly counterstained with hematoxylin to enable visualization of nuclei. CD11b⁺ monocytes/dendritic cells (left) CD3⁺ T cells (middle), and CD20⁺ B cells (right) were detected in the spleen of Rag1 hu- mice.

A) Adult Rag1 hu-mice **B) Neonate Rag1 hu-mice**

Figure 2. Infectivity in adult and neonatal hu-mice

Six adult and six neonatal Rag1 mice were infected with HTLV-1 by injecting them with irradiated MT-2 cells and MT-2/CD34⁺ co-culture respectively. The proviral Load (PVL) was measured from blood and organ DNA samples using ddPCR. **(A)** At 2 weeks post infection (wpi) blood from one control and five infected adult Rag1 mice was analyzed for detection of PVL (left). At 5 wpi two Rag1 mice were euthanized for tissue collection and DNA was extracted from the blood, brain and spleen of the mice to measure the PVL (right). RNA samples from plasma obtained from Rag1 mice 5 wpi were reverse transcribed and amplified by real-time PCR using primers specific for spliced Tax mRNA and human GAPDH for normalization (center). **(B)** Blood PVL measured at weeks 11, 13, 15 and 17 post infection in neonatal Rag1 mice is represented on the left while PVL detected in blood, brain, liver and spleen is shown on the right. Blood collected at these time points was also analyzed for Tax copies by ddPCR (center). Statistical significance (** $p < 0.01$) between control and infected mice was determined by Student's t test. Error bars represent standard error of mean. **(C)** (i) Seven adult BLT mice were infected with HTLV-1 by injecting them intraperitoneally with irradiated MT-2 cells. PVL was measured from blood and organ DNA samples using ddPCR. Blood PVL was measured at weeks 2, 4, 6, 8 and 10 wpi. (ii) At 2

wpi PVL in the blood and other organs of one control and one infected BLT mice were plotted (lower panel). Error bars represent standard error of mean. (iii) Engraftment and infection was performed as described. Human CD4⁺ T cells (green) and Tax (red) were seen by immunofluorescence staining in spleen sections of hu-mice. (N.D. stands for 'Not Detected').

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Figure 3. Detection of immune infiltration and resultant Tax expression within the CNS of infected hu-mice

At 4.4 and 10 weeks post-infection Rag1 and BLT mice were euthanized followed by the collection of brain and spinal cord. **(A)** Immunofluorescence revealed the presence of human CD45⁺ cells (green arrow) in infected hu-mice within the spinal cord. **(B)** Immunofluorescence imaging showed CD3⁺ cells and Tax expression in the spinal cord of infected hu-mice **(C)** CD45 cells and Tax speckles in Rag1 and BLT hu-mice were quantified using NIH Image J software and depicted as bar diagram. Error bars represent standard error of mean. Data is representative of 12 Rag1 and 10 BLT hu-mice.

Figure 4. Presence of demyelination of spinal cord sections of hu-mice

Mice were sacrificed and spinal cords were collected under aseptic conditions. 4 μ m sections were cut and sections were stained. Luxol Fast Blue and MOG peptide staining revealed regions of myelin degeneration in spinal cord sections of infected (A) Rag1 and (B) BLT mice compared to the control. Data is representative of 12 Rag1 and 10 BLT hu-mice.

Figure 5. HTLV-1 infection led to increased expression of PD-1 in both the CNS and periphery of infected hu-mice

Lymph node, spleen, thymus, brain and spinal cord were isolated, frozen in O.C.T. compound and sectioned. Following fixation the slides were permeabilized and stained for antibodies against CD8 and PD-1. (A) Representative figures showing CD8 and PD-1 expression in lymph node (A) and spinal cord (B) of control and infected hu-mice. (C) NIH Image J software was used to quantify the intensity of CD8 staining. Results are shown as bar graphs. (D) Quantification of PD-1 expression using NIH Image J software.

Figure 6. HTLV-1 infection led to increase in TIGIT expression in infected hu-mice
Lymph node, spleen, thymus, brain and spinal cord were isolated, frozen in O.C.T. compound and sectioned. Following fixation the slides were permeabilized and stained for antibodies against CD8 and TIGIT. Representative figures showing CD8 and TIGIT expression in lymph node (A) and spinal cord (B) of control and infected hu-mice. (C) Quantification of TIGIT expression using NIH Image J software.

Figure 7. HTLV-1 infection led to differential expression of Tim-3 in CNS and periphery of infected hu-mice

Lymph node, spleen, thymus, brain and spinal cord were isolated, frozen in O.C.T. compound and sectioned. Following fixation the slides were permeabilized and stained for antibodies against CD8 and Tim-3. Representative figures showing CD8 and Tim-3 expression in lymph node (A) and spinal cord (B) of control and infected hu-mice. (C) Quantification of Tim-3 expression using NIH Image J software.

Table 1

Details of HTLV-1 humanized mice utilized in the study along with their demyelination score.

ID	Strain	Treatment	Date of infection	%CD45	%PVL	Euthanasia	Demyelination score
B742	BLT	PBS-Control	5/29/15	30	ND	8/7/15	0
B745	BLT	PBS-Control	5/29/15	39	ND	8/7/15	0
B734	BLT	MT-2 HTLV-1	5/29/15	55	99	7/3/15	2
B737	BLT	MT-2 HTLV-1	5/29/15	40	91	7/3/15	NA
B739	BLT	MT-2 HTLV-1	5/29/15	40	58	8/7/15	3
B743	BLT	MT-2 HTLV-1	5/29/15	49	87	8/7/15	2
B744	BLT	MT-2 HTLV-1	5/29/15	45	47	7/10/15	2
J1621	Rag1	PBS-Control	11/1/13	53	ND	12/5/13	0
MT2-C	Rag1	CD34 Control	11/8/13	45	ND	5/9/14	0
J1647	Rag1	MT-2 HTLV-1	11/1/13	63	9	12/2/13	0
J1696	Rag1	MT-2 HTLV-1	11/1/13	12	138	12/2/13	3
MT2-1	Rag1	MT-2/CD34 co-culture	11/8/13	65	20	2/14/14	NA
MT2-3	Rag1	MT-2/CD34 co-culture	11/8/13	51	13	4/18/14	0
MT2-4	Rag1	MT-2/CD34 co-culture	11/8/13	66	32	5/9/14	2

NA; Not Available

ND; Not Detected