Investigating the role of Nef in HIV infection-induced dysregulation of innate immune cell function

by

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Nef is an accessory protein of HIV that is associated with promoting HIV pathogenesis through increasing replication and immune evasion. This has been proven through delta Nef mutation studies which caused low viral load and no development of AIDS. However, Nef isn't an easy target for creating a therapeutic drug due to its lack of enzymatic activity. Here we examined HIV expression in its wildtype and delta Nef mutated forms in humanized mice. The BLTS model, which has been a cornerstone in aiding HIV studies, was re-examined here for functionality. This was done using immunohistochemistry (IHC) staining of healthy BLTS spleen tissues to analyze characterize the major human immune cells, especially T and B cells. The model was then infected with WT and delta Nef HIV types to analyze the differences in infectivity. This was detected through IHC which detects proteins and RNAScope which detects RNA genome. Wildtype HIV was found to have a higher viral infectivity and tissue damage within 2 weeks post infection (wpi) whereas delta Nef mutated HIV took nearly two months post infection (mpi) to reach lesser infectivity, viral genome load and almost no structural damage. Here, we also introduce the HSC-humanized mice model which has different transplant origins from the BLTS mice. This model was phenotyped using flow cytometry and assessed for its functionality through T cell stimulation assays, which gave a positive functionality indicating that this model, once further optimized, can be used for HIV studies. Finally, we also determine the tissue resident presence of natural killer (NK) cells and dendritic cells (DCs) in the BLTS spleen and thymus and their localization within the tissue. This will allow us to further study the effect of Nef on the localization of these crucial innate immune cells and determine how innate immunity combined with defective Nef could potentially be used to suppress the viral reservoirs of HIV.

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Preface

This Thesis is dedicated to my parents who made it all possible.

- I wish to extend my gratitude to my advisor, Dr. Bility for supporting my research and for the constant guidance.

- To Dr. Mailliard, I would like to extend my heartfelt thanks for guiding me the right way in life and in this project.

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1.0 INTRODUCTION

1.1 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) is a retrovirus that primarily infects and kills CD4+ T-cells, known as helper T-cells of the human immune system. This leaves the host with a weak immune system that can no longer control the virus or any other pathogen that may enter (1). HIV is the causative agent of the disease called acquired immunodeficiency syndrome or AIDS in 1984 (2). Nearly 84.2 million people have become infected with HIV since its first report in 1981 out of which nearly 40.1 million people have died from AIDS or AIDS related illnesses (3). There has also been a significant increase in the number of people living with HIV (PLWH) who now have access to antiretroviral therapy (ART).

1.1.1 HIV virology and pathogenesis

There are two major subtypes of HIV, namely HIV type-1 and type-2. (4). HIV entry occurs through binding the CD4 protein on CD4+ T cells and other immune cells such as macrophages and DCs with a second helper in the form of co-receptors like CCR5 and CXCR4, to provide effective entry into the cells (5). HIV is a lentivirus with an RNA genome. This is contained in a capsid and viral envelope derived from host cell membranes with glycoproteins gp41 and gp120 on their surface. The capsid which encloses two strands of HIV RNA is mainly composed of the protein p24. Nef is another major HIV protein. It is one of six regulatory viral proteins (1). Some of the notable functions of Nef include enhancing HIV infectivity, decreasing CD4 and MHC class

1 cell surface expression. Nef is also associated with aiding disease progression of AIDS. These functions were determined through the study of Nef defective HIV, better known as delta Nef mutation, and the ability to control the viral pathogenesis makes Nef a suitable candidate for therapeutic studies (6). This is also the main reason why my thesis employs delta Nef mutation as a suitable control in the path to determining the relationship of innate immunity with HIV.

1.1.2 Existing treatments and HIV latency

Since the time that HIV was first discovered, many advancements in HIV treatments have t been made without achieving a complete cure. The antiretroviral therapies developed against HIV can be classified based on their target of inhibition or control like the NRTIs (nucleoside reverse transcriptase inhibitor). Employment of more than one class of inhibitors was more effective in viral control compared to just one, leading to combination antiretroviral therapy (cART), which is still the standard of treatment today. It consists of a combination from a choice of 30 drugs in 6 classes of inhibitors and antagonists. These therapies tend to diminish viral load and PLWH can lead near-normal lives so long as they administer these drugs daily (7). Due to suppression of viral load and absence of viral protein production, HIV genetic material is able to stay in the host cells' DNA through the process of integration, without any interference from the human immune system. This is known as viral latency and the latent viral cellular reservoir is the major focus of most HIV therapeutic research. One of the most common principles of HIV vaccines is the "kick and kill" concept, which targets latently infected cells and using latency reversal agents (LRAs) in the presence of ART to induce viral protein production (the 'kick). and immunologically targeting the infected cells expressing the transcribed viral protein antigen (the "kill"). LRAs can be extremely useful in identifying the reservoirs but have mostly been unsuccessful in elimination of the virus

by themselves. Incapacitating Nef protein along with this technique could be the combination needed to eliminate HIV with zero remission (8).

1.2 Natural killer (NK)- dendritic cell (DC) crosstalk

One of the aims of this project is to explore the ability of innate immune cells to affect and control the initial immune response to HIV infection and its subsequent communication with the adaptive immunity. Natural killer (NK) cells are known for their functionality of killing infected, modified or protein-tagged cells especially, in the control of a viral infection, through proinflammatory cytokine / enzyme production like granzymes. NK cells are usually triggered by production of IL-2 and it, in-turn triggers CD4+ T cells to differentiate into helper cell subsets signaling the adaptive immune response has been alerted (9). DCs are major phagocytes and antigen presenting cells (APCs) in the immune system. These APCs can activate other immune cells upon encountering certain antigens on infected cells. An example of this is dendritic cells using MHC class 2 molecules to present HIV proteins to CD4+ T cells, effectively contributing to the spread of the infection, since HIV infects CD4+ T cells. When activated, DCs can produce molecules like IL-12, IL-15, which in turn can activate NK cells and helper T cells (10).

NK-DC crosstalk is a usual reaction to encountering virus particles leading to DC upregulation, which triggers NK effector functions such as cytotoxic killing and subsequent cytokine signaling (12). These in turn, trigger DC maturation which can occur through both cell-cell interactions and communication through cytokines (9). NK cell-mediated lysis provides apoptotic bodies that sometimes plays a role in DC maturation to help fulfill their presentation of

viral antigens to T cells and by producing IFN-γ, activated NK cells can also promote helper T cell polarization (12).

1.2.1 HIV immune response

HIV expresses the regulatory protein named negative regulatory factor (Nef) which suppresses the production of certain Human Leukocyte Antigen (HLA) classes such as HLA-A and B. This leads to successful evasion of the cytotoxic immune response, while also protecting the infected cells from NK cell intervention. Nef is also seen reducing NKG2D ligand production on invaded cells to reduce binding to NK cells (13). The role that NK cells play alone and in relationship with DCs in controlling HIV infection and pathogenesis is currently unclear. This sets up the major basis for my thesis work.

1.3 Cell-line and animal models to study HIV

The most common cell line models to name a few, used to study the mechanism of HIV and its latency are Bosque/Planelles model, Greene model, Siliciano model and so on. In my thesis work, the HIV infection induced post-activation of CD4+ T cell model, i.e., Bosque/Planelles model was employed a lot to study differences in infectivity of the viral strains and to generate enough HIV stock for the rest of the study (14).

1.3.1 The SBBC study

To understand Nef in-depth, the history of the Sydney blood bank cohort (SBBC) study must be explained (40). This was a cohort of 8 recipients who were infected with a new strain of HIV-1 due to receiving blood products from a common donor. The donor virus has a genetic deletion of about 150 bp or more at the Nef-LTR overlap region which lead to nonprogressive infection. The outcomes can be divided into slow progressors (SP) and elite long term non-progressors (LNTPs). A mix of both types succumbed to death within the 1990's- some due to HIV and some completely unrelated. One of the major findings from this study are that Nef attenuated HIV is not a safe vaccine component. It was also found that subjects with a high viral load (SP) had elevated CD8+T cell response and a gradual decline in CD4+ T cells. The patterns of Nef deletion were genetically non-similar amongst the recipients despite having a common donor. Delayed IgG response was observed in a few subjects, but it didn't protect them against disease progression in SP subjects. Finally, T helper cell responses were detected in the LNTPs whereas it was declined in the SPs. These findings have helped further our knowledge of the naturally occurring Nef mutation in HIV and how to exploit this to develop a therapeutic against the virus.

1.3.2 SIV-macaque models

Study of Simian immunodeficiency Virus (SIV) in non-human primates has been used as a model to learn about HIV due to their similarities in genome. Simian-Human Immunodeficiency Virus (SHIV) which is SIV incorporated with HIV reverse transcriptase or the envelope protein, have also been developed to study in Macaque models (35,34,33). A recent study has also shown that Nef plays a role in localization of the SIV infection in the B cell follicles in macaques (37).

The study shows us that delta Nef mutation in SIV causes a dip in viral load at 10wpi and AIDS doesn't occur during their time of study. They've also discussed that Nef deleted SIV localizes in the B cell follicles whereas wildtype SIVmac239 localized in the paracortex. This model, however close it may come to HIV, genetically, still has differences in latency regulation in the host, CTL epitopes, and in the env protein which led to non-accurate similarities or drug trials being drawn to HIV (15). Study of HIV in an animal model is rather difficult due to the specificity of viral entry factors and subsequent non progression of disease. This was however soon over come through stimulating a human immune system in a rodent model which can be readily infected by HIV.

1.3.3 Humanized rodent models

The achievement of establishing a human immune system in a naturally or induced immunodeficient rodent through xenografts of human cells or tissues, has allowed the study of HIV to progress along with certainty and replicability. Most commonly used mice strains to portray immunodeficiency are the $scid^{-/-}$, NOD.Cg-$scid^{-/-}$ $IL2R\gamma^{-/-}$ (NSG), NOD/Shi-scid $IL2rg^{-/-}$ (NOG) and so on, which are usually mutations on genes that produce certain kinases or are responsible for whole functions that, once absent/deterred, severely affect the innate immune system. Some of the notable mentions go to hu-PBL-SCID mice, Hu-HSC mice, Bone marrow-liver-thymus (BLT) mice explained further below (14).

HUPBL mice model was the first to be established (1983) and was done using immunodeficient SCID mice with a mutation on $Prkdc^{scid}$ leading to absence of an innate immune system of the mice and injecting them with human peripheral blood lymphocytes (huPBL), after exposure to irradiation dosage. This facilitates functional T cells in these mice along with some

sub populations of B cells. This, however, lasts only for a short-term before the cells turn on the murine host (16).

HIS (human immune system) mice model was developed next by injecting immunodeficient mice with CD34+ hematopoietic stem cells (HSCs). The origin of these HSCs is either from fetal liver, cord blood (high natural presence of HSCs) or stimulation of blood using GM-CSF. Our lab employs the first two sources heavily to derive HSCs. The T cells that develop in this model are rather underdeveloped due to their maturity attained through training from the murine MHC molecules (16).

The Bone marrow, liver, and thymus (BLT) mice model involves intravenous CD34+ HSC injection along with transplantation of fetal liver and fetal thymus tissues on the renal capsule. The major improvement in this model is the proper development of T and B cells, not just in the BLT organoid but also in mucosal and GI tracts.

The perfection of the BLTS humanized mouse model and testing its ability to reconstitute human immune cells is therefore of major importance to study Nef. Humanized mice, however, typically tend to only have a lifespan of 6 months to a year when subjected to HIV. While, this has its own advantages, it is a strength to have a long-term study of HIV as it provides a more accurate representation of the natural HIV entry and AIDS progression. This can be achieved through the usage of rats as a model of study; however, humanized rats are rarely established.

2.0 SPECIFIC AIMS

2.1 SPECIFIC AIM 1: Development of HSC-humanized-mice model and characterizing their immune cell constitution

Hypothesis: The development of HSC-Humanized mice through the transplantation of a Fetal Liver Organoid onto immunodeficient and irradiated mice in addition to an injection of hematopoietic stem cells can develop a functionally active human spleen.

Rationale: Although, the development of BLTS humanized mouse models was the crucial edge we needed to study HIV in its natural infection progression to develop a therapeutic, it still has its drawbacks. An HSC-humanized model will be able to overcome the ethical challenges that come with direct transplantation of fetal tissues since it employs an organoid grown in a laboratory setting. This also gives us more control over the development of the tissue, and ease to introduce a genetic deletion or insertion mutation if needed to tailor the immune system being generated to the scientists' specific needs.

2.2 SPECIFIC AIM 2: To determine the role of NK-DC crosstalk in live attenuated HIV infected humanized mouse tissues

Hypothesis: We hypothesize that the initial innate immune response to HIV infection contributes more towards the control of HIV than literature currently supports. Natural killer cells

and dendritic cells will be co-localized near sites of HIV reservoirs in the B cell follicles of humanized mouse spleen.

Rationale: In previous experiments performed at our lab using gene expression profiling techniques, a nano-string analysis of total RNA isolated from Humanized mice spleen tissue, showed us that NK signaling, NK-DC crosstalk and IL-15 production were highly upregulated in Delta Nef and Y115D variants of HIV compared to that in wildtype. These insights the fact that absence or dysregulation of the viral regulatory protein Nef is causing upregulation of the innate immunity taking over. Natural killer cells, due to their unique ability to partake in both types of immunity and to perform anti-viral killing, and to communicate with DCs in an intricate web of signaling through chemokines and cytokines. This, when well understood, will open avenues of immunotherapeutic using NKs and DCs against HIV, as the kill functionality of the "kick and kill" method.

3.0 METHODS AND MATERIALS

3.1 Construction of the Humanized mouse model

3.1.1 Equipment

- Hemocytometer
- Scalpels
- Microcentrifuge tubes
- Sanitized paper towels
- Hydrogen Peroxide
- Operating scissors
- 14ml polystyrene tubes

3.1.2 Reagent setup

- **Cell quench buffer: Freshly prepare using** 500 ml RPMI, 5ml Strep/Pen, 5ml Fungizone, 15 ml FBS and 500 µl Gentamicin.
- **Cell wash buffer:** Freshly prepare using 500 ml RPMI, 5ml Strep/Pen, 5ml Fungizone and 500 µl Gentamicin.
- **Resuspension medium:** 100 µg/ml DNase 1 in wash buffer.
- **Human CD34+ Selection:** EasySep^{TM} Human CD34 Positive Selection Kit II (Catalog #17856).

- **Magnet:** The Big Easy (Catalog #18001).
- **Recommended medium:** RoboSep™ Buffer (Catalog #20104).
- **Freezing solution:** ATCC® Serum-Free Cell Freezing Medium (Catalog #30-2600).

3.1.3 Protocol

Tissue preparation (Duration: 1 h)

1. Warm up the wash buffer in a 37 °C water bath.
2. Add 2.5 µl of DNase for every 50 ml of liver digestion medium (this creates the liver digestion solution). Prepare 100 ml per liver to be digested.
3. Pour a single 15- to 18-week gestation period human fetal liver into a 10-cm dish; use scalpels to separate the connective tissue from the parenchyma. (Fetal tissues were obtained from the Health Sciences Tissue Bank at the University of Pittsburgh).
4. Discard the connective tissue and chop the parenchymal tissue into small pieces (1–2 mm^3).
5. Transfer the tissue to a 50-ml tube with a 25-ml pipette; wash the dish with 10 ml of liver digestion solution and add it to the same tube.
6. Fill the tube with liver digestion solution to a 40-ml total volume.
7. Wrap the tube cap with Parafilm. **Critical step:** This will prevent contamination of the sample.
8. Shake the tube for 5–10 s, place the tube in the 37 °C water bath for 30 min and shake the tube every 5 min.
9. The larger pieces will settle to the bottom; remove the liquid portion and filter it through a 70-µm filter into a new 50-ml tube; place the new tube on ice.

10. Add 35 ml of liver digestion solution to the tube (from Step 9) containing the larger pieces of liver tissue and repeat Steps 8 and 9.
11. Spin the two tubes with liver cells at 300*g* for 10 min at 4 °C.
12. Decant the supernatant, resuspend the cells with 40 ml of wash buffer and combine the tubes into one 50-ml tube.
13. Alternatively, for spleen and thymus, the human samples need only be chopped into 1–2 mm^3 pieces and can be frozen down using the freezing solution in an LN2 tank.

Lymphocyte purification (Duration: 1 h)

14. Carefully thaw bone marrow sample form liquid nitrogen by spinning them in a centrifuge at 400*g* for 5 min at 4 °C and discarding the supernatant. (One wash step with wash buffer is encouraged but not necessary).
15. If thawing multiple tubes, combine the pellets and resuspend in a total volume of 35ml of resuspension medium and incubate for 15 min at room temperature (24 °C).
16. Aspirate a 10-ml pipette with 14 ml of Ficoll-paque reagent and eject 11 ml very slowly at the bottom of the tube under the cell suspension.

    [**Critical step:** Hold the 50 ml tube at a slight 70° slanted angle to the basal hood floor and eject the Ficoll with a slow, constant flow, being extremely careful not to disturb the interface. Retain 3 ml of Ficoll in the pipette to prevent air bubble disturbance].
17. Carefully move the tube to the centrifuge to not disturb the layers, and then spin the suspension down at 2000 *g* for 30 min at room temperature (24 °C), with the brake function off.

18. Using a 10-ml pipette tip, remove the cells at the interface, trying to remove as little of the Ficoll as possible.
19. Transfer the collected cells into a 50-ml tube and add wash buffer up to 40 ml.
20. Remove 20 µl and add it to 180 µl of wash buffer in a 1.5-ml microcentrifuge tube and place the tube on ice. Count the cells by staining with trypan blue dye using a hemocytometer.

CD34+ Hematopoietic Stem Cell purification (**Duration: 2 h)**

21. Once cell count is confirmed, spin the suspension at 400*g* at 4 °C for 5 min, discard the supernatant and resuspend the pellet in 10 ml of wash buffer. (Wash step 1)
22. Spin the suspension at 400*g* at 4 °C for 5 min and discard as much of the supernatant as possible without disturbing the pellet. (Wash step 2)
23. Resuspend the cells in 250 µl of wash buffer for < 5 x 10^7 cells counted by trypan blue in Step 20.
24. Add your cell sample into a 14 mL (17 x 95 mm) polystyrene round-bottom tube (e.g., Catalog #38008).
25. Add 100 µl of the EasySep™ Human CD34 Positive Selection Cocktail (in the kit) for every 1 × 10^8 cells counted in Step 20 and mix well.
26. Mix and incubate for 20 minutes.
27. Vortex the EasySep™ Dextran RapidSpheres™ (50100) tube for a full 30 seconds.
28. Add 75 µL of spheres/mL of sample and let incubate for 10 minutes for the beads to bind any CD34+ HSCs in the sample.

29. Top-up the tube with the recommended medium with 3mL for <1mL sample and 10mL for >1mL sample.
30. Place the tube without the lid, inside the magnet and let it incubate for 15-20 minutes
31. Discard the supernatant into another tube while holding the tube inside the magnet through one continuous motion. The original tube inside magnet has the positive cells.
32. Repeat steps 28-31 as needed on the supernatants (up to 3 times).
33. Resuspend cells from sides of the tube into desired medium and count them.
34. Combine all positive fractions (CD34+ HSCs, if more than one) and label the tubes with the tissue identification number.
35. Spin down the negative and positive fractions separately at 400*g* at 4 °C for 5 min. Count the cells and determine the yield of CD34+ HSCs. A yield of >5% CD34+ HSCs is considered high concentration.

Cell preparation for injection (Duration: 30–40 min)

36. Spin down the CD34+ HSCs obtained from Step 35 at 400*g* for 5 min at 4 °C and resuspend them with wash buffer to 1 × 10^6 mL^{-1}.
37. Prior to transplantation, spin down CD34+ HSCs at 400*g* and liver cells at 100*g* for 5 min at 4 °C and resuspend each cell type with wash buffer to 0.5 to 1 ×10^6/17.5µl. Mix approximately 0.5-1×10^6 HSCs and 0.5-1×10^6 liver progenitor cells (35 µl total volume) for each mouse to be injected. [**Critical step:** Cell suspension needs to be approximately 35-µl volume per mouse and not more.]

Transplantation of human cells (Duration: 1.5 h)

38. Take the cells from Step 37 to the animal room.
39. Irradiate 2- to 5-d-old newborn or adult NSG mice in a sterilized paper bag with a single non-lethal dose of 200 rad using a cesium irradiator.
40. Prepare an ice bucket wrapped with autoclaved aluminum foil in the hood. **[Critical step:** Be careful when handling the foil so it remains sterilized throughout the procedure].
41. Set up the warming light in the hood and put a cage lid covered with sterile paper towels under it. This is for mouse recovery after cold anesthesia and irradiation.
42. Place the pups in a clean cage in the hood, separating them from the mother.
43. Place one pup on the foil in the ice bucket and let the animal cool for about 1 min. [**Critical step:** This is for light anesthesia].
44. Resuspend the cells immediately prior to injection each time. Load the syringe (27-gauge needles − 0.5 ml insulin syringes) with the cell suspension at a volume of 30 µl per mouse.
45. Remove the first pup and place the next on ice, in the meantime so cooling process can being by the time the previous one pup is injected.
46. Restrain the pup using one hand applying gentle pressure downwards and outwards (just enough to pull the skin taut without injuring the animal).
47. Begin by vertically positioning the needle angled slightly toward the right lobe; push the needle in about halfway, and then deposit the cells into the right lobe slowly. The liver should be visible as a dark area located cranially to the stomach; it should be the largest organ in young pups. The stomach is identifiable by a white color on the animal's heart

side. Release the pressure immediately with your fingers. Thereafter, remove the needle, being careful to avoid contact with your finger.

48. House the mice in a pathogen-free BSL-2 facility and microisolator cages. Feeding the animals with autoclaved or irradiated food and maintain them on acidified autoclaved water for the duration of the animals' lives is critical.
49. For adult mice, lymphoid tissues are transplanted after HSC injection into blood via the retroorbital route. Lymphoid tissues can also be transplanted into previously HSC-transplanted neonatal mice at 4-6 weeks via implantation beneath the kidney capsules.
50. Human immune cell reconstitution was detected through flow cytometry performed after or at the 10 week-mark after HSC and tissue transplantation.

3.2 Flow Cytometry

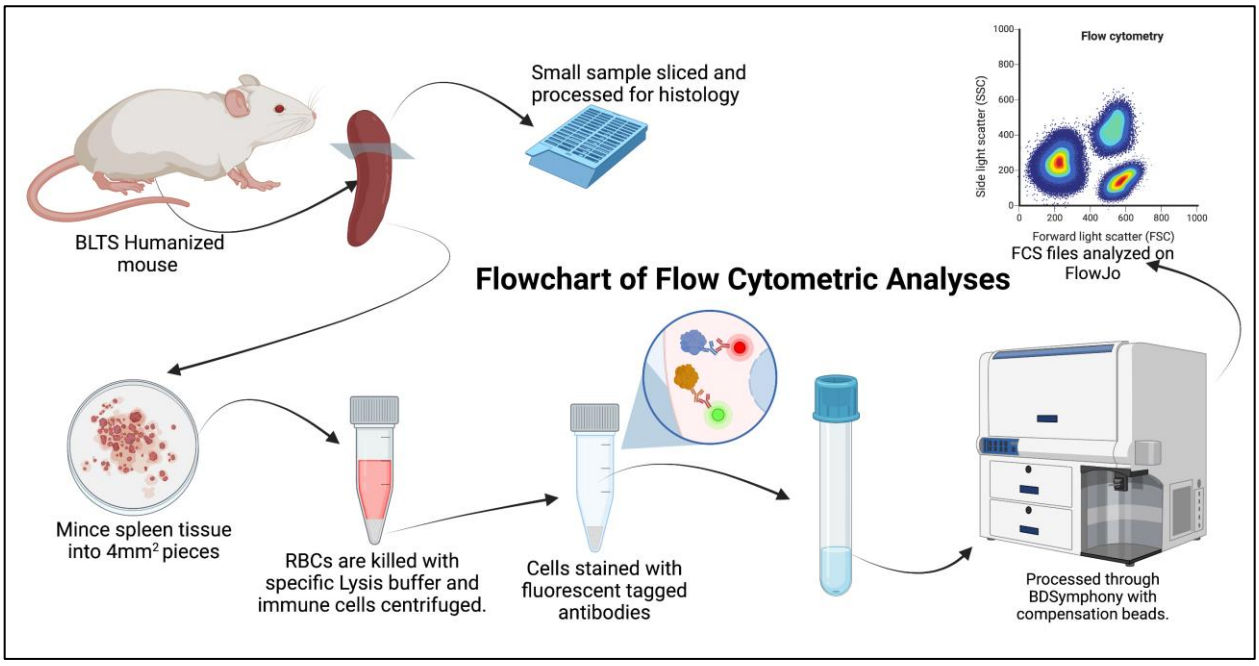


Figure 1: Representative flowchart of flow cytometric analyses: Image shows retreival of humanized spleen from the BLTS mouse, which is then cut into two pieces, the smaller one being used for histology and the larger used to determine the immune cell reconstitution through flow cytometric staining and analysis.

3.2.1 Sample preparation

Peripheral blood cells and lymphoid tissue cells were analyzed using flow cytometry.

- Briefly, peripheral blood was collected from humanized mice and mixed with 20 mM Ethylenediaminetetraacetic acid (EDTA) at a 1:1 ratio. Single cell preparation of blood cells was prepared after removal of the plasma layer, via red blood cells lysis using Ammonium Chloride-Potassium (ACK) buffer.
- Lymphoid tissues such as spleen and thymus were homogenized into single cell suspensions by

3.2.2 FC Staining and analysis

Single-cell suspensions were stained with a LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific), fluorochrome-conjugated antibodies {anti- mouse CD45-BioLegend Cat. No. 103126, anti-human CD45-BioLegend Cat. No. 304014, anti- human CD3-BioLegend Cat. No. 300312, anti-human CD4-BioLegend Cat. No. 317410, anti-human CD8-BioLegend Cat. No. 300906, anti-human CD19-BioLegend Cat. No. 302232, anti-human HLA-DR, BD, cat. No. 562304, anti-human CD25, BD, cat. No.740397, Anti-human CD56 clone N901; Beckman Coulter, CD16, Cat. No. 302027 BD Pharmingen, anti-human CD69, BD, cat. No. 562645, Anti-human CD14 Cat. No. 301819, IFN-γ, BD Biosciences Cat. No. 557995, Anti-HIV-1 p24, Dako, Cat. No. M0857}, fixed and analyzed on a FACSymphony™ A5 Cell Analyzer-flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Dako).

Briefly, leukocytes or granulocytes were selected based on forward and side scatter. Single cell and live cell gates were selected for further analysis of the percentage of human leukocytes (anti-human CD45+, hCD45+) and mouse leukocytes (anti-mouse CD45+, mCD45+) in the peripheral blood. Subsequent analysis of the various human lymphocyte populations and subsets were gated on human leukocytes. The same was repeated for other tissue samples.

3.3 Immunohistochemistry (IHC)

Antibodies used to detect various proteins using IHC included CD3 MAb (OKT3) – Invitrogen Catalog #MA1-10175, CD4 (4B12) - BioCare Catalog #ACI3148A, CD8(CD8/144B)

- BioCare Catalog #ACI3160A, anti-HIV p24 – Dako Catalog #M0857, DC-SIGN (CD209) Polyclonal Antibody – Invitrogen Catalog #PA5-78968.

3.3.1 Protocol

DAY 1

1. Deparaffinize slides in xylene for 10 minutes.
2. Submerge slides in EtOH100% for 10 minutes.
3. Submerge slides in EtOH95% for 15 minutes.
4. Finally, submerge slides in EtOH75% for 10 minutes.
5. Submerge slides in Water in the slide tub for 10 minutes.
6. Lay slides flat onto slide-holder box and cover tissue with 2-3 drops of Biocare Peroxidase-1 (stored in 4ºC fridge) for 5 minutes.
7. In this time, make diluted (1:10) rodent de-cloaker.
8. Submerge the slides in ddH_2O for 5 minutes.
9. Fill up a separate slide tub with rodent de-cloaker solution and transfer the slides into this.
10. Cook the slides in manual mode for 18 minutes at high pressure (Insta-pot).
11. Remove the slides and let them cool for 20-30 mins.
12. Rinse the slides in ddH_2O for 5 mins.
13. Wipe the slides around the tissue section using Kimtech wipes and circle each tissue area with a PAP pen to create a hydrophobic boundary.
14. Prepare 1x solution of TBS.
15. Submerge slides in a slide tub with TBS for 5 minutes.

16. Lay slides flat and add 2-3 drops of Background punisher (stored in 4ºC fridge) for 10 minutes.
17. Rinse off slides by submerging them back in TBS for 5 minutes.
18. Add the primary antibody (diluted accordingly with a diluent/PBS) [~ 200 µL / slide] and incubate overnight in the slide box at 4ºC.

DAY 2

19. Rinse slides with 1x TBS for 5 minutes.
20. Apply MACH 4 Mouse Probe for 5-15 minutes at RT (for Mouse Antibodies only).
21. Apply MACH 4-HRP Polymer for 10-20 minutes (Mouse Antibodies) OR 30 minutes (Rabbit Antibodies) at RT.
22. Wash with 1x TBS for 5 minutes.
23. Make sufficient Betazoid DAB: 1drop DAB Chromogen per 1mL DAB substrate buffer.
24. Apply Betazoid DAB to slides (lay flat) for 5 minutes at ~250 µL/slide.
25. Wash with ddH_2O for 5 minutes.
26. Add 2-3 drops of Cat Hematoxylin for 15-20 seconds.
27. Rinse with ddH_2O for 10 minutes.
28. Immerse tissues in Tacha's Bluing solution for 30 seconds.
29. Rinse with ddH_2O for 5 minutes.
30. Submerge slides in increasing concentrations of alcohols as follows:

a. 75% Et-OH for 5 seconds.
b. 95% Et-OH for 10 seconds.
c. 100% Et-OH for 10 seconds.
d. Xylene for duration of 1 strong dip.

31. Immediately mount slides (upon alcohol evaporation) with a tiny drop of Ecomount and place the appropriate size of coverslip.
32. Dry slides overnight (lay flat) at RT.
33. Photograph morphology under 20x magnification using the inverted microscope and label appropriately.

3.4 Immunofluorescence (IF)

Immunofluorescence (IF) is the process of labelling target antigens with specific antibodies with a fluorescent dye such as a cyanine dye. IF was combined with non-antibody methods of fluorescent staining (e.g., labeling DNA using DAPI). The fluorescent dye allows visualization of the target distribution in the tissue culture samples under a fluorescent microscope. Direct IF uses an antibody directed against the target. The primary antibody is directly conjugated to a fluorophore. In contrast, indirect IF uses a secondary antibody as well. The primary antibodies used: anti-HIV p24 – Dako Cat. No. M0857, NKp46 (CD335) Polyclonal Antibody – Invitrogen Catalog # PA5-79720, LAMP3 Polyclonal Antibody – Invitrogen Catalog # PA5-84069 are unconjugated, and a fluorophore-conjugated secondary antibody directed against the primary antibody is used for detection. Coverslip slides with Prolong® Gold Antifade Reagent (Cell Signaling Technology).

3.4.1 Protocol

1. Deparaffinize slides in xylene for 10 minutes.

2. Submerge slides in EtOH100% for 10 minutes.
3. Submerge slides in EtOH95% for 15 minutes.
4. Finally, submerge slides in EtOH75% for 10 minutes.
5. Boil slides for 6 minutes under pressure in (1x) eBioscience™ IHC Antigen Retrieval Solution Low pH (Catalog: 00-4955-58). After antigen retrieval, tissues shouldn't be allowed to dry out.
6. Allow the slides to cool down to room temperature for 30-40 minutes.
7. Rinse slides in PBS for 5 minutes.
8. Gently dry area of slide around the tissue and use a pap pen to create a hydrophobic outline. Wait a few seconds and then cover tissues with blocking buffer (2% BSA-PBS).
9. Block for 30 minutes in a humidified chamber at RT (can also be done overnight at 4ºC).
10. Make primary antibody cocktails with their recommended dilutions using blocking buffer to dilute.
11. Flick off blocking buffer and add primary cocktail, incubate for 1 hour RT or overnight at 4ºC.
12. Flick off primary antibody, wash 3-4 times with IHC wash buffer or 1x PBS.
13. Make secondary cocktails that are specific to the isotype of primary antibodies.
14. Add secondary antibody cocktail and wash 3-4 times with IHC wash buffer or 1x PBS.
15. Gently dry around the tissue sections, add 50 µL of Prolong® Gold Antifade Reagent with DAPI (Cell Signaling Technology), to stain the nuclei and to mount the coverslips.
16. Allow medium to cure and acquire images with a fluorescence microscope.

3.5 PBMC isolation

Peripheral blood mononuclear cells (PBMC) were obtained from a buffy coat and were isolated by gently overlaying the blood over Ficoll-Paque Medium (density gradient) in a 50mL falcon tube at 400g centrifugation for 30 minutes with the brake off. The plasma is either discarded or stored for further analysis in -20 ºC. The middle cloudy-white layer consists of the leukocytes and is isolated carefully without disturbing the bottom layer of PBMCs. This layer, upon centrifugation at 400g for 10 minutes, pellets down the PBMCs which can then be isolated and resuspended in 1mL cold PBS. The isolated cells were counted using a hemocytometer and cryopreserved until future use.

3.6 T-cell functionality assay

HSC-humanized spleenocytes were directly used for these assays, instead of T-cell isolation, due to the very high composition of T cells in spleen. The functionality assays were done to determine T cell-functionality derived from the HSC- humanized mice spleen through determining activation, proliferation, and cytokine production. This was performed using two methods to analyze replicability.

Method 1 involved culturing the humanized mouse tissue immune cells in a T-25 culture with warm (25 ºC - 37 ºC) IL-2 Growth medium (RPMI-1640 containing 20% heat-inactivated FBS, 5% IL-2 and 50 µg gentamicin/ml) and Phytohemagglutinin-P or PHA-P (5 µg/mL) for every 1 mL of medium. After incubation for one day at 37 ºC incubator with 5% CO_2, cells typically

divide once. After day 3 of culture, cells can be seeded into a 24-well plate at approximately 1million cells/ 1 mL of IL-2 medium without PHA-P.

Method 2 involved similar cell culture without PHA addition. It involves similar seeding into the plate and then addition of 1:1 ratio of Human T-activator CD3/CD28 Dynabeads, with similar incubating conditions.

Both methods will be allowed to culture till day 7, on which day, cells are split into two and restimulation will be executed on one set. Restimulation involves incubation of cells in IL2-GM with addition of 20µl stimulation medium (50µl diluted ionomycin (50µg/ml), 5µl diluted PMA (0.5µg/ml), 445µl IL-2 GM) for 3 hours. After this, the beads are removed from cells, containing beads, using the "Big easy" magnet and the supernatant is harvested for future use and cell are pelleted out. These cells are then stained intracellularly for IFNgamma and TNFalpha and extracellularly for h.CD45 and CD3 and analyzed through flow cytometry.

3.7 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 obtained in 293T cells (ATCC; CRL-3216) after transfection and amplified in the T-cell line MT2 (AIDS Reagent Program). This stock virus was then used to infect PBMCs previously isolated from human blood, after stimulation using PHA, at a 2ng p24Ag per 1 million cells. This additional step was done to ensure that the virus used in further experiments have a physiological similarity to HIV-1 virus that naturally infects humans

(viral envelopes similarity). Viral titers were quantified by HIV-1 p24 antigen assay 2.0 (Zepto Matrix; 0801002) according to the manufacturer's protocol.

3.8 Generation of Monocyte Derived-Dendritic cells

PBMCs were harvested from human blood through Ficoll-Paque density gradient separation and counted using a hemocytometer. Monocytes were separated out using CD14 MicroBeads (Human, catalog: 130-050-201) and then seeded into a 24 well plate with 60,000 monocytes per well for 10 wells. Next, immature DCs were generated and cultured for 5 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). Wild type and Delta Nef Infections of the immature DCs was started on day 6 at an infection rate of 2ng of p24 Ag/ 1*10^6 cells. Culture was harvested on day 9 using ice-cold PBS incubations to release the Dendritic cells from the well bottoms. Differentiation of DC was confirmed by assessing the DC-specific markers such as CD83, CD86, CCR-7 by flow cytometry.

4.0 RESULTS

4.1 Reconstitution of human immune cells in the Bone marrow Liver Thymus Spleen (BLTS) model

To confirm the reconstitution of human immune cells in the BLTS Hu.Mice model (based on our protocol established in publication: https://doi.org/10.1038/nprot.2012.083), a series of immunohistochemistry (IHC) staining were performed on serially cut sections of the humanized spleen by testing for the subpopulations of CD3+T cells, such as the helper CD4+ T cells which have the capability to recognize MHC class 2 antigen presentation, cytotoxic CD8+ T cells which recognize MHC class 1 antigen presentation and CD20+ B cells which control humoral immunity (Fig.2). The brown coloration on certain cells, indicate positivity for the antibody with which the slide was stained, linked through antibody specificity. The results adhere to the normal ratio of CD4:CD8 (ratio should be greater than 1) which indicates the proper reconstitution of a healthy humanized spleen. The T cells are also placed surrounding the marginal (white pulp) zones which represent accurate distribution compared to a human spleen (18). CD20 which is a marker for mature B cells which are usually found heavily populated in the germinal centers were also observed in similar locations in the Humanized spleen. I also performed IHC staining on multiple organ tissues from the BLTS Hu.Mice (published as: doi: 10.3389/fimmu.2022.881607) to identify gamma-delta T cells, which are a lesser common subpopulation of T cells found to be targeted first during natural HIV infection even before CD4+ T cells. This paper was published to explore the effect of HIV on their phenotype, function, and distribution in the BLT Hu. Mice model (Fig. 3).

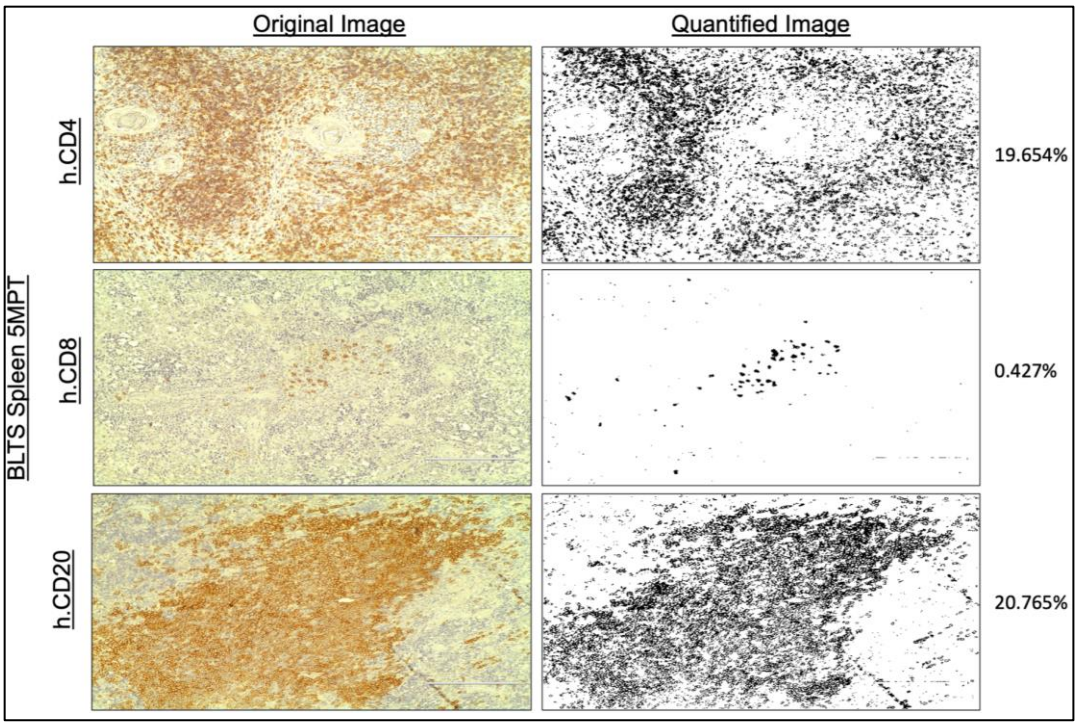


Figure 2: Reconstitution of human T and B cells in the Bone marrow Liver Thymus Spleen (BLTS) model: Sections of humanized spleen in BLTS huMice were stained with anti-Human CD4, anti-Human CD8 and anti-Human CD20 antibodies via immunohistochemistry. CD4+, CD8+ and CD20+ cells are stained dark brown using DAB- 3, 3'-diaminobenzidine and quantified using ImageJ software to determine %area of positive cells.

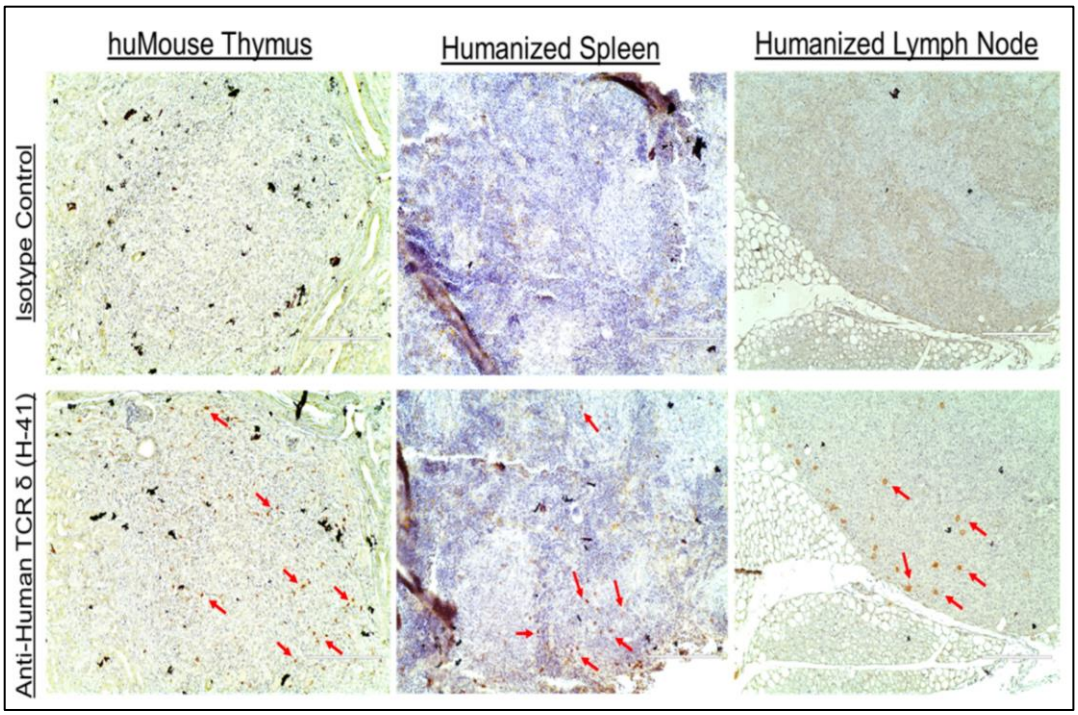
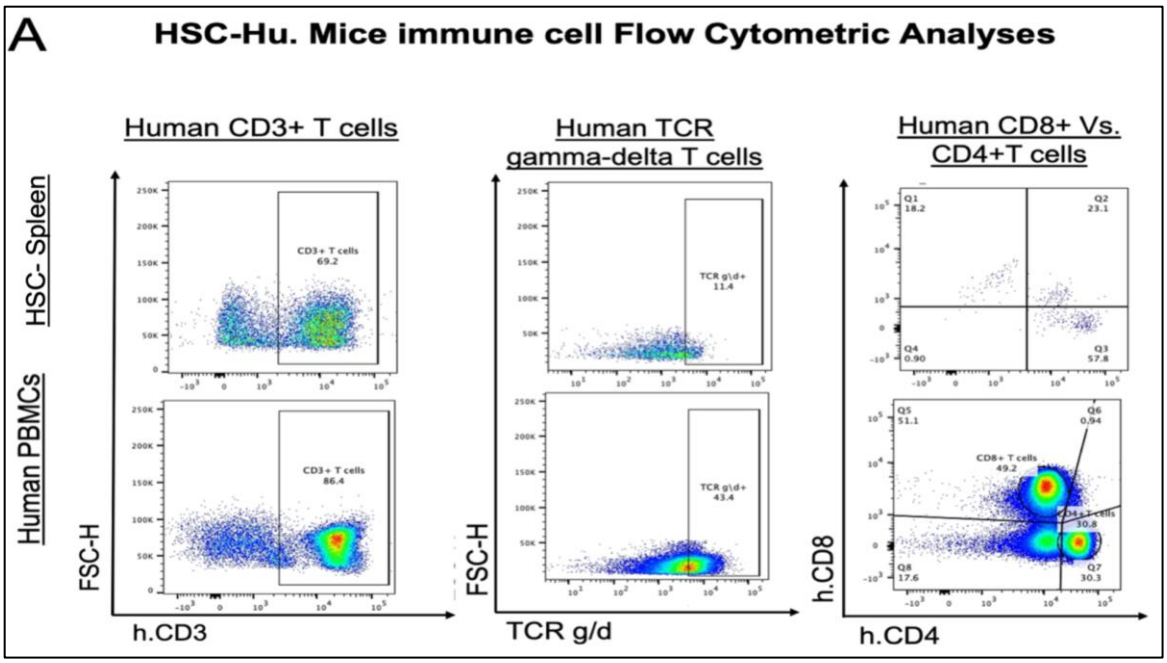


Figure 3: Reconstitution of gamma-delta T cells in BLT humanized Spleen: Image taken from the supplementary figures of our lab publication: doi: 10.3389/fimmu.2022.881607 (17). Employs IHC stained with Anti-Human TCR δ (clone H-41) and isotype control. Gamma-delta T cells are the lesser populated subsection of T cells that have various functions. Positive cells are indicated through red arrows. The images were captured using an inverted microscope using a 20x magnification.

4.2 Reconstitution of human immune cells in Fe-Lo transplanted HSC-humanized mice

Through a collaboration, we then worked on merging the development of the Fetal Liver Organoid (from Hematopoietic stem cells or HSCs in fetal liver) through GATA6 expression (19), with our lab's humanized mouse model, through transplantation. This HSC-humanized mouse model gives an alternative path to mice humanization and can be further explored for HIV studies to avoid ethical issues that come with the territory of direct transplantation of human fetal tissues. I analyzed 7 mice from these series of optimization experiments held by our labs, to phenotypically characterize the reconstituted cells from the humanized spleen. Below, Fig. 4 focuses on the direct

comparison of the major immune cell percentage (out of live single cell subsets) of the Spleen Vs. Human PBMCs. The myeloid cell markers were gated from $CD3^-$ live singlet lymphocytes and live singlet granulocytes. The rest were gated from live singlet lymphocytes. Fig. 4A discusses the frequency of the major T cell markers in 5 mice. Fig. 4B discuss representative myeloid markers in HSC-Hu. mice against human PBMCs as control, respectively. Fig.5 shows graphical representation of all three- T, B, and myeloid cell marker panels as percent live cells positive for the markers.



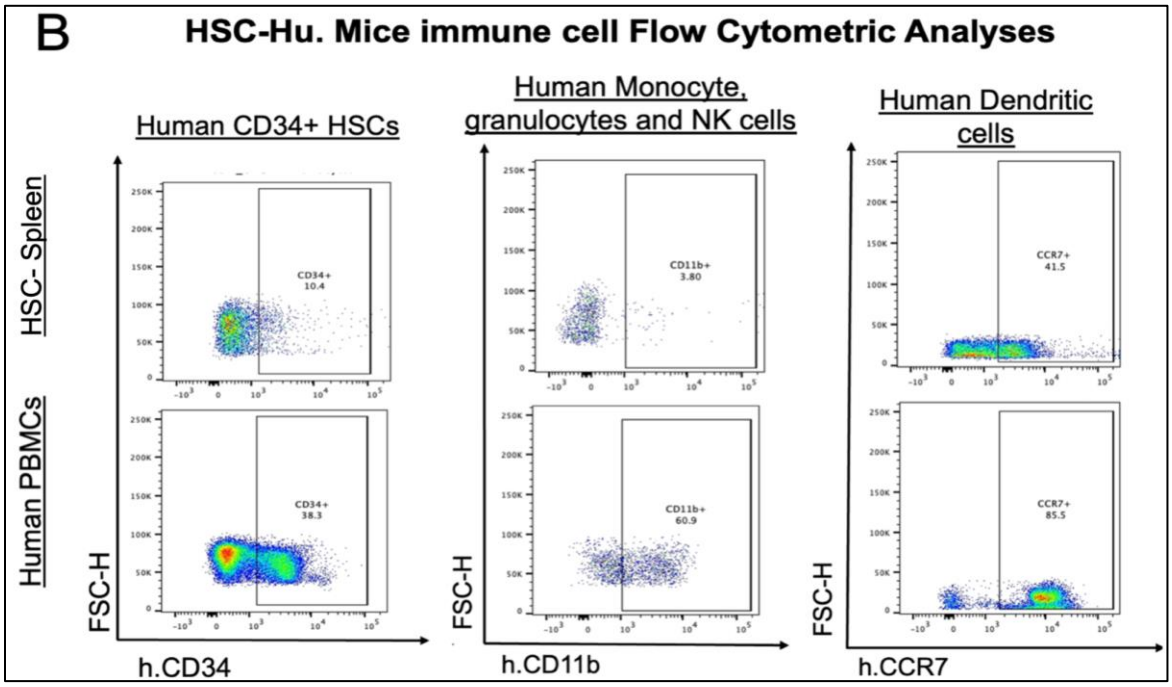


Figure 4: Phenotypic characterization of reconstituted human immune cells in Fe-Lo transplanted Hu.Mice: (A and B) Representative flow cytometry data gated from Lymphoctes, Singlets, Live cells compares human immune cell percentages in Fe-Lo transplanted Hu.mice Vs. Human blood PBMCs as control.

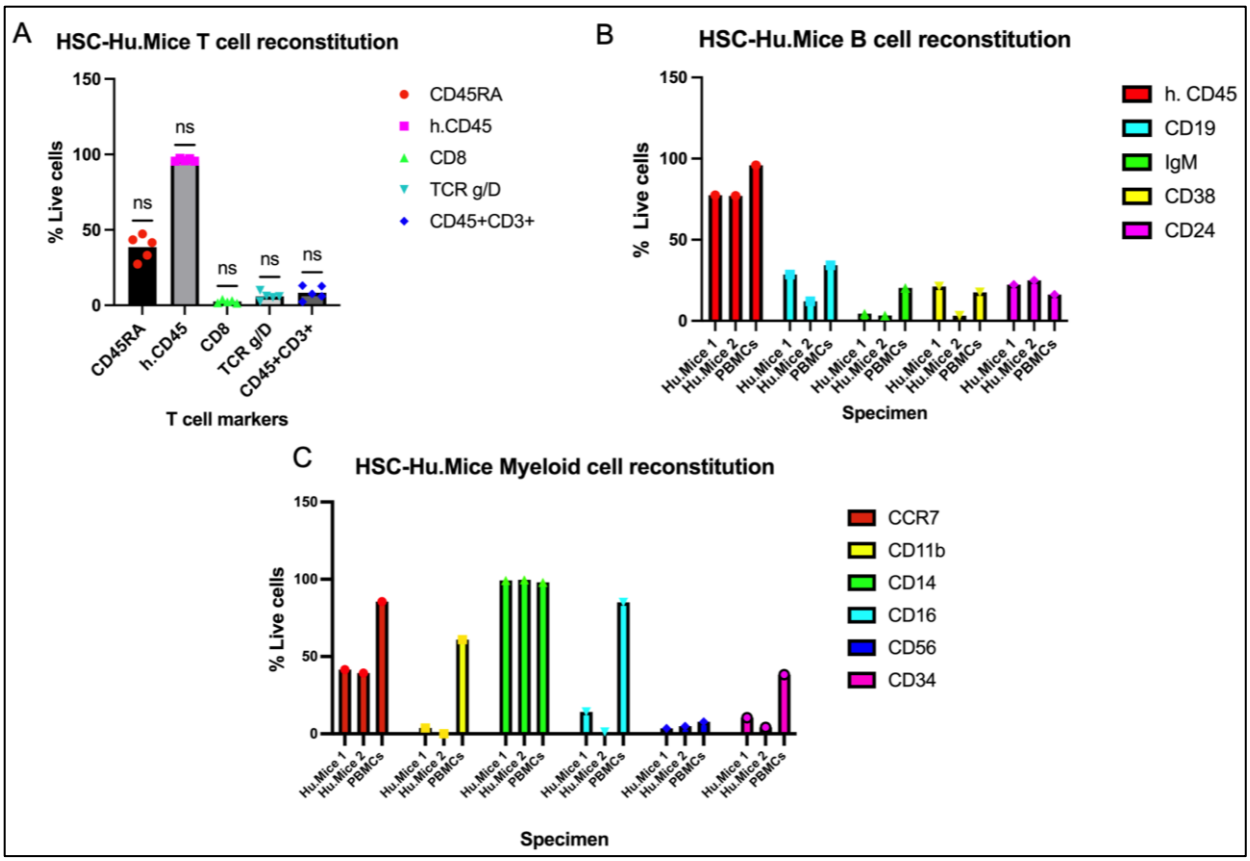


Figure 5: Graphical summary of immune cell reconstitution in HSC-humanized mice: (A) % T cell marker Frequency distribution out of the live, single cell Lymphocyte population (n=5). (B) %B cell marker frequency distribution in Hu.Mice spleen compared to human PBMCs as control (n=2). (C) %Myeloid cell marker frequencies in Hu.Mice Spleen compared to human PBMCs as control (n=2). Flow Cytometric analyses were performed on FlowJo V.10.

4.3 Delta Nef mutation causes lower infectivity and cell death compared to wild type in the BLTS humanized mouse model

The next step was to explore the ability of the BLTS model to be infected with wildtype HIV and delta Nef mutated HIV to test its infectivity, damage to morphology and locations of infection as well. This was done in two methods to ensure accurate results- IHC and RNA-scoping. Figures 6 and 7 are replicates of the IHC staining of HIV-p24 protein on slides that consisted of

BLTS Spleen from mice that were infected with Wildtype HIV, delta Nef HIV, in comparison to a healthy BLTS Hu. Mice (Mock).

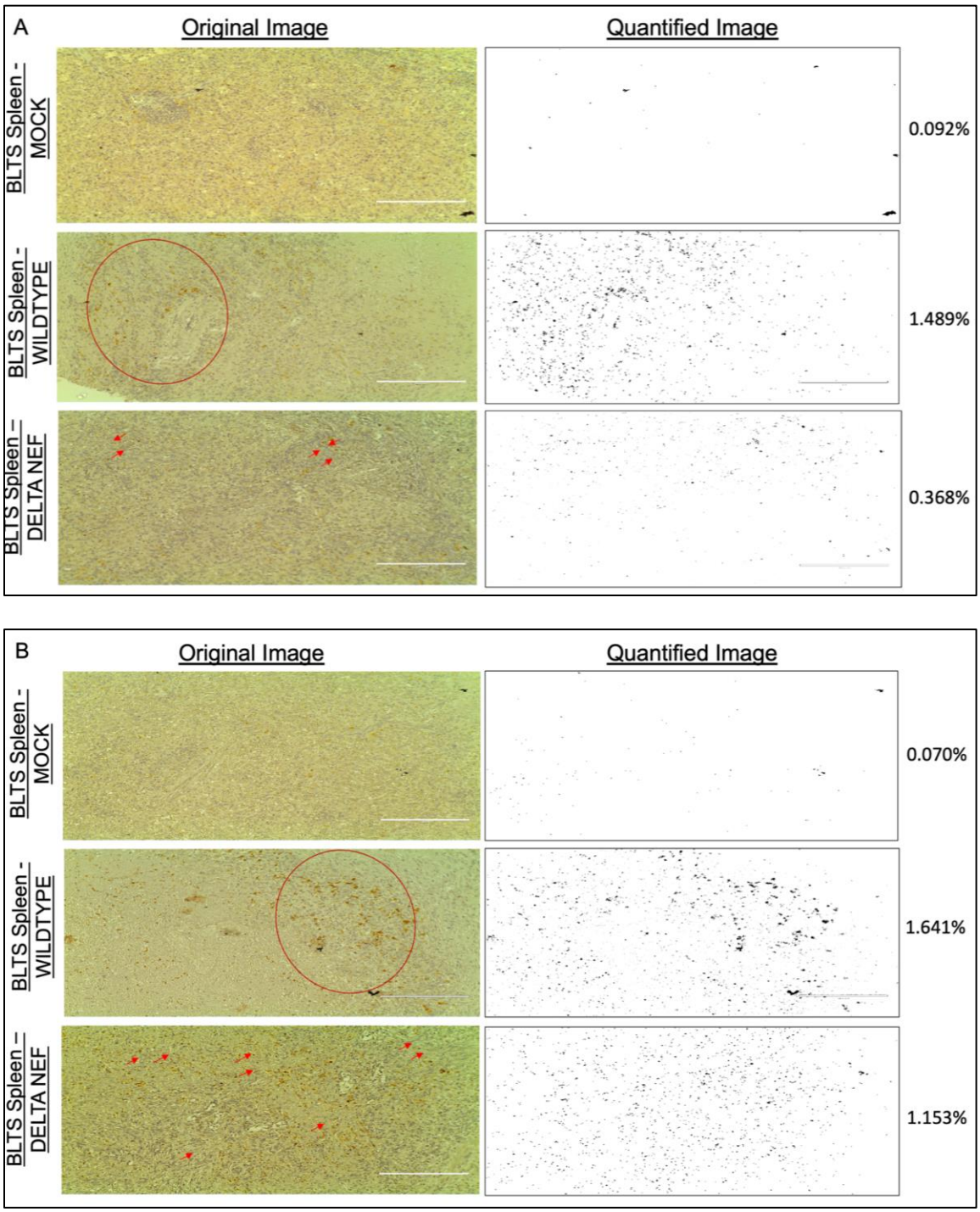


Figure 6: Comparison between the capability of Wildtype and Delta nef HIV strains to infect BLTS model: Sections of BLTS humanized spleen stained using IHC to detect HIV-p24 ImageJ analysis of sub-figures (A and B) were done to analyze duplicates to determine percentage of area posititve for the respective stain/antibodies.

Wildtype HIV has higher infectivity within lesser duration of infection, reaching sufficient infection upon the 6-week mark. delta Nef has a slower infection route and takes nearly 20 weeks to achieve the same amounts of infectivity calculated in %Area infected Fig. 6A supported by the less than 50 % of the infectivity in Fig. 6B due to lesser duration of infection (6 weeks same as wildtype). Fig. 7 explores the infectivity of the different viral strains by detection of HIV-1 viral RNA using certain conserved region primers using the ACD RNAScope detection kit (Catalog: 446551). Similar results of Wildtype having stronger infectivity, quicker was detected. From these experiments, it is also possible to detect morphological deformities present in animal tissues infected with wildtype and none observed in delta Nef infection.

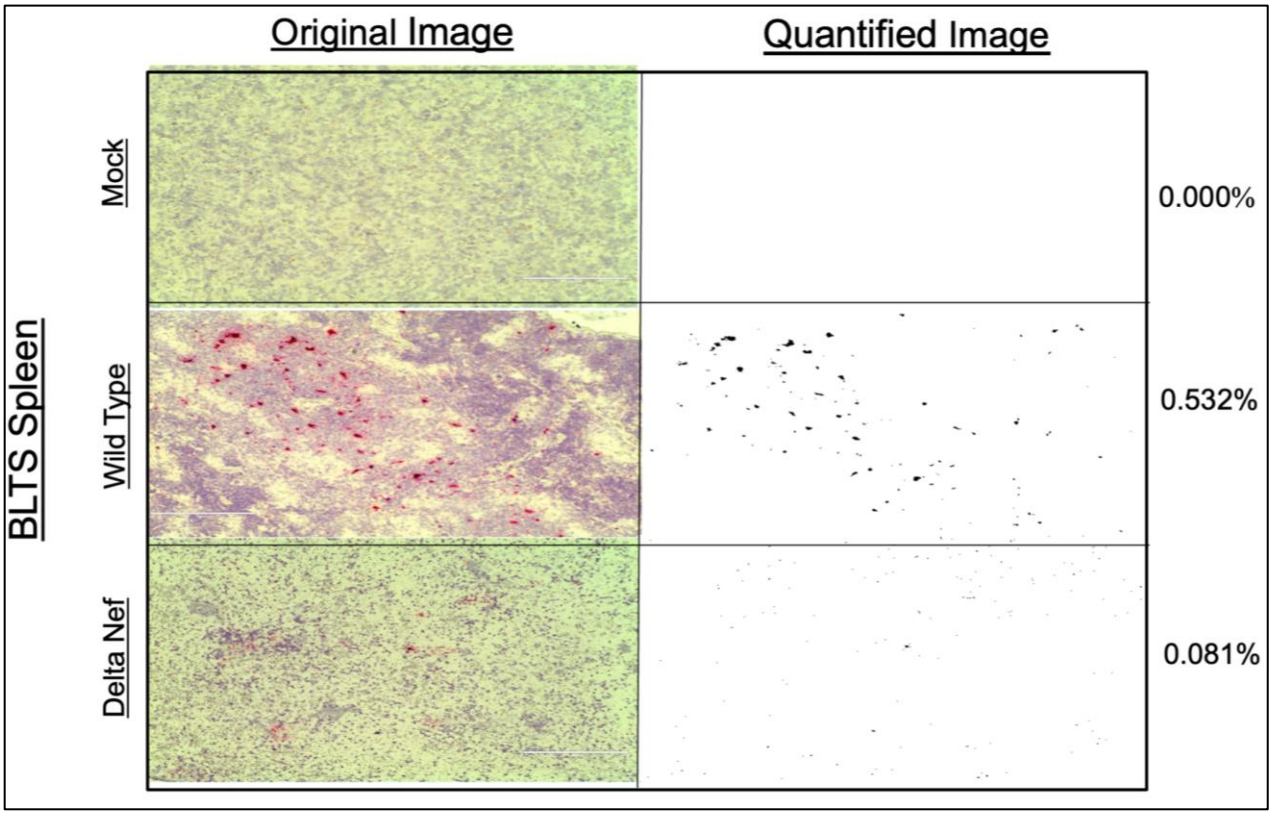


Figure 7: RNA-scope analysis of BLTS spleen tissues targeting HIV-1 RNA: Sections of BLTS humanized spleen stained using RNA-Scope to detect HIV-RNA. ImageJ analysis of each slide was done to determine percentage of area posititve for the respective HIV RNA primers.

4.4 Stimulation of T cells from the HSC-Mice Spleen in tissue culture produced high cytokine functionality

T cell stimulation is known to be achieved through a multi-faceted route. Naïve T cells, upon leaving the thymus, undergo their training to become mature T cells depending on whether they have a CD4 or CD8 marker on them, which dictates their role in either being a helper T cell or a cytotoxic one. But T cells, during this training, must communicate with Antigen Presenting Cells (APCs) like Dendritic cells. They do so by using their T-Cell Receptors (TCRs) and co-receptors like CD28 with also the use of cytokines using feedback loop-triggered activation (20). Therefore, a commercial kit that employs magnetic beads that replace APCs, bearing anti-CD3 and anti-CD28 antibodies (Catalog: 11131D) on their surface were used as a method for T cell activation. The other method used, was the use of a superantigen Phytohemagglutinin or PHA which is a T cell mitogen that has the capability to bind surface proteins like the TCR through their sugars, thereby aiding in cross-linking them. This in turn leads to a strong series of signals leading to t cell activation and has already been used as a method of HIV-1 production before (21). This experiment was done to decide the functionality of the spleenocytes in HSC-humanized mice.

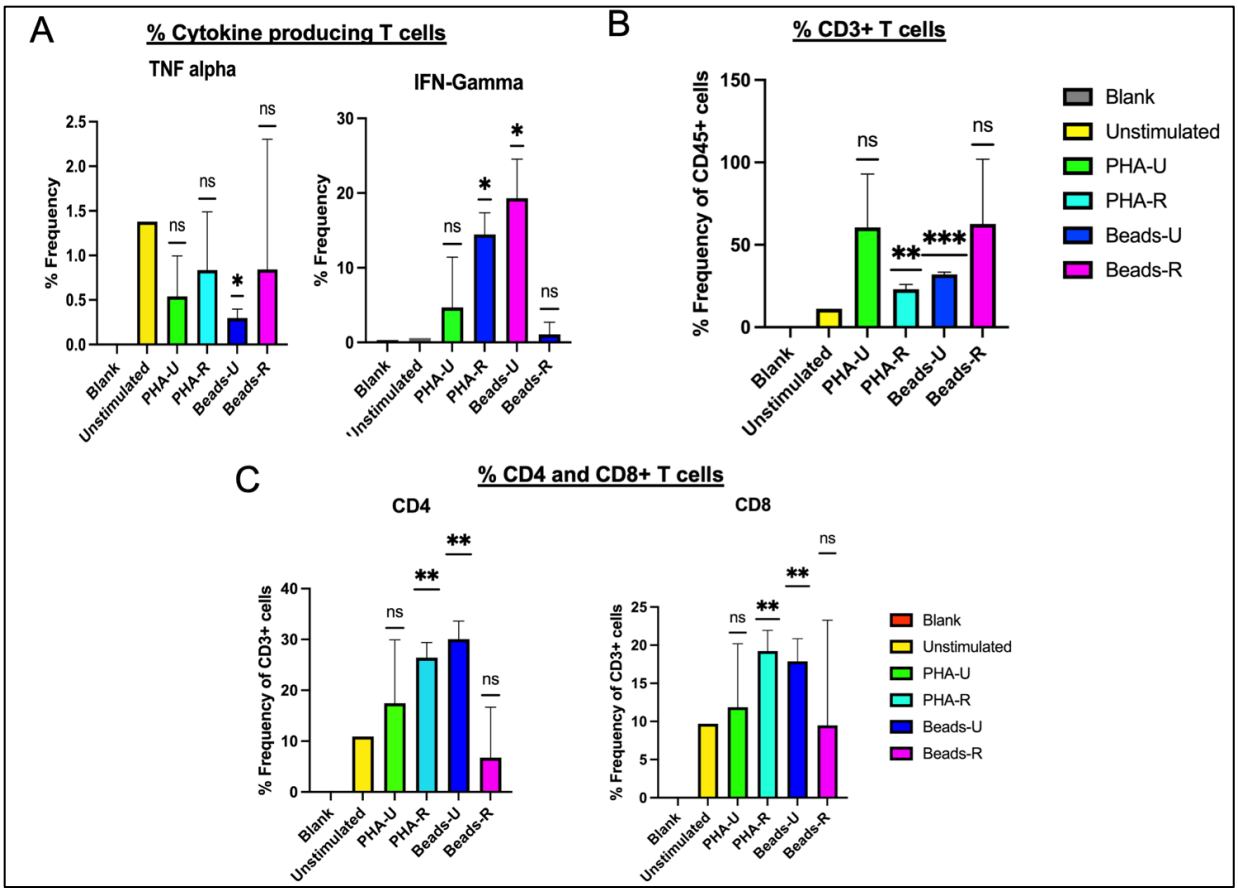


Figure 8: Graphical summary of T cell functionality assay of HSC-humanized mice spleen: (A) Graphs showing %Frequency of cytokine producing T cells across all methods, quantified for TNF-Alpha and IFN Gamma. (B) %CD3+ T cell frequency out of the CD45+ immune cell population across all methods. (C) % CD4 and CD8+ T cell subpopulation out of the CD3+ T cells for all methods. Graphical analysis- One sample t-tests (n=2) was performed on Graphpad PRISM.

Some of these protocols also call for re-stimulation of T cells after nearly 5 days in a cell culture with a stimulating medium. All these factors lead to the four conditions cited in Figure 8, the two main methods being CD3/28 beads Vs. PHA stimulation and the sub-methods of whether they were restimulated on day 6 or on the day of the harvest. Fig.8B looks at the dot plots of CD45+ CD3+ T cells taken from culture and the culture with PHA stimulation with restimulation on day 6 was found to be the highest with 84% of live cells compared to the lowest yield which was Beads-with restimulation at 19.2% of live cells. The activity of the T cells post-stimulation was measured through flow cytometry as well, by intracellular staining for cytokines TNF-alpha

and IFN-gamma which are associated with the process of T cell activation. Fig.8A shows that nearly 0.5-1.0% of TNF-alpha was the highest produce, was found in both the restimulated cultures, but when it came to IFN-gamma, nearly 20% of T cells produced it through the Bead technique alone, which was the highest. Fig.8C summarizes the percentages of CD4+ helper T cells and CD8+ cytotoxic T cells that were found out of the total CD3+T cells in all four conditions. Not unsurprisingly, CD4 markers were more abundant than CD8 markers, keeping these cultures' CD4/CD8 ratio well above 1, however the highest producer varied with Bead, when non-restimulated produced the highest CD4+T cells and PHA with restimulation produced the highest CD8+T cells. Overall, from these analyses, both methods shows that HSC-Hu.Mice spleen produce functionally active T cells.

4.5 Activated NKs and mature DCs identified in BLTS model compares to human counterpart

I then wanted to analyze the presence of tissue resident-NKs and DCs in the BLTS humanized mouse model to determine with contrast to its human counterpart to determine efficacy of the model to further my thesis studies. Fig. 9A shows the high NK cell count of 5 to a low 1

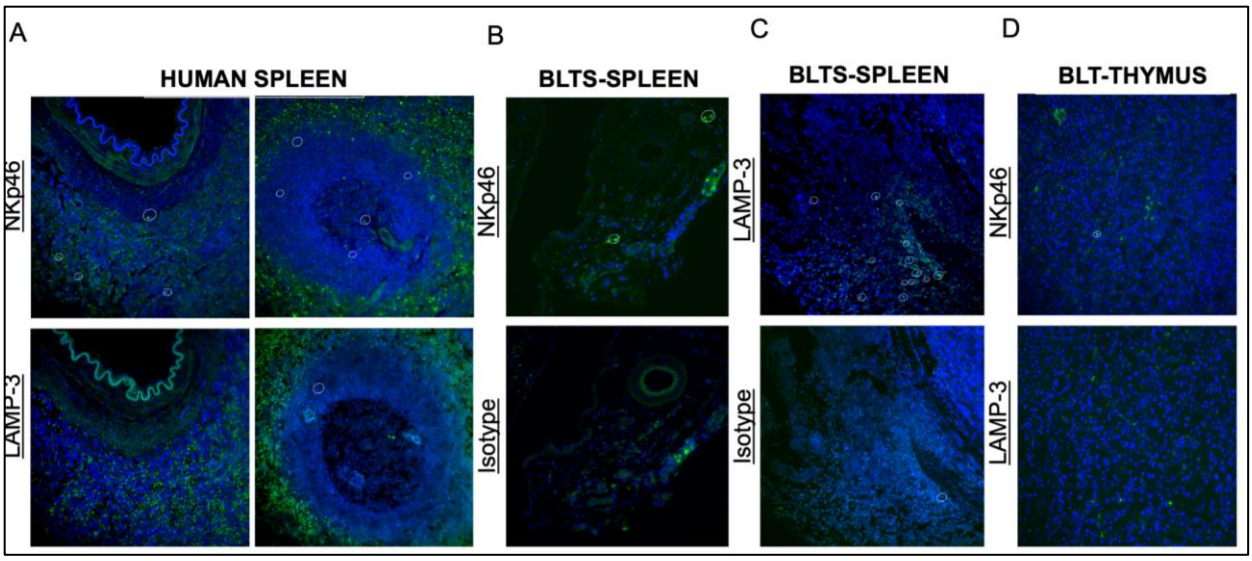


Figure 9: Localization of tissue resident-NKs and DCs in the BLTS mouse model: (A) NKp46 Vs. LAMP3 staining was applied on serial sections of human spleen as control for all samples. Serial sections of BLTS humanized Spleen stained with (B) NKp46 Vs. Isotype and (C) LAMP-3 Vs. Isotype using Immunofluoresence technique. (D) NKp46 Vs. LAMP3 staining was applied on serial sections of BLTS-Thymus. The secondary fluorophore used is Alexa Flour 488 (FITC) and cellular nuclei were stained with DAPI diffused in the mounting medium. Images were captured using a fluorescense microscope with FITC and DAPI filters.

dendritic cell in these areas of cross section under 20x magnification, in serial human spleen sections. Figures 9B and C were done initially to determine the efficacy of the antibodies used to detect them-NKp46, which is a marker for activated natural killer cells and LAMP-3, which is a marker of mature dendritic cells, by comparing serial sections of the BLTS-Spleen tissues through isotype staining. The location of the NKs inside the follicles and dendritic cells all throughout the rest of the tissue gives comparable identity to the human spleen. Lastly, these cells were found to be in lower concentration in the BLT thymus tissues (Fig. 9D), which translates to the low presence of these tr-NKs and DCs in human thymus from previous studies. Overall, the cells weren't over-expressed due to absence of any infection as well. In conclusion, the BLTS model can successfully replicate similar tissue resident- NK and DC distribution to the human immune organs and can be infected to study their crosstalk further.

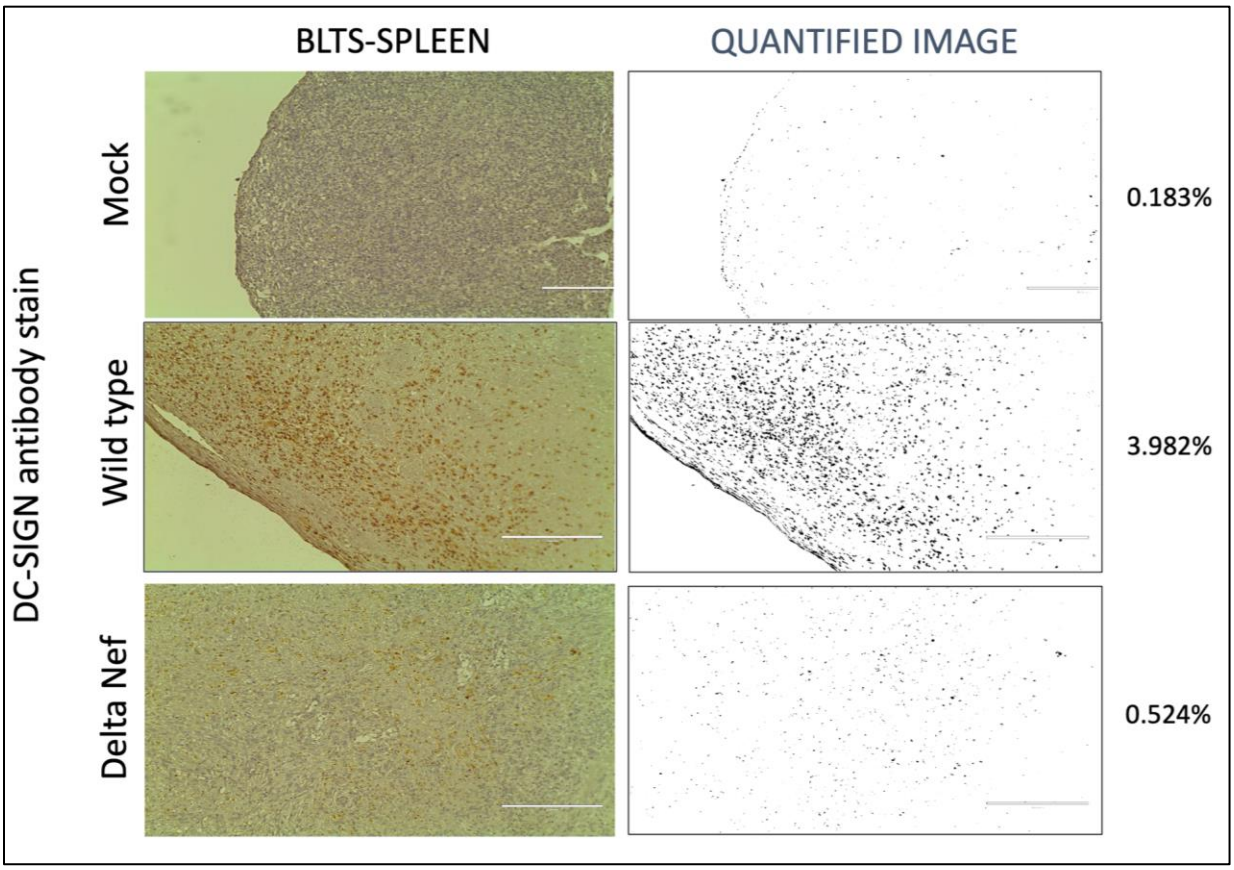


Figure 10: Quantification of DCSIGN in wild type compared to delta nef mutation: IHC staining was performed on BLTS spleen tissues to compare the spread of DCSIGN-receptor in wildtype, delta Nef HIV compareed to Mock. Positive areas were quantified using ImageJ analysis.

Figure 10 captures the differences that each infection has on the amounts of DCSIGN found in BLTS spleen at 12 weeks post infection. Mock is the positive control and has a 0.183% of the receptor spread out. Compared to this healthy state, we see an increase in DCSIGN no matter which mutation of HIV. Wildtype infection has an overly increased population of DCSIGN with a 3.98% coverage compared to delta Nef which was only 0.52%.

5.0 DISCUSSION

One of the major roadblocks on the path to identify therapeutics for HIV cure is HIV latency and its subsequent reversal. This is due to the early establishment of latency during the acute infection phase, thereafter which it remains unaffected even if treatments like ART, HAART are administered early (23). Therefore, achieving latency reversal in a less harmful method has been the goal of HIV research in recent years due to its potential to gain permanent clearance of the virus from the hosting human being. Most cells harboring latent provirus are CD4+T cells (major), γδ T cells and CD4+ naïve T cells (minor) that we know of thus far (24). Another rather older roadblock to solving HIV, is the inability to study the virus in animal models due to the high specific infectivity to only humans. This saw the advent of multiple humanized animal models, of which the BLTS model has been an established model to study HIV in our lab. Fig.2 and Fig.3 confirm the efficiency of reconstitution of the major immune cells in the BLTS model. CD3+, CD4+, CD8+ T cells were distributed as found in nature. CD4 to CD8 ratio was comfortably above 1.0 which indicates appropriate replication of natural T cell subtype distribution (28).

Nef is a viral accessory protein coded for and carried in the HIV-1 RNA, that is known to facilitate HIV replication and help evade destruction of the virus by the innate immune system through mechanisms such as CD4 downregulation (26). This has been confirmed through real life cases (27) and laboratory humanized models where Nef defective HIV fails to replicate well and doesn't progress to AIDS. This was confirmed through percentage area findings from Fig.6 and Fig.7 wherein Nef defective HIV produced forth a lower viral load, lesser infection and virtually no damage to the BLTS spleen tissue morphology when compared to the wildtype infection detected through p24 protein detection and HIV-RNA detection, respectively. This, in a dual

functionality, also confirms the efficiency of the BLTS model developed in our lab, to sustain a clinically accurate infection of HIV and its variants.

Previously, it was believed that the human innate immune cells didn't play much of a role in HIV pathogenesis due its quick transfer to the adaptive immune system as its course of infection. However, interestingly, through gene profiling studies done in our lab previously (under review), reveal that in HIV infected BLT Hu. mice models show increased NK-DC crosstalk signaling amongst other observations. It was also noted that IL-15 production seemed to be upregulated in Nef defective strains of HIV when compared to the wild-type strain. This indicates elevated NK activation through IL-5 cytokine production by HIV-activated monocytes. These observations suggest that the absence/disruption of Nef protein seems to increase the innate immune response which in-turn suggests that Nef is directly or indirectly responsible for the dysregulation of innate immune response upon initial HIV entry. Herein lies the crux of my thesis and the center of Aim 2 which is to explore exactly how Nef is able to achieve this.

On the road to achieve this, my work also encompassed the development of a Fe-Lo (19) incorporated humanized mice model termed HSC-humanized mice, to bypass the usage of fetal tissues. The HSC-humanized spleenocytes were then preliminarily analyzed for their ability to activate and produce cytokines, through T cell functionality assays. A confirmation of comparable immune cell reconstitution in the HSC-humanized spleen was done through flow cytometric analyses of the humanized spleen compared to human PBMC control in Fig.5 suggesting a viable HIV animal model. Fig.8 shows the summary of the flow cytometric analyses done using two types of stimulation to confirm the functionality of production of TNF-alpha and IFN-gamma at a comparable level, indicating the proper activation of T cells present in the spleen and the

plausibility to use this model as a HIV-study model (30). This model is a work in progress, but initial results are promising.

It was then apparent to identify and locate the positioning of NKs and DCs in the healthy BLTS humanized spleen and thymus to determine closeness in comparison to a human immune organ (in this case: human spleen). Since activated NKs have the capability to in-turn activate DCs to communicate with CD8+T cells, through inflammatory cytokine production, these cells were my target cells. Fig.9 A shows the density of activated NKs to be more than mature DCs and that they have been located in and just around the B cell germinal centers, which is the actual case from clinical literature on structure of a human spleen (18). Human spleen also shows us that NKs and DCs seems to be closely located with each other which could be one of the reasons for better crosstalk function. BLTS spleen which seems to possess lower tissue resident-NKs and DCs, nevertheless, seems to more accurate in terms of location of these cells (Fig. 9 B, C). BLT Thymus however, possessed very little thymic NKs and DCs (Fig. 9 D), which agrees with the composition of healthy human thymus (31). This confirms that the BLTS model is an accurate model to study the DC-NK crosstalk in-vivo and in-vitro through a co-culture (32). DCSIGN or CD209 is a C-type lectin family of receptors found on DCs majorly and are known to perform adhesion and pathogen recognition in these immune cells. In dendritic cells, they mediate the contact to ICAM-3 found on T cells for antigen presentation (39). HIV is known to use DCSIGN to attach onto dendritic cells using its gp120 to then be delivered to their target cells in the lymph nodes (38). It therefore makes sense that DCSIGN appeared to be vastly increased in the BLTS spleen infected with wildtype HIV and absence of Nef accounts to a significantly lower increase of DCSIGN around areas of infection, indicating presence of the viral load indirectly as well (Fig.10).

5.1 Public health significance

The importance of Nef defectiveness in facilitating an appropriate adaptive response has been further illustrated by this data and understanding the consequences of HIV-mediated dysfunction on the NK cell repertoire is critical for continued research into restoring immune functionality and improving the long-term health outcomes of PLWH. This is due in large part to the particularly unique nature of NK cells to mediate both arms of the immune system in addition to their innate antiviral capabilities. The significance of determining the role of Nef (or lack thereof) in HIV with respect to the control of the innate immune system will in, a small way contribute to our collective knowledge pool of the HIV viral mechanisms. This in turn can take us a step closer to improving current HIV vaccine designs such as the 'kick and kill' method in terms of employing cells from the human immune system like dendritic cells to "kick" HIV out of its latent hiding reservoirs.

5.2 Future directions

With the limited time allocated on my MS degree, this was the extent of findings that were accomplished. This, however small it maybe, is sufficient seed data for future studies to come. The next order of events is to identify the DC-NK crosstalk on infected BLTS humanized spleen through immunofluorescence. To confirm this, we also plan to perform a series of infections on co-cultures with T cells, NKs, DCs to identify through a secondary method, how Nef dysregulates these immune cell interactions. The BLTS humanized rat model is a work in progress and needs to be established in terms of its immune cell reconstitution in both blood and spleen. Optimization

of this model is the next crucial step, since achieving a humanized rat model would allow longer duration HIV studies, due the natural longer lifespan of rats (up to a year).

Bibliography

1. Chinen, Javier, and William T. Shearer. "Molecular Virology and Immunology of Hiv Infection." Journal of Allergy and Clinical Immunology 110, no. 2 (2002): 189-98. https://doi.org/10.1067/mai.2002.126226.

2. Chapter 25 - Human Immunodeficiency Virus. F. Cassis-Ghavami, M. Curlin, R. Geise and A. DuerrIn: Vaccines for Biodefense and Emerging and Neglected Diseases, edited by A. D. T. Barrett and L. R. Stanberry. Academic Press 2009. https://www.sciencedirect.com/science/article/pii/B9780123694089000251.

3. Global HIV & AIDS statistics - https://www.unaids.org/en/resources/fact-sheet.

4. Fanales-Belasio, E., M. Raimondo, B. Suligoi, and S. Buttò. "Hiv Virology and Pathogenetic Mechanisms of Infection: A Brief Overview." [In eng]. *Ann Ist Super Sanita* 46, no. 1 (2010): 5-14. https://doi.org/10.4415/ann_10_01_02.

5. Chun, Tae-Wook, and Anthony S. Fauci. "Hiv Reservoirs: Pathogenesis and Obstacles to Viral Eradication and Cure." AIDS 26, no. 10 (2012): 1261-68.

6. Staudt, R. P., J. J. Alvarado, L. A. Emert-Sedlak, H. Shi, S. T. Shu, T. E. Wales, J. R. Engen, and T. E. Smithgall. "Structure, Function, and Inhibitor Targeting of Hiv-1 Nef-Effector Kinase Complexes." [In eng]. *J Biol Chem* 295, no. 44 (Oct 30 2020): 15158-71. https://doi.org/10.1074/jbc.REV120.012317.

7. Dionne, B. "Key Principles of Antiretroviral Pharmacology." [In eng]. *Infect Dis Clin North Am* 33, no. 3 (Sep 2019): 787-805. https://doi.org/10.1016/j.idc.2019.05.006.

8. Tanaka, K., Y. Kim, M. Roche, and S. R. Lewin. "The Role of Latency Reversal in Hiv Cure Strategies." [In eng]. *J Med Primatol* 51, no. 5 (Oct 2022): 278-83. https://doi.org/10.1111/jmp.12613.

9. Fernandez, N. C., A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, *et al.* "Dendritic Cells Directly Trigger Nk Cell Functions: Cross-Talk Relevant in Innate Anti-Tumor Immune Responses in Vivo." [In eng]. *Nat Med* 5, no. 4 (Apr 1999): 405-11. https://doi.org/10.1038/7403.

10. Thomas, R., and X. Yang. "Nk-Dc Crosstalk in Immunity to Microbial Infection." [In eng]. *J Immunol Res* 2016 (2016): 6374379. https://doi.org/10.1155/2016/6374379.

11. Boggiano, C., N. Manel, and D. R. Littman. "Dendritic Cell-Mediated Trans-Enhancement of Human Immunodeficiency Virus Type 1 Infectivity Is Independent of Dc-Sign." [In eng]. *J Virol* 81, no. 5 (Mar 2007): 2519-23. https://doi.org/10.1128/jvi.01661-06.

12. Altfeld, M., L. Fadda, D. Frleta, and N. Bhardwaj. "Dcs and Nk Cells: Critical Effectors in the Immune Response to Hiv-1." [In eng]. *Nat Rev Immunol* 11, no. 3 (Mar 2011): 176-86. https://doi.org/10.1038/nri2935.

13. Cohen, G. B., R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, and D. Baltimore. "The Selective Downregulation of Class I Major Histocompatibility Complex Proteins by Hiv-1 Protects Hiv-Infected Cells from Nk Cells." [In eng]. *Immunity* 10, no. 6 (Jun 1999): 661-71.

14. Whitney, J. B., and R. Brad Jones. "In Vitro and in Vivo Models of Hiv Latency." [In eng]. *Adv Exp Med Biol* 1075 (2018): 241-63. https://doi.org/10.1007/978-981-13-0484-2_10.

15. Letvin, N L, K A Eaton, W R Aldrich, P K Sehgal, B J Blake, S F Schlossman, N W King, and R D Hunt. "Acquired Immunodeficiency Syndrome in a Colony of Macaque Monkeys." *Proceedings of the National Academy of Sciences* 80, no. 9 (1983): 2718-22. https://doi.org/doi:10.1073/pnas.80.9.2718. https://www.pnas.org/doi/abs/10.1073/pnas.80.9.2718.

16. Skelton, J. K., A. M. Ortega-Prieto, and M. Dorner. "A Hitchhiker's Guide to Humanized Mice: New Pathways to Studying Viral Infections." [In eng]. *Immunology* 154, no. 1 (May 2018): 50-61. https://doi.org/10.1111/imm.12906.

17. Biradar, Shivkumar, Yash Agarwal, Michael T. Lotze, Moses T. Bility, and Robbie B. Mailliard. "The Blt Humanized Mouse Model as a Tool for Studying Human Gamma Delta T Cell-Hiv Interactions in Vivo." [In English]. Original Research. *Frontiers in Immunology* 13 (2022-May-20 2022). https://doi.org/10.3389/fimmu.2022.881607. https://www.frontiersin.org/articles/10.3389/fimmu.2022.881607.

18. Lewis, Steven M., Adam Williams, and Stephanie C. Eisenbarth. "Structure and Function of the Immune System in the Spleen." *Science Immunology* 4, no. 33 (2019): eaau6085. https://doi.org/doi:10.1126/sciimmunol.aau6085. https://www.science.org/doi/abs/10.1126/sciimmunol.aau6085.

19. Velazquez, Jeremy, Ryan LeGraw, Farzaneh Moghadam, Yuqi Tan, Jacquelyn Kilbourne, Joseph Maggiore, Joshua Hislop, *et al.* "Gene Regulatory Network Analysis and Engineering Directs Development and Vascularization of Multilineage Human Liver Organoids." *Cell Systems* 12 (12/01 2020). https://doi.org/10.1016/j.cels.2020.11.002.

20. Roh, Kyung-Ho. "Artificial Methods for T Cell Activation: Critical Tools in T Cell Biology and T Cell Immunotherapy." In *Biomimetic Medical Materials: From Nanotechnology to 3d Bioprinting*, edited by Insup Noh, 207-19. Singapore: Springer Singapore, 2018.

21. Blasi, M., B. Balakumaran, P. Chen, D. R. Negri, A. Cara, B. K. Chen, and M. E. Klotman. "Renal Epithelial Cells Produce and Spread Hiv-1 Via T-Cell Contact." [In eng]. *Aids* 28, no. 16 (Oct 23 2014): 2345-53. https://doi.org/10.1097/qad.0000000000000398.

22. Perot, Brieuc P., Victor García-Paredes, Marine Luka, and Mickaël M. Ménager. "Dendritic Cell Maturation Regulates Tspan7 Function in Hiv-1 Transfer to Cd4+ T Lymphocytes." [In English]. Original Research. *Frontiers in Cellular and Infection Microbiology* 10 (2020-February-28 2020). https://doi.org/10.3389/fcimb.2020.00070. https://www.frontiersin.org/articles/10.3389/fcimb.2020.00070.

23. Mbonye, Uri, and Jonathan Karn. "The Molecular Basis for Human Immunodeficiency Virus Latency." *Annual Review of Virology* 4, no. 1 (2017): 261-85. https://doi.org/10.1146/annurev-virology-101416-041646. https://www.annualreviews.org/doi/abs/10.1146/annurev-virology-101416-041646.

24. Kim, Y., J. L. Anderson, and S. R. Lewin. "Getting the "Kill" into "Shock and Kill": Strategies to Eliminate Latent Hiv." [In eng]. *Cell Host Microbe* 23, no. 1 (Jan 10 2018): 14-26. https://doi.org/10.1016/j.chom.2017.12.004.

25. Staudt, R. P., J. J. Alvarado, L. A. Emert-Sedlak, H. Shi, S. T. Shu, T. E. Wales, J. R. Engen, and T. E. Smithgall. "Structure, Function, and Inhibitor Targeting of Hiv-1 Nef-Effector Kinase Complexes." [In eng]. *J Biol Chem* 295, no. 44 (Oct 30 2020): 15158-71. https://doi.org/10.1074/jbc.REV120.012317.

26. Mariani, R., and J. Skowronski. "Cd4 Down-Regulation by Nef Alleles Isolated from Human Immunodeficiency Virus Type 1-Infected Individuals." [In eng]. *Proc Natl Acad Sci U S A* 90, no. 12 (Jun 15, 1993): 5549-53. https://doi.org/10.1073/pnas.90.12.5549.

27. Rhodes, D. I., L. Ashton, A. Solomon, A. Carr, D. Cooper, J. Kaldor, and N. Deacon. "Characterization of Three Nef-Defective Human Immunodeficiency Virus Type 1 Strains Associated with Long-Term Nonprogression. Australian Long-Term Nonprogressor Study Group." [In eng]. *J Virol* 74, no. 22 (Nov 2000): 10581-8. https://doi.org/10.1128/jvi.74.22.10581-10588.2000.

28. CD4:CD8ratio https://www.urmc.rochester.edu/encyclopedia/content.aspx?contenttypeid=167&contentid=cd4_cd8_ratio#:~:text=A%20normal%20CD4%2FCD8%20ratio,HIV.

29. Tolstrup, Martin, Lars Ostergaard, Alex L Laursen, Skou Finn Pedersen, and Mogens Duch. "HIV / SIV Escape from Immune Surveillance: Focus on Nef." Current HIV Research 2, no. 2 (2004): 141–51. https://doi.org/10.2174/1570162043484924.

30. Zappasodi, R., S. Budhu, M. Abu-Akeel, and T. Merghoub. "In Vitro Assays for Effector T Cell Functions and Activity of Immunomodulatory Antibodies." [In eng]. *Methods Enzymol* 631 (2020): 43-59. https://doi.org/10.1016/bs.mie.2019.08.012.

31. Blackburn, Clare, and Nancy Manley. "Developing a New Paradigm for Thymus Organogenesis." *Nature reviews. Immunology* 4 (05/01 2004): 278-89. https://doi.org/10.1038/nri1331.

32. Svanberg, Cecilia, Sofia Nyström, Melissa Govender, Pradyot Bhattacharya, Karlhans F. Che, Rada Ellegård, Esaki M. Shankar, and Marie Larsson. "Hiv-1 Induction of Tolerogenic Dendritic Cells Is Mediated by Cellular Interaction with Suppressive T Cells." [In English]. Original Research. *Frontiers in Immunology* 13 (2022-August-10 2022). https://doi.org/10.3389/fimmu.2022.790276. https://www.frontiersin.org/articles/10.3389/fimmu.2022.790276.

33. Thippeshappa, Rajesh, Jason T. Kimata, and Deepak Kaushal. "Toward a Macaque Model of Hiv-1 Infection: Roadblocks, Progress, and Future Strategies." [In English]. Review. *Frontiers in Microbiology* 11 (2020-May-13 2020). https://doi.org/10.3389/fmicb.2020.00882. https://www.frontiersin.org/articles/10.3389/fmicb.2020.00882.

34. George, M. P., A. Brower, H. Kling, T. Shipley, J. Kristoff, T. A. Reinhart, M. Murphey-Corb, *et al.* "Pulmonary Vascular Lesions Are Common in Siv- and Shiv-Env-Infected Macaques." [In eng]. *AIDS Res Hum Retroviruses* 27, no. 2 (Feb 2011): 103-11. https://doi.org/10.1089/aid.2009.0297.

35. Balzarini, J., E. De Clercq, and K. Uberla. "Siv/Hiv-1 Hybrid Virus Expressing the Reverse Transcriptase Gene of Hiv-1 Remains Sensitive to Hiv-1-Specific Reverse Transcriptase Inhibitors after Passage in Rhesus Macaques." [In eng]. *J Acquir Immune Defic Syndr Hum Retrovirol* 15, no. 1 (May 1 1997): 1-4. https://doi.org/10.1097/00042560-199705010-00001.

36. Bănică, L., O. Vlaicu, R. Jipa, A. Abagiu, I. Nicolae, E. Neaga, D. Oţelea, and S. Paraschiv. "Exhaustion and Senescence of Cd4 and Cd8 T Cells That Express Co-Stimulatory Molecules Cd27 and Cd28 in Subjects That Acquired Hiv by Drug Use or by Sexual Route." [In eng]. *Germs* 11, no. 1 (Mar 2021): 66-77. https://doi.org/10.18683/germs.2021.1242.

37. Sugimoto, C., K. Tadakuma, I. Otani, T. Moritoyo, H. Akari, F. Ono, Y. Yoshikawa, *et al.* "Nef Gene Is Required for Robust Productive Infection by Simian Immunodeficiency Virus of T-Cell-Rich Paracortex in Lymph Nodes." [In eng]. *J Virol* 77, no. 7 (Apr 2003): 4169-80. https://doi.org/10.1128/jvi.77.7.4169-4180.2003.

38. Geijtenbeek, T. B., G. C. van Duijnhoven, S. J. van Vliet, E. Krieger, G. Vriend, C. G. Figdor, and Y. van Kooyk. "Identification of Different Binding Sites in the Dendritic Cell-Specific Receptor Dc-Sign for Intercellular Adhesion Molecule 3 and Hiv-1." [In eng]. *J Biol Chem* 277, no. 13 (Mar 29 2002): 11314-20. https://doi.org/10.1074/jbc.M111532200.

39. Geijtenbeek, T. B., D. J. Krooshoop, D. A. Bleijs, S. J. van Vliet, G. C. van Duijnhoven, V. Grabovsky, R. Alon, C. G. Figdor, and Y. van Kooyk. "Dc-Sign-Icam-2 Interaction Mediates Dendritic Cell Trafficking." [In eng]. *Nat Immunol* 1, no. 4 (Oct 2000): 353-7. https://doi.org/10.1038/79815.

40. Learmont, Jennifer C., Andrew F. Geczy, John Mills, Lesley J. Ashton, Camille H. Raynes-Greenow, Roger J. Garsia, Wayne B. Dyer, *et al.* "Immunologic and Virologic Status after

14 to 18 Years of Infection with an Attenuated Strain of Hiv-1 — a Report from the Sydney Blood Bank Cohort." *New England Journal of Medicine* 340, no. 22 (1999): 1715-22. https://doi.org/10.1056/nejm199906033402203.