DEVELOPMENT OF A HUMAN IMMUNE SYSTEM FROM HEMATOPOIETIC STEM CELLS IN A HUMAN/MOUSE XENOGENEIC MODEL

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DEDICATION

I dedicate my Ph.D. to my parents, Francis and Mary Melkus. Their love and support for my siblings and me, can not be measured by any scientific standard. You have brought me many glories and riches! Thank you for eternally being there for me.

I wish to thank Drs. Michael San Francisco and Richard Larry Blanton for providing me the skills and the encouragement to attend the University of Texas Southwestern Medical Center. I praise the entire Garcia laboratory members for their friendship, support, hard work and encouragement through all the years. Without Angel Padgett-Thomas and Joel Gatlin, I wouldn't have accomplished it. Thank you to Drs. Danny Douek, Don Sodora, David Margolis and Laurie Davis laboratories for scientific incite, mentoring and most importantly friendship. Especially, thank you to Drs. Jacob Estes and Ashley Haase for their endeavors, hard work, and scientific incite in this collaboration. I appreciate all the endeavors and knowledge my committee provided me, Drs. Michael Bennett, David Farrar, John Schatzle, Phillip Shaul and Victor Garcia. Thank you to Dr. Nancy Street for all of her encouragement. Prost to J. Victor Garcia. I learned much more than science. I appreciate all of your endeavors, hardships and opportunities you provided me in your lab. Thank you! And to my dearest friend Dr. Cheryl Vahling, thank you for all of your support, friendship, good times and more to surely come.

DEVELOPMENT OF A HUMAN IMMUNE SYSTEM FROM HEMATOPOIETIC STEM CELLS IN A HUMAN/MOUSE XENOGENEIC MODEL

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DISSERTATION

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T cells play a central role in the development of immune responses. Patients lacking T cells due to genetic defects such as DiGeorge or Nezelof's syndrome and individuals infected with the human immunodeficiency virus are highly susceptible to infections and cancers. The lack of adequate *in vivo* models of T cell neogenesis has hindered the development and clinical implementation of effective therapeutic modalities aimed at treating these and other clinically important maladies. Transplantation of severe combined immunodeficient (SCID) mice with human hematopoietic stem cells results in long-term engraftment and systemic reconstitution with human progenitor, B and myeloid cells but curiously, human T cells are rarely present in any tissue. While the implantation of SCID mice with human fetal thymus and liver (SCID-hu thy/liv mice) allows for the development

of abundant thymocytes that are localized to the human organoid implant, there is minimal systemic repopulation with human T cells. Here I present evidence that transplantation of autologous human hematopoietic fetal liver $CD34^+$ cells into NOD/SCID mice previously implanted with fetal thymic and liver tissues results in long-term, systemic human T cell homeostasis. In addition to human T cells, these mice have systemic repopulation with human B cells, monocytes/macrophages and dendritic cells (DC). This mouse model of the human immune system has been designated as **BLT** for a **B**one marrow transplant in fetal **L**iver and **T**hymus implanted mice. T cells in these mice generate human MHC Class I and Class II restricted adaptive immune responses to Epstein Barr virus infection and are activated by human DCs to mount potent T cell immune response to super antigens. Administration of the super-antigen toxic shock syndrome toxin-1 (TSST-1) resulted in the specific systemic expansion of human $V\beta2^+$ T cells, release of human pro-inflammatory cytokines and localized specific activation and maturation of human $CD11c^+$ dendritic cells. These results represent the first demonstration of long-term systemic human T cell reconstitution *in vivo* allowing for the manifestation of the differential response by human DCs to TSST-1.

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PRIOR PUBLICATIONS

1. **Melkus, M.W.**, Estes, J.D., Padgett-Thomas, A., Gatlin, J., Denton, P.W., Othieno, F., Wege, A.K., Haase, A.T. and Garcia, J.V., "Humanized Mice Mount Specific Adaptive and Innate Immune Responses to EBV and TSST-1", Nature Medicine 2006, Vol. 12 (11): 1316-1322.

2. Bente, D.A., **Melkus, M.W.**, Garcia, J.V., Rico-Hesse, R., "Dengue Fever in Humanized NOD/SCID Mice", Journal of Virology 2005. Vol. 79 (21): 13797-13799.

3. Cravens, P.C., **Melkus, M.W.**, Padgett-Thomas, A., Islas-Ohlmayer, M., Martin, M., Garcia, J.V. "Development and Activation of Human Dendritic Cells *In Vivo* in a Xenograft Model of Human Hematopoiesis", Stem Cells 2005, Vol. 23: 264-278.

4. Islas-Ohlmayer, M., Padgett-Thomas, A., **Melkus, M.W.**, Cravens, P., Martin, M., Garcia, J.V.. "Experimental Infection of NOD/SCID Mice Reconstituted with Human $CD34^+$ Cells with Epstein-Barr Virus." Journal of Virology 2004, Vol. 78 (24): 13891-1300.

5. Palucka AK, Gatlin J, Blanck JP, **Melkus M.W.**, Clayton S, Ueno H, Kraus ET, Cravens P, Bennett L, Padgett-Thomas A, Marches F, Islas-Ohlmayer M, Garcia JV, Banchereau J., "Human Dendritic Cell Subsets in NOD/SCID Mice Engrafted with $CD34^+$ Hematopoietic Progenitors." Blood 2003, Nov 1;102(9): 3302-10.

6. Gatlin J., **Melkus M.W.**, Padgett A, Petroll WM, Cavanagh HD, Garcia JV, Jester J. "In Vivo Fluorescent Labeling of Corneal Wound Healing Fibroblasts." Experimental Eye Research 2003 Mar:76(3) 361-71.

7. Gatlin J., Padgett, A., **Melkus M.W.**, Kelly P. F., and Garcia J.V.**. "**Long-Term Engraftment of NOD/SCID Mice with Human $CD34^+$ Cells Transduced by a Self-Inactivating (SIN) HIV-1 Vector." Human Gene Therapy 2001 Vol. 12 (9):1071-1090.

8. Gatlin J., **Melkus M.W.**, Padgett A., and Garcia J. V., "Engraftment of NOD/SCID Mice with Human $CD34^+$ Cells Transduced by Concentrated Oncoretroviral Vector Particles Pseudotyped with the Feline Endogenous Virus (RD114) Envelop Protein." Journal of Virology 2001 Vol. 75: 9995-9999.

9. Hasseloff B. J., Freeman T. L., Valmeekam V., **Melkus, M.W.**, Oner F., Valacovic M. S. and San Francisco M. J. D., "The *exuT* Gene of *Erwinia chrysanthemi* EC16: Nucleotide Sequence, Expression, Localization, and Relevance of the Gene Product." Molecular Plant Microbe Interactions 1998 Vol. 11, (4): 270-276.

10. Valmeekam V., **Melkus M.W.**, Hasseloff B. J.and San Francisco M. J. D., "Transport of Plant Cell Wall Molecules in Phytopathogenic Bacteria." Trends in Plant Pathology 1998 Vol. 1: 71-75.

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ABBREVIATIONS/ACRONYMS

APC – allophycocyanin

AZT – azidothymidine

BLT mouse – **B**one marrow transplant in fetal **L**iver and **T**hymus implanted mouse

BSA – bovine serum albumin

CB – cord blood

CBA – cytokine bead array

CD – cluster of differentiation

cDMEM - complete Dulbecco's Modified Eagle's Medium

CFU – colony forming unit

CGys – centi-Greys (radiation dose)

CPD – citrate phosphate dextrose

cRPMI – complete RPMI 1640 medium

EBV – Epstein-Barr virus

EDTA – ethylenediaminetetraacetic acid

EGFP – enhanced green fluorescent protein

FACS – fluorescent activated cell sorting

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

FL – fetal liver

FT – fetal thymus

G-CSF – granulocyte colony stimulating factor

GM-CSF – granulocyte macropahge colony stimulating factor

HIV – human immunodeficiency virus

HSCs – hematopoietic stem cells

Ig – immunoglobulin

IL – interleukin

IMDM – Iscove's Modified Dulbecco's Medium

INF – interferon

i.p. – intraperitoneal

i.v. – intravenous

LDL – low density lipoprotein

mAB – monoclonal antibody

μg – micrograms

μl – microliter

mg – milligrams

ml – milliliter

MNCs – mononuclear cells

MOI – multiplicity of infection

NOD/SCID mouse – non-obese diabetic/severe combined immunodeficent mouse

Organoid – a developed fetal thymus/fetal liver/fetal thymus implant

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PE – phycoerythrin

PerCP – peridinin chlorophyll protein

PHA – phytohemagglutinin

SCF – stem cell factor

SRBCs – sheep red blood cells

TCR – T cell receptor

TH – T helper

Thy/Liv – fetal thymus/fetal liver/fetal thymus implant

TSST-1 – toxic shock syndrome toxin-1

UCB – umbilical cord blood

Vβ TCR – V beta T cell receptor

VSVG – vesicular stomatitis virus G protein

PREFACE

Human stem cells have become a focal forefront of scientific research due to their potential for curing human diseases, but yet remain one of the most morally controversial topics within the scientific and political communities. Although embryonic stem cells have been identified to potentially alleviate/cure diseases such as Alzheimer's disease, Parkinson's disease, spinal cord injury, diabetes and many more, the use of embryonic stem cells has continued to raise much debate about whether these cells are an ethically viable source to study and more importantly, use to correct cellular/molecular deviances in human development, tissue damage, and disease states of human ailments. The use of these cells was even a central issue in the 2004 presidential campaign. Leading scientists, such as Irving Weissman, political movie star activists, Christopher Reeves and Michael J. Fox, as well as the conservative former first lady Nancy Reagan advocated the use of embryonic stem cell research in order to develop cures for human diseases. President George W. Bush Jr., shortly after being elected president, restricted the use of federal funds used for embryonic stem cell research to the limited 21 cell lines created before August 9, 2001. In response to his decision, California developed its own funding for stem cell research through a bond measure (Proposition 71) that devotes $3 billion to human embryonic stem cell research. Although I agree we must be critical in our decisions both morally and our scientific initiatives in unraveling the knowledge of the full potential of embryonic stem cells, I believe these cells retain curative promise that will one day be used routinely in the clinic to treat human ailments.

A pluripotent embryonic stem cell has the ability to self renew and give rise to lineage committed progenitor stem cells, which generates new cellular organization leading to tissue development and ultimately organized structures generating an entire living organism. Hence, these same pluripotent stem cells may provide the key in cellular plasticity needed to correct defective development and ultimately tissue damage. Primitive organisms such as starfish, salamanders and lizards have the capacity to regenerate limbs and tails following dismemberment as a survival mechanism to predator attack. How these primitive organisms monitor and invoke new regeneration of organized structures still eludes the scientific community. Do these organism possess stem cells which recognize tissue damage signals to regenerate new limbs? Much research has focused on identifying how these organisms are capable of identifing tissue damage and regenerate the appropriate biochemical gradients to induce new tissue-regeneration. Likewise, do mammals have the same capacity to self regenerate or correctively repair tissue damage if the proper stem cells are identified and appropriately transplanted (or triggered)? Although several adult stem cells have been identified in organ development, little is understood about how these cells generate and maintain cellular/organ development and most importantly their role to regenerate new tissue following organ damage. Are these stem cells exhausted during our normal development due to long life span, telomere shortening, maintenance of the tissues or the lack of tissue damage signaling? More importantly can we identify these stem cells and tap their resources for curative purposes? These are the questions for the next decade.

At present, the only identified stem cell routinely used in the clinical setting is the $CD34^{+}$ hematopoietic stem cell which is isolated from the bone marrow or G-CSF treated

donors for transplantion to recipient patients. Several companies are cryo-preserving cells from the cord blood after birth and charge minimal fees for storage for the possible use of the HSCs for future autologous treatments of disease. A new controversial trend that is developing is where a married couple will have another child in order to isolate and use cord blood $CD34^+$ cells from that child to perform a stem cell transplant to cure hematopoietic abnormalitities for their afflicted child. The use of $CD34^+$ cells has been extremely successful in the clinical setting and their potential plasticity for corrective procedures continues to be explored.

Due to the controversial issues of using embryonic stems cells, I have focused my research using human hematopoietic stem cells ($CD34^+$). $CD34^+$ cells have been previously demonstrated to engraft small animal models and generate many of the components of the human immune system. However, it is still unclear of the identity of the true T cell progenitor and the environmental factors needed to generate *de novo* T cells. A shortcoming in the field of human hematopoiesis is failure of human T cell development in animal models. It is critical to develop models of human hematopoiesis in which all lineages of the immune system develop in which we will be able to study immune development and manipulate it to generate cures for human diseases. Although no animal will fully recapitulate the complete human immune system, they will all provide new insight into pertinent biological and clinical questions from which cures to human disease may be developed.

CHAPTER I

INTRODUCTION/LITERATURE REVIEW

A. Stem cells

A stem cell is defined as an individual cell that has the potential to self renew and gives rise to differentiated progenitor cells which can further develop into a hierarchy of organize tissue(s).[1] Stem cells are part of the repair system for the body since they can divide and differentiate to replenish cell populations as needed to maintain the host organism. Stem cells have been categorized as either embryonic or somatic (adult) stem cells and are classified based on their ability to differentiate into specific cellular or tissue lineages. A fertilized egg (zygote) is considered the true totipotent cell since it contains all of the unbiased genetic information to generate an organism. During development the zygote undergoes various stages of divisions defined as a morula and then a blastula (embryo). If unmanipulated the embryo will develop into a viable organism. *In vitro*, embryonic stem cells have been isolated from the embryos of mice, rats, and more recently, humans[2,3] In contrast, somatic stem cells, which are derived from developed tissues, have also been isolated and are currently being pursued for therapeutic treatments of human diseases. The full potential of stem cells for curative approaches of human disease is still in its infancy.

A.1. Embryonic stem cells

Embryonic stem cells (ES) are isolated from the inner cell mass of the blastulocyst stage (day 5-7) of the developing embryo (Fig.1.1). ES cells have the

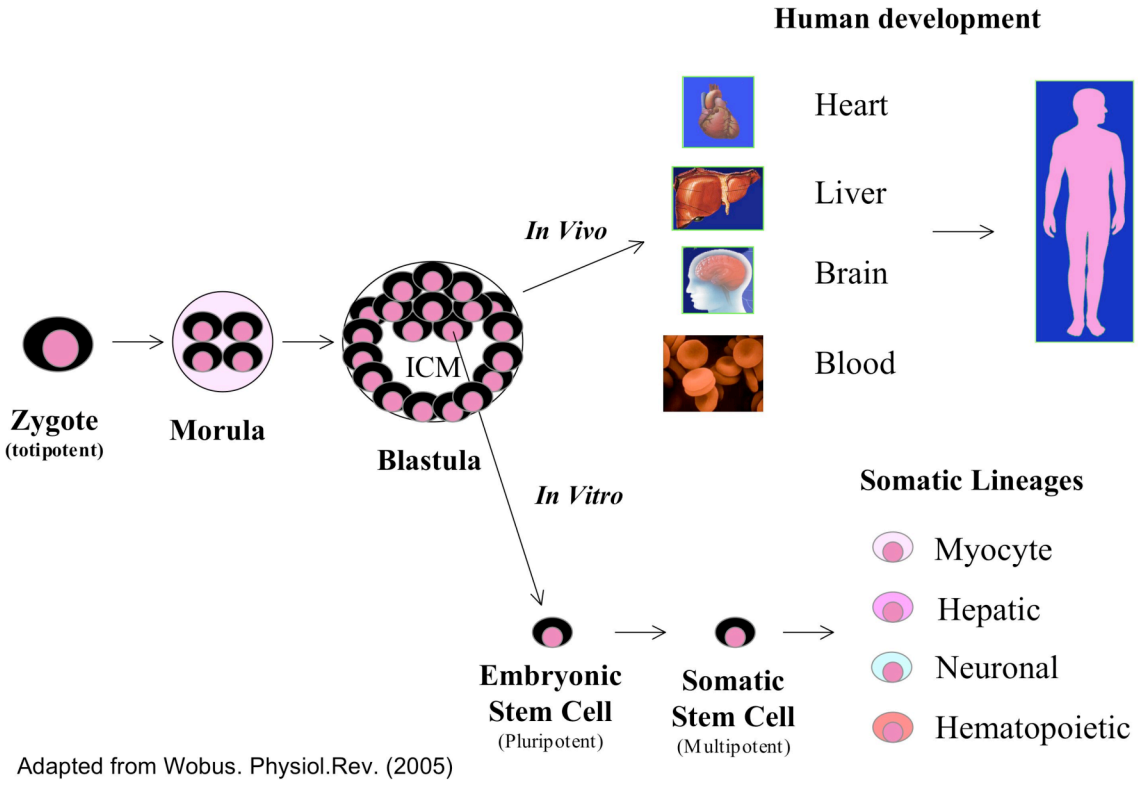


Figure 1.1 Embryonic stem cells and human development.

A fertilized vertebrate egg is termed a zygote. A zygote is a totipotent cell that has the capacity to divide (self renew) and give rise to all of the pluripotent stem cells needed to develop organized tissues to generate a viable organism (human). ES cells can be isolated from the inner cell mass of the blastula that retain the pluripotent capacity to generate endoderm, mesoderm, and ectoderm and have been demonstrated *in vitro* and in *in vivo* mouse models to generate specific tissues that differentiate into muscle, liver, neuronal and hematopoietic lineages.

capacity to self renew and are pluripotent in that they can generate endodermal, mesodermal and ectodermal cell lineages.[4] Mouse ES cell lines were first established in the early 1980's and relied on mouse embryonic fibroblast (MEFs) feeder cells in order to maintain the undifferentiated ES phenotype. It was identified that MEFs secrete leukemia inhibitory factor (LIF), a soluble glycoprotein which suppresses differentiation.[4,5] This advance has led to using serum free media supplemented with LIF rather than using MEFs decreasing the influence of contaminating cells in studying ES cells. Mouse ES cells have been characterized to express the stage specific antigen SSEA-1, gp130 and have both alkaline phosphatase and telomerase activity.[5] Mouse ES cells constitutively express the transcription factor Oct-3/4 which maintains pluripotency, however a decreased level of Oct-3/4 leads to trophectodermal development and an increase of Oct-3/4 induces endodermal and mesodermal commitment.[4,5] Mouse ES cells can be expanded *in vitro* and be reintroduced into a new embryo contributing to all germ layers as a chimera. This property has made these cells an interesting target for the treatment of disease.

In 1998 the first human ES cell line was generated.[6] hES cells share many of the same identifying characteristics as mES cells but have been demonstrated to also express the stage-specific embryonic antigens SSEA-3, SSEA-4 and display the proteoglycan tumor recognition antigens TRA-1-60, TRA-1-81 and GCTM2.[4,7] Due to ethical restrictions, hES have not been determined for their ability to incorporate into a new embryo or whether they can contribute to the transmissible germ line cells. Human ES cells have been expanded *in vitro,* however unlike mES cells where the media can be supplemented with LIF, hES cells require feeder cells.[4] Unfortunately, most of the available hES cell lines now available for

research supported by federal funds are contaminated with mouse feeder cells which inhibit the ability to study their therapeutic potential. More recently, hES lines have been established using human feeder cells or are cultured in serum free media.[8] Although several technological advances have been made, much is to be learned before human ES cells can be utilized in human therapeutic applications.

A.2. Somatic stem cells

Somatic stem cells, often referred to as "adult" stem cells, have the capacity to self renew and generate progenitors that then can differentiate into a variety of lineage specific cells. Unlike embryonic stem cells that are pluripotent, somatic stem cells are multipotent and generate only a closely related family of cells. Somatic stem cells have been identified and described in a variety of human tissues such as the blood, heart, muscle, and brain.[9] Somatic stem cells tend to have limited cellular plasticity and give rise to and maintain specific tissues. A question that remains is whether somatic stem cells contain the same pluripotent potential as ES cells to either differentiate into new tissues or dedifferentiate into a primordial stem cell in which to generate new cells and tissues.[10] The ability of somatic stem cells to cure human ailments has been routinely used in bone marrow transplantations and used in radical experimental clinical procedures for heart therapy (Vescell™ therapy, TheraVitae, Bangkok, Thailand) and recently approved pre-clinical trials of human brain regeneration. A specific pan marker(s) identifying all somatic stem cells has not been identified, but markers have been identified for several tissue specific somatic stem cells. The most thoroughly characterized somatic stem cell is the hematopoietic stem cell.

A.3. Hematopoietic stem cells

Somatic stem cells, such as blood-forming stem cells in the bone marrow (hematopoietic stem cells, or HSCs), are currently the only type of stem cell routinely used to treat human diseases such as leukemia, lymphomas and other inherited blood disorders. HSCs are functionally defined as self renewing and have the capacity to generate all of the mature hematopoietic lineages. [3,11-13] Doctors have been transferring HSCs in bone marrow transplants for over 40 years. John Dick in 1988 demonstrated that the immunodeficient bg/nu/xid mouse would engraft with human bone marrow cells and reconstitute with both myeloid and B cells.[14] During this same time period, Irving Weissman and collaborators began focusing on identifying potential mouse hematopoietic stem cell markers. Through both *in vitro* and *in vivo* experiments a SCID repopulating cell (SRC) was identified to reside within the $CD34^+$ cell fraction. [15-17] Initially, this cell population was termed a SRC since the T cell component did not develop in SCID mice. Since then, $CD34^+$ cells have been shown to generate human T cells in both mouse and human *in vitro* thymic organ cultures and *in vivo* in human thymus implanted mice.[18-21] Weissman in 2000 proposed a set of phenotypic cell markers for both the mouse and human for identifying long-term hematopoietic stem cells (Fig 1.2).[9,22] CD34 is a glycoprotein found on the surface of hematopoietic stem cells and the function of CD34 still has not been elucidated.

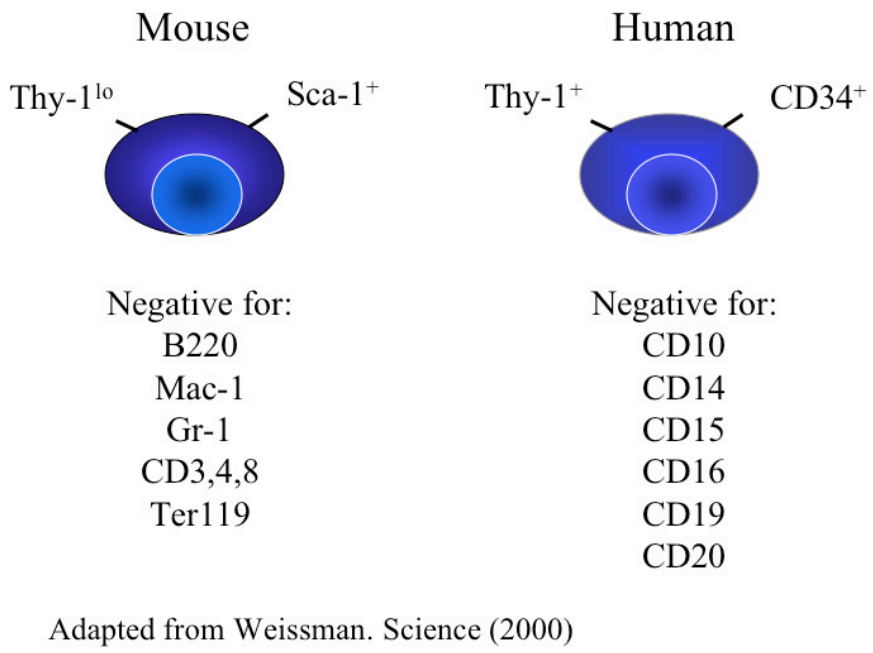


Figure 1.2 Hematopoietic stem cells.

Phenotypic comparison of the mouse and human hematopoietic stem markers. Both the mouse and the human hematopoietic stem cells lack lineage markers of differentiation.

$CD34^+$ cells have readily been identified and isolated from the bone marrow, umbilical cord blood and fetal liver as well as from patients treated with GM-CSF to mobilize $CD34^+$ cells into the peripheral blood.[23,24] Not all tissue $CD34^+$ cells have the same engraftment and reconstitution capacity. Pre-conditioned NOD/SCID mice were transplanted with $CD34^+CD38^-$ cells from human fetal liver, CB, and adult BM and the types and numbers of human cells were measured in the bone marrow at 6-8 weeks, for *in vitro* progenitor assays and secondary transplant endpoints. Holyoake demonstrated an ontogeny difference of HSCs in which the earlier the developmental state the higher number of BM colony forming units (CFU), whereas in later ontogeny a greater number of mature CFU cells develop.[25]

It has been identified that a more primitive HSC is $CD34^{+}CD38^{-}$ which engrafts and provides long-term reconstitution of the recipient transplanted host. The $CD34^{+}CD38^{-}$ cell population is significantly enriched for cells with high clonogenic and long-term culture potential. [17,26,27] A new potential primitive HSC is a $CD34^{-}$ cell, which has also been identified in the SP (side population) of cells that excludes Hoechst dye, and are able to engraft immunodeficient mice.[28,29] Although these cells may be a new therapeutic target for correcting hematopoietic abnormalities, little is understood in their role of human hematopoiesis .

The identification of the $CD34^{+}$ cells, through the use of small animal models, as the HSC has revolutionized the field of human hematopoiesis. HSCs have been demonstrated to generate all the components of the blood system and their use has been implemented clinically to treat a variety of hematopoietic ailments. Due to the unique properties of $CD34^{+}$ cells to engraft and reconstitute a recipient host, these cells have become a major target for gene therapeutic approaches against hematological disorders.[30-33] Several ongoing studies are investigating how to expand this cell population without losing their engraftment potential[34-36] and whether $CD34^{+}$ cells have trans-differential potential to cure/alleviate other tissue disorders.[37-40]

B. Hematopoiesis

Hematopoiesis is the regulation and generation of all of the blood lineages from a common hematopoietic stem cell (Fig1.3).[9] Initially, the multipotent HSCs differentiate into either a short-term HSC or a multipotent progenitor (MPPs).[41] Progenitor cells have limited

self-renewal capacity but can give rise to many cell types. Clonogenic MPPs have been identified from the bone marrow to generate either myeloid or lymphoid lineages. Although poorly understood, it appears during differentiation that HSCs commit to either a common myeloid progenitor (CMP) giving rise to granulocytes, monocytes, erythrocytes and megakaryocytes or a common lymphoid progenitor (CLP) giving rise to NK, B and T cells.[22,42,43] Both CLPs and CMPs generate $CD123^+$ and $CD11c^+$ DCs, respectively.[44,45]The primitive CLP is still being investigated to determine when CLPs become committed to the T cell lineage.

The earliest identified hematopoietic activity in a developing vertebrate is the formation of blood islands in the mesoderm of the extraembryonic yolk sac.[46] The mesoderm of the human yolk sac appears to thicken at about day 16 which may be representative of primordial blood islands.[47] In the formation of the blood islands and the yolk sac vascularization, CD34 has been identified on HSCs and on developing endothelial cells suggesting a common precursor for blood and endothelial cell lineages.[48,49] Functional studies of human yolk sac hematopoiesis showed clonogenic progenitors at 4.5 weeks of development.[50] By 6 weeks of gestation, the progenitor cells dissappear. During this time HSCs and committed progenitor cells migrate and colonize the fetal liver begining around gestation week 5 contributing to hematopoiesis.[50] By the 11^{th} week of human development, hematopoiesis begins in the bone marrow mesodermal structures.[51] As previously discussed, the bone marrow HSC was identified as a $CD34^+$ cell. The earliest differentiated cells in the bone marrow are $CD15^+$ myeloid cells followed by erythocytes.[51] B cells develop and are educated against self in the BM where as T cells are generated and educated in the thymus.

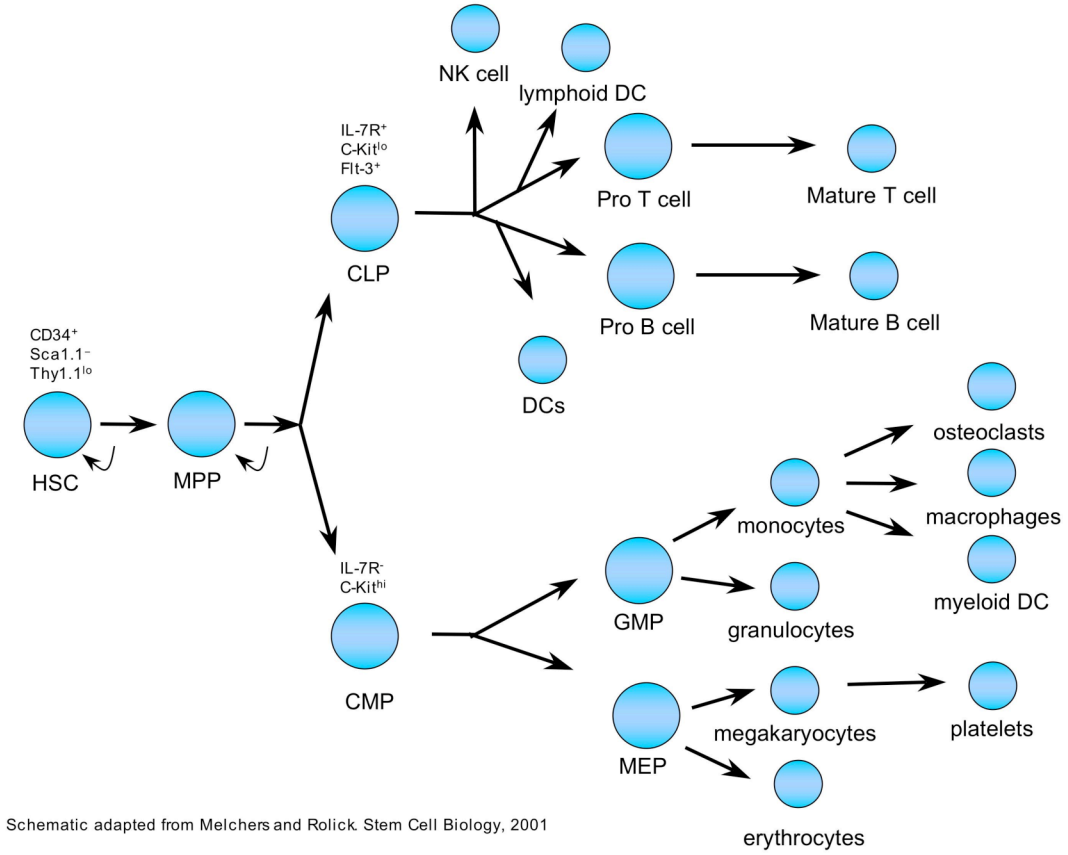


Figure 1.3 Hematopoiesis

A hematopoietic stem cells (HSC) is capable of self renewing or generating multipotent progenitors (MPPs). MPPs have limited self renewal and give rise to the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). CLPs give rise to lymphoid DCs, NK cells, B and T cells. CMPs give rise the short-term progenitor granulocyte/monocytic progenitors (MEP) and the megakaryotic/erythroid progenitors (MEPs). These progenitors give rise to myeloid DC, monocytes, macrophages, granulocytes, megakaryocytes, platetets and erythrocytes.

C. Progenitor T cell seeding of the thymus

The human thymus is seeded by progenitor cells which, through positive and negative selection, led to the generation of mature T cells. The precursor cells that seed the human thymus at gestation week 7 are initially derived from the fetal liver. The first mature T cells

in the human fetal thymus can be detected at week eight, approximately a week after HSC precursors have migrated to and entered the thymus. By week 22, thymus progenitors are derived from the bone marrow rather than the fetal liver.[52] It has been demonstrated, in the mouse, that there is an ontogeny difference for progenitor activity of fetal and adult T cell progenitors.[53] The progenitor activity of the fetal precursor cells is much greater giving rise to T cells faster than that of adult precursors. The percentage of multipotent precursor cells within the human fetal thymus is remarkably higher than the percentage of the same subset of the postnatal thymus. Precursors within the thymus express CD34 and several studies have investigated whether pre-thymus $CD34^+$ cells contain a committed T cell progenitor.[53] A $CD34^+$ cell population has been described in the fetal liver and bone marrow that possesses hematopoietic developmental potential restricted to lymphoid cells.[42] This population expresses CD10 and high levels of CD45RA but is negative for other early T cell markers such as surface CD7 and TCR gene rearrangement molecules. It has been argued that the T lineage marker CD7 appears even before thymus seeding since some fetal liver and bone marrow cells are $CD34^+CD7^+$.[54] Utilizing human fetal thymic organ cultures, Plum *et al* demonstrated transplantation of primitive $CD34^+CD38^-$ ($CD4^-CD7^-CD3^-HLA^-DR^{-/+}$) cells can develop through two distinct pathways: $CD4^+CD7^+$cytoplasmic $CD3^+$ cells differentiated into mature dendritic cells where as a $CD4^-CD7^+$cytoplasmic $CD3^+$ cells are able to generate both NK or T cell lineages.[20,55] The specific environmental and cellular factors that allow for distinct lineage commitment have not been elucidated. At present, the biology of human progenitor T cells is lacking and the committed T cell progenitor remains to be identified.

D. Human T cell development

Following entry of precursor T cells into the thymus, lymphoid committed progenitors cells begin their development into T cells. Our general knowledge of T cell development has been derived from various mouse strains and mouse knockout/transgenic systems and human thymus organ cultures. The primary knowledge of the development of mouse T cells has remarkable similarities to the limited human studies. The architecture of the human fetal thymus and initial T cell developmental events have been characterized and described by Barton Haynes.[52,56] Human thymocytes at approximately 8 weeks of gestation were $CD4^{+}CD8\alpha^{+}$ and cytoplasmic $CD3\varepsilon^{+}CD3\delta^{+}CD8\beta^{+}$ and $CD3\zeta^{-}$.[52,56] At this developmental stage, only 5% were $TCR\beta^{+}$ and < 0.1% $TCR\gamma^{+}$. Appearing around week nine, the expression of CD2 and CD8β, then TCRβ (followed by TCRγ), CD45RA and CD45RO isoforms, CD28 at week 10, and CD3ζ (week 12). At week 16, CD4,CD8,CD3ε and TCR β were expressed in the fetal thymus with similarities to the post natal thymus to give rise to the human T cell repertoire.[57]

Naïve T cells continue to be renewed through thymopoiesis even into late adulthood, although the level of new naïve T cells is diminished due to atrophy of the thymus during aging.[58] For a brief review, thymocytes develop and undergo education in the thymus through a series of TCR gene re-arrangements and positive and negative selection in the cortex and medulla. T cells develop from a triple negative CD3,CD4,CD8 (TNP) precursor that enters the thymus and develops into a potential thymocyte expressing CD4.[59] [60] After commitment to the T cell lineage, thymocytes begin TCR gene re-arrangement and the upregulation of CD8. The level of surface CD3 expression within the $CD4^{+}CD8^{+}$ (DP)

population increases as the thymocytes mature. At the $CD3^+CD4^+CD8^+$ (TP) stage, thymocytes are educated to antigens from both thymic DCs and epithelial cells in which the thymocytes are eliminated due to neglect (weak MHC recognition) or high affinity binding (strong MHC recognition). The TP thymocytes that recognize MHC I or MHC II expressing cognate antigen with an intermediate threshold of activation subsequently mature into naïve $CD8^+$ and $CD4^+$ T cells, respectively. As mature naïve T cells exit the thymus, the level of *de novo* naïve $CD3^+$ T cells can then be measured based on the number of TCR excision circles (TREC analysis) as a result of TCR re-arrangement.[58] The diversity of an individual's Vβ TCR repertoire is based on the genetic TCR selection in the thymus and peripheral expansion.

E. Small animal models of hematopoiesis

The majority of our understanding of the human hematopoietic system has been extrapolated from mouse hematopoietic studies. Although many similarities of immune ontology are shared between mice and men, distinct differences have been observed. Therefore, it is important to develop animal models of human hematopoiesis in which to study the development of the human immune system and hematopoietic dysfunctions. Primitive human HSCs have been demonstrated to engraft the bone marrow of various types of immunodeficient xenogeneic hosts, such fetal sheep, canines and certain strains of mice.[61-65]

E.1. The Beige/Nude/XID (BNX) Mouse

The BNX mouse was the first immunodeficient mouse strain to demonstrate human hematopoietic cell engraftment and reconstitution.[14] Noalta *et al* further demonstrated BNX mice co-transplanted with $CD34^+$ cells and stromal cells expressing human IL-3 engraft and reconstitute long-term (up to 18 months) with human myeloid, B and T cells. The T lymphocytes in these mice develop by extrathymic mechanisms and are abnormal. The percentage of CD8 T cells exceed the CD4 T cells and the Vβ repertoire is severely skewed.[66,67] The BNX mouse led to the development of new immunodeficient mouse strains for the study of human hematopoiesis.

E.2. The Severe Combined Immunodeficient (SCID) Mouse

SCID mice homologous for the *scid* mutation lack both mouse B and T cell development. This defect is due to a failure to activate a DNA recombinase enzyme that requires a functional Prkdc gene. [68] The $Prkdc^{scid}$ gene encodes a nonsense mutation which causes an insertion of a te rminal codon rendering this protein non-functional. As a result, these mice fail to generate functional BCR and TCR gene rearrangement. Due to this phenotype, the SCID mouse was quickly adopted as a model for studying human hematopoiesis and immunology since these mice failed to reject foreign tissue transplants. Three primary SCID models have been developed and implemented to study various aspects of human hematopoiesis and immunology.

E.2.1 SCID-hu PBL Mouse

The SCID-hu PBL mouse is generated by i.v. or i.p. administration of human peripheral blood MNCs or T cells .[69,70] In this model, the mice do not reconstitute long-term with human cells. Generally, most of the human cells can not be detected within a few weeks with only transient B and expanded T cells remaining.[71] Both CD4 and CD8 T cells show signs of activation and expansion of the $CD45RO^+$ memory population. Human T cells isolated from SCID-hu PBL mice and restimulated *in vitro* had poor responses to antigen compared to human donor PB MNCs. It has been suggested that the T cells in these mice may be undergoing chronic stimulation leading to T cell exhaustion or anergy.[72,73] Another possibility for T cell anergy in this model is that the human T cells do not recognize the murine MHC class I as a stimulatory signal and therefore do not receive the necessary survival/stimulatory signals. However, this human/mouse xenograft model has significantly contributed in human studies of HIV infectivity and pathogenesis.[74-76]

E.2.2 SCID-hu BM Transplant Mouse

The SCID-hu BM transplant mouse was developed to study bone marrow transplantation and hematopoiesis. The intravenous injection of human HSCs or bone marrow cells into sublethally irradiated SCID mice resulted in the engraftment of primitive human cells that proliferate and differentiate into multiple lineages in the bone marrow. [77-79] The transplanted human HSCs home to the mouse bone marrow and produce immature and mature myeloid cells, immature erythroid cells and a high percentage of B cells without administration of exogenous human growth factor.[80] Mouse thymus derived human T cells do not develop in this system. The overall levels of human peripheral reconstitution are low, but this system has allowed for *in vivo* hematopoietic stem cell engraftment studies.

E.2.3 SCID-hu thy/liv Mouse

An *in vivo* SCID-hu model was developed in which to study human thymopoiesis and HIV infection. Constructing the SCID-hu thy/liv mouse involves the co-implantation of human fetal thymus and fetal liver fragments under the kidney capsule. The conjoint organ develops and phenotypically and functionally represents the human thymus. The fetal liver provides hematopoietic progenitors that enter the thymus and undergo T cell development.[81,82] The thy/liv implants from SCID-hu mice have been shown to represent a normal fetal thymus with double and single positive CD4 and CD8 thymocytes. The single positive CD4 versus CD8 populations exists in a 3-4/1 ratio respectively and express a Vβ TCR repertoire indistinguishable from the thymic donor prior to implantation. [83] Hematopoiesis is relegated to the thymic organoid that develops, however low numbers of naïve T cells have been observed in the circulating peripheral blood. The initial development of the SCID-hu thy/liv model was intended for the evaluation of HIV pathogenesis and for the development of therapeutic approaches to treat HIV. HIV infection can be accomplished by direct thymic or intraperitoneal injection of viral supernatants resulting in productive infection of human cells.[81,84] Productive infection in the thy/liv implants results in depletion of $CD4^+$ T cells within a few weeks.[85] The observed pathology closely resembles that seen in fetal, pediatric and adult thymi from HIV infected individuals. The SCID-hu thy/liv system fulfills many of the basic parameters for a model to study human thymopoiesis but the lack of systemic reconstitution hinders its utility.

Although these SCID models have been instrumental in our understanding of human hematopoiesis, the low level of reconstitution and the lack of systemic T cells have hindered further progress in understanding human thymopoiesis and potential therapeutic cures of T

cell ailments. Therefore new mouse strains/systems have been sought to increase the levels of human reconstitution and an environment that allows for human T cell development.

E.3. NOD/LtSz-scid (NOD/SCID) Mouse

The NOD/SCID mouse was generated by crossing the *scid* mutation onto the NOD/LtSz mouse strain.[86] The NOD/SCID mouse lacks B and T cells and remain diabetes-free. These mice have low NK cell activity, the innate immune response to LPS is diminished and lack detectable hemolytic complement. These mice do not appear to be as "leaky" for T or B cells like SCID mice. In addition, NOD/SCID mice have an approximate two-fold reduction of bone marrow cell counts and a slight reduction in erythrocytes. These mice must be kept in a clean barrier facility because of their extreme immune deficiencies. However, these mice are easy to breed and have large litters.

NOD/SCID mice transplanted with human HSCs engraft with long-term systemic reconstitution resulting in myeloid cells and high percentages of B cells.[15,78,80,86,87] NOD/SCID mice transplanted with PB $CD34^+$ cells were demonstrated to provide the necessary microenvironment that supports megakaryocytopoiesis and platelet production.[88] In addition, the NOD/SCID mouse has been used to study human DC ontogeny[89] and to demonstrate that both $CD11c^+$ and $CD123^+$ DCs are functional and mature in response to LPS and Flu even in the absence of T cells.[89,90] Kalberer demonstrated in CB $CD34^+$ cell transplanted NOD/SCID mice that administration of IL-15 and Flt-3 ligand was sufficient for *in vivo* human NK cell development. Human $CD56^+CD3^-$ NK cells were detected in the BM, spleen, and PB and were phenotypically and functionally similar to human PB NK cells.[91]

Although many of the cells of the human immune system are generated, human T cells do not develop in NOD/SCID mice. Therefore, a variety of experimental approaches and mouse strains have been generated to address this shortcoming of human hematopoiesis in immunodeficient mice.

E.4. NOD/SCID Rag1^{null} Mouse

As a new model to investigate human hematopoiesis, Shultz *et al* backcrossed the null allele of the recombinase gene (*Rag1*) onto the NOD/LTSz-scid strain. These mice are deficient in V(D)J recombination rendering the mice T and B cell deficient. They have a longer life span compared to NOD/SCIDs, are not leaky for mouse Igs, are more radioresistant (750 cGys) and have diminished NK cell cytotoxic activity.[92] When transplanted with PB MNCs these mice demonstrated engraftment of up to 40% with human CD45$^+$ cells in the spleen with the majority being CD3$^+$. This NOD/SCID Rag1^{null}-hu PBL model was readily infected with HIV with the detection of high levels of viral RNA from the plasma. However, NOD/SCID Rag1^{null} mice transplanted with CB CD34$^+$ cells engraft with up to 7 % CD45$^+$ cells in the BM and reconstituted with human B cells and myeloid cells, but human T cells were not detected.

E.5. NOD/SCID β2 Microglobin-deficient (β2m^{null}) Mouse

NOD/SCID β2^{null} mice have reduced innate immunity compared to the NOD/SCID mouse due to the complete lack of MHC class I expression and NK cell activity.[93] The permissiveness of these mice for xenograft acceptance allows for higher levels of human T cell engraftment following i.p. injection of PB MNCs. NOD/SCID β2^{null} mice had a six to

seven fold increase in CD4 T cells compared to the NOD/SCID-hu PBL mouse. [93] However, the evaluation of SRC engraftment of CB MNCs only resulted in the engraftment and reconstitution of human ($CD45^+$) $CD33^+$myeloid, $CD19^+$ B and $CD56^+$ NK cells. In addition, breeding this strain is difficult and the mice have a shorter life span than the NOD/SCID strain due to the early development of thymomas.[94,95]

E.6. NOD/Shi-scid (NOD/SCID) Mouse

The *scid* mutation was backcrossed onto the NOD/Shi mouse strain to develop a NOD/SCID model for investigating human hematopoiesis.[96] The NOD/Shi-*scid* (NOD/SCID) mouse has similar characteristic to the NOD/LtSz-*scid* (NOD/SCID) mouse except the NK cell activity was not diminished. Yoshino demonstrated that anti-asialo GM1 antibody treatment enhanced human CB $CD34^+$ cell engraftment but did not affect their ability to differentiate into $CD33^+$ and $CD19^+$ hematopoietic lineages.[97] Human T cells still failed to develop. Human engraftment was only enhanced for the initial treatment with anti-asialo GM1 antibody.

F. Experimental approaches to generate human T cells in NOD/SCID mice

F.1. NOD/SCID mice transplanted with human bone fragments

SCID mice implanted with human bone fragments sustained active hematopoiesis for as long as 20 weeks.[98] The human cells present in the periphery were $CD33^+$ and $CD19^+$ cells. Fujiki *et al.* set out to develop an *in vivo* model in which human T cells develop and function normally. NOD/SCID mice were implanted with human BM fragments and analyzed for human reconstitution at 3 months post implant.[99] Dominant human T cell

lymphocyte expansion was observed in which up to 40% of the PB was $CD3^+$. The T cells in these mice exhibited similar levels of $CD4^+$ vs $CD8^+$ T cell ratios as observed to normal human PB controls. No obvious graft versus host disease reaction was observed and the T cells developed a broad Vβ TCR repertoire. They further demonstrated that this model of T cell homeostasis was susceptible to HIV-1 (NL4-3) infection with a decline of $CD4^+$ cells and the detection of serum p24 *gag* 2 weeks post virus inoculation. Although Fujiki did not elaborate on the expansion of possible contaminating human BM T cells in their model or show mouse thymic data for T cell development, the title explains their results: "Dominant expansion of human T cells".

F.2. NOD/SCID mice administered TNFα engraft with human T cells.

NOD/SCID mice were assessed for T cell development after administration of TNFα before transplantation of CB or G-CSF-mobilized PB MNCs, $CD3^+$ T cell depleted MNCs or CB $CD34^+$ cells.[100] T cell engraftment was only observed in mice that received total MNCs. The mice had low levels of human immature CD4CD8 double positive T cells in the bone marrow, spleen, and thymus. The T cells in this system were $CD45RO^+$ suggesting T cell activation and expansion rather than *de novo* generated human T cells.

F.3. NK cell depleted NOD/SCID mice generate human T cells.

In this NOD/SCID model of hematopoiesis, Kerr *et al* investigated how to optimize the development of human T cells. Previous observations have shown on rare occasions that human T cells develop in NOD/SCID mice. This group tested the hypothesis that

endogenous NK cells are responsible for the lack of T cell development. NOD/SCID mice were treated with the murine interleukin-2Rβ monoclonal antibody to deplete the residual mouse NK cells.[101] Human thymopoiesis occurred in up to 60% of the NOD/SCID mice with normal polyclonal, mature and functional single positive T cells. Furthermore, the peripheral T cells in these mice were shown to have a naïve phenotype as determined by TREC analysis. Although not all mice generated human T cells and the level of peripheral T cells is low (specifically the spleen), this was a hallmark paper in which many groups began to explore other immune deficient mice that lacked endogenous mouse NK cells.

G. Human T cell development in immune-deficient mice

G.1. NOD/Shi-scid γc^{null} Mouse

The NOD/Shi-*scid* mouse was backcrossed with the IL2r mutation that truncates the common cytokine receptor γ chain (γc). This mutation lacks its cytoplasmic region. [102,103] In this strain, all mouse lymphoid cells (NK, B, and T cells) are devoid since they lack the heterodimer component for many cytokine receptors (IL-2, IL-7, IL-9, IL-12, IL-15, and IL-21). The transplantation of CB CD34$^+$ in these mice results in human myeloid and B cells in the bone marrow and human T cells that develop within the mouse thymus. [102,104] The T cells demonstrated a polyclonal TCR repertoire, were functional in response to PHA and IL-2 stimulation and responded to allogeneic human cells. [102,104] Matsumura *et al* demonstrated that CD5$^+$ B cells develop in these mice from transplanted BM, CB, or mobilized PB CD34$^+$ cells and were able to generate antigen specific IgM but not IgG to DNP-KLH in immunized mice. [105] The NOD/Shi-*scid* γc^{null} mouse is the first consistent model of human reconstitution

and functional human T cells that develop in the mouse thymus without exogenous cytokines or antibody treatment.

G.2. Rag2$^{-/-}\gamma c^{null}$ Mouse

C57BL/6 Rag2$^{-/-}\gamma c^{null}$ mice developed by Mazurier *et al* demonstrated complete absence of mouse B, NK, αβ and γδ T, and NK-T cells.[106] These mice engraft with human CB CD34^{+} cells, and when treated with exogenous human cytokines, resulting in human reconstitution comparable to the NOD/SCID mouse. However, without administration of human cytokines, these mice failed to engraft and reconstitute with human leukocytes even with high levels of radiation (700 cGy) conditioning.

More recently, Manz and Spits demonstrated that 1 day-old BALB/c Rag2$^{-/-}\gamma c^{-/-}$ mice (lacking endogenous B, T and NK cells) transplanted intrahepatically or i.p. with CD34^{+} cells reconstituted with human CD45^{+} cells.[107-109] These mice generate *de novo* human B, T, NK cells, monocytes and DCs with the formation of structured primary and secondary lymphoid organs. Humanized BALB/c Rag2$^{-/-}\gamma c^{-/-}$ mice challenged with tetanus toxoid or EBV developed specific human adaptive T and B cell immune responses that closely resembled human controls. [108] Germinal centers developed in the lymph nodes and specific IgG for tetanus toxoid and EBV was determined , albeit at lower levels than human controls. The discrepancy in these results supports the notion that not only depleting the endogenous mouse innate immune system allows for increased human reconstitution, but the necessity for appropriate environmental conditions providing for human leukocyte development and reconstitution.

G.3. NOD/LtSz-*scid* IL2Rγ^{null} Mouse

NOD/LtSz-*scid* IL2Rγ^{null} mice have a complete γc null mutation and are deficient in mouse B, T and NK cells and survive for longer than 16 months.[110] NOD/LtSz-*scid* IL2Rγ^{null} were characterized for their ability to engraft with HSCs and develop into multilineage human hematopoietic cells. Mice transplanted with mobilized PB CD34^{+} HSC reconstituted with a 6-fold higher percentage of human CD45^{+} cells in the BM compared to NOD/LtSz-*scid* mice. The reconstitution of human cells consisted of myeloid cells, plasmacytoid DC, B cells, NK cells and low percentages of periphery CD3^{+} T cells.[110,111] The administration of human FC-IL7 fusion protein resulted in human double and single positive thymocytes within the mouse thymus and resulted in predominantly human T cells in the periphery.[110] The mice demonstrated a complex Vβ TCR repertoire and proliferated in response to PHA and the superantigen SPE-C (*streptococcal* pyrogenic exotoxin).

Table 1.1 Mouse strains used to study human stem cell engraftment.

Strain	Mouse Characteristics	Human Engraftment
Beige/Nude/XID	T, B, and NK cell deficiencies [112] (can be leaky)	$CD34^+$ cells engraft and reconstitute with B and myeloid cells. Extra-thymic T cells development. Human cytokines required. [14,67]
C57BL/6-*scid*	T and B cell deficiencies [68] (can be leaky)	$CD34^+$ cells engraft with low systemic reconstitution of B and myeloid cells. Low level of PB MNC engraftment. [70,76-78,113]
NOD/LtSz-*scid*	T and B deficiencies, low NK cell activity, macrophage dysfunction, defective complement. Diabetes does not develop. Thymomas develep at 8 months.[86]	$CD34^+$ cells engraft with moderate to high peripheral reconstitution with B cells, monocytes, macrpphages, and DCs Moderate to high MNC engraftment. . [15,78,80,89-91]
NOD/LtSz-*scid*/$Rag1^{null}$	No T or B cells and NK cell deficiencies[92]	Low $CD34^+$ cell engraftment with low peripheral reconstitution with B and myeloid cells.. High level of PB MNC engraftment. [92]
NOD/LtSz-scid $\beta 2M^{null}$	T and B cell deficiencies, relative NK cell deficiencies, short life span[93]	High levels of MNC engraftment. $CD34^+$ reconstitute with myeloid, B and NK cells. [93-95]
NOD/LtSz-scid $IL2R\gamma^{null}$	No T, B or NK cell development No thymoma development. Diabetes does not develop[110]	$CD34^+$ cells engraft in new born pups with high levels of reconstitution of monocytes, DCs, NK, B, and T cells. Human MHC restricted immune responses generated. IL-7 analogue for high T cell levels. [110,111]
NOD/Shi-*scid*	T and B deficiency, NK cells develop[96]	Similar engraftment to NOD/LtSz-scid mice with myeloid and B cells. [96,97]
NOD/Shi-scid/γc^{null}	No lymphocytes, no NK activity and impaired DC function[103]	$CD34^+$ cells engraft with human myeloid and B cells. Human T cells develop in the mouse thymus. [102-105]
C57BL/6 $Rag2^{null}/\gamma c^{null}$	No T, B, or NK cell development[106]	$CD34^+$ cells engraft and reconstitute with T, B and myeloid cells Human cytokines required.[106]
Balb/c/$Rag2^{null}/\gamma c^{null}$	No T, B or NK development[107,108]	$CD34^+$ cells engraft in new born pups and reconstitute with lymphoid NK, B, T $CD123^+$ DC and myeloid monocytes, macrophages and $CD11c^+$ DCs. Adaptive immune responses develop. [107-109]

Table expanded from Thomsen *et al.*[114]

H. Statement of objectives and significance

The goal of my dissertation project was to develop a novel model of human hematopoiesis in which the human T cell component develops and T cell homeostasis is systemically maintained. Xenotransplantation of human hematopoietic $CD34^+$ cells into NOD/SCID mice represented the most thoroughly characterized animal model for the study of human hematopoietic stem cell biology, however T cells do not develop. McCune described a model in which SCID mice implanted with fetal thymus and liver were capable of generating human thymopoiesis. However, this system of hematopoiesis is relegated to the implanted organoid. Therefore I tested the hypothesis that NOD/SCID mice transplanted with human $CD34^+$ cells fail to develop human T cells due to the lack of the appropriate human thymic environment. I implemented an immunodeficient mouse model of hematopoiesis by implanting human thymic tissue and transplantation of $CD34^+$ cells.

The significance of this study is that new relevant and cost-effective models of human hematopoiesis are needed for the development of therapeutic approaches of hematopoietic ailments and the evaluation of the most promising candidates for preclinical trials to treat these ailments. Xenogeneic human/mouse models provide affordable methods for a variety of experimental maneuvers that would be difficult (and in many cases impossible) to carry out in a clinical setting. The BLT mouse model of human hematopoiesis developed is unique in that systemic T cell homeostasis is generated and that the human T cells specifically develop within a human thymic environment. This new model allows for the development of both the myeloid and lymphoid (T and B cells) compartments. Specifically, this model has now been used as an experimental tool to study *in vivo* hematopoiesis, thymopoiesis and

human tropic virus infections such as CMV, EBV, HHV-8, and HIV. In the future, this model will allow for both basic research of human T cell development and for clinical research for the *in vivo* efficacy of potential therapeutic strategies to treat or cure human hematopoietic ailments.

CHAPTER II

MATERIALS AND METHODS

A. Solutions.

Complete DMEM Medium (cDMEM)

1 liter DMEM media (Sigma, St. Louis, MO. Cat. # D5796), 10% fetal bovine serum (FBS) (Mediatech Inc. Herndon, VA. Cat. # 35-010-CV) heat in activated at 56˚ C for 45 minutes, 1% Pen/Strep/Glut [50 U/mL penicillin, 50 mg/ml streptomycin, 2 mM glutamine (Mediatech, Cat. #30-009-Cl], 1 mM sodium pyruvate (Mediatech, Cat. # 25-000-Cl).

Complete RPMI Medium (cRPMI)

1 liter RPMI 1640 media (Sigma, Cat. # R8758), 10% FBS (heat inactivated), 1% Pen/Strep/Glut, 1 mM sodium pyruvate.

Enzyme Cocktail

5mls RPMI, 12 mg Collagenase (Roche Diagnostics GmbH, Mannheim, Germany, Cat. # 11 088 874 103), 100 µg DNAse I (Boehringer Mannheim, Mannheim, Germany, Cat. # 776 785).

FACS Buffer

500 mls PBS (Sigma, Cat # D8537), 2 % FBS**.**

Freezing Medium

10% Dimethyl Sulfoxide (DMSO) (Fisher, Fair Lawn, NJ., Cat # BP231-1) and FBS.

HBSP Buffer (pH 7.12)

140mM NaCl (Fisher, Cat # S271-3), 5 mM KCl (Sigma, Cat # P-1597), 0.75 mM Na2HPO4 (Sigma, Cat # S-0876), 6 mM glucose (Fisher, Cat # BP350-1), 25 mM HEPES (Fisher, Cat # BP310-500).

Luria Bertani Medium (LB)

1 liter H_2O, 20 g Difco LB Broth, (10 g tryptone, 5 g yeast extract, 5 g NaCl) Lennox (BD), Sparks, MD. Cat #, 240210) Sterilize media by autoclaving 121˚C at 19 psi for 15 minutes.

Percoll Stock (100 %)

10 % PBS, and 90 % Percoll (Sigma, Cat. # P1644)

Proteinase K buffer

0.01 M Tris [pH 7.4], 100 μg/ml proteinase K (Roche, Cat # 0 3 654 672)

Solution A

1 liter PBS, 10 mls FBS (heat inactivated), 10 mls Pen/Strept/Glut.

Solution B

1 liter PBS, 5 grams BSA (Fisher, Pittsburg, PA., Cat # BP1605-100), 10 mls Penn/Strept/Glut, 10 mls Citrate Phosphate Dextrose [(26.3 mg sodium citrate (dihydrate), 25.5 mg dextrose (monohydrate), 3 mg citric acid (anhydrous), 2.2 mg monobasic sodium phosphate (monohydrate)] (Baxter Healthcare Corporation, Deerfield, IL., Cat. # 4R0837MC)

Stem Cell Media

IMDM (Mediatech, Cat. # 10-016-CV) 1% Pen/Strept/Glut, 1% BSA, 5 µg/ml insulin (Sigma, Cat. # I5500), 100 µg/ml transferrin (Life Technologies, Rockville Maryland., Cat # 13008), 10 µg/ml low density lipoprotein (Sigma, Cat. # L 7914), 300 ng/ml SCF (PeproTech Inc. Rockyhill, NJ., Cat. # 300-07), 300 ng/ml Flt-3 (R&D Systems, San Jose, CA., Cat. # 308-FK), 10 ng/ml IL-3 (R&D Systems, Cat. # 203-IL), 10 ng/ml IL-6 (R&D Systems, Cat. # 206-IL), 10^{-4} M β-mercapto-ethanol (Fisher, Cat. # M6250-250ML).

Tissue Wash Buffer

500 mls PBS supplemented with 10% Pen/Strept/Glut and 20 mls Amphotericin B (250mg/ml) (Fungizone® Hyclone, Logan, UT., Cat # SV30078.01).

10X Lysis Buffer

500 mls dH_2O, 41.5 g NH_4Cl (Fisher, A661-500), 5 g $KHCO_3$ (Sigma, P-9144), 0.19 g EDTA (Sigma, BP120-1).

B. Antibodies.

Becton Dickenson (San Jose, CA.): CD3 PerCP (SK7, Cat. # 347344), CD4 PerCP (SK3, Cat. #347324), CD8-PE (SK1, Cat. # 340046), CD11c-APC (S-HCL-3, Cat. # 340544), CD19-PerCP-Cy5.5 (SJ25C1, Cat. # 340950), CD33-PerCP-Cy5.5 (P67.6, Cat. # 341640), CD34-PE (8G12, Cat. # 348057), CD45-FITC (2D1, Cat. # 347463), CD45-PerCP-Cy5.5 (2D1, Cat. # 340952), CD45RA-FITC (L48), CD123-PE (9F5, Cat. # 340545), Lineage cocktail (lin 1) (CD3, CD14, CD16, CD19, CD20, CD56), (SK7, Mop9, 3G9,

SJ25C1, L27, NCAM16.2, respectively, Cat. # 340546), anti-HLA-DR-PE (L243, Cat. # 347364), Streptavidin-APC (Cat. #349024), Streptavidin PerCP (Cat. # 340130), Mouse IgG_1,γ –FITC/PE/PerCP/ PerCP–Cy5.5/APC (Cat. # 349041/349043/349044/347202/ 340422), IgG_{2a},κ-FITC/PE/PerCP/APC (Cat. # 349051/349053/349054/340473).

BD Pharmingen (San Diego, CA.): CD3-FITC (HIT3a, Cat. # 555339), CD3-APC (UCHT1, Cat. # 555342), CD4-PE (RPA-T4, Cat. # 555347), CD4-APC (SK3, Cat # 555349), CD11c-PE (B-ly6, Cat. #, 555392), CD14-APC (M5E2, Cat. #555399), CD16-PE (3G8, 555407), CD19-PE (HIB19, Cat. # 555413), CD25-PE (M-A251, Cat. # 555432), CD25-APC (M-A251, Cat. # 555434), CD27-PE (MT-271, Cat. # 555441), CD34-APC (581, Cat. # 555824), CD40-APC (5C3, Cat. # 555591), CD45-FITC (H130, Cat. #), CD45-APC (H130, Cat. # 555485), CD45RA-FITC (HI100, Cat. # 555488), CD45RO-FITC (UCHL1, Cat. #55549), CD45RO-PE (UCHL1, Cat. #555493), CD56-PE (B159, Cat.# 555516), CD56-APC (B159, Cat. # 555518), CD80-Biotin (BB1, Cat. # 555682), CD83-APC (HB15e, Cat. # 551073), CD86-APC (FUN-1, Cat. # 555660), CD123-PE (7G3, Cat. # 554529), Mouse IgG_1,κ –FITC/PE/PerCP-Cy5.5/APC (Cat. # , 555909/555749/550795/555751), IgG_{2a},κ-FITC/PE/PerCP-Cy5.5/APC, 555573/555574/550972/555576), IgG_{2b},κ-FITC/PE/APC (Cat. # 555742/555743/555745).

Immunotech (Beckman Coulter, Fullerton, CA.): Vβ1-FITC (BL37.2, Cat. # 2406), Vβ5.1-PE (MPB2D5, Cat. # 2285), Vβ3-FITC (CH92 Cat. # 2372), Vβ8.1 and Vβ8.2-PE (56C5, Cat. # 2289), Vβ14-PE (CAS1.1.3, Cat. # 22047), Vβ17-FITC (E17.5F3, Cat. #

1234), Vβ21.3-FITC (IG125, Cat. # 1483), Pan TCR γδ-PE (IMMU 510, Cat. # PN IM1418), IgG_1- FITC (RAT, Cat. # PN IM3040), IgG_1-FITC (Cat. # PN IM0639), and IgG_1-PE (Cat. # PN IM0670).

Isotype controls: The appropriate mouse/rat isotype control antibodies (IgG_1-γ, IgG_1-κ, IgG_{2a}κ, IgG_{2b}κ) were used as controls to determine positive staining for anti-human specific antibodies.

C. Mice.

Non-Obese Diabetic/Severe Combined Immunodeficient (NOD/SCID) NOD/LtSz-Prkdc<scid> mice (Jackson Laboratories, ME., Stock # 001303) and NOD/SCID Rag 1^{null} NOD/LtSz-Rag<tmImom> mice (Jackson Laboratories, Stock # 003729) were housed and bred in a specific pathogen-free facility at the University of Texas Southwestern Medical Center at Dallas according to Institutional Animal Care and Research Committee approved protocols. Mice were maintained in micro-isolators and were fed sterile food and sterile chlorinated water.

D. Cord blood $CD34^+$ cell isolation.

Human umbilical cord blood was obtained from Parkland Memorial Hospital (Dallas, TX.) from uncomplicated births and was processed as previously described [31,32] according to IRB approval. Mononuclear cells were isolated by Ficoll (Amersham Biosciences, Piscataway, NJ. (GE Healthcare Cat. # 17-1440-03)) gradient separation and were enriched

for $CD34^+$ cells by positive immuno-magnetic isolation according to manufacturer's instructions (Miltenyi Biotech, Auburn, CA., Cat # 130-046-705). MNCs were resuspended in Solution A and blocked with 100 µl FcR Block per 10^8 cells at 4˚C. MNCs were then labeled with 100 µl $CD34^+$ MicroBeads per 10^8 total cells, mixed well and incubated at 4˚ C for 30 minutes. At the same time, LS columns (Miltenyi, Cat. # 130-042-401) were placed in the magnetic holder and equilibrated with 3 mls of cold degassed Solution B. The cells were passed through the column twice and washed 3x with 3 mls Solution B. The column was removed from the magnet and placed on a 15 ml Falcon Tube. Five mls of Solution B was added to the column and by applying pressure with the supplied plunger the cells were obtained. The cells were passed over a second column for increased purity. Isolated $CD34^+$ cells were then stained with mouse anti–human monoclonal antibodies to CD34-APC (clone 581, Pharmingen, 555824) and CD3FITC (clone HIT3a, Pharmingen, 555339) or labeled isotype controls and analyzed by flow cytometry on a FACSCalibur using CellQuest Pro (version 4.0.2) (BD) for CD34 expression and residual $CD3^+$ T cells. This protocol yielded 90-95% pure $CD34^+$ cells. The cells (2×10^5 to 1×10^6 per mouse) were infused intravenously into sub-lethally irradiated (325-350 cGy by a ^{137}Cs source Room NB3.406) 8-10 week old NOD/SCID mice. Mice were maintained in micro-isolators and were fed sterile food and water. Reconstitution with human cells was evaluated by either PCR for human β-globin or flow cytometry as indicated below.

E. Counting and freezing viable mononuclear cells.

Mononuclear cells were isolated according to individual tissue protocols and counted. Cell counts were determined by 1:10 serial dilutions of the cell preparation with Trypan Blue (Cambrex BioScience, Walkersville, MD., Cat. # 17-942E) (1:10 dilution in PBS) exclusion dye for live cells.Ten microliters of the MNCs were resuspended in 90 µl of the trypan blue diltion in a microcentrifuge tube and vortexed before the next (1:10) dilution with a clean pipette. The appropriate cell dilution was aliquoted onto a hemacytometer slide. Cells were counted in the large (16) squares for each of the four sections. The cell count was repeated on the opposite side of the hematocytometer for accuracy and averaged. The cell concentration was calculated with the formula: (average cell count ÷ 4) x dilution factor x 10,000 cells per milliliter.

MNCs and $CD34^+$ cells were resuspended in Freezing Medium and aliquoted into 1.2 ml Cryogenic Vials (Corning Incorporated, Corning, NY. Cat. # 430487) using a Nalgene™ Cryo 1˚ C Freezing Container (Cat. # 5100-0001) filled with 70% isopropyl-alcohol (Fisher, Cat. # A416-4) and placed at -80˚ C. This process allows cells to freeze at a rate of 1˚C per minute for maximum cellular viability. Twenty-fours later the cells were transferred to liquid nitrogen.

F. NK cell depletion.

In order to eliminate residual mouse NK cell activity, mice were administered 200 µl i.p. of the NK cell depleting antiserum asialo GM-1 (Wako Pure Chemical Industries, Ltd. Richmond, VA., Cat. # 986-10001) (antibody concentration was based on titration of anti-

asialo GM-1 in vitro for each lot preparation according to the manufactures specifications) twenty-four hours prior to irradiation (325 or 700 cGys). Mice were transplanted with 1 x 10^6 CB $CD34^+$ cells to allow for high levels of human bone marrow engraftment and reconstitution. As indicated in the result section, two separate experiments, NOD/SCID mice (*n*=5) and NOD/SCID $Rag1^{null}$ mice (*n*=5) were treated every 11 days with asialo GM-1 antiserum for 5 treatments to inhibit and deplete de novo mouse NK cell activity. The peripheral blood was monitored for human reconstitution and T cell development by flow cytometry as described below. Mice were sacrificed at the indicated times and bone marrow, spleen, and mouse thymus was analyzed for human engraftment and T cell thymopoiesis as described in Chapter II, Sections M-Q.

G. Infant thymus and thymus implantation.

Postnatal thymuses were obtained according to IRB approval from patients undergoing either repair of congenital cardiac abnormalities with incidental partial thymectomy (newborn and infants) or thymectomy for myasthenia gravis (adult) (Children's Medical Center or Parkland Memorial Hospital, Dallas, TX). Newborn, infant and adult thymuses were processed within 6 hours of harvest as described in Chapter II, Section H.

Mice were anesthetized with 150-250 µl of a 1:5 dilution of Nembutal Sodium Solution (50 mg/ml) (Ovation Pharaceuticals, Deerfield, IL., NDC 67386-501-55) in PBS by i.p. injection. The mice were also administered 30 µl of a 1:10 dilution of the analgesic buprenorphine hydrochloride (0.3 mg/ml) (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA., NDC 12469-0757-1) in PBS. The mice were prepared for surgery in a

laminar flow hood by shaving the left side and sterilizing the skin with betadine (Purdue Frederick Company, Stamford, CT.). A ½ to ¾ inch incision was made through the skin and peritoneum in order to access the kidney. The kidney was lifted up to the surface and held in place with a Kelly's hemostat (curved hemostat). A small incision was made to the kidney capsule. Gently, the kidney capsule was lifted up with 0.5 mm forceps and 2-3 fragments (1-3 mm) of human infant thymus were inserted with a second set of 0.5 mm forceps. The kidney capsule is then laid over the implanted tissue holding it in place. The kidney was then lowered back into the peritoneum cavity. The peritoneum was closed with 3-5 stitches using polydioxanone suture with an F2 cutting needle (Ethico Inc., Somerville, NJ., Cat # Z422H) and then the body cavity was closed with an additional 3-5 stitches. The mice were monitored for side effects, discomfort and stitches replaced as necessary. All procedures followed IRB approved guidelines.

H. Thymocyte depletion.

Newborn, infant or adult thymuses were processed within 6 hours of harvest. Thymus tissue was cut into 2-3 mm pieces and cultured on 0.4-μm membranes suspended in cRPMI medium. For thymocyte depletion studies, the culture medium was supplemented with 0.06, 0.3 or 1.5 mM 2-deoxyguanosine (dGuo) (Sigma, Cat. # D-7145). Thymus fragments were disrupted at 3, 5 and 7 days and analyzed by flow cytometry for live cells and the percentage of CD4 and CD8 double positive cells. For mouse experiments, thymic tissue was cultured with 0.3 mM dGuo for 72 hours and washed for 3 to 4 hours with fresh cRPMI medium to remove residual drug and then surgically implanted under the mouse kidney capsule as

previously described above.

Thymocyte depletion by various doses of radiation was also determined. Thymus fragments were subjected to 500, 800 and 1000 cGys of radiation and cultured in cRPMI for forty-eight hours. The thymic tissue was disrupted as previously described and viability versus dead cells was determined by flow cytometry for side scatter vs. forward scatter and thymocyte depletion was determined by analyzing $CD3^+$, $CD4^+$ and $CD8^+$ double positive and single positive cell populations. The optimal level of thymocyte depletion was determined to be 1000 cGys.

I. Fetal tissue preparation for implantation.

Fetal thymus and liver tissue (18-24 weeks gestation) were obtained from Advanced Bioscience Resources (Alameda, CA). The tissue was removed from their original containers and transferred to new sterile 50 ml conical tubes. Thirty to 40 mls of Tissue Wash Buffer was added and the tubes gently inverted 15-30 times to mechanically disrupt contamination from the tissues and then incubated for 15 minutes at room temperature. This process was repeated twice to clean the tissue of bacterial and fungal contamination before implantation into immune compromised mice. After the third wash, a piece of fetal liver was excised for use in the surgical implants of the co-joint liver thymus sandwich. The rest of the fetal liver was then processed for isolating $CD34^+$ cells as described in Chapter II, Section J. A fragment of fetal thymus was removed to isolate thymocytes for CD3-FITC, CD4-PerCP and CD8-PE analysis as described Chapter II, Section J and indicated in the results section.

J. Fetal thymus/liver/thymus sandwich implants, FL CD34$^+$ cell isolation and transplantation.

Six to eight week old NOD/SCID mice were anesthetized and surgically implanted with human fetal thymus and liver (1-3 mm fragments) under the kidney capsule as described in Chapetr II, Section G. [81] CD34$^+$ cells were obtained by incubating fetal liver tissue at 37˚C in RPMI supplemented with 1 mg/mL collagenase/dispase (Roche, Mannheim, Germany, Cat. # 269 638), and 0.5 U/mL DNase I (Roche, Cat. # 776 785). The liver tissue was disrupted by cutting it into small pieces, incubated at 37˚ C to allow connective tissue digestion and the cellular supernatant pipetted [10 ml pipette (BD Falcon, Cat. # 357551)] up and down every 15 minutes for 1-2 hours (depending on the size of the tissue). The cell suspension was filtered through a 70-µm nylon mesh filter (BD Falcon, 352350), and mononuclear cells were isolated by Ficoll gradient separation. CD34$^+$ cells were isolated using immunomagnetic beads (Miltenyi Biotech, Auburn, CA.) as previously described in Chapter II, Section D.[31,32] Isolated CD34$^+$ cells were then subsequently stained with the mouse anti–human monoclonal antibodies to CD34-APC (clone 581, Pharmingen) and CD3-FITC (clone HIT3a, Pharmingen) or similarly labeled isotype controls and analyzed by flow cytometry on a FACSCalibur using CellQuest Pro for CD34 expression and residual CD3$^+$ T cells. This protocol yielded greater than 90-95% pure CD34$^+$ cells and less than 0.6% CD3$^+$ T cells. Cells were immediately frozen (-80˚ C) in freezing medium following isolation and stored in liquid nitrogen until transplanted into mice. Three to six weeks post implant, mice were sub-lethally radiated (325 or 700 cGy by a ^{137}Cs gamma radation source) and transplanted within 24 hours intravenously with 0.2-2.5 x 10^6 CD34$^+$ cells. Mice engrafted

and reconstituted with human cells were assessed periodically for up to 26 weeks post transplant by monitoring the percentage of human $CD45^+$ cells in whole peripheral blood samples using the whole blood lysis kit (BD, Cat. # 349202) according to manufacturer's instructions. Specific human cell subsets were phenotypically characterized by flow cytometry as described below. Mice were sacrificed and tissues harvested for human analysis at the indicated time points post transplant essentially as described Chapter II, Section Q.[31,32]

K. Human reconstitution and EGFP expression from peripheral blood.

Human reconstitution and EGFP expression was determine using mouse peripheral blood. Blood was collected from mice that were pre-warmed using a heating lamp and the right lateral tail vein nicked using a razor blade (American Safety Razor Company, Staunton, VA., Cat # 74-0001). Seventy microliters of mouse peripheral blood was collected in a 75 mm EDTA treated capillary tube (Drummond Scientific, Broomall, PA., Cat # 1-000-7500EC). The blood was either stained CD3-FITC, CD19-PE, CD33-PerCP, and CD45-APC) at room temperature and red blood cells lyzed using the BD lysis kit or resuspended in 1 ml PBS and pelleted at 1500 rpm for 2 minutes for PCR analysis. The PBS was carefully aspirated off and the cells were immediately prepped for DNA isolation or stored at –80˚C. To isolate DNA, the cell pellet was resuspended in 50 µl of proteinase K buffer for 1 hour at 56˚C with vigorous shaking. Proteinase K was heat inactivated at 95˚C for 20 minutes, and the cell suspension was vortexed briefly and frozen immediately for 30 minutes at –80˚C. The thawed cell lysate was spun at 14,000 rpm to pellet cell debris and 10 µl of the cell

supernatant was used per PCR reaction. The PCR reactions were designed to detect the presence of human reconstitution using primers (forward 5'- GGG CAA GGT GAA GGT GGA TGA-3' and reverse 5'- CCA TCA CTA AAG GCA CCG AGC-3') to the human β−globin gene or vector intergration using primers (forward 5'- CTC GTG ACC ACC CTG ACC TAC GG-3' and reverse 5'- ATG CCC TTC AGC TCG ATG CGG TT-3') to the EGFP gene. The PCR reaction consisted of 1 µl of a 10 mM stock of dNTP stock of four nucleotide bases (Invitrogen, Cat. # 18427-013), 5 µl of 10 X PCR buffer, 0.2 µl Taq platinum polymerase (Invitrogen, Cat. # 10966-034) , 50 pmol of each of two primers, 3 µl of 50 mM Mg^{+2} and brought up to 50 µl with PCR grade water. The contents were amplified under the following conditions, 95˚C for 1 minute, annealed at 64˚C for 1 minute, elongated for 1.5 minutes, and repeated for 38 cycles. Following the PCR reaction, the samples were loaded onto a 2% agarose (Invitrogen, Cat. # 15510-027) gel and elctrophoresed for analysis.

L. Bone marrow mononuclear cell isolation.

Femurs, pelvic girdles, and spine were harvested from mice and muscular tissue removed with sterile gauze (Medical Action Industries Inc. Arden, NC., Cat. # 44422). The bones were subsequently ground in a mortar with a pestle to release bone marrow cells for mononuclear cell isolation. The cell suspension was filtered through a 70 µm filter (BD Falcon, 352350) and washed in Solution B. The red blood cells were lyzed with 1X lysis buffer buffer (1:10 dilution of the 10 X lysis buffer stock in dH_2O) for 10 minutes at 4˚C, spun down at 1500 RPM at 4˚C, for 5 minutes. The mononuclear cells were then washed and resuspended in 1 ml of Solution B. Cells were counted by trypan blue exclusion for live cells

and analyzed for human reconstitution and for both myeloid and lymphoid phenotypic characterization by flow cytometry (CD3-FITC, CD19-PE, CD33-PerCP, CD45-APC).

M. Spleen mononuclear cell isolation.

Spleens were removed and mononuclear cells isolated by disrupting the tissue with a 3 ml syringe plunger (BD, Cat. # 309585) and filtering the cells through a 70 μm filter. The cells were washed with Solution B and the red blood cells were lyzed with 1X lysis for 10 minutes at 4˚. The cells were spun at 1500 RPM, washed with Solution B and filtered again when necessary. The mononuclear cells were resuspended in 0.5 mls of Solution B and counted. Cells were then analyzed for human reconstution and phenotypic analysis as further indicated.

N. Thy/Liv/Thy Organoid mononuclear cell isolation.

The human thymic organoid was carefully excised from the kidney. The tissue was finely minced into small fragments and disrupted in 5 mls Solution B using a 3 ml syringe plunger and the supernatant filtered through a 70 μm nylon mesh filter. The RBCs were lyzed with 1X lysis buffer for 10 minutes. The cells were spun down at 1500 rpm, 4˚C, for 10 minutes and washed with Solution B. The thymocytes were then resuspended in 0.5-1 ml Solution B determined by the size of the thymic organoid. The cell suspension was counted and analyzed for double and single positive CD4-PerCP (BD) and CD8-PE (BD) markers, and CD3-FITC (Pharmingen) expression and a diverse T cell receptor repertoire by flow cytometry. Specific stains: [(Vβ3-FITC, Vβ2-PE, CD3-PerCP, CD45-APC), (Vβ1-FITC,

Vβ14-PE, CD3-PerCP, CD45-APC), (Vβ17-FITC, Vβ5-PE, CD3-PerCP, CD45-APC), (Vβ21-FITC, Vβ8-PE, CD3-PerCP, CD45-APC)].

O. Isolation of liver mononuclear cells.

(Protocol adapted from Dr. Theile's lab at UTSW.)

Human reconstituted mice were anestitized and the peritoneum opened to expose the liver. The anterior vena cava was tied off with suture and the portal vein cut to allow for peripheral blood drainage. The mouse liver was perfused through the vena cava with PBS or RPMI (37˚C) at 3 mls/minute (10 ml syringe (BD, Cat. #309604) with a 21 gauge needle (BD, Cat. # 305167)). Following perfusion, the liver was removed and diced into small pieces, and the tissue disrupted using a 3 ml plunger in a 100 mm Petri dish. The cell suspension was filtered through a 70 μm nylon filter and spun down at 1500 rpm, 4˚C for 5 minutes. The cells were then resuspended in 5 mls 40% percoll (100% stock diluted in cRPMI) in a 15 ml conical tube (BD Falcon, Cat. # 352097) and underlayed with an equal volume of 70% percoll and spun at 2400 rpm, 20˚C for 20 minutes. The lymphocytes were isolated from the 40% -70% interface and washed in Solution B. The red blood cells were lysed with 1X lysis buffer 10 min at 4˚ C and washed with solution B. The cells were resuspended in Solution B, counted and analyzed by flow cytometry.

P. Preparation of lung mononuclear cells.

(Protocol adapted from Dr. Mehrad's at UTSW.)

Intact lungs of anestitized mice were removed and perfused through the trachea with 1X PBS to remove peripheral blood mononuclear cells as described for the liver in Chapter II, Section O. The lungs were minced into small pieces with scissors and treated with 5 mls of collagenase enzyme cocktail and incubated for 30 minutes at 37˚ C. The tissue supernatant was further disrupted by pressing the cell suspension through a 16 gauge needle (BD, Cat. # 305198) and filtered through a 70 μm filter. The cells were washed with cRPMI and brought up to 30 mls in cRPMI. The cell suspension was underplayed with 12 ml of 70% Percoll and spun at 2000 rpms (Jouan CR422 bench-top centrifuge) for 20 minutes at 25˚ C. The resident lung cells were collected from the interface and washed with cRPMI. Red blood cells were lysed and washed as previously described. Isolated mononuclear cells were resuspended in 5 mls RPMI and filtered again through a 70 μm filter. Lung mononuclear cells were counted and phenotypic analysis for human reconstitution was performed by flow cytometry.

Q. Human reconstitution analysis of immunodeficient mice by flow cytometry.

Mononuclear cells from BLT mice or control mice were isolated from the bone marrow, spleen, lymph nodes, lung, liver and thymic organoid tissues as described above. The percentage of human leukocytes ($CD45^+$) and hematopoietic lineages was determined by 4 color flow cytometry using antibodies to specific human hematopoietic markers CD3-FITC, CD19-PE, CD33-PerCP, and CD45-APC as previously described[31,32,115]. MNCs (2 x

10^5 to 1 x10^6) were aliquoted into 5 ml polypropylstyrene round-bottom tubes (BD, Cat. # 352052) and blocked with 10 µg per 10^6 cells mouse IgG (Sigma, Cat. # I5381) for 10 minutes on ice and subsequently stained for specific lineages. For further characterization, subsets of myeloid and lymphoid cells were stained with appropriate antibodies and analyzed by flow cytometry on a FACSCalibur (BD) collecting a minimum of 10,000 live cell gated events. Live cells were identified based on their characteristic side scatter versus forward scatter. Subsequently, live human MNCs were identified with mouse anti-human $CD45^+$ (clone HI30, Pharmingen) to determine the percentage of human reconstitution. Myeloid cells were identified by expression of human CD33 (clone P67.6, BD) and analyzed for the monocyte and macrophage markers CD14 (clone M5E2, Pharmingen) and CD16 (clone 3G8 Pharmingen). Dendritic cells were identified using the BD DC Kit gating through mononuclear cells that were Lineage negative, HLA DR bright and further characterized for CD11c (clone B-ly6, Pharmingen) and CD123 (clone 7G3, Pharmingen) expression. DC were characterized for expression of the activation markers CD40 (clone 5C3, BD), CD80 (clone BB1, Pharmingen), CD86 (clone FUN-1, BD) and the maturation marker CD83 (clone HB15e, Pharmingen). Lymphocytes were gated through human CD45-APC cells and CD19-PE (clone HIB19, Pharmingen) for B cells, $CD3^-$-(FITC) $CD56^+$-(PE) (clone B159, Pharmingen) for natural killer cells and $CD3^+$ cells for T cell subsets. T cells were further analyzed for CD4-PerCP (clone SK3, BD) and CD8-PE (clone SK1, BD) subsets, γδ TCR-PE (clone B1.1, BD) expression, CD25-PE (clone M-A251, Pharmingen) expression and for naïve versus memory T cell subsets using CD45RA-FITC (clone HI100, Pharmingen), CD27-PE (clone MT-271, Pharmingen) and CCR7-biotintlated (clone 3D12, Pharmingen) .

The T cell Vβ repertoire (Vβ1 clone BL37.2, Vβ2 clone MPB2D5, Vβ3 clone CH92, Vβ8.1 and Vβ8.2 clone 56c5 clone, Vβ14 clone CAS1.1.3, Vβ17 clone E17.5F and Vβ21.3 clone IG125, Immunotech, France) was monitored by gating through $CD45^+$ and $CD3^+$ cells. All flow cytometry data was isolated on a FACs Calibur and analyzed using CellQuest Pro (version 4.0.2). Specific stains: [(CD3-FITC, CD19-PE, CD33-PerCP, CD45-APC), (CD3-FITC, CD56-PE, CD45-PerCP, CD16-APC), CD14-FITC, CD33-PE, CD45-PerCP, CD16-APC), (Lin^--FITC, CD123-PE, HLA-DR-PerCP, CD11c-APC), (CD3-FITC, CD8-PE, CD4-PerCP, CD45-APC), (CD3-FITC, CDγδ-PE, CD4-PerCP, CD45-APC), (CD3-FITC, CD25-PE, CD4-PerCP, CD45-APC), and (CD45RA-FITC, CD27-PE, CD4-PerCP or CD8-PerCP, CD3-APC).

R. Plasmid preparations.

DH5α bacterial cultures were transformed with the lenti-virus plasmids $pRtat^+EGFP$, $pRtat^-EGFP$, pBH10 ψ^-env^-, and pLVSV-G and grown up over night in LB media with ampicillin (100 μg/ml). Cells were spun down and plasmid DNA isolated using the Qiagen DNA maxiprep kit (Qiagen, Maryland, USA, Cat. # 19763) according to the manufacturers protocol. The plasmids $pRtat^+EGFP$ and $pRtat^-EGFP$ were digested with HindIII generating four bands of approximately 570, 1800, 1900, and 3000 base pairs. pBH10 ψ^-env^- digested with HindIII generated 3 fragments approximately 450, 600, and 6500 base pairs and pLVSV-G digested with PvuII generated 3 fragments approximately 2500, 4300, 5000 base pairs. All plasmids were confirmed by restriction enzyme digestion analysis on 1-2%

agarose gels. These plasmids were subsequently used to generate the lentivirus vectors used in this study.

S. Vector stock preparation and CD34$^+$ cell transductions.

Lentivirus vectors used in this study were produced by cotransfecting 293T cells (2 x 10^6 cells/10 cm dish) with the plasmids pRtat$^-$EGFP, pBH10 ψ^-env$^-$, and pLVSV-G (18,18, and 6 μg per dish, respectively) or pRtat$^+$EGFP, pBH10 ψ^-env$^-$, and pLVSV-G (18,18, and 6 μg per dish, respectively) by calcium phosphate transfection in the presence of 50 μl of 2.5 M $CaCl_2$ and 0.5 ml of 2X HBSP buffer, pH 7.12. Vector containing media supernatants were harvested over two consecutive days, pooled, filtered through a 0.45 μM stericup filter (Millipore, Cat. # 190-2545), and concentrated once by centrifugation at 100,000 x g for 90 minutes (Beckman Optima LE-80k Ultracentrifuge). The viral pellet was resuspended in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies) supplemented with 1% bovine serum albumin and 1 % penn/strept/glut by vortexing the tube every 30 minutes for 3 hour and stored at –80˚ C until use. Vector stocks were titered on HeLa cells for Enhanced Green Fluorescent Protein (EGFP) expression by flow cytometry as described in Chapter II, Section T. Isolated human fetal liver 2x 10^5 CD34 cells$^+$ were maintained in 200 μl stem cell medium per well in a 48-well plate and transduced with the vector Rtat$^-$EGFP (multiplicity of infection [MOI] of 5. Retronectin (Takara Biomedicals, Kyoto, Japan, Cat. # T100B) coated plates (20 μg/cm2) were prepared in house by coating 48-well tissue culture plates (BD Falcon, Cat. # 353078) with Retronectin (100μg/ml) for 2 hours followed by a 30 minute block with 2% BSA in PBS. Plates were rinsed with IMDM prior to the addition of cells.

Transductions were performed at 0, 16, and 48 hours. Forty-eight hours after the last addition of vector, both transduced and mock tranduced (no vector) cells were isolated and analyzed for CD34 and EGFP expression by flow cytometry. Transduced and mock transduced $CD34^+$ cells were immediately frozen as described above until time of transplant. Preconditioned NOD/SCID mice and NOD/SCID-hu Thy/Liv mice were then transplanted with $4x10^5$ cells per mouse.

T. Vector titration

HeLa cells were plated in a six well plate at 1 x 10^5 cells per well in 3 mls cDMEM (day 1). On day two, one well of cells was removed, resuspended in 1 ml of cDMEM and counted at a 1:10 dilution to be able to calculate the multiplicity of infection (MOI). The remaining wells were treated with polybrene (Sigma, Cat. # H9268) at a concentration of 8 μg/ml. One well served as a mock transduction control, a second well for a vector inhibitor control by pretreating the cells with 50 μM AZT at 37˚C for 30 minutes before the addition of vector. The remaining 3 wells were transduced with 0.01 μl, 0.1 μl and 1 μl of concentrated vector supernatant. At 24 hours post transduction the media was aspirated and replaced with fresh medium. On day 5, the cells were wash with 3 ml PBS, incubated with 200 μl trypsin (Mediatech, Cat. # 25-053-Cl) for 1 minute and resuspended in 1 ml cDMEM. The cells were washed in 2 mls FACs buffer and the cell pellet resuspended in 500 μl FACS buffer and analyzed by flow cytometry for EGFP expression. The vector infectious units was calculated based on the total percentage of cells expressing EGFP and the cell counts from

the plated cells on day 2 using the formula: (Number of cells plated x percentage of EGFP expression) ÷ mls of added vector supernatent = Infectious Units per milliliter.

U. Immunohistochemistry analysis.

Tissues were collected and placed in fresh phosphate buffered 4% paraformaldehyde for 4 to 6 hours, washed with 80% ethanol and stored in 80% ethanol until embedded in paraffin. All immunohistochemistry was performed by Dr. Jacob Estes (University of Minne sota) using a biotin-free polymer approach (MACH-3™; Biocare Medical, Concord, CA) to analyze all BLT and human control tissues. Immunohistochemistry was performed on 5 μm tissue sections mounted on glass slides, dewaxed and rehydrated with PBS (pH 7.4). Antigen retrieval was performed by heating sections in 1X EDTA Decloacker™ reagent (Biocare Medical) in a 95^{o}C water bath for 20 minutes followed by cooling to room temperature. Nonspecific Ig-binding sites were blocked with Blocking Reagent (Biocare Medical) for 1h at room temperature. Endogenous peroxidase was blocked with 3% (v/v) H_2O_2 in PBS (pH 7.4). Primary antibodies were diluted in 10% Blocking Reagent in TNB and incubated overnight at 4^{o}C. The sections were then analyzed using either the mouse or rabbit MACH-3™ polymer systems (Biocare Medical) according to the manufacturer's instructions and developed with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). Sections were counterstained with Harris Hematoxylin (Surgipath, Richmond, IL), mounted in Permount (Fisher Scientific, Pittsburg, PA) and examined by light microscopy. Primary antibodies used for immunohistochemistry were mouse anti-human CD3 (clone F7.2.38, DakoCytomation, Denmark), mouse anti-human CD4 (clone 1F6, Novocastra Laboratories,

Newcastle-upon-Tyne, UK), mouse anti-human CD8 (clone 295, Novocastra Laboratories) or rabbit monoclonal anti-human CD8 (SP16, NeoMarkers, Freemont, CA), mouse anti-human CD14 (clone 223, Novocastra Laboratories), mouse anti-human CD20 (clone L26, DakoCytomation), mouse anti-human CD45 LCA (clone 2B11 + PD7/26, DakoCytomation), mouse anti-human CD68 (clone KP1, DakoCytomation), mouse anti-human CD83 (clone 1H4b, Novocastra Laboratories), anti-human BLC/BCA-1/CXCL13 (clone AF801, R&D Systems, Minneapolis, MN) and anti-human 6Ckine/SLC/CCL21 (clone AF366, R&D Systems). Isotype matched negative control antibodies used were mouse IgG1 (clone X 0931, DakoCytomation), mouse IgG2a (clone X 0943, DakoCytomation), goat ChromPure IgG (Jackson Immuno-Research, Westgrove, PA) and rabbit ChromPure IgG (Jackson Immuno-Research).

V. Sheep Red Blood Cell (SRBC) administration.

Sheep Red Blood Cells (Quadfive, Ryegate, MT., Cat. # 643) were washed with pyrogen free saline (B. Braun Medical Inc., Irving, CA., Cat. # R5201-01) and resuspended at a concentration of 20% SRBCs in pyrogen free saline. Mice were anesthetized and inoculated with 50 µl per rear footpad, 50 µl subcutaneous in the scruff of the neck, and 200 µl intraperitoneal.[116] Mice were sacrificed at 5 days and PB, BM, thymic organoid, spleen and lymph nodes (axillary, cervical, inguinal,popliteal and mesenteric) were isolated and analyzed by flow cytometry for human reconstitution and immunohistochemistry (Dr. Jake Estes) for $CD45^+$, $CD4^+$, $CD8^+$,$CD20^+$ $CD68^+$ and $CD83^+$ for germinal center formation in secondary lymphoid tissues.

W. Establishment of autologous EBV-transformed B Cell Lines (LCLs).

For lymphoblastic cell line (LCL) generation, fetal liver MNCs (2×10^5) obtained from the flow through fraction of the $CD34^+$ isolation column were plated in 48-well plates in cRPMI and infected with EBV supernatent (50-100 µl) obtained from the Akata cell line (kindly provided by J. Sixbey) as previously described.[115,117] All cell lines generated were cultured in complete RPMI medium supplemented with 1 µg/ml cyclosporine A (LC Laboratories, Woburn, Ma., Cat. # C-6000). Established LCL cultures were fed at weekly intervals with cRPMI and supplemented with 100 µM acyclovir (Sigma, Cat. # A4669) two weeks prior to ELISPOT analysis to inhibit virus production.

X. EBV infection of BLT mice and ELISPOT analysis for MHC restricted T cell responses.

Fourteen weeks post-transplant, reconstituted BLT mice were infected with EBV essentially as we have previously described[115]. Briefly, BLT mice were anesthetized and then injected directly in the spleen with either 50 or 100 µl of Akata virus stock. Five weeks post-infection, mice were sacrificed and cells isolated from different tissues for ELISPOT analysis. Human T cells were enriched by negative selection to exclude mouse $CD45^+$ and human $CD19^+$ cells using magnetic beads according to manufacture's protocol (Miltenyi) essentially as described in Chapter II, Section D. The ELISPOT assay was performed using a Human IFN-γ ELISPOT kit (e-Biosciences, San Diego, CA., (10 plates, Cat. # 88-7386-88) according to the manufacture's protocol. Multiscreen 96-well plates (Millipore, Bedford,

MA., Cat. # MSIPS4W10,) were pre-coated with capture antibody against IFN-γ (overnight at 4˚ C (diltion according to instructions on the Certificate of Analysis provided with the reagent set). The wells were washed 2x with Coating Buffer and blocked with cRPMI for 60 minutes. LCLs (4×10^5) and enriched human T cells (1×10^5) were cultured in 200 µL cRPMI/well. In each assay, negative controls included media alone, LCLs alone, T cells alone and, for a positive control, T cells were stimulated with *staphylococcus* enterotoxin B (SEB). Human MHC Class restriction was established using blocking monoclonal antibodies specific for human MHC I (clone W6/32, eBiosciences, Cat. #14-9983-82) and/or human MHC II (cone CR3/43, Dako Cytomation, Cat. #M0775) added to LCLs 30 minutes before culturing with autologous BLT Tcells. Thirty-six hours later, plates were washed 3x with Wash Buffer (PBS/0.05% Tween 20) and incubated for 2 hours at room temperature with biotinylated detection antibody against IFN-γ. Plates were washed 5x with Wash Buffer and incubated at room temperature with avidin-HRP complex (according to instructions on the Certificate of Analysis provided with the reagent set) for 45 minutes. The plates were then washed 3x with Wash Buffer and 2x with PBS. The plates were developed in the dark with 100 µl of freshly prepared AEC (3-amino-9-ethyl carbazole) (Sigma, A5754-10G) Substrate Solution [100 mg AEC in 10ml of N,N Dimethylformamide (Piece, Cat. # 20672) add 333 µl of AEC stock Solution to 10 ml 0f 0.1 Acetate Solution (pH 5.0), filter through 0.45 µg filter, and just before using add 5 µl of 30% H_2O_2] and the reaction stopped by washing the plate 3x with distilled water. The spots were manually counted using a dissection microscope.

Y. Administration of TSST-1.

TSST-1 (endotoxin free) (Sigma, Cat. # T5662) was resuspended in endotoxin free 0.9% sodium chloride solution (B. Braun Medical Inc.). Mice were administered by i.p. injection with 6 μg of TSST-1 in a 100 μl volume of endotoxin free 0.9% sodium chloride solution. Mice were bled or sacrificed at 0 hours, 1 hour, 18 hours, 3 days and 5 days post injection for human plasma cytokine analysis and phenotypic analysis of human cells as indicated in the result section in Chapter V.

Z. Human cytokine analysis.

The presence of human cytokines in the plasma of TSST-1 administered BLT mice and saline control BLT mice were quantitated using the Human Cytokine 10-Plex kit (Biosource, Camarillo, CA., Cat. # LHC0001) and the results were confirmed by Cytokine Bead Array (CBA) TH1/TH2 (BD, Cat. # 550749) and Inflammation (BD, Cat. # 551811) kits according to manufacture's instructions. Data was acquired (by Petra Cravens) and analyzed using a Luminex 100 (Luminex, San Antonio, TX) and a FACSCalibur (BD) according to the manufacture's instructions. No cross-reactivity to mouse cytokines was detected in NOD/SCID mouse control samples.

AA. Statistical analysis.

GraphPad Prism (San Diego, CA) version 3.0 was used for comparisons of parameters between groups by unpaired Student t-test (either single tailed or doubled tailed

as indicated). Numerical values are expressed as mean ± S.D. or S.E.M. as indicated in the text. A value of $P < 0.05$ was considered significant. The P values are indicated.

CHAPTER III

HUMAN HEMATOPOIESIS IN A HUMAN/MOUSE XENOGRAFT MODEL

A. Introduction

Due to ethical concerns and the rarity of pluripotent hematopoietic stem cells, human studies of bone marrow transplantation, hematopoietic disorders, gene therapeutic approaches to treat hematopoietic ailments (SCID and Sickle Cell Anemia) and the study of pathogenesis of human specific pathogens (EBV, CMV, HTLV, HHV8 and HIV) have been significantly hindered from the lack of available *in vivo* models to conduct appropriate experiments. Small animal models of human hematopoiesis have been used as surrogates to study a variety of important aspects of human transplantation, immune function, microbial and viral infections, tumor genesis, gene transfer and the *in vivo* repopulating potential of embryonic and somatic stem cells [18,81,90,115,118-120]. Such animal models are prospective candidates for investigating novel approaches for vaccine development, therapies against bioterrorism agents and preventative measures of HIV transmission. Despite their enormous success in a multitude of applications each of these systems has significant limitations that curtail their usefulness due to the lack of human T cell development in these models.

Initial studies of human hematopoiesis were conducted in the SCID mouse which contains a mutation in the DNA-dependent protein kinase gene (DNA PK) that renders these mice defective for productive TCR and BCR gene rearrangement.[121,113,122] Therefore, mouse T and B cells do not develop. Due to this phenotype, SCID mice fail to reject MHC mismatched tissues and have been extensively used to study graft transplantation, tumor

immunobiology, autoimmunity and for transplantation of HSCs.[17,65,123-129] SCID mice transplanted with human bone marrow engrafted and reconstituted with both human myeloid and B cells.[14] Through both *in vitro* culturing systems and *in vivo* experiments the $CD34^+$ cell or SCID repopulating cell was identified as a somatic stem cell that is capable of engrafting the mouse bone marrow and reconstituting the hematopoietic system.[12,14,119,130] Human hematopoietic stem cells have been isolated from bone marrow, mobilized peripheral blood, fetal liver, and the most available source umbilical cord blood.[12,130] Although systemic human reconstitution occurs in SCID mice, the overall level of human repopulation is relatively low and human T cells fail to develop in this model system. Since then the NOD/SCID mouse has been adopted as the primary mouse recipient for human HSC studies due to higher levels of bone marrow engraftment and systemic reconstitution.

The NOD/SCID mouse has been shown to have a defective complement system, lower myeloid and NK cell activity than SCID mice and T and B lymphocytes do not develop. [65] There are few reports that human T cells develop in NOD/SCID mice transplanted with highly purified HSCs. Nevertheless, this model has proven to be extremely useful to study a variety of important aspects of hematopoeisis, the pathogenesis of human-specific virus infection and the ontogeny and function of the human immune system *in vivo*.[89,90,115,131,132] Therefore, the greatest challenge in the field of human hematopoeisis is to generate a small animal model in which systemic human reconstitution, including T cells and recapitulating all of the components of both the innate and adaptive human immune system.

B. Results

B.1 Isolation of CB $CD34^+$ cells and human reconstitution in NOD/SCID mice.

Initial studies were designed to characterize the mouse model system of human hematopoiesis and determine whether human engrafted NOD/SCID mice developed human T cells under our experimental conditions. Human mononuclear cells were isolated from umbilical cord blood donors by Ficoll gradient centrifugation and phenotypically analyzed for $CD34^+$ (stem cells), $CD3^+$ (T cells), $CD19^+$ (B cells), and $CD33^+$ (myeloid cells) percentages prior to $CD34^+$ cell isolation (Figure 3.1). $CD34^+$ cells were between 1-3% of the total mononuclear cell preparation. $CD34^+$ cells were isolated by positive magnetic selection that resulted in 95.6% (± 1.6 SD, (*n*=5) range 93.7 - 97) purity (Figure 3.1). The $CD34^+$ cells were further characterized for the lineage cell markers $CD19^+$, $CD33^+$ or $CD3^+$. The isolated $CD34^+$ cells contained 1% ± 0.7 SD (*n*=5 range 0.2-1.9) $CD3^+$ T cells. The majority of the $CD33^+$ and $CD19^+$ cells were also positive for CD34 expression suggesting an immature hematopoietic state of lineage development. These results suggest that the differential state of maturation of the hematopoietic $CD34^+$ cells in umbilical cord blood is driven toward a B cell or myeloid cell phenotype. More importantly, these results demonstrate that the $CD34^+$ cell preparation contains few contaminating human T cells.

The isolated $CD34^+$ cells (2×10^5 cells/mouse) were transplanted into preconditioned (325 cGys of gamma radiation) eight to ten week old NOD/SCID mice. Mice were monitored at various times for human reconstitution by peripheral blood analysis for hCD45 (pan leukocyte marker for human cells). At 8-12 weeks mice were sacrificed and analyzed for human reconstitution in the mouse bone marrow, spleen and peripheral blood (Fig. 3.2a).

The percentage of total $CD45^+$ human mononuclear cells in the bone marrow was 22 ± 16.4 SD (*n*=17), range 2-60. Although these mice had a variable level of human reconstitution, no $hCD45^+CD3^+$ cells were detected in any of the NOD/SCID mouse tissues examined. When specifically examining the mouse thymus (*n*=17) no human $CD45^+$ single or double positive CD4 or CD8 cells were detected (Fig. 3.2b). This result suggests human progenitor T cells do not seed the mouse thymus or that there is a lack of an appropriate microenvironment for them to develop.

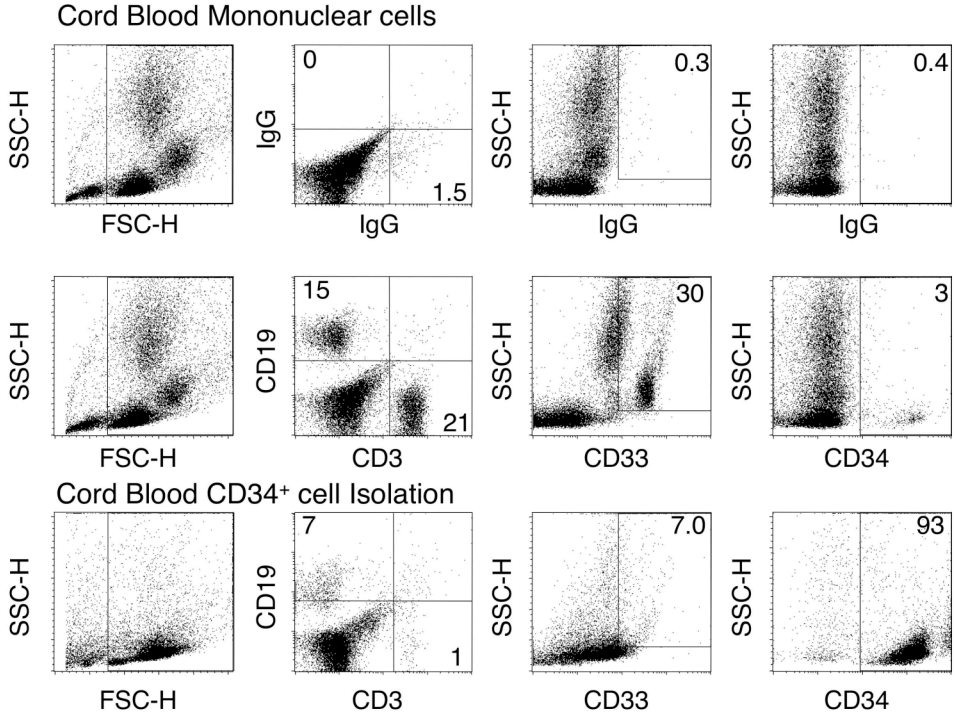


Figure 3.1 $CD34^+$ cell isolation from umbilical cord blood.

Cord blood mononuclear cells were isolated by Ficoll gradient centrifugation and red blood cells lysed. $CD34^+$ cells were further purified by magnetic positive selection and analyzed by flow cytometry. The top panel shows representative data for live cell gating and the isotype Ig controls used for subsequent cell gating. The middle panel shows the percentage of lymphocytes, myeloid and $CD34^+$ cells in total cord blood. The bottom panel shows the purity of $CD34^+$ cells following the isolation.

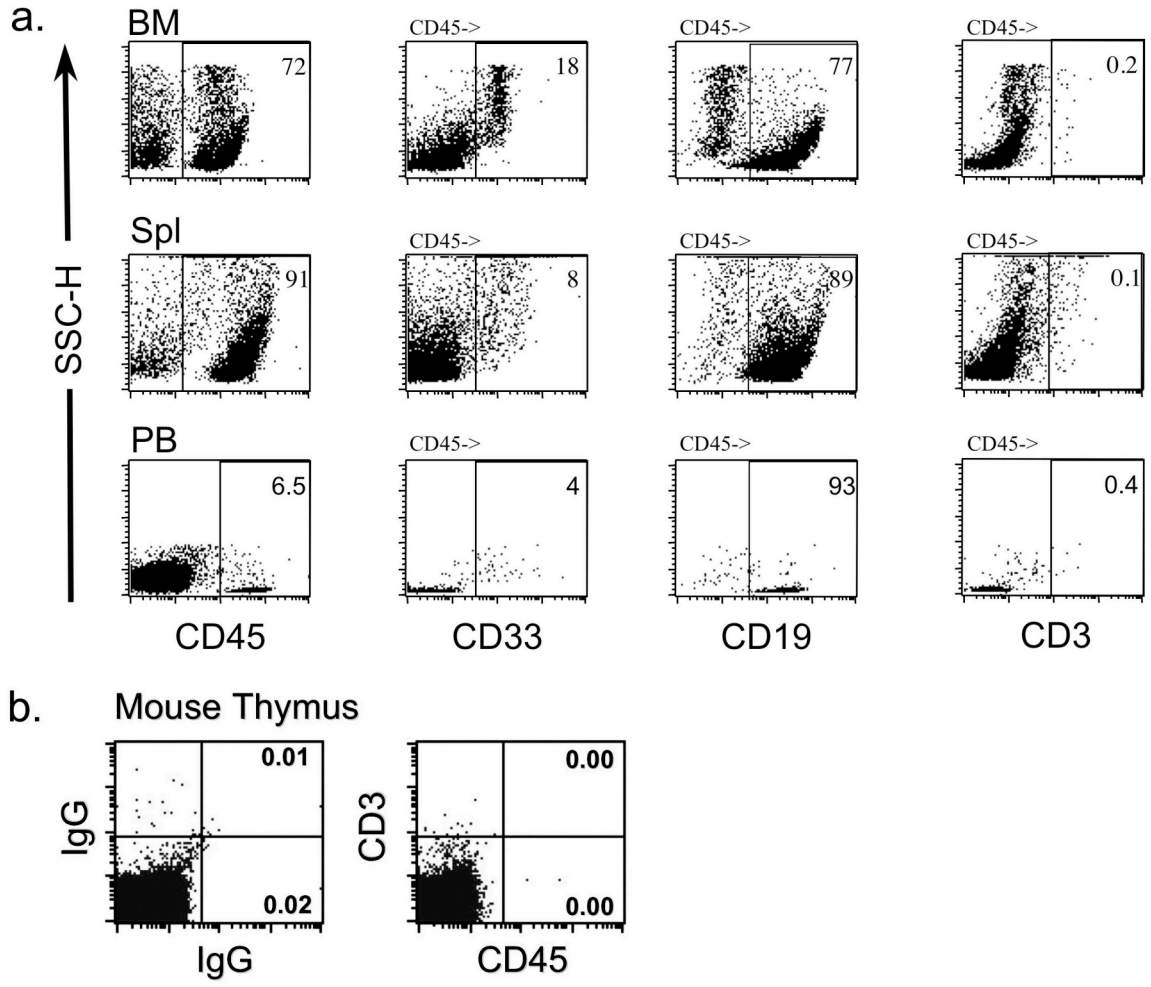


Figure 3.2 Human hematopoiesis in NOD/SCID mice transplanted with CB CD34$^+$ cells.

Eight to twelve week old NOD/SCID mice were preconditioned with 325 cGys of gamma irradiation (^{137}Cs source) and transplanted with 2x10^5 CB CD34$^+$ cells. At twelve weeks post transplantation the mice were sacrificed and analyzed for bone marrow engraftment and peripheral tissue reconstitution for human (CD45$^+$) cells. CD45$^+$ cells were further analyzed for both human myeloid (CD33$^+$) and lymphoid (CD19$^+$ and CD3$^+$) cells. a) NOD/SCID mouse bone marrow, spleen and peripheral blood analysis for human CD45$^+$ reconstitution. No human CD3$^+$ were identified. b) Analysis of human cells in the NOD/SCID mouse thymus. No human cells (CD45$^+$) were observed in the mouse thymus.

B.2. Human reconstitution of anti-asialo GM1 antibody treated NOD/SCID and NOD/SCID/Rag 1^{null} mice transplanted with CB CD34^{+} Cells.

The result that human T cells do not develop in NOD/SCID mice transplanted with CB CD34^{+} cells (Fig 3.2) was consistent with most previously published results. [14,65,113,115,133-135] There are a multitude of reasons why human T cells might not develop in this system, for example, the underdeveloped mouse thymus is incapable of supporting thymopoiesis. Several groups have demonstrated that the fetal NOD/SCID mouse thymus provides an appropriate environment for human CD34^{+} cells to develop into single positive T cells in thymic organ cultures and *in vivo* culture systems. [20,136,137] However, the adult NOD/SCID mouse thymus is very involuted and may not be able to provide the appropriate growth factors or thymic architecture required for thymopoiesis. It has been demonstrated that during human aging the thymus involutes and the level of thymopoieses declines.[58,138] Second, human T cell progenitors may traffic or migrate differently to the mouse thymus than to a human thymus. Alternativily, human T cells might fail to develop due to residual mouse NK cell activity, specifically, the ability of NK cells to target T cell progenitors [101]. Although this hypothesis is still controversial, Kerr *et al.,* demonstrated that NOD/SCID mice preconditioned with anti-TMβ, a monoclonal antibody against the murine interleukin-2R beta (IL-2R beta) known to decrease natural killer cell activity, were capable of developing human T cells within the mouse thymus. However, the systemic levels of human CD3^{+} T cells were very low. Therefore, I tested the hypothesis that NK cell depleted NOD/SCID mice would allow for higher human reconstitution allowing for progenitor T cells to seed the mouse thymus and generate human CD3^{+} thymocytes. In addition, to test

this hypothesis, we selected to use NOD/SCID Rag 1^{null} NOD/LtSz-Rag 1 mice which have been reported to be devoid of T and B cells and have little to no NK cell activity and are more resistant to higher doses of radiation.[92] NOD/SCID and NOD/SCID/Rag 1^{null} mice were preconditioned with anti-asialo GM1 antibody and subsequently transplanted with highly purified $CD34^+$ cells from the same CB preparation for direct comparison of the mouse strains in their ability to generate human T cells within the mouse thymus.

B.2.1. Isolation and transplantation of $CD34^+$ cells into NK depleted immuno-defiecient mice.

In order to determine if human $CD3^+$ T cells develop in NK cell depleted NOD/SCID or NOD/SCID/Rag^{null} mice, highly purified $CD34^+$ cells (97% $CD34^+$ and < 0.1 $CD3^+$) (Fig 3.3) were isolated from CB. NOD/SCID mice (*n*=5) and NOD/SCID/Rag^{null} mice (*n*=5) were preconditioned 24 hours before transplantation with 325 cGys and 700 cGys (respectively) and administered anti-asialo GM1 antibody i.p. The two cohorts were subsequently transplanted with 1 X 10^6 $CD34^+$ cells (i.v.). The mice were administered anti-asialo GM1 antibody i.p. every 11 days for 5 treatments to prevent new NK cell development and to allow for higher levels of systemic human reconstitution. Mice were bled and monitored monthly for human reconstitution by flow cytometry and sacrificed at 20 weeks. Although the NOD/SCID mice had high levels of human reconstitution in the bone marrow (52 ± 16 SD, range 35-78 (*n* =5)), no human T cells were detected (Fig. 3.4). The mouse thymus was further examined for human reconstitution and thymocyte development. As shown in figure 3.5, no CD4CD8 double positive thymocytes were present and no human

$CD45^+$ cells were detected. In contrast, the NOD/SCID/Rag^{null} mice had very low to no human reconstitution in the bone marrow (1 ± 1 SD, range 0.2-2.5, (*n*=5)) (Figure 3.4). No T cells were detected in the BM, spleen, and I was unable to find and analyze the NOD/SCID/Rag^{null} mouse thymus due to the fact that it had involuted. These results demonstrate that human T cells do not develop in NOD/SCID or NOD/SCID/Rag^{null} mice under our experimental conditions. Based upon these results, endongenous NK cells probably only play a minor role in preventing human T cells from being generated, but rather there is a lack of initial seeding of the mouse thymus or an inappropriate micro-environment for T cell development.

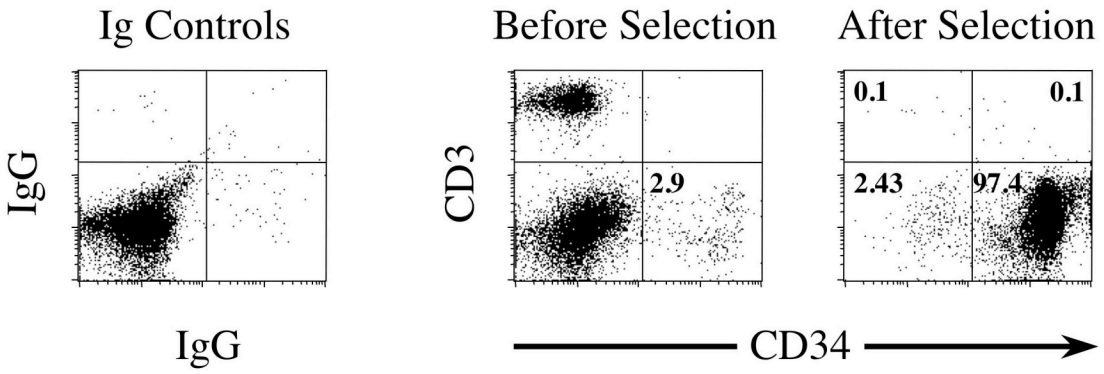


Figure 3.3 Highly purified $CD34^+$ cells from umbilical cord blood.

CB $CD34^+$ cells were isolated using magnetically labeled beads. The magnetically labeled $CD34^+$ cells were further purified by passing the CD34+ cells over a second set of columns. The isolated cells were analyzed for CD34 expression and the percentage of contaminating $CD3^+$ T cells. This procedure yielded 97% pure $CD34^+$ cells and 0.1% $CD3^+$ cells. 1 x 10^6 cells were transplanted into precondition NOD/SCID and NOD/SCID/$Rag1^{null}$ mice.

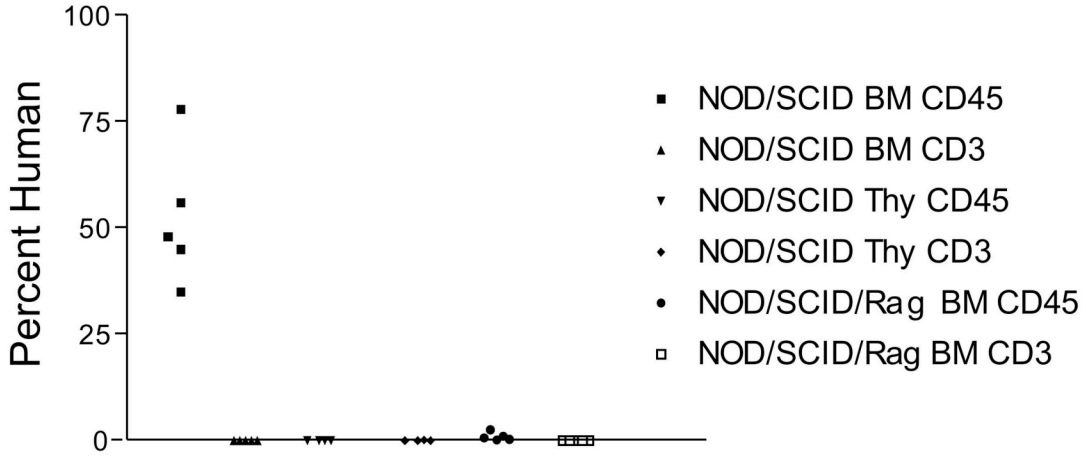


Figure 3.4 Human reconstitution in the bone marrow of NOD/SCID and NOD/SCID/Rag1^{null} mice preconditioned with α-asialo GM1 Antibody.

NOD/LtSz-Scid (*n*=5) and NOD/LtSz-Scid/Rag1^{null} (*n*=5) mice were preconditioned with α-asialo GM1 antibody to deplete endogenous mouse NK cells and sub-lethally irradiated to allow for bone marrow engraftment. Mice were transplanted with 1 x 10^6 CB CD34$^+$ cells and administered α-asialo GM1 antibody every 11 days to prevent *de novo* NK cell activity. At 20 weeks the mice were sacrificed and analyzed for human CD45$^+$ cell BM reconstitution and human T cell development. No human T cell development or T cell expansion was observed.

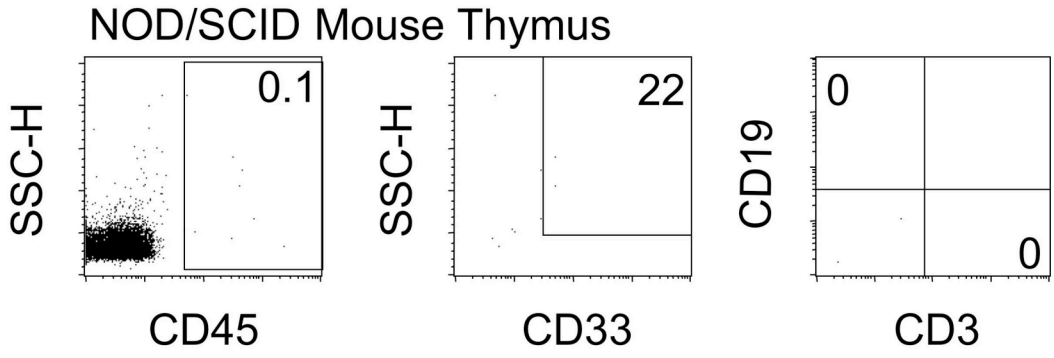


Figure 3.5 Human T cells do not develop in NK cell depleted immunodeficient mice. NOD/SCID and NOD/SCID Rag1^{null} mice were depleted of mouse NK cells with anti-asialo GM1 antibodies. Mice were transplanted with human CD34^{+} cells and allowed for human reconstitution for 20 weeks. The mice were sacrificed and analyzed for human thymopoiesis within the mouse thymus by gating for human CD45 and myeloid and lymphoid markers (CD33, CD19, CD3). In contrast to the bone marrow where human cells were abundant, no human cells were identified.

B.3. Analysis of cord blood CD34^{+} transplanted NOD/SCID mice followed by infant thymus implantation.

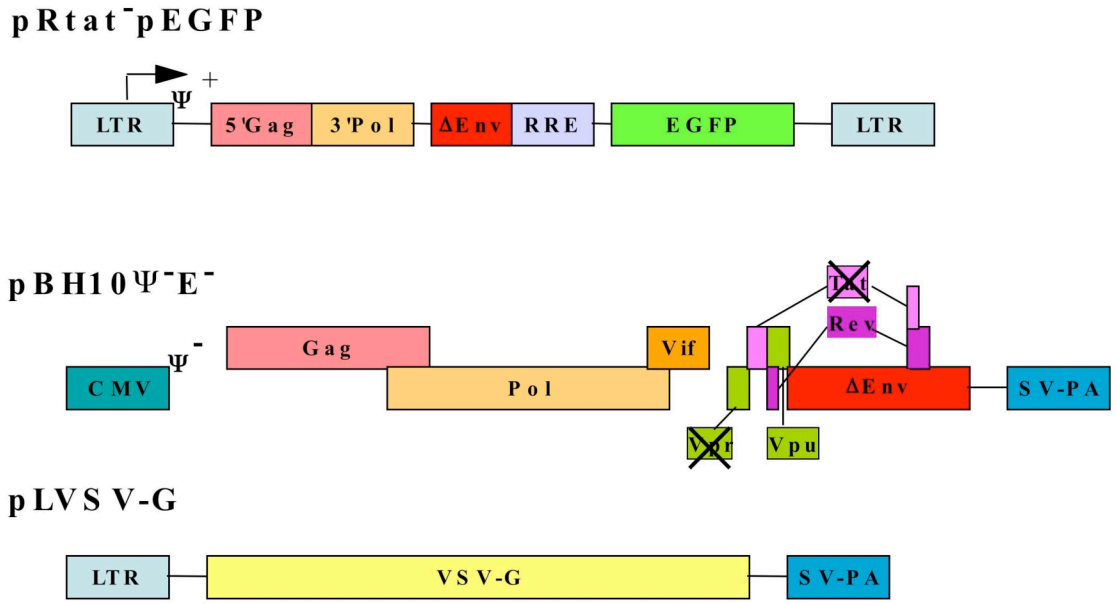
Individuals born with complete DiGeorge syndrome have a defective thymus in which T cells fail to develop.[139] However, all other components of the hematopoietic system appear to be intact and functional. Markert *et al.* demonstrated that patients with DiGeorge syndrome transplanted with allogeneic human infant thymus developed circulating peripheral blood T cells which resulted in some level of immune protection.[140,141] This advance was made possible by knowledge derived from *in vivo* experimentation in mice without other available systems that could serve to bridge the transition between animal experimentation *in vivo* and the clinical implementation of this knowledge to humans.[142]

Based upon the primary literature and my previous results, I decided to take an alternative approach. Human reconstituted NOD/SCID mice reconstituted with $CD34^+$ cells closely mimick the described immune phenotype of DiGeorge patients in that both myeloid and B cells develope in the absence of T cells. A possible reason why NOD/SCID mice do not have human T cells could be due to the lack of an appropriate microenvironment for progenitor T cells to develop into mature T cells. I tested the hypothesis that NOD/SCID mice previously engrafted in the bone marrow with $CD34^+$ and systemically reconstituted with $CD33^+$ and $CD19^+$ human cells would allow bone marrow progenitor T cells to seed implanted human thymic tissue to generate human T cells.

B.3.1 CB $CD34^+$ cells marked with a Lentivirus vector expressing EGFP

To test this hypothesis, CB $CD34^+$ cells were isolated and marked with a lentivirus vector that expresses EGFP under a CMV promotor. The schematic describing the plasmids used to generate the lentivirus vector are shown in figure 3.6.[143] $CD34^+$ cells were transduced 3X at a multiplicity of infection (MOI) of 5 with the $Rtat^-$pEGFP vector and frozen until time of transplant. A portion of the ttransduced cells were examined for EGFP expression at 48 hours after the last transduction. The cells were 37 % $EGFP^+$ and 20% $CD34^+EGFP^+$. NOD/SCID mice were transplanted with 2 x 10^5 transduced cells (n=10) or mock transduced $CD34^+$ cells (n=4) for controls and allowed to reconstitute the recipient hosts. Human reconstitution was confirmed by PCR for EGFP and human β-globin. At 12 weeks post transplant, reconstituted mice were implanted with human thymic tissue under the

mouse kidney capsule to allow for an appropriate microenvironment for *de novo* T cell development.



Adapted from Douglas (Human Gene Therapy) 1999

Figure 3.6 Schematic of the lentivirus vector system.

Lentivirus vector was produced by cotransfecting 293T cells with the plasmids pRtat⁻pEGFP, pBH10ψ⁻ env⁻, and pLVSVG. (18 µg, 18 µg, 6 µg, respectively) as described in Chapter II, Sections R and S. pRtat⁻pEGFP generates the expression cassette for EGFP expression, pBH10ψ⁻env⁻ produces the packaging construct for the viral RNA, and pLVSVG is the envelope protein. Following transfection, media supernant was collected 3 times at 24 hour intervals for 72 hours and concentrated for viral vector by ultra-centrifugation. The viral pellet was resuspended in IMDM, titered on HeLa cells and frozen until time of $CD34^+$ cell transductions.

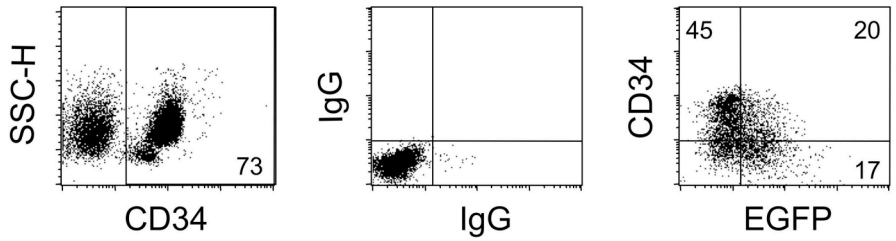


Figure 3.7 Efficiency of cord blood CD34$^+$ cell transduction by a lentivirus vector. Cord blood CD34$^+$ cells were isolated and plated in a retronectin coated 48-well plate. The cells were transduced 3x (MOI =5) with Rtat$^-$pEGFP. Forty-eight hours after the last transduction the cells were isolated and analyzed for EGFP and CD34 expression. The cells were frozen at -80˚ C until time of bone marrow transplant.

B.3.2 NOD/SCID mice transplanted with transduced CB CD34$^+$ cells and implanted with human infant thymus.

The reconstituted NOD/SCID mice were divided into 4 experimental cohorts. The mice transplanted with mock transduced HSCs were divided into CD34$^+$ cell only controls (*n*=2) and CD34$^+$ transplanted and implanted with allogeneic thymus controls (*n*=2). The mice that were transplanted with transduced HSCs cells were divided into marked CD34$^+$ cells only (*n*=2) and marked CD34$^+$ cells and implanted with allogeneic infant thymus (*n*=8). The mice were allowed 12 weeks for human reconstitution and *de novo* T cell development. The mice were sacrificed and mononuclear cells were isolated from the bone marrrow, spleen, and thymic implants and analyzed for human reconstitution (CD45$^+$ cells) and both myeloid and lymphoid cells. The cohort of NOD/SCID mice transplanted with CD34$^+$ cells only had high levels of human (CD45$^+$ cells) (55.6 ± 16 SD, range 40-71). The experimental group of mice transplanted with CD34$^+$ cells and implanted with allogeneic infant thymus

had a minimal amount of $CD45^+$ cells (4.1 ± 4.6 SD, range 0-10.5). The majority of the human cells in these mice were $CD3^+$ in the bone marrow (74.9 ± 27.9 SD, range 13.6-96) as demonstrated in Figure. 3.8. The thymic tissue was involuted and appeared to be only a thymic epithelial layer. Examination of the thymic implants for human CD4 and CD8 double positive thymocytes (12 ± 19.6 SD, range 1-54) demonstrated severe impairment of sustained thymopoiesis. There were no $CD45^+CD3^+EGFP^+$ cells in the bone marrow, spleen or implanted allogeneic infant thymus (Fig. 3.8). These results suggest the bone marrow progenitor T cells did not seed the implanted allogeneic thymic tissue to generate MHC tolerant T cells. The hypothesis is that endogenous thymocytes exited the thymus graft which resulted in the depletion of the previous reconstituted human hematopoietic cells.

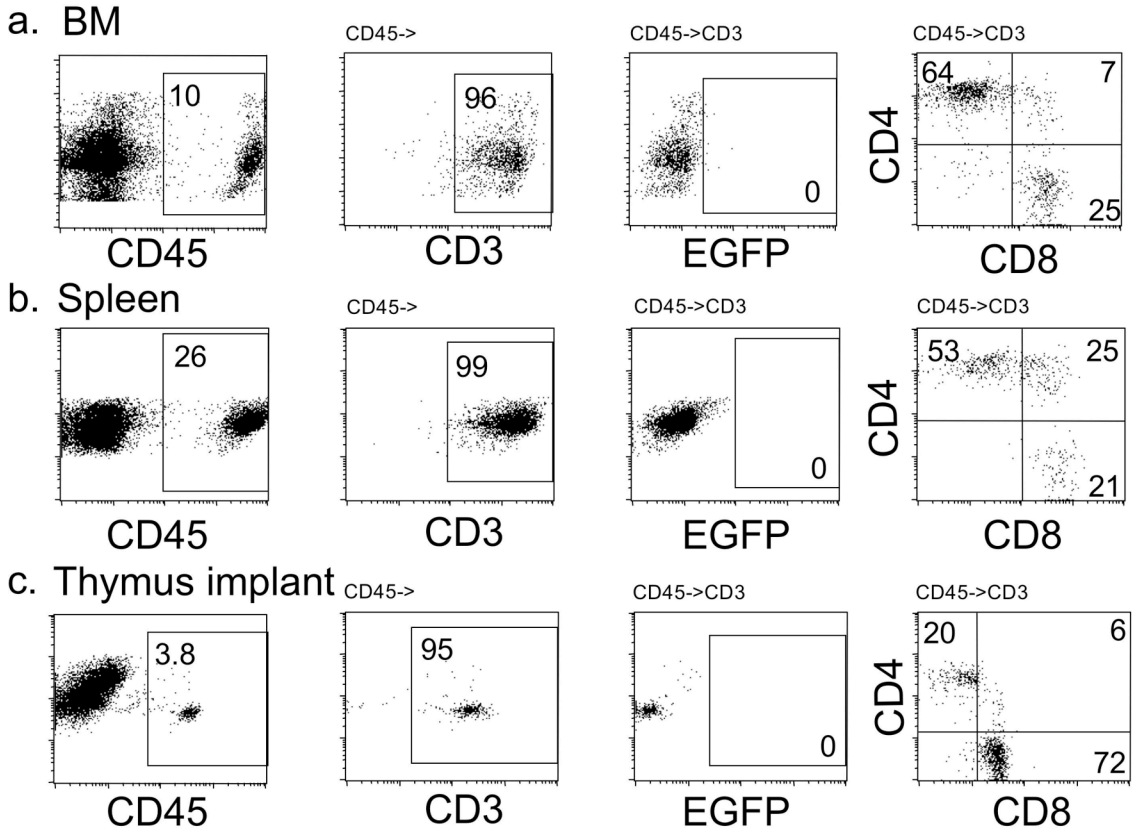


Figure 3.8 NOD/SCID mice reconstituted with EGFP marked CD34$^+$ cells and implanted with infant thymus tissue.

Preconditioned NOD/SCID mice were transplanted with CD34$^+$ cell marked with a lentivirus vector expressing EGFP. At 12 weeks, reconstituted mice were implanted with 3mm^3 fragments of infant thymus under the kidney capsule to allow for bone marrow T cell progenitors to seed the implanted thymus for *de novo* thymopoiesis. Mice were sacrificed at 12 weeks post thymus implantation and analyzed for human reconstitution and EGFP$^+$ T cells in the BM, spleen and implanted thymic tissue. a and b) Analysis of a representative mouse bone marrow and spleen for human reconstitution. The majority of the human cells were CD3$^+$ T cells. c) Analysis of a representative thymus implant. The thymus contained low levels of human cells with a small proportion of CD4CD8 double positive thymocytes. No EGFP$^+$CD3$^+$ T cells were detected in any of the tissues examined.

B.4. Analysis of NOD/SCID mice reconstituted with human CB $C34^+$ cells implanted with thymocyte depleted infant thymus.

The previous results suggest that the endogenous allogeneic thymocytes exited the implanted infant thymic tissue and depleted the initial human graft. This is most likely due to the MHC mismatch between the implanted thymic tissue and the previously transplanted human $CD34^+$ cells. Therefore, I hypothesized that human reconstituted NOD/SCID mice implanted with human thymic tissue depleted of endogenous thymocytes would provide the necessary thymic micro-environment for the engrafted human bone marrow progenitor T cells to seed the implanted thymus and generate tolerant T cells. In addition the depleted thymic tissue creates a niche for immigrant T cell progenitors allowing for *de novo* $CD34^+$ derived thymocytes to develop. The experimental approach was transplantation of NOD/SCID mice with UBC $CD34^+$ cells and allow for human reconstitution and then implant mice with either γ-irradiated or deoxyguanosine treated human thymus.

B.4.1 Thymocyte depletion of human infant thymus.

Infant thymus was obtained from donors undergoing corrective heart repair and depleted of human thymocytes by deoxyguanosine treatment *in vitro* or radiation conditioning. Deoxyguanosine is a DNA nucleotide analog of guanosine that incorporates into the DNA of replicating thymocytes inhibiting the correct guanosine-cytosine DNA base pairing resulting in thymocyte depletion. [140,144] The infant thymus (2 month old) was treated with 0.3 mM deoxyguanosine for 72 hours in thymic organoid cultures.[19] Infant thymus tissue was also irradiated at various doses (500, 800, 1000 cGys) to deplete thymocytes and

further cultured for 72 hours in thymic organ cultures. 1000 cGys was the most effective dose for depleting the human thymocytes as determined by flow cytometery for the percentage of live cells and double positive CD4 and CD8 and single positive $CD3^+$ thymocytes. In order to distinguish if the T cells in these mice were endogenous to the implanted allogeneic thymus or derived from the BM engrafted HSC cells, $CD34^+$ cells and thymocytes were frozen as viable cells for MHC typing.

B.4.2 Human reconstitution of NOD/SCID transplanted mice implanted with thymocyte depleted infant thymus.

NOD/SCID mice (*n*=15) were preconditioned and transplanted with $1.5x10^5$ CB $CD34^+$ cells and allowed to reconstitute for 9-10 weeks. Mice were tested for human engraftment by PCR for human β-globin and divided into experimental cohorts (Figure 3.9). The mice with the lowest level of β-globin were used as controls for sustained human reconstitution (*n*=5). Reconstituted mice were implanted with untreated infant thymus (*n*=3), irradiated (1000 cGys) infant thymus (*n*=4), or deoxyguanosine treated infant thymus (*n*=3). Mice were bled and monitored for human $CD45^+$ reconstitution throughout the experiment. At 26 weeks, the mice were sacrificed and analyzed for human reconstitution and human T cells in the bone marrow and the implanted thymic tissue by flow cytometry.

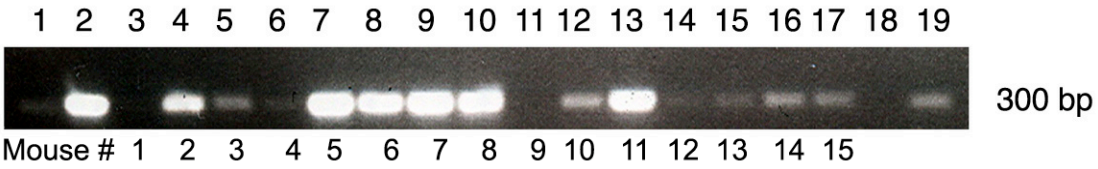


Figure 3.9 Human reconstitution in transplanted NOD/SCID mice.

Cord blood $CD34^+$ cells were isolated and transplanted into NOD/SCID mice and allowed to reconstitute the recipient for 9-10 weeks. The mice were bled and analyzed for human reconstititution by PCR for human β-globin. Lane 1 and 19: DNA ladder, Lane 2: human β-globin postive control for PCR reaction, Lane 18: no DNA negative control, Lane 3-17: CB $CD34^+$ transplanted mice. The reconstituted mice were divided into four experimental cohorts; CB 34^+ only cohort: M4, M8, M10; CB $CD34^+$ + 1000 cGys treated thymus cohort: M2, M5, M6, M11: CB 34^+ + deoxyguanosine treated thymus cohort: M3, M7, M15; and CB 34^+ + no treatment thymus: M12, M13, M14.

The BM of engrafted and reconstituted mice with human $CD45^+$ cells (50 ± 29 SD range 21-80 (*n*=3)) consisted of both myeloid and B cells as previously demonstrated. The reconstituted mice implanted with irradiated allogeneic thymus had high levels of human $CD45^+$ cells in the BM (80 ± 6 SD range 74-88) and virtually no human T cells (0.9 ± 1.5 SD, range 0.1-3.0)(Figure 3.10). Likewise, the mice that were implanted with deoxyguanosine treated thymic tissue had high levels of human CD45 reconstitution (73 ± 7.5 SD, range 64-78) with no human T cells detected by flow cytometry analysis (Figure 3.10). In human reconstituted NOD/SCID mice implanted with untreated infant thymus, the levels of BM reconstitution with $CD45^+$ cells varied (29 ± 39 SD range 3.6-74), but were lower than mice receiving deoxyguanosine or radiation treated thymic tissue. These mice had high percentages of human $CD3^+$ T cells (30 ± 30 SD range 2-60) in the bone marrow. In the implanted thymic tissue, under all the experimental conditions, no human $CD45^=$ cells

or double positive $CD3^+$ thymocytes were detected. The thymic tissue under all of these conditions was severely involuted and appeared as a white epithelial layer on the kidney rather than a robust organ filled with cells. These results suggest that the implanted infant thymus depleted of thymocytes does not graft (vascularize) or is not seeded from bone marrow progenitor T cells to sustain thymopoiesis.

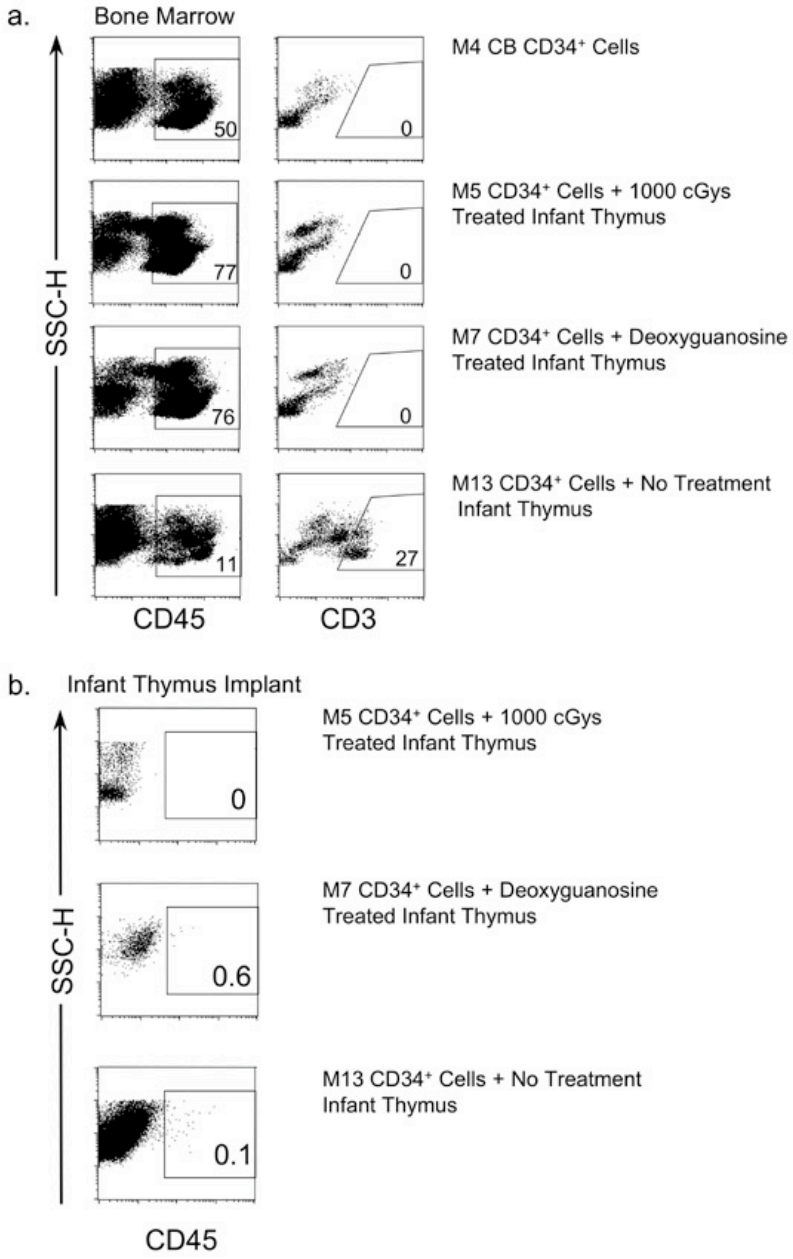


Figure 3.10 Human reconstituted NOD/SCID mice implanted with thymocyte depleted infant thymus.

NOD/SCID mice were transplanted with CB $CD34^+$ cells and allowed to engraft. Reconstituted mice were subsequently implanted with infant thymus (no treatment) or thymocyte depleted infant thymus (1000 cGys or deoxyguanosine treated). The mice were allowed 12 weeks for seeding of the thymus and T cell development. The mice were sacrificed and analyzed for *de novo* human thymocytes and T cells in the peripheral tissues.

B.5. EGFP Marked $CD34^+$ cells injected directly into the Thy/Liv organoid of NOD/SCID/$RAG1^{null}$ mice develop $CD3^+$ thymocytes.

Previous studies demonstrated that allogeneic human $CD34^+$ cells injected directly into the thymic organoid of SCID-hu thy/liv mice develop into $CD3^+$ T cells.[18] To test the hypothesis that CB $CD34^+$ cells were able to develop into single positive $CD3^+$ thymocytes in a human thymic microenvironement *in vivo* under our experimental conditions and that there is not an intrinsic defect in thymocyte development. NOD/SCID/$Rag1^{null}$-hu thy/liv mice were generated since this mouse strain develops large thy/liv organoids. The thymic organoid was allowed to develop for 8 weeks before $CD34^+$ cell injection into the organoid of irradiated preconditioned mice. Allogeneic CB $CD34^+$ cells were isolated from the same fetal liver and transduced 3x at an MOI of 5 with the lentiviral vector $Rtat^{+p}$EGFP expressing EGFP (vector provided by Angela Pagett-Thomas and Joel Gatlin). $CD34^+$ cells were analyzed at 48 hours after the last transduction and the percent EGFP and CD34 expression determined by flow cytometry. The cells were immediately frozen at -80˚C until thymic injection. To test this hypothesis, 1 x 10^5 $CD34^+$ cells marked with EGFP (94% $CD34^+$ and 24% $CD34^+EGFP^+$) were injected directly into the thymic organoid of preconditioned (700 cGys) NOD/SCID/RAG^{null} mice (*n*=4). At 8 weeks post thymic organoid injection, mice were sacrificed and the bone marrow and thymic organoid MNCs were isolated and examined for human reconstitution and *de novo* thymocyte development. As predicted, no human reconstitution was observed in the mouse bone marrow of these mice. The thymic organoids contained 55 ± 26.5 SD, range 37.2-93.6 (*n*=4) $CD45^+$ cells of which 1.1 ± 0.6 SD, range 0.4 - 1.8 (*n*=4) percent were double positive for CD3 and EGFP expression. Although

only low levels of $CD3^+EGFP^+$ cells were detected, the majority of these cells were double positive for CD4 and CD8 with a minor fraction of CD4 or CD8 single positive cells (Figure 3.11). These results demonstrate that $CD34^+$ cells can develop into $CD3^+$ thymocytes in an appropriate microenvironment *in vivo* generating single positive thymocytes.

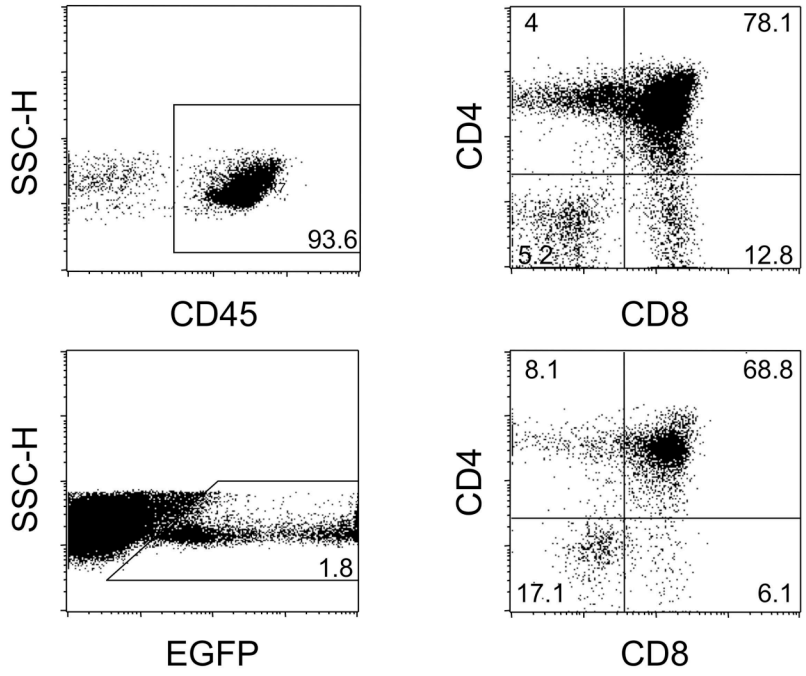


Figure 3.11 $CD34^+$ cells generate human thymocytes in an *in vivo* NOD/SCID/$Rag1^{null}$-hu thy/liv model.

NOD/SCID/$Rag1^{null}$ mice were implanted with fetal thymus/liver/thymus under the kidney capsule and the thymic organoid allowed to develop. Eight weeks later the mice were irradiated and injected with 1 x 10^5 EGFP marked allogeneic CB $CD34^+$ cells directly into the thymic organoid. At 8 weeks post injection the mice were sacrificed and the thymic organoid analyzed for human thymopoiesis.(top panels). Although low levels of $EGFP^+$ $CD45^+$ cells were observed (bottom left), the majority of these cells were double positive for CD4 and CD8 demonstrating $CD34^+$ cell generate human T cells (bottom right).

C. Conclusion

Several animal models of human hematopoiesis have been developed. All of these models have contributed to our understanding of human hematopoiesis. However, new, affordable, easy to manipulate and practical models are needed to understand human hematopoiesis and develop curative approaches to immune system ailments and disease states. Immune deficient mice have been used to study various aspects of human transplantation and diseases due to the fact they do not reject transplanted tissue. This revolutionary scientific contribution has led to many scientific discoveries in human hematopoiesis and pioneered the development of the SCID-hu thy/liv mouse model which has been instrumental in understanding HIV pathogenesis.[81] Both the SCID- hu and the SCID-hu thy/liv mouse models of human hematopoeisis and thymopoiesis have been extensively used to study the *in vivo* function of hematopoietic stem cell engraftment and reconstitution, important aspects of transgene expression studies for gene therapy, the replication of human specific pathogens, the role of human cytokines in hematopoietic T cell development and homeostasis and more recently to study the ontogeny and function of different components of the human immune system, including dendritic cells (DC)[65,119,120]. My results of transplanted NOD/SCID mice with $CD34^+$ cells are consistent with previously described results that human T cells do not develop. I pursued a variety of hypothesis driven approaches from NK cell depleted immunodeficient mice to implantation of human infant thymus depleted of thymocytes to generate human T cells. All of these experimental approaches resulted in no *de novo* human T cell generation from bone marrow engrafted $CD34^+$ cells.

Although it has been suggested that endogenous mouse NK cells may be the reason that human T cells do not develop[101], the logic of why NK cells would target T cell progenitors is unexplainable. I would predict that any transplanted MHC mismatched cell that reconstitutes a mouse not expressing the appropriate MHC class I restriction would be subject to endogenous NK cell killing. It is difficult to explain my results in light of the Kerr[101] experiment describing T cell development in NK cell depleted mice. One discrepancy is the use of different NK cell depleting antibodies. I used anti-asialo GM-1 and the Kerr group used anti-TMβ. Although we were not able to detect human T cells in anti-asialo GM-1 treated and $CD34^+$ transplanted NOD/SCID mice, it has been demonstrated that endogenous mouse NK cells play a significant role in human T cell development in immunodeficient mouse models.[97,101]

I further tested a variety of approaches of reconstituting NOD/SCID mice with human $CD34^+$ cells followed by implantation of allogeneic infant thymic tissue to generate human T cells. None of the experimental approaches tested were able to generate a systemic hematopoietic system that maintained both myeloid and lymphoid cells including T cells. I hypothesized that the reason was a graft versus transplant reaction due to MHC mismatch of the implanted thymus tissue to the engrafted $CD34^+$ cell resulting in clearance of the bone marrow graft. This was supported by the presence of T cells in the bone marrow of animals transplanted with allogeneic $CD34^+$ cells. However, direct injection of CB $CD34^+$ cells into a thy/liv implant did allow for human single and double positive thymocytes which suggest there is not an intrinsic defect for human T cell development in the implanted thymic tissue.

Based on these experiments I concluded that T cells do not generally develop in NOD/SCID or NOD/SCID RAG 1^{null} mice. I hypothesized that an appropriate human micro-environment would allow for human T cells to develop. Our results did not confirm this hypothesis but led me to believe that an implanted graft versus a transplant graft reaction developed in which the endogenous human thymus T cells depleted the original human bone marrow $CD34^{+}$ cell graft. Therefore, I hypothesized that MHC matched $CD34^{+}$ cells and implanted human thymus would allow for human reconstitution with *de novo* T cell development and T cell homeostasis in the peripheral tissues.

CHAPTER IV

GENERATION AND CHARACTERIZATION OF THE BLT MOUSE MODEL OF HUMAN HEMATOPOIESIS

A. Introduction

One important improvement to the original transplantation model using SCID mice was the use of other immunodeficient strains, like NOD/SCID, as recipients for transplantation. Transplantation of NOD/SCID mice with human $CD34^+$ cells results in dramatically higher levels of systemic repopulation with human $CD45^+$ cells. However, despite the higher levels of human reconstitution, T cells generally fail to develop in this system[65,115].

In contrast to the lack of human T cells in SCID or NOD/SCID mice transplanted with purified $CD34^+$ cells, in SCID-hu thy/liv mice there is an abundance of human thymocytes. However, except for the spleen where low levels (< 1%) of human T cells (and rarely human B cells) can be found, virtually all human cells are confined to the thymic organoid that develops after implantation[81,118,145]. Thus, SCID-hu thy/liv mice do not have significant systemic repopulation with human T cells and are virtually devoid of human B cells, monocytes/macrophages and dendritic cells.

Since both the SCID-hu thy/liv model and the human $CD34^+$ cell transplanted NOD/SCID model seem to complement each other in very important aspects, the hypothesis was that a combination of both systems would result in a new model that incorporates the best aspects of each in order to develop a significantly more complete human immune

system. When autologous or allogeneic human $CD34^+$ progenitor cells are provided with an appropriate thymic microenvironment, such as being injected directly into the thymic organoid that develops in the SCID-hu thy/liv model, they can mature into naïve single positive human T cells.[18,146]. I therefore asked whether $CD34^+$ cells introduced via bone marrow transplantation could systemically reconstitute the mouse and sustain thymopoiesis in the implanted human thymic tissue. In order to maximize the likelihood of seeding the transplanted thymus with human T cell progenitors originating from the bone marrow I used NOD/SCID mice instead of SCID mice or NOD/SCID Rag 1^{null} mice, as NOD/SCID mice support significantly higher levels of reconstitution following transplantation with human $CD34^+$ cells due to low endogenous mouse NK cell activity.[65] In addition, I originally used allogeneic cord blood $CD34^+$ cells because of their accessibility and high *in vivo* repopulating potential [16]. However, regardless of their source, transplantation with allogeneic $CD34^+$ cells failed to sustain the implanted thymic tissue and resulted in clearance of the bone marrow graft in this model.

B. Results

B.1. Generation of the BLT mouse.

I proceeded to determine if autologous fetal liver $CD34^+$ cells could contribute to the overall levels of human reconstitution when transplanted after implantation of MHC matched human fetal thymus and liver. The overall scheme is illustrated in figure 4.1. In essence, NOD/SCID mice were first implanted with a human fetal liver and thymic tissue sandwich under the mouse kidney capsule and allowed to recover from surgery for 3-6 weeks. I found

inclusion of the piece of fetal liver to be critical in allowing for vascularization and initial development of the human thymic graft. Implanted mice were then preconditioned with a sub-lethal dose of gamma radiation (325 cGys) and transplanted with autologous $CD34^+$ cells (2×10^6), depleted of human $CD3^+$ cells, obtained from a portion of the same fetal liver used for the implant. My results show that transplantation with autologous FL $CD34^+$ cells into thy/liv/thy implanted mice results in a dramatic increase in the overall levels of systemic reconstitution with human hematopoietic cells. Specifically, human T, B, and myeloid cells develop with maintained human immune homeostasis in the bone marrow, peripheral blood, and spleen. Human cells represented a minor proportion of cells in the blood of NOD/SCID mice that were co-implanted with human thymus and liver alone. However, mice that in addition to the implanted fetal thymic and liver tissue also received a bone marrow transplant with autologous $CD34^+$ cells had readily detectable levels of human cells in the peripheral tissues. To emphasize the importance of the reconstitution of the mouse **B**one marrow with human transplanted hematopoietic stem cells to repopulate mice previously implanted with fetal **L**iver and **T**hymic tissue we have adopted the designation NOD/SCID-hu BLT mice (or **BLT** mice) to distinguish them from the SCID-hu thy/liv model.

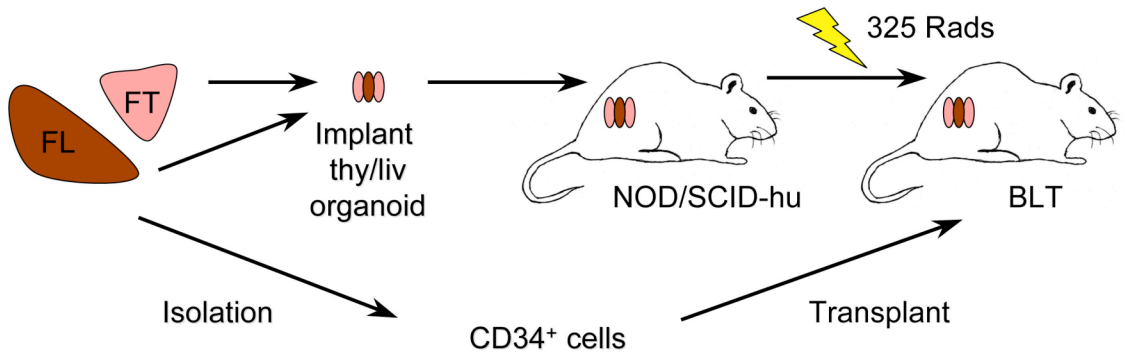


Figure 4.1 Schematic for generating the Bone marrow/Liver/Thymus (BLT) mouse model of human hematopoeisis.

1-3 mm^3 pieces of fetal thymus, fetal liver and fetal thymus are assembled and implanted as a conjoint sandwich under the kidney capsule of a NOD/SCID mouse to develop a human thymic organoid. At the same time, autologous fetal liver $CD34^+$ cells are isolated and stored frozen at -80˚ C until time of bone marrow transplant. The implanted sandwich is allowed to vascularize and develop for 3-6 weeks before mice are non-lethally irradiated at 325 cGys. Mice were then transplanted with purified fetal liver $CD34^+$ cells by i.v. injection.

B.2. Chararacterization of the BLT mouse.

Since human hematopoietic cells were readily found in the peripheral blood of BLT mice we proceeded to determine the presence of human cells in other primary and secondary lymphoid tissues. Overall levels of reconstitution with human cells in different mouse tissues are summarized in figure 4.2. In sharp contrast to SCID-hu thy/liv mice, BLT mice demonstrated high levels of human $CD45^+$ cells in bone marrow (48% ± 14 SD, range 23-75, *n*=24), spleen (42% ± 21 SD, range 5-84, *n*=24) and lymph nodes (71% ± 22 SD, range 6-95, *n*=14) (Fig. 4.2). In addition, we observed that the percentage of human BM engraftment also contributed to the overall levels of human reconstitution in the peripheral tissues. Upon

lineage analysis of these tissues, we noted the presence of human T cells, B cells, monocyte/macrophages and dendritic cells (Fig. 4.3, Fig. 4.4 and Fig. 4.5). With respect to the human T cell subsets present in the different tissues, the mean of $CD4^+$ cells was 71% (±11 SD, range 47-91, *n*=73) and the mean of $CD8^+$ T cells was 21% (±11 SD, range 4-48, *n*=73) (Table 4.1) for all tissues examined. The mean percentages of human $CD4^+$ versus $CD8^+$ T cells obtained from BLT mouse peripheral blood compared to human peripheral blood control subjects was not significantly different: BLT mice $CD4^+$ 72% (±12 SD, range 52-88, *n*=18) and $CD8^+$ 24% (±11 SD, range 11-42, *n*=18) versus human $CD4^+$ 75% (±10 SD, range 62-86, n=5) and $CD8^+$ 23% (±10 SD, range 14-38, *n*=5) (Table 4.1). We also determined the differentiation state of the human T cells in the peripheral blood of BLT mice by analyzing the expression of CD45RA and CD27; approximately 62% (± 15 SD, range 40-87, *n*=11) of the T cells in the periphery exhibited a naïve phenotype ($CD45RA^+CD27^+$) and 32% (±14 SD, range 13-56, *n*=11) exhibited a central memory phenotype ($CD45RA^-CD27^+$) (Fig. 4.3 and Table 4.2). As expected BLT mice kept under sterile conditions had a higher percentage of naïve T cells when compared to healthy human controls (Fig. 4.3 and Table 4.2).

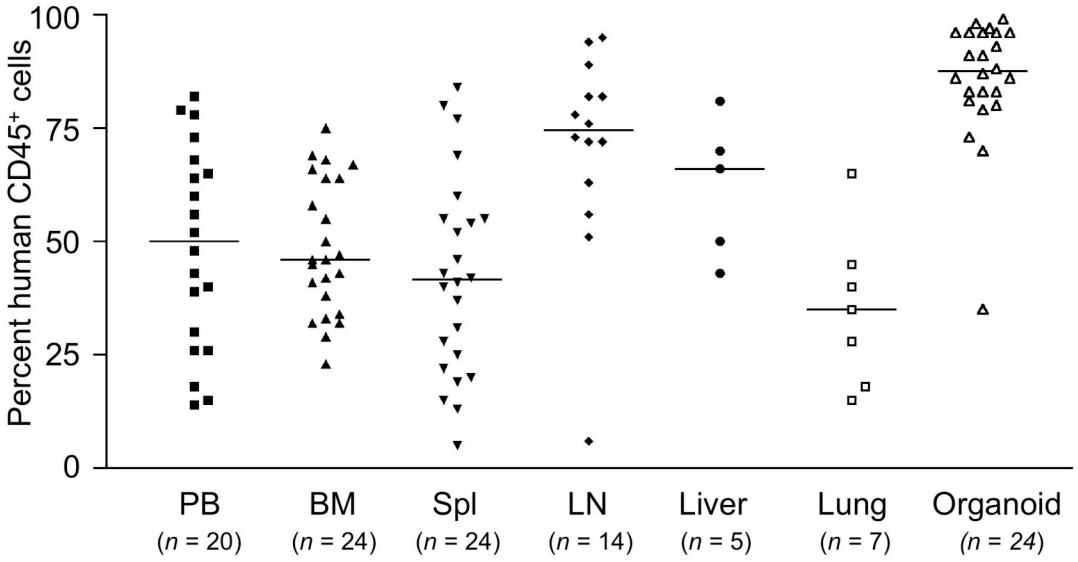


Figure 4.2 Human ($CD45^+$ cell) reconstitution of BLT mice.

BLT mice were generated as described in Chapter IV section B.1. BLT mice were sacrificed between 20 and 26 weeks post bone marrow transplant. Mononuclear cells were isolated from the bone marrow, spleen, lymph node, liver, lung and the thymic organoid and analyzed for the percentage of human $CD45^+$ cells mononuclear cells. The peripheral blood was prepared using the BD whole blood lysis protocol and analyzed by flow cytometry. The bar is the median of each tissue of all BLT mice analyzed.

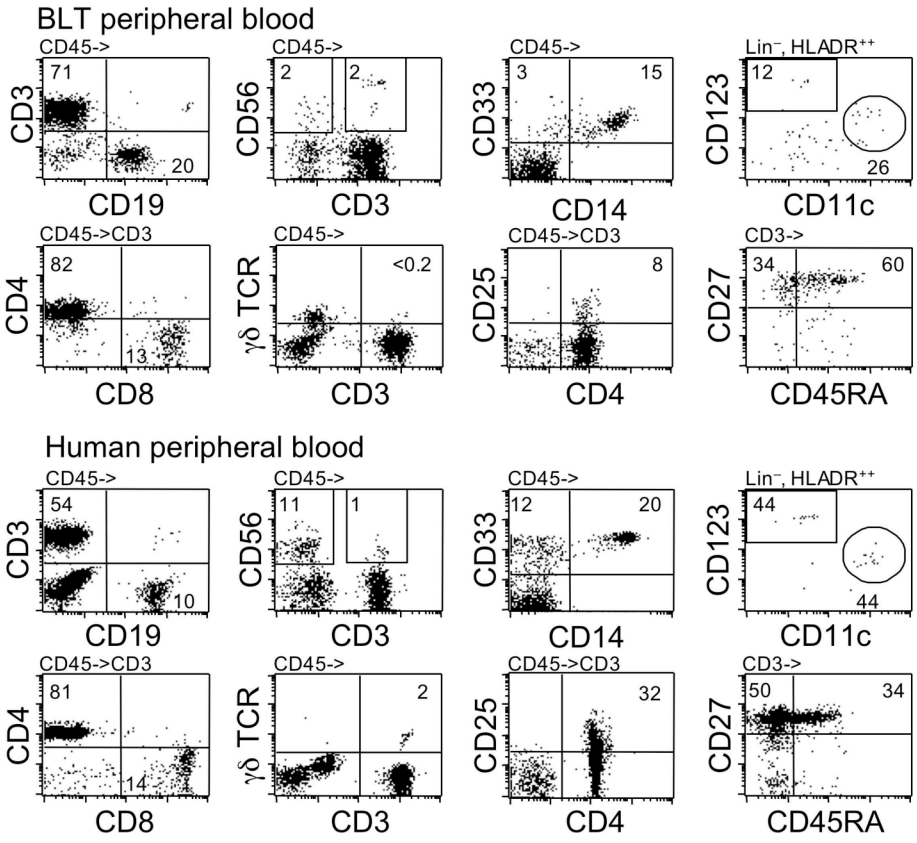


Figure 4.3 Phenotypic analysis of peripheral blood from a representative BLT mouse at 26 weeks post transplant and a normal healthy human donor.

Live cells were gated by side scatter versus forward scatter and subsequently gated for human cells (CD45). Human populations were first gated for hCD45 and then for the respective lineage specific marker (i.e. CD45>CD3, CD19, CD33, etc). Individual sub-populations were analyzed by gating on CD45, the lineage marker and then the specific antigen (i.e. CD45>CD3>CD4 or CD8, CD45>CD3>CD4 and CD25, etc). Human DCs were defined as lineage negative and HLA-DR^{++} cells expressing either CD123 or CD11c (i.e. lin^{-}, HLA-DR^{++}>CD123 or CD11c). All data presented were derived from a single BLT mouse and a single human control. Indicated numbers refer to the percentage of total human cells for the indicated lineage (CD3 vs CD19) or to the percentage of the indicated sub-population in any individual lineage (such as the percentage of CD4 versus CD8 cells of the CD3 gate). Specific stains: [(CD3-FITC, CD19-PE, CD33-PerCP, CD45-APC), (CD3-FITC, CD56-PE, CD45-PerCP, CD16-APC), CD14-FITC, CD33-PE, CD45-PerCP, CD16-APC), (Lin^{-}-FITC, CD123-PE, HLA-DR-PerCP, CD11c-APC), (CD3-FITC, CD8-PE, CD4-PerCP, CD45-APC), (CD3-FITC, CDγδ-PE, CD4-PerCP, CD45-APC), (CD3-FITC, CD25-PE, CD4-PerCP, CD45-APC), and (CD45RA-FITC, CD27-PE, CD3-PerCP, CD4-APC).

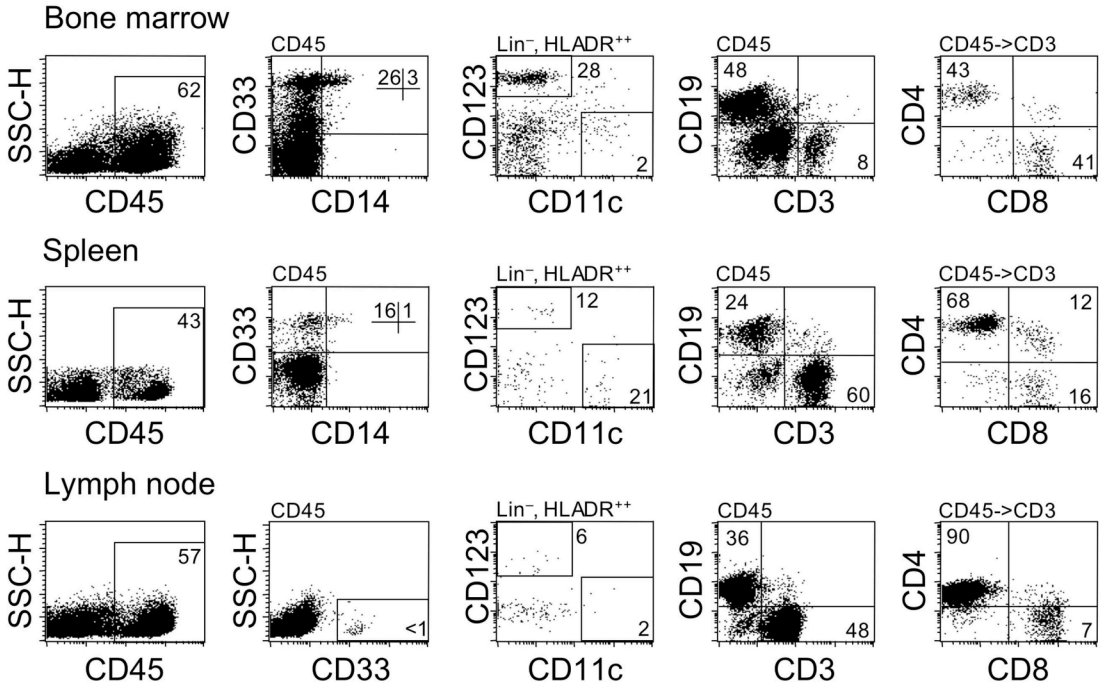


Figure 4.4 Characterization of the human cells in the bone marrow, spleen, and lymph node of BLT mice.

BLT mice were sacrificed between 20 and 26 weeks post autologous $CD34^+$ cell transplant and analyzed for human reconstitution and immune cell lineages. BLT mouse tissues were analyzed by flow cytometry as described in figure 4.3. All human reconstituted BLT mice contained both myeloid and lymphoid cells. Specifically, BLT mice reconstituted with human T cells.

Table 4.1 Analysis of human $CD4^+$ and $CD8^+$ T cells in different tissues of BLT mice.

BLT T cells	Human T cells
Peripheral Blood (*n* = 18)	Peripheral Blood (*n* = 5)
† $CD3^+CD4^+$ 71.9 ± 11.8 (52-88)	$CD3^+CD4^+$ 75.4 ± 9.7 (62-86)
$CD3^+CD8^+$ 23.8 ± 10.9 (11-42)	$CD3^+CD8^+$ 23.0 ± 9.7 (14-38)
BM (*n* = 12)	
$CD3^+CD4^+$ 66 ± 7.5 (52-81)	
$CD3^+CD8^+$ 26 ± 8.2 (13-43)	
Spleen (*n* = 15)	
$CD3^+CD4^+$ 67.6 ± 10.3 (48-81)	
$CD3^+CD8^+$ 22.3 ± 9.5 (11-46)	
LN (*n* =12)	
$CD3^+CD4^+$ 78.1 ± 7.4 (64-91)	
$CD3^+CD8^+$ 15.3 ± 7.4 (8-30)	
Lung (*n* = 10)	
$CD3^+CD4^+$ 68.7 ± 12.8 (47-87)	
$CD3^+CD8^+$ 17.7 ± 13.2 (4-48)	
Liver (*n* = 6)	
$CD3^+CD4^+$ 75.7 ± 12.9 (60-91)	
$CD3^+CD8^+$ 20.3 ± 13.2 (7-40)	

† Mean percentage of $CD4^+$ and $CD8^+$ human T cells with S.D. and ranges.
P = 0.28 for comparison of $CD4^+$ peripheral blood T cells of BLT mice to healthy human controls.
P = 0.44 for comparison of $CD8^+$ peripheral blood T cells of BLT mice to healthy human controls
No significant differences were observed based on a single tailed Student t test comparing BLT to human PB T cells.

Table 4.2 Naïve versus memory phenotype of human peripheral blood T cells in BLT mice.

† BLT peripheral blood		Human peripheral blood	
Naïve T cells $\%CD3^+CD45RA^+CD27^+$	Memory T cells $\%CD3^+CD45RA^-CD27^+$	Naïve T cells $\%CD3^+CD45RA^+CD27^+$	Memory T cells $\%CD3^+CD45RA^-CD27^+$
78.0	21.0	42.0	50.0
87.0	13.0	22.0	41.0
67.0	32.0	50.0	34.0
80.0	20.0	43.0	54.0
53.0	38.0	37.0	52.0
40.0	56.0		
60.0	34.0		
64.0	13.0		
40.0	51.0		
50.0	45.0		
65.0	33.0		
62.2 ± 15.6	32.4 ± 14.6	38.8 ± 10.5**	46.2 ± 8.4*

† Mean values for each T cell population are indicated with the S.D.. (Memory effector and resting memory T cell percentages not included)
Significant differences of T cell phenotypes determined using a single tailed Student t test are indicated with asterisks.
** $P = 0.005$ for comparison of naïve T cells of BLT mice to normal human donors.
* $P = 0.035$ for comparison of memory T cells of BLT mice to normal human donors.

Examination of other tissues important in immune regulation and mucosal immunity such as liver, lung and gut-associated lymphoid tissue (GALT) for the presence of human ($CD45^+$) hematopoietic cells. These tissues from BLT mice contained significant levels of human hematopoietic cells ($CD45^+$) as determined by flow cytometry and/or immunohistochemistry analysis (Fig. 4.5). Lineage analysis by flow cytometry confirmed the presence of human T cells, B cells, monocytes/macrophages and both $CD11c^+$ and $CD123^+$ DCs in the liver and lung (Fig 4.5a). Multi-lineage reconstitution was also observed in the GALT of BLT mice analyzed by immunohistology (Fig. 4.5b). Collectively, these data demonstrate that NOD/SCID mice implanted with human thymus and liver tissue prior to receiving autologus human $CD34^+$ cells can generate a remarkable state of sustained

systemic multilineage reconstitution with human hematopoietic cells that can persist up to 26 weeks post transplant (the longest time point I analyzed).

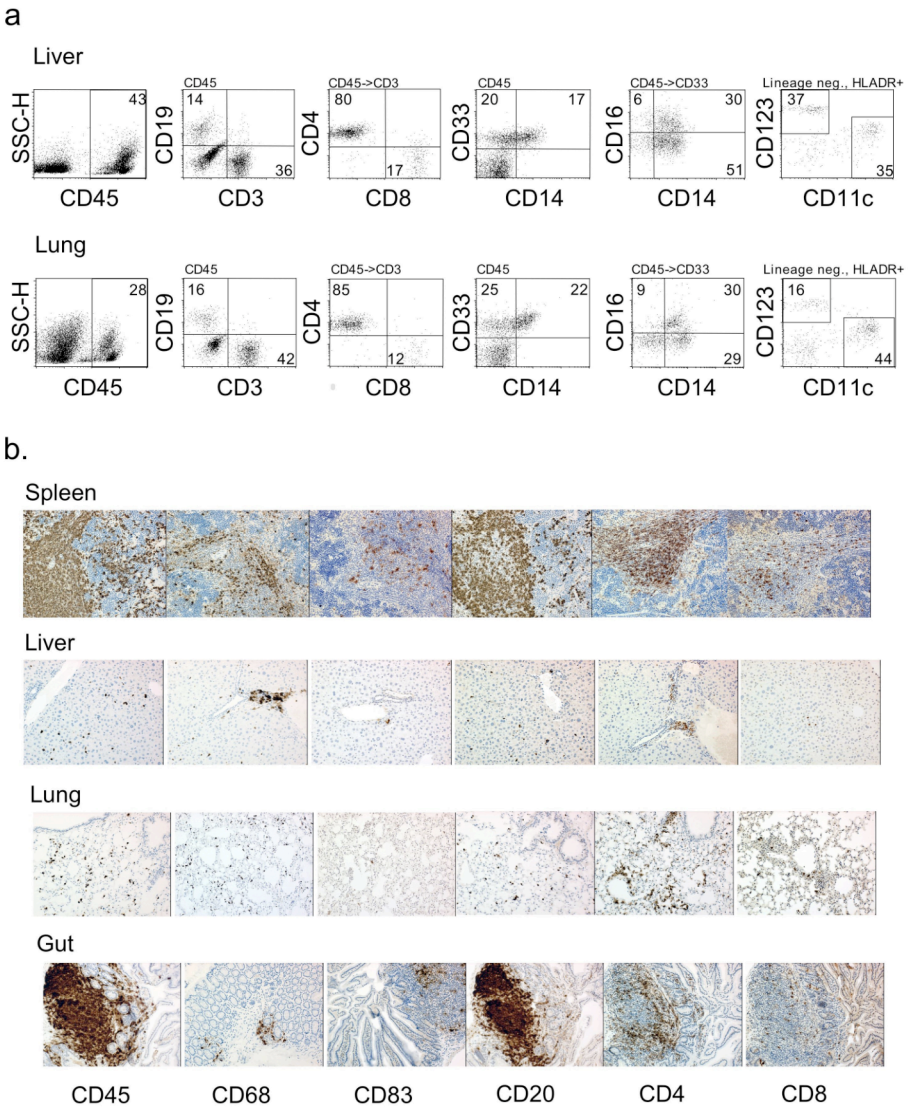


Figure 4.5 Systemic human reconstitution of BLT mice with human leukocytes.

BLT mice were generated and examined for human reconstitution in secondary and tertiary lymphoid tissues. a) BLT mouse liver (site of immune regulation) and lung (site of mucosal immunity) were analyzed for human $CD45^+$ cells and both myeloid and lymphoid cell subsets. All BLT mice examined were reconstituted with human $CD3^+$ T cells. b) Immunohistochemistry of the spleen, liver, lung and gut demonstrating human reconstitution and organization of the human $CD45^+$ cell subsets.

B.3. Germinal Center-Like Structures

NOD/SCID mouse secondary lymphoid tissues are small to non-existent [113]. The BLT mouse lymph nodes (LN) are small but recognizable and clearly reconstituted with human ($CD45^+$) hematopoietic cells (Fig. 4.6). Furthermore, immunohistochemical analysis for the human chemokines CXCL13 (B cell-attracting chemokine 1) and CCL21 (secondary lymphoid tissue chemokine) (Fig. 4.6b) in BLT LN indicate that human hematopoietic cells are organizing rudimentary secondary lymphoid structures. It should be noted that there was no positive staining with isotype matched control antibodies in any tissues examined. In contrast to the spleens of BLT mice where B and T cell zones can develop (Fig. 4.5b) there was not yet discrete lymphoid compartmentalization in lymph nodes (Fig 4.6). Therefore BLT mice (n=3) were immunized with sheep red blood cells (SRBC) to determine if germinal center formation would be induced in secondary lymphoid tissues. BLT mice were anesthetized and inoculated with 50 µl per rear footpad, 50 µl subcutaneous in the scruff of the neck, and 200 µl i.p. with sheep red blood cells .[116] Five days post SRBC administration, BLT mice were sacrificed and the secondary lymphoid tissues (spleens and lymph nodes) were isolated for immunohistochemistry analysis. B cell containing follicular-like structures were identified in the spleen and more organized T and B cell zones developed in the lymph nodes (Fig. 4.6). These results not only demonstrate that BLT mice reconstitute with high levels of multi-lineage human cells, but that the lymphoid cells generate appropriate secondary lymphoid structures.

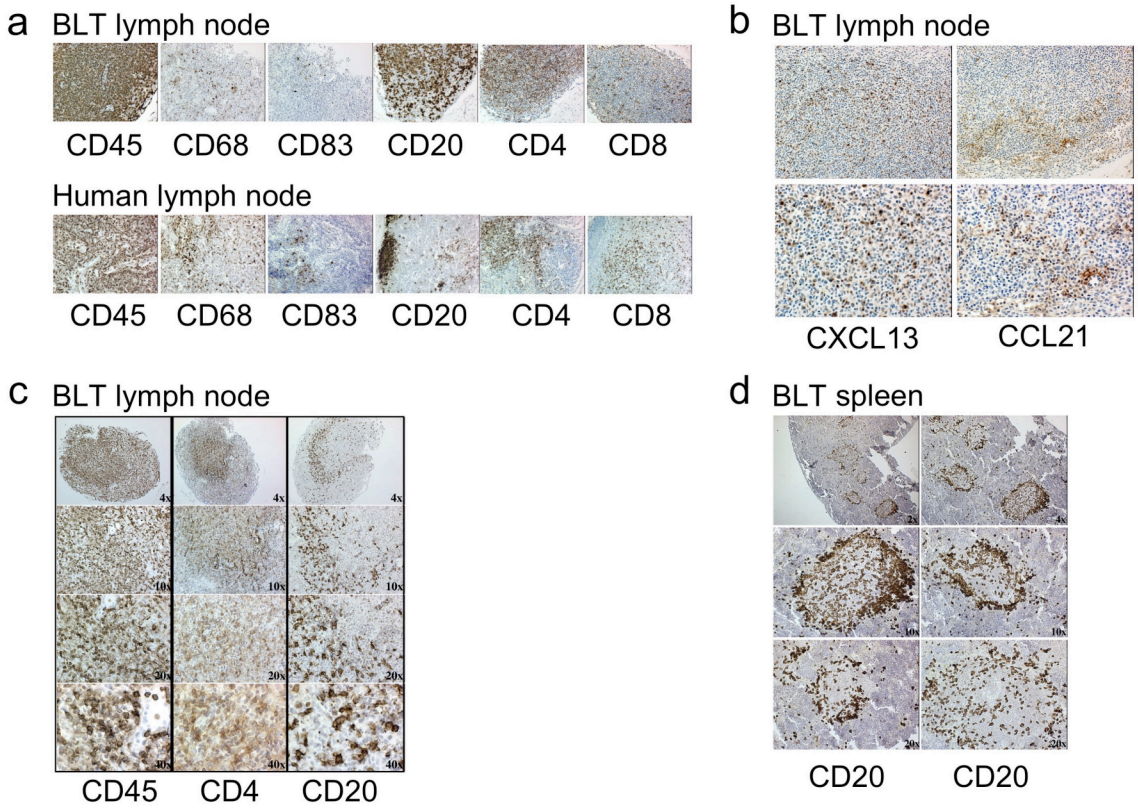


Figure 4.6 Immunohistological analysis of secondary lymphoid tissues of BLT mice.

a) Immunohistological staining comparing the human reconstitution of BLT mouse lymph node to human lymph node tissue controls (100x). b) Immunohistological staining of BLT lymph node for human CXCL13 and CCL21 (100x top and 200x bottom). c) BLT mice were administered sheep red blood cells to induce germinal center formation and lymphoid tissues were analyzed by immunohistochemistry 5 days later. BLT mouse lymph nodes stained with anti-human CD45, CD4 and CD20 showed organization of T and B cells into discrete cellular compartments reminiscent of B and T cell zones. d) BLT spleens develop distinct germinal center-like structures.

B.4. Human T cell neogenesis.

In order to determine if thymopoiesis was sustained in BLT mice, I first examined the composition of the thymic organoid post irradiation and hematopoietic stem cell transplant. For comparison, we also determined the proportion of human single and double positive thymocytes in the original fetal thymus and in the thymus of a 6-year-old subject. The relative proportions of double and single positive thymocytes of all three samples are remarkably similar (Fig. 4.7) with greater than 80 % double positive thymocytes, demonstrating long term sustained thymopoiesis in BLT mice. I then evaluated progenitor seeding of the implanted thy/liv organoid from transplanted bone marrow stem cell progenitors and their contribution to sustain thymopoiesis in generating *de novo* T cells. For this purpose, autologous fetal liver $CD34^+$ cells were transduced *ex-vivo* with the lentivirus-based $Rtat^-EGFP$ vector (3 x at an MOI of 5) expressing the enhanced green fluorescence protein (EGFP). $2x10^5$ transduced cells (~30% $EGFP^+$) (Fig. 4.8) were then transplanted into NOD/SCID-hu thy/liv implanted mice (*n*=5). Twelve weeks post transplant, mice were sacrificed and hematopoietic tissues were analyzed for the presence of human hematopoietic cells expressing EGFP. Human cells expressing CD45 and EGFP were found in all the tissues examined (bone marrow, spleen and thymic organoid) albeit at relatively low levels (Fig. 4.9). A more detailed analysis of EGFP expression in different hematopoietic lineages, as well as T cell subsets, further demonstrated that the transplanted EGFP marked $CD34^+$ cells contributed to the overall levels of engraftment and reconstitution of all human lineages examined, including the seeding of the thymic organoid therefore contributing to *de novo* thymopoiesis and T cell homeostasis (Fig. 4.9).

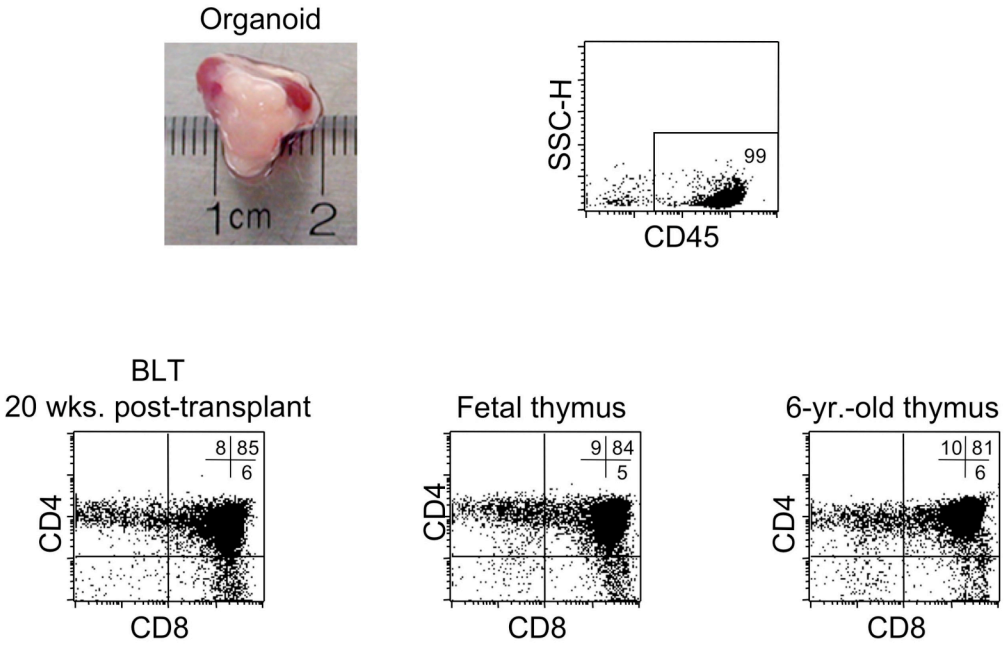


Figure 4.7 Analysis of a BLT thymic organoid and comparison to human fetal and infant thymi.

Top: BLT thymic organoid harvested 20 weeks post transplant (left) consists almost exclusively of human $CD45^+$ cells (right). Bottom: Comparison of single and double positive CD4 and CD8 thymocytes in the BLT mouse organoid, a fetal thymus and the thymus of a 6 year old infant. Thymocytes were first gated for live cells, human CD45 expression and then for human CD4 and CD8 expression. Numbers represent the percentage of cells in each population.

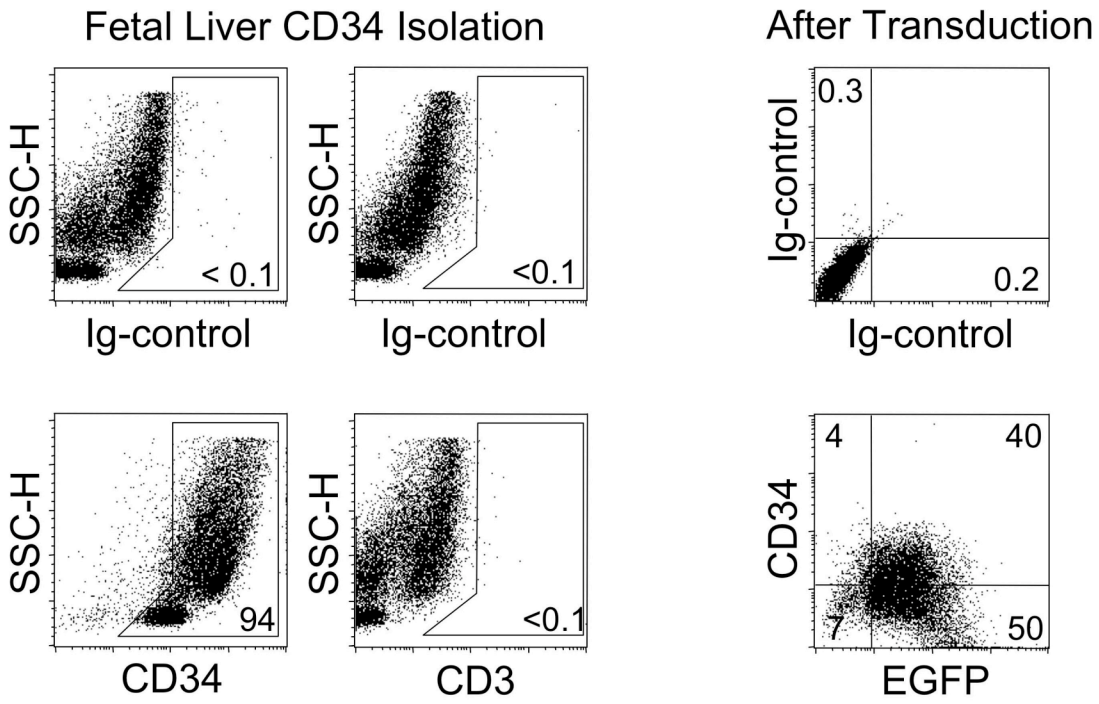


Figure 4.8. Fetal liver CD34$^+$ cell isolation and transduction efficiency with a lentivirus vector expressing EGFP.

Fetal liver tissue was disrupted and CD34$^+$ cells were isolated by magnetic positive selection and analyzed for CD34$^+$ cell purity. The contaminating CD3$^+$ cell population in the CD34$^+$ cell preparation was less than 0.1 %. The isolated cells were transduced 3x with the lentivirus vector Rtat$^-$EGFP (MOI=5). The cells were frozen at -80˚C until time of bone marrow transplant. For analysis of the transduction efficiency, a sample was analyzed 48 hours after the last transduction. The cells were isolated and analyzed for EGFP and CD34 expression. The cells were 40% double positive for EGFP and CD34.

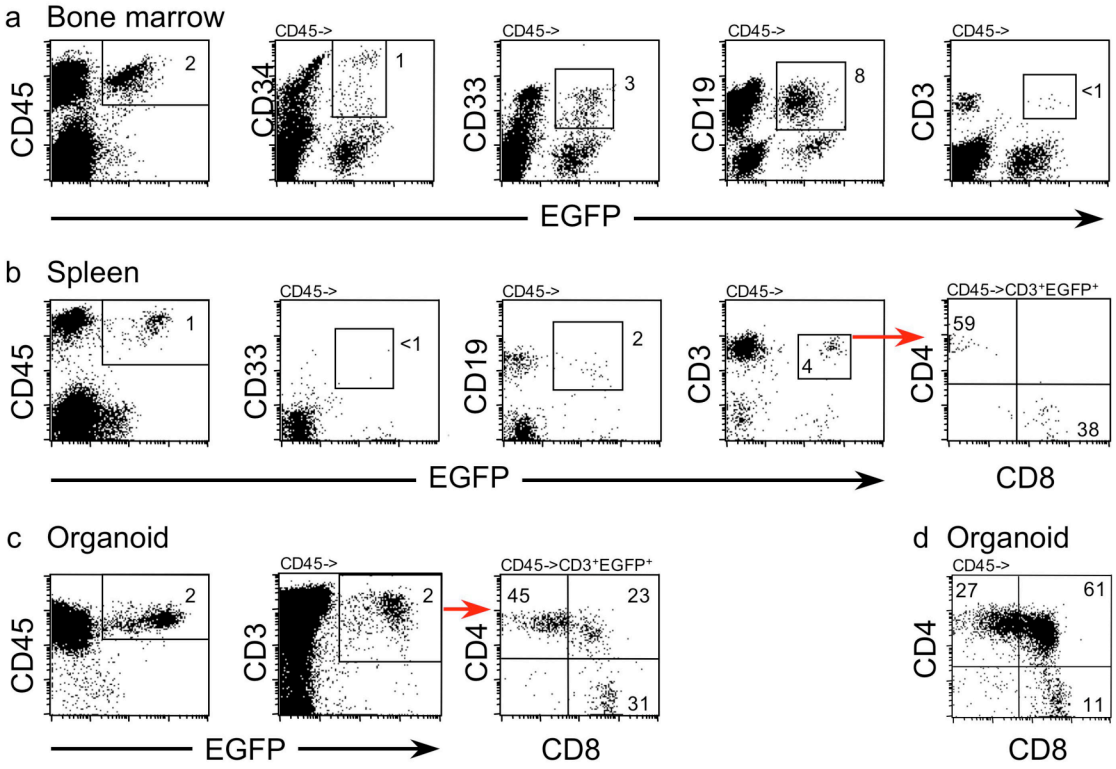


Figure 4.9 Analysis of EGFP expression in different hematopoietic cell subsets after transplantation of BLT mice with CD34$^+$ cells transduced with a lentivirus-based vector.

NOD/SCID mice implanted with human fetal thymic and liver tissue were preconditioned (325 cGys) and transplanted with autologous FL CD34$^+$ cells transduced with a Rtat$^-$pEGFP as described in Figure 4.8. Human reconstituted mice were harvested 12 weeks post transplant for BM, spleen, and thymic organoid.. Analysis shows EGFP expression in mononuclear lineages in the bone marrow (a), spleen (b) and thymic organoid (c). T cells expressing EGFP were found in all hematopoioetic tissues examined demonstrating stem cell engraftment and seeding of the thymic organoid in generating peripheral T cell homeostasis. (d) Total human thymopoiesis in the thymic organoid.

B.5. Human MHC restricted T cell responses to EBV in BLT mice.

A major advantage of the BLT model is that human T cells develop properly in an autologous human thymic environment (in contrast to a xenogeneic mouse thymus environment in other systems[103,104,108,110,111]). To determine whether human T cells recognize peptide in the context of human MHC and generate specific T cell responses, we used a clinically relevant approach, namely the development of a T cell response to Epstein-Barr virus (EBV). In parallel to the implantation of the fetal liver/thymic tissue and the isolation of autologous $CD34^+$ cells, we infected fetal liver B cells from the MNCs to establish autologous lymphoblastoid cell lines (LCLs) to serve as antigen presenting cells. Then 14 weeks after $CD34^+$ cell transplant, we infected the same set of BLT mice with EBV. Five weeks later infected animals were bled and peripheral blood T cells analyzed for their naïve versus memory phenotype. As shown in figure 4.10, there was a dramatic increase in the percentage of $CD45RA^-CD27^+$ memory T cells in infected animals (Fig. 4.10a and Table 4.3). This is consistent with the pattern of expansion of human T cells in peripheral blood during acute EBV infection.[147] I then examined human T cells isolated from different organs to determine whether they would respond to EBV presented by the previously established autologous LCLs in the context of human MHC. ELISPOT analysis for γ-interferon production was used as readout. As shown in Fig. 4.10b and c, isolated human T cells only produced γ-interferon in the presence of LCLs. In addition, γ-interferon production was inhibited when LCLs were pre-treated with anti-human MHC Class I and/or Class II blocking antibodies. These results demonstrate that human T cells developed in BLT mice

can produce a human MHC restricted response and highlight the dynamic interaction between human T and B cells in this model.

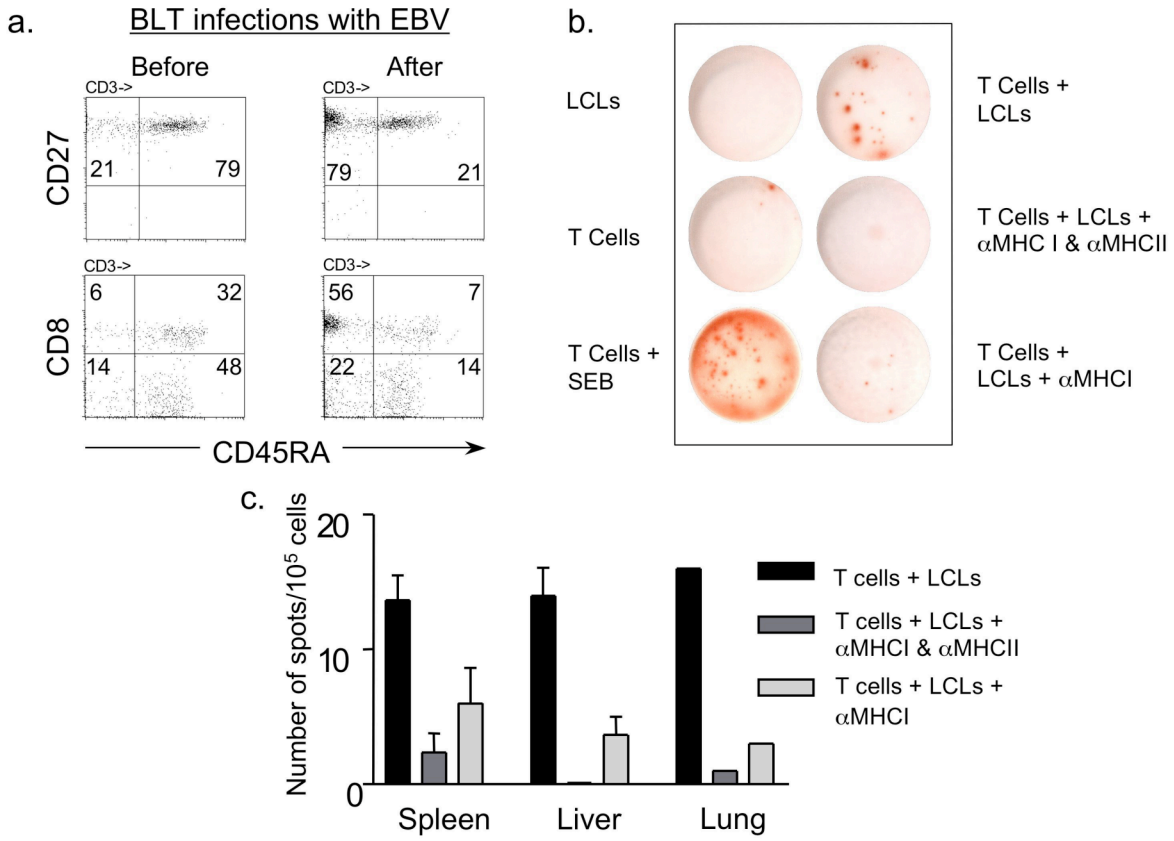


Figure 4.10 Analysis of the human T cell immune response to EBV infection in BLT mice.

BLT mice were infected with EBV (Akata) and five weeks later tissues were harvested for analysis. a) Naïve versus memory phenotypic analysis of peripheral blood human T cells before and 5 weeks post infection. Top panel depicts the inversion of the naïve versus memory phenotype of the $CD3^+$ human T cells and lower panels the inversion of the percentage of naïve to memory effector phenotype of $CD8^+$ T cells after infection. b) ELISPOT analysis for γ-interferon secretion from human T cells isolated 5 weeks post infection stimulated by autologous LCLs. Representative ELISPOT data for human T cells isolated from the liver. c) Analysis of ELISPOT data for spleen (secondary lymphoid tissue), liver (site of immune regulation) and lung (mucosal tissue). Human MHC restriction was determined using inhibitory antibodies specific to human MHCI and MHCII. Error bars represent standard deviations of triplicate determinations.

Table 4.3 Peripheral blood T cell analysis of BLT mice infected with EBV.

BLT Mice Before EBV Infection		BLT Mice After EBV Infection	
Naïve T cells $\%CD3^+CD45RA^+CD27^+$	Memory T cells $\%CD3^+CD45RA^-CD27^+$	Naïve T cells $\%CD3^+CD45RA^+CD27^+$	Memory T cells $\%CD3^+CD45RA^-CD27^+$
79.0	21.0	21.0	79.0
87.0	13.0	64.0	36.0
67.0	32.0	9.0	90.0
80.0	20.0	40.0	50.0
78.3 ± 8.3	21.5 ± 7.9	33.5 ± 24.0*	63.8 ± 25.0**

Average values for each T cell population are indicated with the SD.
Significant changes in T cell phenotypes using a single tailed Student t test are indicated with asterisks.
* p=0.01 for comparison of naïve T cells before and after EBV infection.
**p=0.01 for comparison of memory T cells before and after EBV infection.

C. Discussion

The implantation of fetal thymus/liver/thymus followed by transplantation of MHC matched fetal liver $CD34^+$ cells into NOD/SCID mice resulted in a novel small animal model of human hematopoiesis. We have designated the term BLT mice to distinguish them from previous established models. BLT mice contained both the myeloid and lymphoid (T and B cell) components of the human immune system. In all tissues examined: primary (BM and human thymus), secondary (spleen and lymph nodes) and tertiary (PB, liver, lung and gut) lymphoid tissues reconstituted with all of the major components of the human innate and adaptive immune system. BLT mice contained human myeloid monocytes, macrophages and both $CD11c^+$ and $CD123^+$ DCs and lymphoid $CD19^+$ B and $CD3^+$ T cells. The implanted human thymic tissue maintained a high percentage of CD4CD8 double positive thymocytes which contributed to long term systemic T cell homeostasis as demonstrated by $EGFP^+$ single $CD4^+$ and $CD8^+$ T cells in the periphery. In all tissues examined I was able to detect

human $CD45^+$ cells and $CD3^+$ T cells. This model is unique compared to previously described models since a human thymus develops in the mouse which allows for the BLT model to be utilized to address new relevant scientific questions which were not previously possible.

One observation I made in this model was human NK cells in BLT mice are detected at low levels in various tissues and the percentage of NK cells are not consistent as seen in humans. There does not appear to be an intrinsic defect for human NK cells to develop in immuno-deficient mice, but rather the requirement of human cytokines to induce the NK cell lineage. Kalberer *et al* demonstrated CB $CD34^+$ cell transplanted in NOD/SCID mice with a combination of IL-15 and Flt-3 ligand administration was sufficient for mature and functional NK cells to develop.[91] This cytokine requirement may help explain why we see sporadic levels of human NK cells in BLT mice, since the humanized mice may not produce the levels of the needed cytokine(s) to induce and maintain NK cells. The NK cells observed contained a mature $CD3^-CD56^+CD16^+$ phenotype population but have not been evaluated their functional potential in our system. We will address this question since NK cells play a vital role in innate immunity, tumor surveillance and BM transplantation. Specifically, we do not understand the role of NK cells in regulating the homeostasis of the developing human immune system. Almost all of the knowledge we have about human NK cells has been elucidated *in vitro* or from the mouse hematopoietic system. However, mouse and man are still very different in that there is no murine homologue of CD56 and there are also differences in inhibitory and activating receptors. [148,149] The BLT mouse will allow us to investigate human NK cell ontogeny and function *in vivo*.

Like SCID mice, NOD/SCID mice secondary lymphoid tissues are very under developed due to the lack of lymphoid cells to fill the niche and produce the necessary lymphotoxins to generate organ architecture development.[150-152] Similarly, NOD/SCID mice transplanted with human $CD34^+$ cells only have small lymphoid tissues and poorly developed follicular structure organization (data not shown) due to the lack of human T cells. In BLT mice, although the spleen and lymph node organs are not completely normal for lymphoid architecture , distinguishable human $CD45^+$ cells are present and secondary lymphoid structures develop. In the secondary lymphoid tissues (spleen and lymph node) CXCL13 and CCL21 expression was detected by immunohistochemistry allowing for the recruitment of human B and T cells. BLT mice did develop rudimentary follicular structures in the spleens of the mice comparable to human spleen control samples. However, the lymph nodes appeared to still have diffuse organization of the T and B cells. When BLT mice were administered SRBC (immunogen known to induce follicular formation), germinal center-like structures were more definitive suggesting that the human cells are interacting with each other. These results suggest APCs took up the antigen at the site of inoculation and migrated to the secondary lymphoid tissues to induce germinal center formation allowing for presentation of the antigen to the T cells. These results are powerful in that not only are the appropriate human cells present in the mouse tissues, but the human immune cells generate the appropriate lymphoid organization for immune cell interactions.

Previously, our laboratory demonstrated that NOD/SCID mice transplanted with $CD34^+$ cells only and infected with EBV developed tumors in the spleen. [115] In this model, since no T cells are present, the transformed B cells are not controled and tumors develop.

The tumors were of human origin and expressed EBNA1, LMP1 and LMP2a EBV mRNAs which is consistent to type II latency. This mouse model of EBV infection is representative of a human subject that is immune suppressed due to AIDS or undergoing a bone marrow or solid organ transplant. Therefore I took advantage of the fact that transplanted $CD34^+$ cell mice were able to be infected and had similar pathology to humans to determine whether BLT mice were able to mount human specific T cell immune responses to EBV. BLT mice infected with EBV did not develop spleen tumors and by 5 weeks the human T cells had a decrease in the percentage of $CD45RA^+CD27^+CD8^+$ naïve T cells and an increase in the memory $CD45RA^-CD27^+CD8^+$ population. This result is consistent with acute EBV infection in humans where an expansion of greater than 10-fold of memory $CD8^+$ T cells was observed with a substantial portion being EBV-peptide specific.[147] We further demonstrated by isolating *in vivo* developed T cells from BLT mice infected with EBV, were able to mount human specific MHC restricted T cell responses. *In vitro* generated EBV transformed autologous human B cell LCLs were used to challenge BLT $CD3^+$ T cells in an ELISPOT assay for interferon-γ production for EBV antigen specific T cells. As predicted, BLT mice mounted a specific adaptive T cell response to EBV antigens as demonstrated by the production of γ-interferon. In addition, APCs blocked with anti MHC I and/or MHC II were unable to stimulate the human T cells. These results demonstrate the functionality of the human T cells in the model. However, only a minor fraction of the total T cells produced γ-interferon in the ELISPOT assay compared to the ($CD45RA^-CD27^+$) effector phenotype observed *in vivo* during the acute response to EBV. This contradictory result may be due to latent viral peptides are preferentially presented by the established LCLs, were as *in vivo* we

observed the acute infection in which many viral peptides (lytic and latent) are presented generating a more dynamic T cell response. Therefore, the BLT mouse will allow for more detailed and in-depth experiments than are possible in human subjects.

CHAPTER V

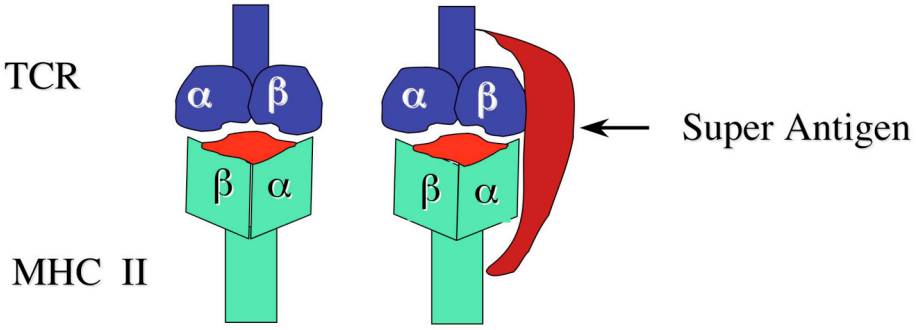
BLT MICE MOUNT A POTENT AND SPECIFIC *IN VIVO* IMMUNE RESPONSE TO TOXIC SHOCK SYNDROME TOXIN-1

A. Introduction

Superantigens (SAg) have been identified as causative agents of food poisoning, scalded skin disease, Kawasaki Syndrome and Toxic Shock Syndrome (TSS).[153-156] SAg have also been suggested to be inducers of several autoimmune diseases by non-clonotypic expansion of T cells bearing specific Vβ T Cell Receptors (TCR).[157] SAg are non-conventional agents that rather than being processed and presented, cross-link MHC class II molecules on Antigen Presenting Cells (APCs) to the Vβ TCR region on T cells (Figure 5.1).[155,158-160] As a result, a potent immune response is generated in which a high proportion of T cells (5-30%) are activated and a massive induction of pro-inflammatory cytokines are secreted.[161,162] Toxic shock has been associated with these cytokines (IL-1, IL-2, IL-6, INFγ and TNFα) to induce pathological effects resulting in fever, shock and death.[162]

The staphylococcal SAg toxic shock syndrome toxin-1 (TSST-1) has been identified as the primary initiator of toxic shock.[163] TSST-1 stimulates T cells expressing Vβ15 in the mouse or Vβ2 in humans. Mouse models of TSS have been extensively used to determine pyrogenic and toxic activity, however mice do not develop a disease resembling TSS even after high doses of SAgs.[164] Typically, mice are sensitized with agents such as hepatotoxin D-galactosamine to induce SAg lethal activity resulting in fulminant liver failure, which has not been reported in humans. The rabbit model has been deemed a more appropriate model since a disease similar to TSS as described in humans develops. However, rabbits are also

resistant to SAgs intravenenously and require continuous infusion of SAgs at low doses to get an effect. Therefore more relevant models of human TSS are needed to directly correlate how SAgs cause disease in humans and to be able to develop preventative measures such as vaccines and conventional therapeutic treatments for TSS which may also help prevent autoimmunity.



Antigen	Vβ affected	Accessory Cells
SEA	1,3,10,11,17	yes
SEB	3,7,8,17	yes
TSST-1	2	yes

Adapted from Janeway (Immunobiology 4th ed.) 1999

Figure 5.1 Schematic of superantigen binding to T cell receptor and MHC molecule.
Superantigens are non-conventional agents that rather than being processed and presented, cross-link MHC class II molecules on Antigen Presenting Cells to the Vβ TCR region on T cells. As depicted on the left shows convential antigen presentation where the peptide is presented in the groove of the MHC molecule of an APC to the T cell receptor. On the right is how a super cross-links the MHC II molecules of an APC to the TCR. Most superantigens require an accessory APC to activate T cells.

The purpose of animal models of human hematopoiesis is to eventually develop a fully functional immune system in which to explore fundamental questions of basic immunology, to culture/study human specific pathogens and to evaluate possible treatments/cures and preventative measures of disease such as vaccine development. Over the past decade several immune compromised mouse strains have been developed to allow for bone marrow transplantation with human hematopoietic stem cells ($CD34^+$ cells) in which both the myeloid and lymphoid systems develop. The majority of the mouse strains developed allow for human engraftment generating reconstitution of myeloid and B cells and under specific conditions the possibility for low level T cell development or T cell expansion.[65,80,92,93,99,134,165,166] More recently, several groups have developed mouse strains that allow for T cell development within the mouse thymus generating human T cells with low levels of systemic T cell reconstitution.[108,110,111] Although each system has their own caveats, these models have allowed for the development of the key components of the human immune system and have been demonstrated to generate adaptive immune responses to Tetanus Toxoid and EBV.[108] However, these systems have been focused on the development of the model and not the true utility for which they were originally designed: to address biologically relevant questions.

I developed and have described a novel model of the human immune system in which all the major components of the innate and adaptive immune system are present. Specifically, human T cells develop within a human thymic environment. We have designated this model as the BLT mouse. The present work was undertaken to better understand the *in vivo* human immune response to TSST-1. In these experiments I used the

BLT mouse model of the human immune system to evaluate the T cell immune response to TSST-1 in generating toxic shock. TSST-1 is a model antigen to use to evaluate the BLT T cell response due to the extensive documentation of the known human pathogenic T cell responses in humans. Specifically, TSST-1 will allow for direct *in vivo* comparison of the human T cell immune response in both BLT mice and humans. If the human T cell immune response in the mouse recapitulates that is seen in humans, we can then utilize this model to study other immune cells in their participation in eliciting human disease. I hypothesized that the inflammatory T cell response in BLT mice would closely mimic the T cell response observed in humans where $V\beta2^+$ T cells expand and an increase of human inflammatory cytokines are released in the plasma. Since BLT mice have most of the components of the human immune system I wanted to address a new relevant question in regard to TSS that has been difficult to impossible to address in humans. DCs are the most potent APC to naive T cells encountering antigen and are an important component in activating T cells. DCs may play a significant role in inducing TSS. At present, there is no published *in vivo* human DC information for their role in inducing TSS pathogenesis. Langenkamp *et al.* demonstrated *in vitro* that TSST-1 pulsed DC prime high avidity $V\beta2^+$ and low avidity $V\beta2^-$ T cells.[167] Since any cell expressing class II MHC has the ability to cross-link with the T cell receptor with TSST-1, I hypothesized that both human $CD123^+$ and $CD11c^+$ DCs would be activated and mature if they participated in inducing TSS. We therefore extended our studies to investigate whether human DCs participate in TSS by evaluating the activation (CD40, CD80, and CD86) and maturation (CD83) state of both $CD11^+$ and $CD123^+$ DCs. The BLT mouse model of the human immune system provides a unique and remarkable tool in which I can

evaluate, *in vivo,* human DCs in their implications in inducing human toxic shock syndrome with TSST-1.

B. Results

B.1. Vβ TCR diversity in BLT mice

Bacterial superantigens are known to stimulate T cells bearing specific TCR β chain variable region gene products. T cells are generated within the thymus through positive and negative selection and successful mature T cells exit to the periphery. The diversity of the T cell V beta repertoire in the thymus is generated through VDJ T cell rearrangement and at the genetic level of the individual where MHC restriction is unique to that individual. A diverse V beta repertoire is maintained in the periphery depending on the antigens encountered and the immune response elicited. I therefore initially determined whether BLT mice expressed a diverse TCR repertoire of Vβ recombination recognized by SAgs. The peripheral blood, as well as, primary and secondary lymphoid tissues of BLT mice were analyzed for seven V beta families including Vβ2 which is specifically activated by TSST-1. As shown in figure 5.2, all V beta families examined were present in the thymic organoid, spleen and peripheral blood of BLT mice. The T cell repertoire in BLT mice resulted in diversity without clonal expansion of a single Vβ family dominating.

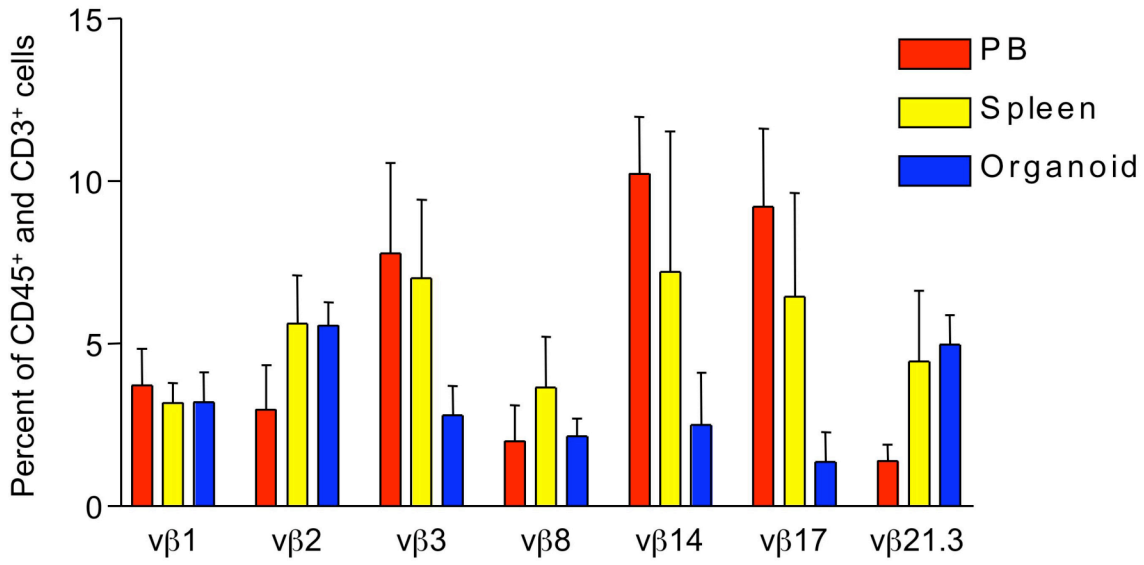


Figure 5.2 Analysis of the Vβ TCR repertoire in BLT mice.

BLT mice were analyzed for human $CD45^+CD3^+$ cells for the generation of a diverse Vβ TCR repertoire. BLT mice were examined for T cells expressing Vβ TCRs in the thymic organoid (*n*=3), spleen (*n*=4) and peripheral blood (*n*=3) tissues. BLT mice express a diverse T cell repertoire with no expansion of a single dominant T cell Vβ receptor family.

B.2. TSST-1 induced specific down regulation of Vβ2 TCR expression and expansion of $V\beta2^+$ T cells

Following identification of $V\beta2^+$ T cells, BLT mice were pre-bled for plasma to look at human cytokines and the relative percentage of peripheral blood human $V\beta2^+$ T cells. BLT mice were divided into experimental cohorts and administered a one-time dose (6 µg) of TSST-1 i.p. and monitored for lethal toxic shock. The mice exhibited signs of distress within 18 hours. The mice were lethargic, had ruffled fur and some mice were noted to have loose stool. At 1 hour, 18 hours, 3 days, and 5 days mice were bled and/or sacrificed for peripheral blood, bone marrow, and spleen T cell phenotyping and cytokine analysis. As

demonstrated in figure 5.3 there was almost complete down regulation of peripheral blood $V\beta2^+$ TCR, gating on $CD3^+$ T cells, by 18 hours. These results are consistent with *in vitro* experiments showing that human peripheral blood mononuclear cells cultured continuously with TSST-1 had remarkably lower $V\beta2^+$ TCR surface expression.[168,169] BLT mouse peripheral blood T cells analyzed at 3 days and 5 days showed an increased percentage of $V\beta2^+$ T cells suggesting a massive expansion of this T cell subset. In fact, 5 days post TSST-1 administration up to 36% of the human $CD3^+$ T cells in the peripheral blood of BLT mice were $V\beta2^+$ (Fig 5.3 and 5.4) These results are similar with patients exhibiting TSS.[161] As an internal control, the level of $CD3^+$ T cells expressing the human Vβ17 TCR (which is not stimulated by TSST-1 but the SAg SEB) in the same mice was also monitored and, as expected, the percentage of human $V\beta17^+$ cells of these mice was unaltered in response to TSST-1 (Fig 5.3 and 5.4). Furthermore, a systemic increase in the percentage of $V\beta2^+$ T cells was observed in the bone marrow and spleen of all BLT mice inoculated with TSST-1, but not in BLT mice that received mock saline injections (Fig 5.4 and data not shown). The *in vivo* specificity of the response to TSST-1 was highlighted in each of these tissues by the fact that the overall percentage of human $V\beta2^+$ T cells expanded while the percentage of $V\beta17^+$ T cells remained relatively constant for five days post TSST-1 administration.

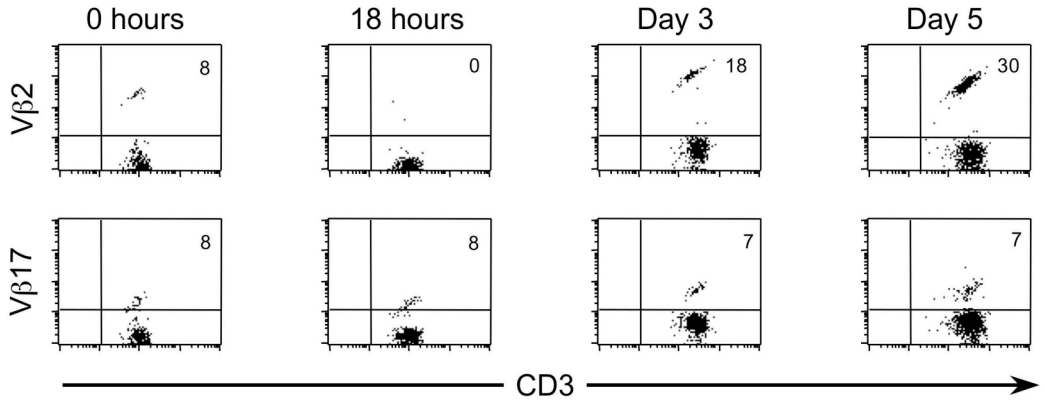


Figure 5.3 Peripheral blood Vβ2 T cell response to TSST-1.

BLT mice were inoculated i.p. with 6 µg of TSST-1 per mouse. Mice were monitored and bled at 18 hours, 3 days, and 5 days and analyzed for Vβ2 T cell expansion. As an internal control, Vβ17 T cells were monitored. At 18 hours there was surface down regulation of the Vβ2 TCR and by day 5 the $V\beta2^+$ T cells increased up to 30% of the $CD3^+$ T cell population. The $V\beta17^+$ T cell population was not affected by TSST-1.

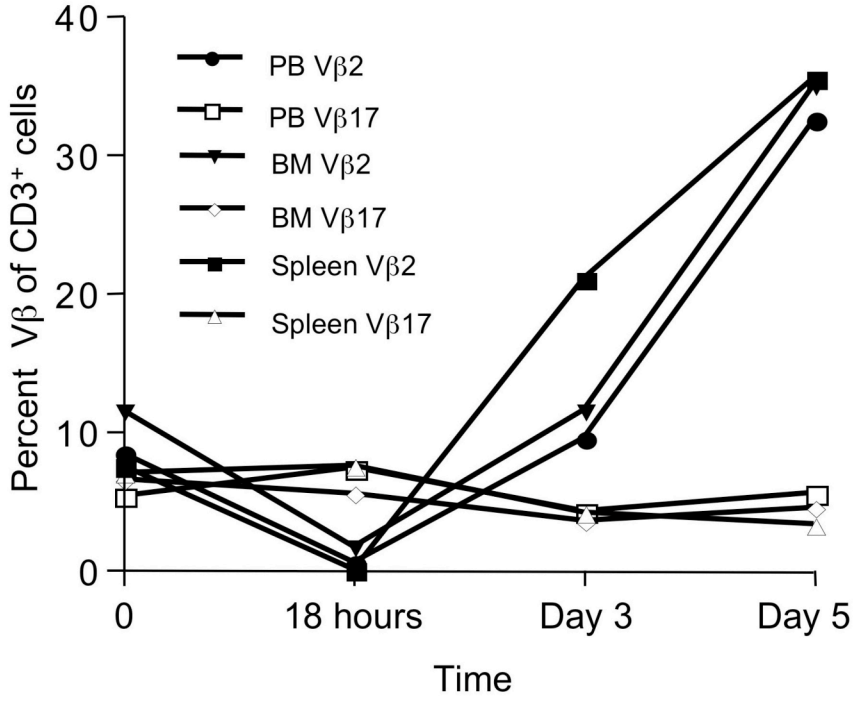


Figure 5.4 Systemic affect of TSST-1 on the Vβ2$^+$ T cells.

Human reconstituted BLT mice were administered 6 μg of TSST-1 i.p., and the PB, BM and spleen were analyzed for the percentage of Vβ2 T cells at 0 hr, 18 hr, 3 days and 5 days. As an internal control Vβ17$^+$ T cells were analyzed to determine the specificity of TSST-1 to activate Vβ2$^+$ T cells.

B.3. TSST-1 elicits a massive human cytokine response.

A massive cytokine production is a key factor in the pathogenesis of toxic shock syndrome in response to TSST-1.[162] Therefore we measured the plasma levels of human cytokines in control and TSST-1 treated BLT mice. One hour after administration of TSST-1 there was a significant increase in the systemic levels of human INF-γ, IL-10, IL-2, IL-6 and TNFα (Fig. 5.5). By 18 hours post TSST-1 injection, there was an even more dramatic increase in the levels of INF-γ, IL-10, IL-2 and IL-6, with more modest but statistically significant increases in the levels of IL-5 and IL-8 (Fig. 5.5). Therefore the cytokine profile in response to TSST-1 in BLT mice resembles that seen in humans, suggesting that human T cells within BLT mice are capable of exerting effector functions following TCR stimulation.

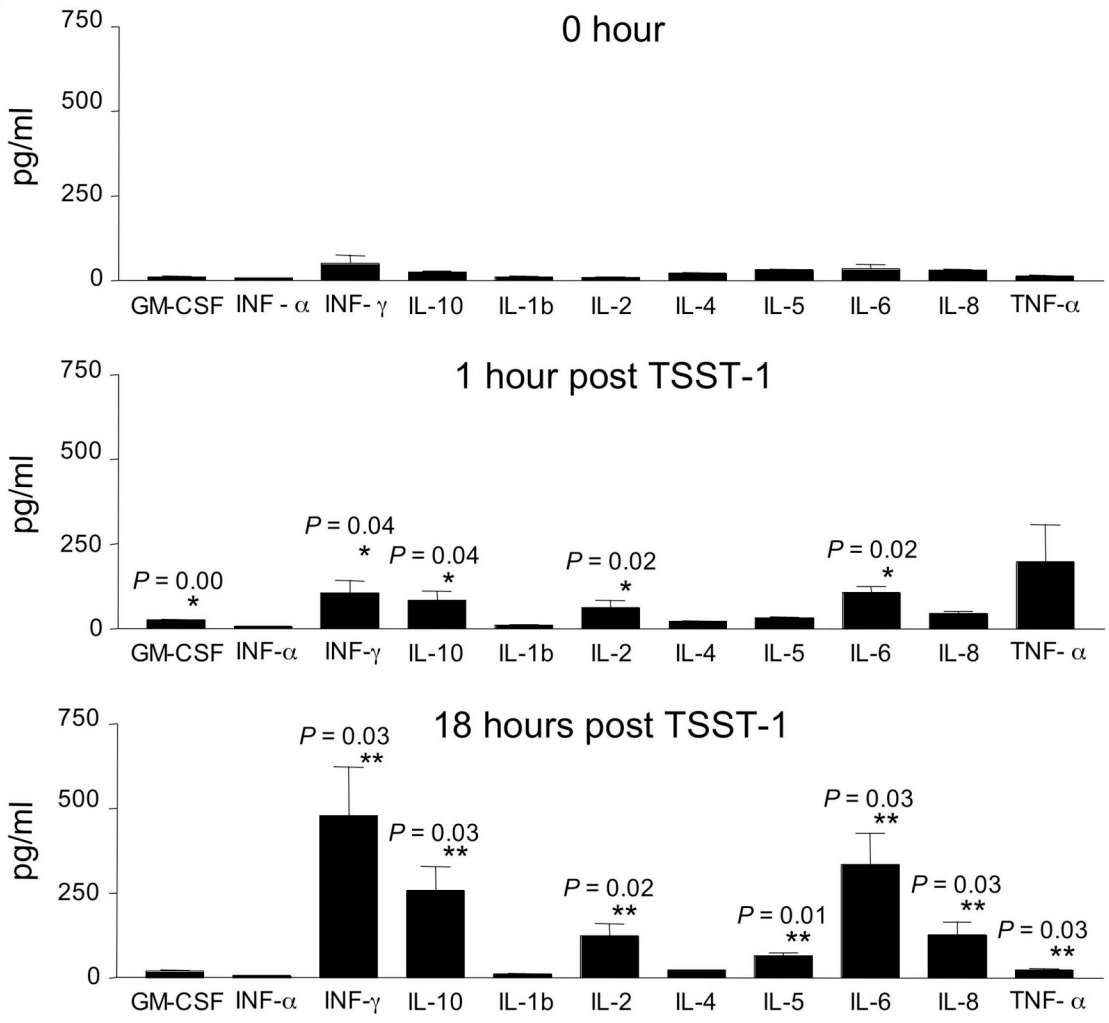


Figure 5.5 TSST-1 induces the systemic release of human cytokines in BLT mice.

Plasma from BLT mice (*n*=9) before and after TSST-1 administration was analyzed for the presence of human cytokines at 0 (pre-injection), 1 and 18 hours post injection. Results shown are averages (± SEM). Statistically significant differences between the cytokine levels at 0 and 1 hour are indicated by *. Statistically significant differences between the levels at 0 and 18 hours are indicated by **. (Unpaired, two-tailed Student's t test)

B.4. Bone marrow DC response to TSST-1

TSST-1 activation of Vβ2 TCR^+ cells is mediated by antigen presenting cells. While abundant information regarding the effect of TSST-1 on $V\beta2^+$ human T cells exists, there is little information regarding its effect on antigen presenting cells, specifically human DC. To determine the extent and the kinetics of possible phenotypic changes to human DC in response to systemic administration of TSST-1 *in vivo*, we evaluated changes in the expression pattern of human DC activation and maturation antigens. Prior to TSST-1 administration, $CD123^+$ and $CD11c^+$ DCs in the bone marrow had an immature resting phenotype as determined by either low levels or the absence of human CD40, CD80, CD86 and CD83 surface expression. Eighteen hours post TSST-1 injection there was no significant change in the activation phenotype of human $CD123^+$ or $CD11c^+$ DC in the bone marrow.

B.5. *In vivo* activation of human spleen DC in response to TSST-1

Similarly to the BM, $CD123^+$ and $CD11c^+$ DC in the spleen of control animals had an immature resting phenotype (Fig. 5.6). However in contrast to the BM, a dramatic upregulation of CD40, CD80, CD86 and CD83 was observed in the $CD11c^+$ DC present in the spleen of BLT mice treated with TSST-1 (Fig. 5.6 top). The cell surface levels of all these markers returned to basal levels by day 3 post injection and remained at their basal levels for the duration of the experiment (five days; data not shown). To confirm that these effects were indeed due to the specific interaction between T cells and dendritic cells and not due to contamination with other stimuli such as lipopolysaccharide, we also administered TSST-1 to a set of transplanted NOD/SCID mice that we have previously shown to have a

full complement of DC but that were devoid of human T cells because they were not implanted with human fetal thymic and liver tissue prior to transplant. There was no up regulation of DC activation and/or maturation markers in the mice devoid of T cells that received TSST-1 injections (Fig. 5.6 bottom). These results demonstrate that in BLT mice, human $CD11c^{+}$ DC respond to TSST-1 by up-regulating activation and maturation markers in spleen but not bone marrow.

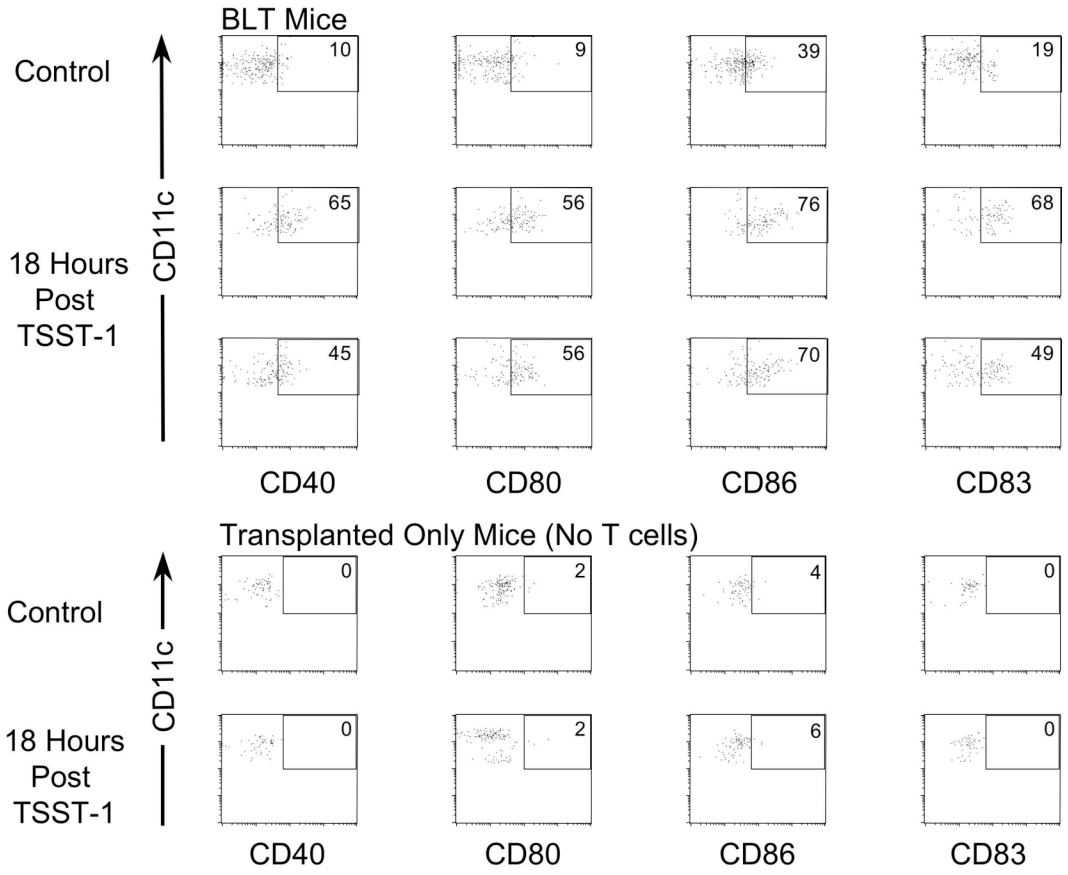


Figure 5.6 Spleen CD11c$^+$ DC are activated and mature in response to TSST-1.

BLT mice were inoculated with 6 μg of TSST-1 i.p. Mice were monitored and sacrificed at 18 hours, 3 days, and 5 days. BLT mice had an increased percentage of human spleen DCs that expressed activation (CD40, CD80 and CD86) and maturation (CD83) markers at 18 hours. Likewise DCs in NOD/SCID mice transplanted with CD34 cells only had no change in phenotypic markers for activation or maturation. Specific stains: [(Lin$^-$- FITC, CD123-PE, HLA-DR-PerCP, Activation marker-APC), (Lin$^-$- FITC, CD11c-PE, HLA-DR-PerCP, Activation marker-APC)].

C. Discussion.

Superantigens such as TSST-1 have been shown to be the causative agents of a variety of human diseases. TSST-1 triggers an excessive cellular immune response that can lead to systemic release of cytokines, the expansion of $V\beta2^+$ T cells and lethal toxic shock. In addition, it has been suggested that bacterial and viral superantigens in general may play a major role in generating a break in immune tolerance and increased epitope spreading in autoimmune diseases. A powerful aspect of the BLT model is demonstrated in figure 5.6 in which the developed human immune system can be dissected to determine not only the role of human T cells but also DCs during an immune response. Our system allows for genetically identical mice to be generated with a complete human immune system or, in non-implanted mice, an immune system devoid of human T cells. This attribute facilitates studies of how human T cells interact with other immune cells, specifically DCs, and how these interactions might result in preventing or inducing disease *in vivo*. Analysis of the effect of administration of TSST-1 to BLT mice demonstrated the systemic and highly specific expansion of human TCR $V\beta2^+$ T cells, a dramatic release of human cytokines into the circulation and the highly specific upregulation of activation and maturation markers on the cell surface of $CD11c^+$ DC in the spleen. Two important aspects of the *in vivo* response to TSST-1 are worth emphasizing. First, no phenotypic changes were noted in $CD123^+$ DC. Second, administration of TSST-1 to animals devoid of human T cells (i.e. reconstituted with human $CD34^+$ cells but not implanted with human thy/liv tissues) did not result in the production of detectable levels of human cytokines or any changes in the phenotype of

human DC (Fig. 5.6 and data not shown). Therefore by using this novel system, we have uncovered a new and unexpected affect of TSST-1 specifically on human $CD11c^+$ DC *in vivo*. Thus, this new human/mouse xenograft model of human hematopoiesis will be useful in dissecting important aspects of human immunology in an *in vivo* setting. These results demonstrate the potential of this system to investigate the cellular interactions of the human innate immune response to superantigens and to evaluate novel therapeutic modalities aimed at preventing the devastating effects of toxic shock syndrome and other superantigens in general.

CHAPTER VI

DISCUSSION

Hematological abnormalities that result in immunodeficiency are generally associated with a multitude of complications and relatively poor prognosis. For example, DiGeorge syndrome is a rare congenital disorder in which the thymus fails to develop. [141] Thus, DiGeorge patients develop all aspects of a functional immune system except they are T-cell deficient, making them susceptible to numerous opportunistic infections. Transplantation of allogeneic thymic tissue into patients with DiGeorge syndrome has been shown to restore T cell production and restore significant immune function.[141] This advance was made possible by knowledge derived from *in vivo* experimentation in mice without other available systems that could serve to bridge the transition between experimentation in mice and the clinical implementation of this knowledge to humans.[142] Although representative experimental models of human diseases have been developed in various mouse strains (knock-outs, knock-ins, transgenics and various other approaches) these mouse systems do not always recapitulate the human aspect since mice and men differ in biological development and cellular function. During the past two decades, the development and implementation of experimental models to study human hematopoietic and immune system dysfunction has been greatly facilitated by the availability of several immune deficient mouse strains capable of accepting human grafts.[65,103,104,108,110,111] Whereas none of these models fully recapitulates all aspects of human hematopoeisis, individually they all have served in the study of a variety of aspects regarding hematopoietic stem cell function as well as immune function.

Transplantation of SCID or NOD/SCID mice with human hematopoietic stem cells recapitulates several important aspects of DiGeorge and Nezelof's syndromes, namely thymic atrophy and the development of multi-lineage human hematopoeisis in the absence of T cells.[141,170] The molecular basis for the T cell lineage restriction in these mice is currently unknown. Therefore, I evaluated the effect of autologous bone marrow transplantation in the development of human hematopoietic homeostasis in mice previously implanted with human fetal thymus and fetal liver (i.e. SCID-hu mice). In SCID-hu thy/liv mice there is long-term sustained thymopoiesis in the graft without systemic peripheral reconstitution preventing a wider utilization of this otherwise very useful model.[81,118,145] This is in contrast to SCID and NOD/SCID mice that are transplanted with human hematopoietic stem cells. In these mice there is systemic reconstitution with hematopoietic cells but T cells are not generated.[65,115] Transplantation of autologous human $CD34^+$ cells into previously implanted mice resulted in systemic repopulation with multilineage human hematopoietic cells including T cells.

These results might seem to contradict previous reports by other investigators to reconstitute SCID-hu mice with human hematopoietic progenitor cells that observed no or only transient levels of human cells in the periphery and spleen.[14,65] However I attribute their results to the use of SCID mice that are a great choice for implantation but that are suboptimal for transplantation because of their low levels of reconstitution after transplantation with human hematopoietic progenitor cells.[65,145] In contrast, NOD/SCID mice have been shown to produce high levels of engraftment with human hematopoietic cells after transplantation. These higher levels of engraftment are likely needed in order to obtain reasonable levels of sustained hematopoiesis and higher overall levels of multilineage

reconstitution. These higher levels of reconstitution will allow for continued seeding of the thymus with progenitor T cells and allow for peripheral APCs to provide survival signals to the T cells.

Phenotypic analysis of human cells in the peripheral blood of BLT mice matched that of cells from a normal human control. In peripheral blood, spleen and bone marrow of BLT mice we were able to identify human single positive T cells, B cells, monocytes, macrophages and both $CD123^+$ and $CD11c^+$ dendritic cells indicating multi-lineage hematopoietic reconstitution in the different primary and secondary lymphoid organs. In the implanted human thymic organoid we were able to identify double negative, double positive and single positive human thymocytes. In contrast to primary and secondary lymphoid organs and other non-lymphoid organs like the gut, liver, lung, and extensive analysis of mouse thymic tissue of fully reconstituted BLT mice did not show repopulation with human hematopoietic cells (Figures 4.2-4.5 and data not shown). This indicates a failure of the human cells to migrate and repopulate the mouse thymus in NOD/SCID mice. Using human $CD34^+$ cells marked with a lentivirus-based vector expressing EGFP we were able to demonstrate the contribution of transplanted human $CD34^+$ cells to human T cell homeostasis in BLT mice. Interestingly, whereas a significant number of single positive cells expressed EGFP only about a third of the double positive thymocytes expressed EGFP. At this point it is unclear if this is due to the specific promoter used for EGFP expression, *in vivo* silencing of the transgene or alternatively if the contribution to thymopoeisis by the transduced $CD34^+$ cells is transient. I believe the low level seeding of the thymic organoid graft is due to low levels of human bone marrow engraftment of marked $CD34^+$ cells. In this experiment, we

transduced FL $CD34^+$ cells with the lentivirus vector $Rtat^-EGFP$ and transplanted NOD/SCID thy/liv mice with 2 x 10^5 $CD34^+$ cells rather than the optimal dose of $2.5x10^6$ $CD34^+$ cells. This change in the experimental protocol was assessed and made due to the limited quantity of vector for the experiment and the necessary controls to evaluate the data. Even though I did not attempt to quantitate the absolute number of bone marrow derived human hematopoietic T cell progenitors seeding the human thymic organoid, I was able to demonstrate multi-lineage reconstitution with human $EGFP^+$ cells, including T cells, in the organs examined.

Recently, other groups have reported multi-lineage human reconstitution including T cells in newly developed strains of immunodeficient mice transplanted as neonates with human $CD34^+$ cells from different sources[107,108,110,111]. The requirements for T cell development in these different strains vary but, for the most part, injection of neonatal mice seems to result in production of T cells whereas transplantation of adult mice of the same strains results in low to undetectable levels of human T cells that in one instance could be increased by repeated administration of a human IL-7 analog[110]. Interestingly, injection of human hematopoietic $CD34^+$ cells into 1 day old NOD/SCID mice did not result in the production of human T cells. Rather, it resulted in reconstitution of the myeloid compartment and in high levels of human B cells (data not shown). These observations highlight the fact that strain differences dictate not only the overall levels of reconstitution with human cells but in addition, they also dictate the human lineages that can be supported by the microenvironment in each mouse strain. Furthermore, the fact that T cells only arise

in neonatal mice indicates the need for a developing system conducive to expansion of the T cell compartment.

The single common denominator in all these recently described systems is the fact that the selection of the human T cell repertoire is believed to take place in the context of the mouse MHC in the mouse thymus[103,104,108,110,111]. Briefly, progenitor T cells develop into mature naïve T cells through positive and negative selection within the mouse thymus. The diversity of the T cell V beta repertoire is generated through VDJ T cell rearrangement and at the genetic level of the individual where MHC restriction is unique to that individual. In these mouse models, positive selection presumably occurs when thymocyte TCRs interact with mouse MHC molecules loaded with self-peptide on murine thymic epithelial cells. In contrast, in BLT mice, thymocytes undergo positive and negative selection in the human thymic organoid in the context of autologus MHC restriction and not in the mouse thymus. In fact, no human T cells were detected in the mouse thymic tissue (very involuted) of any of the fully reconstituted BLT mice evaluated. These results strongly suggest the preferential trafficking of human hematopoietic cells into the human thymic tissue.

Since human T cells developed in the implanted human thymic tissue, we determined whether human MHC restricted T cell responses could occur in BLT mice. By infecting BLT mice with EBV, we were able to demonstrate that specific MHC class I and II restricted human T cell immune responses were mounted (Fig. 4.10). Analysis of secondary lymphoid tissue (spleen), sites of immune regulation (liver) and mucosal sites (lung) all showed significant T cell responses. Even though we can not rule out some level of selection of the developing human thymocytes on murine MHC, the fact that human T cells isolated from

EBV infected mice showed human MHC restriction demonstrates that human T cells in BLT mice are selected in the context of the human MHC.

This aspect of human MHC restriction may be important in vaccine development. In developing vaccines, specific dominant immunogenic epitopes of the pathogen will need to be identified and tested over a diversity of MHC individuals. For a vaccine to be effective it will need to generate a strong TH2 response for antibody to recognize and neutralize the antigen and/or a TH1 response in which cytotoxic T cells recognize and eliminate the infecting antigen. The BLT mouse model allows for identical MHC mice to be generated and also for diversity of MHC individual donors in which we can evaluate novel vaccines and conventional therapeutic approaches to fight human diseases.

Effective vaccines will generate both T and/or B cell memory. In addition, these memory cells of the immune system must be able to recognize and elicit a recall response to clear the antigen (pathogen) for protective immunity. Although human T cell development and adaptive immune functions in immune deficient mice have been demonstrated, we will need to demonstrate true immune memory in which a recall response to antigen takes place in the *in vivo* environment upon secondary challenge. Such as a demonstration will strengthen these models as a tool for the evaluating novel vaccines.

I selected to use TSST-1 to evaluate a human disease state in BLT mice. Toxic shock syndrome has been reported since 1927 and was first described by Todd *et al*. in 1978 as a non-invasive systemic disease associated with *Staphylococcus aureus*.[163] Not until the early 1980's when young women using high-absorbancy tampons developed Toxic Shock Syndrome did superantigens gain attention. In 1981 it was determined that a secreted protein

from *Staphylococcus aureus* was the causative agent of toxic shock syndrome hence the name TSST-1.[7] Superantigens have been associated with several human pathologies and have been implicated as a potential causative agents in inducing auto-immunity.[154,155,157,158,164,171] I demonstrated that BLT mice can be induced to generate a very similar TSST-1 pathological state as described in human[163] where a massive release of inflammatory cytokines are released with a specific expansion of the $V\beta2^+$ T cells. I expanded the scope of this study to address the *in vivo* responsiveness of human DCs as potential activators of the T cells. I determined that i.p. administration of TSST-1 generates a systemic and specific immunological reaction in which $CD11^+$ DCs demonstrate an activated state and mature. I speculated that these DCs and T cell interactions participate in the inflammatory immune response seen during toxic shock syndrome. Since DCs are potent activators of T cells, a new approach in treating TSS and other superantigen induced diseases may be to target the DC cell's ability to stimulate the T cells.

The use of animal models of human hematopoiesis has led to understanding human hematopoietic stem cells, immunology, bone marrow transplantation and viral infections and have been instrumental for the development of novel clinical approaches to treat human diseases. The development of the SCID-hu thy/liv mouse model has provided insight into human thymopoiesis and has been implemented as a powerful tool to investigate HIV infection and therapies. In the SCID-hu thy/liv mouse, HIV infects both thymoctes and thymic epithelial cells resulting in thymocyte depletion. The kinetics of thymocyte depletion are HIV isolate specific.[85,118,172,173] Furthermore, it was demonstrated that some HIV isolates have preferential infectivity and depletion of thymocytes[174] which may affect T cell rebound

after anti-viral therapy. This SCID-hu thy/liv model has been used to evaluate a variety of anti-virals such as azidothymidine, dideoxyinosine, nevirapine, protease inhibitors, antisense and other oligonucleotide compounds,[175-180] some of which are being used in the clinic today. The development of the BLT mouse will allow further preclinical evaluations of HIV therapies and to study other hematopoietic disorders.

Several immunological questions still need to be addressed in humanized mice in order to fully utilize their ability as a tool to study the human immune system. A significant question is whether primary (thymus derived) and/or peripheral tolerance is generated allowing for human T cell development and function without a graft versus xenograft disease (GVXD). Regulatory T cells have been demonstrated to play a significant role in the pathogenesis of autoimmune diseases, tumors and organ transplantation.[181-184] $CD4^+CD25^+$FOX3P regulatory T cells (Tregs) develop in the thymus and immigrate to the periphery.[181-183] Tregs are found primarily in the thymus, PB, LNs, spleen and during homestatic conditions and account for 6-10% of the $CD4^+$ T cells in these tissues. [181,185-187] Therefore we will need to evaluate the developmental stages of thymopoiesis and determine if Tregs develop and exit the thymus into the periphery and their ability to traffic to the appropriate tissues. Furthermore we will need to address the issue whether Tregs use conventional chemokine signaling to home to appropriate peripheral organs. In addition, we can begin to investigate whether Tregs are maintained or generated de novo in the peripheral environment and their contribution in controlling GVXD. An interesting question is whether specific Vβ TCR Tregs are generated which could be initially determined by Vβ sprectra-typing. If a specific Vβ TCR subfamily is identified, then the specific TCR and antigen

could further be identified and evaluated in inducing or preventing disease. These are all difficult questions to address but humanized mice will provide better insight in understanding human Tregs in inducing or controlling human GVHD, tumor development, and autoimmune diseases.

A short coming of all the previously described models is the determination and evaluation of human CMPs to develop into mature and functional basophils and eosinophils *in vivo*. Eosinophils have been implicated as a key component in the development of allergies.[188] Likewise, little is known or understood about the function of human basophils in their role in the human immune system, however it is speculated they have a similar function as eosinophils and mast cells in contributing to allergies.[188] Although these cells are not the focus of this study, it will be intriguing whether or not human eosinophils and basophils develop and are functional in mouse xenograft models. Generation of these cells in an experimental humanized mouse model will provide new knowledge about their ontogeny, their role in immune homeostasis and the elucidation of their specific roles in allergy reactions. Humanized mouse models of eosinophil and basophils development will provide a tool to manipulate the human immune system and evaluate novel therapies to prevent or treat allergies.

In addition, other BM derived $CD34^+$ progenitor cells can be evaluated in humanized mice. Endothelial progenitor cells (EPCs) are $CD45^+$ $CD31^+$ $TIE2^+$ and $CD34^+$ and have been demonstrated to play a pivotal role in vascular generation, maintenance and repair.[189-192] BM derived EPCs have also been identified to contribute to vascularizing new tumors.[193] Previously in the SCID-hu thy/liv mouse, both human and mouse $CD34^+$ cells have been

identified in the vascularization of the thymic organoid. Crisa *et al*., demonstrated that CB-derived cells contributed to the generation of new blood vessels at sites of graft implantation and wound healing.[194] Furthermore, it was demonstrated that CB $CD34^+$ cell transplanted NOD/SCID mice contained EPCs which incorporated into new blood vessels at low levels.[195] Additionally, they observed enhanced vasculogenesis after MNC injection which may be attributed to cytokine induced mobilization of human EPCs from the bone marrow. It is remarkable that human EPCs have been identified in vessel formation in these previous mouse models. I would predict the BLT mouse would also have EPC capabilities contributing to vascularization. In which case, we could utilize this humanized mouse model to study blood vessel formation, treatments targeting tumors angiogenesis and developing new therapeutic approaches to induce vascularization for ischemic heart repair.

Currently, Vescell^{TM} Therapy (Thera Vitae, Bangkok, Thailand) is isolating $CD34^+$ cells from the peripheral blood of patients with heart disease (untreatable by conventional technology), expanding the cells *in vitro* and re-infusing these cells into the patient for heart vascular repair. Although their results are encouraging, identifying specific BM derived EPCs and expanding them *in vitro* before infusion may enhance this technology. The utility of humanized mice to study human disease and to develop new therapeutic applications to treat human diseases is in its infancy. Therefore, appropriate humanized mouse models will dictate the future for the development of novel therapies to treat human diseases.

The initial long-term goal of my dissertation project was to develop the T cell component of the human immune system in a small animal model to evaluate possible gene therapeutic vectors in T cells. Specifically, evaluation of gene therapeutic vectors expressing

siRNA or proteins inhibiting HIV infection that could be evaluated in a laboratory setting before being introduced into clinical trials. During the course of the project, I and the laboratory have taken advantage of the available NOD/SCID transplant system and the newly generated BLT mouse to address biologically relevant questions of human specific virus infections (Epstein-barr virus, Dengue virus, Cytomegalovirus, human herpes virus 8 and human immunodeficiency virus), human DC ontology and the human immune response to superantigens such as TSST-1.[89,90,115,132]

Here, I have described a novel model of human hematopoiesis, designated as the BLT mouse, in which we incorporated the best attributes of two well-established systems: the SCID-hu thy/liv mouse and the NOD/SCID-hu bone marrow transplant mouse. BLT mice develop *de novo* T cells within a human thymic environment and generate a systemic human hematopoietic system, which is maintained long term in all tissues examined and that is capable of mounting human MHC restricted T cell immune responses. Immunohistological analysis for human hematopoietic cells organization showed a striking similarity between BLT mice and human tissue controls. These observations demonstrate not only human hematopoietic reconstitution in BLT mice but also niche development by these cells in primary and secondary lymphoid tissues. Taken in its entirety, this small animal model closely resembles fundamental aspects of the human immune system including the MHC restricted human T cell adaptive response to EBV and the in vivo innate immune response to TSST-1.

Practical models of human hematopoiesis are essential to bridge the gaps in our understanding between the mouse and human immune systems. No animal model will

completely mimic all aspects of human hematopoiesis or the human immune system. However new and improved models that closely recapitulate key aspects of human hematopoiesis will serve to gain insight into fundamental aspects of human stem cell engraftment and reconstitution, immune system development, new strategies to study human pathogenesis and novel therapeutic approaches to alleviate or cure human diseases.

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BIBLIOGRAPHY

1. Till, J.E. & Mc, C.E. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**, 213-22 (1961).
2. Axelrod, H.R. Embryonic stem cell lines derived from blastocysts by a simplified technique. *Dev Biol* **101**, 225-8 (1984).
3. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-6 (1981).
4. Wobus, A.M. & Boheler, K.R. Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* **85**, 635-78 (2005).
5. Smith, A.G. et al. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688-90 (1988).
6. Thomson, J.A. et al. Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-7 (1998).
7. Henderson, J.K. et al. Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells* **20**, 329-37 (2002).
8. Lee, J.B. et al. Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. *Biol Reprod* **72**, 42-9 (2005).
9. Weissman, I.L. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* **287**, 1442-6 (2000).
10. Wagers, A.J. & Weissman, I.L. Plasticity of adult stem cells. *Cell* **116**, 639-48 (2004).
11. Aguila, H.L. et al. From stem cells to lymphocytes: biology and transplantation. *Immunol Rev* **157**, 13-40 (1997).
12. Morrison, S.J., Uchida, N. & Weissman, I.L. The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol* **11**, 35-71 (1995).
13. Szilvassy, S.J. et al. Partially differentiated ex vivo expanded cells accelerate hematologic recovery in myeloablated mice transplanted with highly enriched long-term repopulating stem cells. *Blood* **88**, 3642-53 (1996).
14. Kamel-Reid, S. & Dick, J.E. Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science* **242**, 1706-9 (1988).
15. Larochelle, A. et al. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat Med* **2**, 1329-37 (1996).
16. Vormoor, J. et al. SCID mice as an in vivo model of human cord blood hematopoiesis. *Blood Cells* **20**, 316-20; discussion 320-2 (1994).
17. Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D. & Dick, J.E. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A* **94**, 5320-5 (1997).
18. Akkina, R.K., Rosenblatt, J.D., Campbell, A.G., Chen, I.S. & Zack, J.A. Modeling human lymphoid precursor cell gene therapy in the SCID-hu mouse. *Blood* **84**, 1393-8 (1994).
19. Evans, J.T. et al. Thymocyte differentiation from lentivirus-marked CD34(+) cells in infant and adult human thymus. *J Immunol Methods* **245**, 31-43 (2000).

20. Plum, J., De Smedt, M., Defresne, M.P., Leclercq, G. & Vandekerckhove, B. Human CD34+ fetal liver stem cells differentiate to T cells in a mouse thymic microenvironment. *Blood* **84**, 1587-93 (1994).
21. Weekx, S.F. et al. Generation of T cells from adult human hematopoietic stem cells and progenitors in a fetal thymic organ culture system: stimulation by tumor necrosis factor-alpha. *Blood* **95**, 2806-12 (2000).
22. Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M. & Peault, B. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A* **89**, 2804-8 (1992).
23. Schmitz, N. et al. Transplantation of mobilized peripheral blood cells to HLA-identical siblings with standard-risk leukemia. *Blood* **100**, 761-7 (2002).
24. Benito, A.I., Diaz, M.A., Gonzalez-Vicent, M., Sevilla, J. & Madero, L. Hematopoietic stem cell transplantation using umbilical cord blood progenitors: review of current clinical results. *Bone Marrow Transplant* **33**, 675-90 (2004).
25. Holyoake, T.L., Nicolini, F.E. & Eaves, C.J. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. *Exp Hematol* **27**, 1418-27 (1999).
26. Civin, C.I. et al. Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo. *Blood* **88**, 4102-9 (1996).
27. Szilvassy, S.J., Humphries, R.K., Lansdorp, P.M., Eaves, A.C. & Eaves, C.J. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. *Proc Natl Acad Sci U S A* **87**, 8736-40 (1990).
28. Kim, D.K. et al. Comparison of hematopoietic activities of human bone marrow and umbilical cord blood CD34 positive and negative cells. *Stem Cells* **17**, 286-94 (1999).
29. Uchida, N., Fujisaki, T., Eaves, A.C. & Eaves, C.J. Transplantable hematopoietic stem cells in human fetal liver have a CD34(+) side population (SP)phenotype. *J Clin Invest* **108**, 1071-7 (2001).
30. Aiuti, A. et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**, 2410-3 (2002).
31. Gatlin, J., Melkus, M.W., Padgett, A., Kelly, P.F. & Garcia, J.V. Engraftment of NOD/SCID mice with human CD34(+) cells transduced by concentrated oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. *Journal of Virology* **75**, 9995-9 (2001).
32. Gatlin, J., Padgett, A., Melkus, M.W., Kelly, P.F. & Garcia, J.V. Long-term engraftment of nonobese diabetic/severe combined immunodeficient mice with human CD34+ cells transduced by a self-inactivating human immunodeficiency virus type 1 vector. *Human Gene Therapy* **12**, 1079-89 (2001).
33. Klein, C. & Baum, C. Gene therapy for inherited disorders of haematopoietic cells. *Hematol J* **5**, 103-11 (2004).
34. Civin, C.I. & Small, D. Purification and expansion of human hematopoietic stem/progenitor cells. *Ann N Y Acad Sci* **770**, 91-8 (1995).
35. Denning-Kendall, P.A. et al. Is in vitro expansion of human cord blood cells clinically relevant? *Bone Marrow Transplant* **21**, 225-32 (1998).
36. Shih, C.C., Hu, M.C., Hu, J., Medeiros, J. & Forman, S.J. Long-term ex vivo maintenance and expansion of transplantable human hematopoietic stem cells. *Blood* **94**, 1623-36 (1999).

37. Avots, A., Harder, F., Schmittwolf, C., Petrovic, S. & Muller, A.M. Plasticity of hematopoietic stem cells and cellular memory. *Immunol Rev* **187**, 9-21 (2002).
38. Anderson, D.J., Gage, F.H. & Weissman, I.L. Can stem cells cross lineage boundaries? *Nat Med* **7**, 393-5 (2001).
39. Harder, F. et al. Developmental potentials of hematopoietic and neural stem cells following injection into pre-implantation blastocysts. *Ann Hematol* **81 Suppl 2**, S20-1 (2002).
40. Heike, T. & Nakahata, T. Stem cell plasticity in the hematopoietic system. *Int J Hematol* **79**, 7-14 (2004).
41. Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E. & Weissman, I.L. Identification of a lineage of multipotent hematopoietic progenitors. *Development* **124**, 1929-39 (1997).
42. Galy, A., Travis, M., Cen, D. & Chen, B. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* **3**, 459-73 (1995).
43. Manz, M.G., Miyamoto, T., Akashi, K. & Weissman, I.L. Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci U S A* **99**, 11872-7 (2002).
44. Manz, M.G. et al. Dendritic cell development from common myeloid progenitors. *Ann N Y Acad Sci* **938**, 167-73; discussion 173-4 (2001).
45. Manz, M.G., Traver, D., Miyamoto, T., Weissman, I.L. & Akashi, K. Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* **97**, 3333-41 (2001).
46. Moore, M.A. & Metcalf, D. Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br J Haematol* **18**, 279-96 (1970).
47. Luckett, W.P. Origin and differentiation of the yolk sac and extraembryonic mesoderm in presomite human and rhesus monkey embryos. *Am J Anat* **152**, 59-97 (1978).
48. Tavian, M. et al. Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood* **87**, 67-72 (1996).
49. Tavian, M., Hallais, M.F. & Peault, B. Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. *Development* **126**, 793-803 (1999).
50. Migliaccio, G. et al. Human embryonic hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac----liver transition. *J Clin Invest* **78**, 51-60 (1986).
51. Charbord, P., Tavian, M., Humeau, L. & Peault, B. Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. *Blood* **87**, 4109-19 (1996).
52. Haynes, B.F. & Heinly, C.S. Early human T cell development: analysis of the human thymus at the time of initial entry of hematopoietic stem cells into the fetal thymic microenvironment. *J Exp Med* **181**, 1445-58 (1995).
53. Galy, A.H., Cen, D., Travis, M., Chen, S. & Chen, B.P. Delineation of T-progenitor cell activity within the CD34+ compartment of adult bone marrow. *Blood* **85**, 2770-8 (1995).
54. Barcena, A. et al. Phenotypic and functional analysis of T-cell precursors in the human fetal liver and thymus: CD7 expression in the early stages of T- and myeloid-cell development. *Blood* **82**, 3401-14 (1993).
55. Plum, J. et al. Human T lymphopoiesis. In vitro and in vivo study models. *Ann N Y Acad Sci* **917**, 724-31 (2000).

56. Haynes, B.F., Markert, M.L., Sempowski, G.D., Patel, D.D. & Hale, L.P. The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. *Annu Rev Immunol* **18**, 529-60 (2000).
57. Lobach, D.F., Hensley, L.L., Ho, W. & Haynes, B.F. Human T cell antigen expression during the early stages of fetal thymic maturation. *J Immunol* **135**, 1752-9 (1985).
58. Douek, D.C. et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* **396**, 690-5 (1998).
59. Galy, A., Verma, S., Barcena, A. & Spits, H. Precursors of CD3+CD4+CD8+ cells in the human thymus are defined by expression of CD34. Delineation of early events in human thymic development. *J Exp Med* **178**, 391-401 (1993).
60. Kraft, D.L., Weissman, I.L. & Waller, E.K. Differentiation of CD3-4-8- human fetal thymocytes in vivo: characterization of a CD3-4+8- intermediate. *J Exp Med* **178**, 265-77 (1993).
61. Zanjani, E.D., Almeida-Porada, G. & Flake, A.W. Retention and multilineage expression of human hematopoietic stem cells in human-sheep chimeras. *Stem Cells* **13**, 101-11 (1995).
62. Zanjani, E.D., Almeida-Porada, G., Livingston, A.G., Porada, C.D. & Ogawa, M. Engraftment and multilineage expression of human bone marrow CD34- cells in vivo. *Ann N Y Acad Sci* **872**, 220-31; discussion 231-2 (1999).
63. Zanjani, E.D., Srour, E.F. & Hoffman, R. Retention of long-term repopulating ability of xenogeneic transplanted purified adult human bone marrow hematopoietic stem cells in sheep. *J Lab Clin Med* **126**, 24-8 (1995).
64. Omori, F. et al. Adoptive transfer of genetically modified human hematopoietic stem cells into preimmune canine fetuses. *Exp Hematol* **27**, 242-9 (1999).
65. Greiner, D.L., Hesselton, R.A. & Shultz, L.D. SCID mouse models of human stem cell engraftment. *Stem Cells* **16**, 166-77 (1998).
66. Dao, M.A., Pepper, K.A. & Nolta, J.A. Long-term cytokine production from engineered primary human stromal cells influences human hematopoiesis in an in vivo xenograft model. *Stem Cells* **15**, 443-54 (1997).
67. Nolta, J.A., Hanley, M.B. & Kohn, D.B. Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: analysis of gene transduction of long-lived progenitors. *Blood* **83**, 3041-51 (1994).
68. Bosma, G.C., Custer, R.P. & Bosma, M.J. A severe combined immunodeficiency mutation in the mouse. *Nature* **301**, 527-30 (1983).
69. Mosier, D.E. Adoptive transfer of human lymphoid cells to severely immunodeficient mice: models for normal human immune function, autoimmunity, lymphomagenesis, and AIDS. *Adv Immunol* **50**, 303-25 (1991).
70. Mosier, D.E., Gulizia, R.J., Baird, S.M. & Wilson, D.B. Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* **335**, 256-9 (1988).
71. Saxon, A. et al. Limited B cell repertoire in severe combined immunodeficient mice engrafted with peripheral blood mononuclear cells derived from immunodeficient or normal humans. *J Clin Invest* **87**, 658-65 (1991).

72. Tary-Lehmann, M., Lehmann, P.V., Schols, D., Roncarolo, M.G. & Saxon, A. Anti-SCID mouse reactivity shapes the human CD4+ T cell repertoire in hu-PBL-SCID chimeras. *J Exp Med* **180**, 1817-27 (1994).
73. Tary-Lehmann, M. & Saxon, A. Human mature T cells that are anergic in vivo prevail in SCID mice reconstituted with human peripheral blood. *J Exp Med* **175**, 503-16 (1992).
74. Hesselton, R.M. et al. High levels of human peripheral blood mononuclear cell engraftment and enhanced susceptibility to human immunodeficiency virus type 1 infection in NOD/LtSz-scid/scid mice. *J Infect Dis* **172**, 974-82 (1995).
75. Hesselton, R.M. et al. Human peripheral blood xenografts in the SCID mouse: characterization of immunologic reconstitution. *J Infect Dis* **168**, 630-40 (1993).
76. Mosier, D.E. Viral pathogenesis in hu-PBL-SCID mice. *Semin Immunol* **8**, 255-62 (1996).
77. Cashman, J.D. et al. Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. *Blood* **89**, 4307-16 (1997).
78. Pflumio, F. et al. Phenotype and function of human hematopoietic cells engrafting immune-deficient CB17-severe combined immunodeficiency mice and nonobese diabetic-severe combined immunodeficiency mice after transplantation of human cord blood mononuclear cells. *Blood* **88**, 3731-40 (1996).
79. Kollmann, T.R., Kim, A., Zhuang, X., Hachamovitch, M. & Goldstein, H. Reconstitution of SCID mice with human lymphoid and myeloid cells after transplantation with human fetal bone marrow without the requirement for exogenous human cytokines. *Proc Natl Acad Sci U S A* **91**, 8032-6 (1994).
80. Hogan, C.J., Shpall, E.J., McNiece, I. & Keller, G. Multilineage engraftment in NOD/LtSz-scid/scid mice from mobilized human CD34+ peripheral blood progenitor cells. *Biol Blood Marrow Transplant* **3**, 236-46 (1997).
81. McCune, J.M. et al. The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science* **241**, 1632-9 (1988).
82. Namikawa, R., Weilbaecher, K.N., Kaneshima, H., Yee, E.J. & McCune, J.M. Long-term human hematopoiesis in the SCID-hu mouse. *J Exp Med* **172**, 1055-63 (1990).
83. Vandekerckhove, B.A. et al. Thymic selection of the human T cell receptor V beta repertoire in SCID-hu mice. *J Exp Med* **176**, 1619-24 (1992).
84. Kaneshima, H. et al. Human immunodeficiency virus infection of human lymph nodes in the SCID-hu mouse. *Proc Natl Acad Sci U S A* **88**, 4523-7 (1991).
85. Bonyhadi, M.L. et al. HIV induces thymus depletion in vivo. *Nature* **363**, 728-32 (1993).
86. Shultz, L.D. et al. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol* **154**, 180-91 (1995).
87. Hogan, C.J. et al. Engraftment and development of human CD34(+)-enriched cells from umbilical cord blood in NOD/LtSz-scid/scid mice. *Blood* **90**, 85-96 (1997).
88. Perez, L.E. et al. Xenotransplantation of immunodeficient mice with mobilized human blood CD34+ cells provides an in vivo model for human megakaryocytopoiesis and platelet production. *Blood* **97**, 1635-43 (2001).
89. Cravens, P.D. et al. Development and activation of human dendritic cells in vivo in a xenograft model of human hematopoiesis. *Stem Cells* **23**, 264-78 (2005).
90. Palucka, A.K. et al. Human dendritic cell subsets in NOD/SCID mice engrafted with CD34+ hematopoietic progenitors. *Blood* **102**, 3302-10 (2003).

91. Kalberer, C.P., Siegler, U. & Wodnar-Filipowicz, A. Human NK cell development in NOD/SCID mice receiving grafts of cord blood CD34+ cells. *Blood* **102**, 127-35 (2003).
92. Shultz, L.D. et al. NOD/LtSz-Rag1null mice: an immunodeficient and radioresistant model for engraftment of human hematolymphoid cells, HIV infection, and adoptive transfer of NOD mouse diabetogenic T cells. *J Immunol* **164**, 2496-507 (2000).
93. Christianson, S.W. et al. Enhanced human CD4+ T cell engraftment in beta2-microglobulin-deficient NOD-scid mice. *J Immunol* **158**, 3578-86 (1997).
94. Meyerrose, T.E., Herrbrich, P., Hess, D.A. & Nolta, J.A. Immune-deficient mouse models for analysis of human stem cells. *Biotechniques* **35**, 1262-72 (2003).
95. Prochazka, M., Gaskins, H.R., Shultz, L.D. & Leiter, E.H. The nonobese diabetic scid mouse: model for spontaneous thymomagenesis associated with immunodeficiency. *Proc Natl Acad Sci U S A* **89**, 3290-4 (1992).
96. Koyanagi, Y. et al. High levels of viremia in hu-PBL-NOD-scid mice with HIV-1 infection. *Leukemia* **11 Suppl 3**, 109-12 (1997).
97. Yoshino, H. et al. Natural killer cell depletion by anti-asialo GM1 antiserum treatment enhances human hematopoietic stem cell engraftment in NOD/Shi-scid mice. *Bone Marrow Transplant* **26**, 1211-6 (2000).
98. Kyoizumi, S. et al. Implantation and maintenance of functional human bone marrow in SCID-hu mice. *Blood* **79**, 1704-11 (1992).
99. Fujiki, Y. et al. Dominant expansion of human T cells in non-obese diabetic/severe combined immunodeficiency mice implanted with human bone fragments. *Exp Hematol* **28**, 792-801 (2000).
100. Samira, S. et al. Tumor necrosis factor promotes human T-cell development in nonobese diabetic/severe combined immunodeficient mice. *Stem Cells* **22**, 1085-100 (2004).
101. Kerre, T.C. et al. Adapted NOD/SCID model supports development of phenotypically and functionally mature T cells from human umbilical cord blood CD34(+) cells. *Blood* **99**, 1620-6 (2002).
102. Hiramatsu, H. et al. Complete reconstitution of human lymphocytes from cord blood CD34+ cells using the NOD/SCID/gammacnull mice model. *Blood* **102**, 873-80 (2003).
103. Ito, M. et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* **100**, 3175-82 (2002).
104. Yahata, T. et al. Functional human T lymphocyte development from cord blood CD34+ cells in nonobese diabetic/Shi-scid, IL-2 receptor gamma null mice. *J Immunol* **169**, 204-9 (2002).
105. Matsumura, T. et al. Functional CD5+ B cells develop predominantly in the spleen of NOD/SCID/gammac(null) (NOG) mice transplanted either with human umbilical cord blood, bone marrow, or mobilized peripheral blood CD34+ cells. *Exp Hematol* **31**, 789-97 (2003).
106. Mazurier, F. et al. A novel immunodeficient mouse model--RAG2 x common cytokine receptor gamma chain double mutants--requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. *J Interferon Cytokine Res* **19**, 533-41 (1999).
107. Gimeno, R. et al. Monitoring the effect of gene silencing by RNA interference in human CD34+ cells injected into newborn RAG2-/- gammac-/- mice: functional inactivation of p53 in developing T cells. *Blood* **104**, 3886-93 (2004).

108. Traggiai, E. et al. Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* **304**, 104-7 (2004).
109. Weijer, K. et al. Intrathymic and extrathymic development of human plasmacytoid dendritic cell precursors in vivo. *Blood* **99**, 2752-9 (2002).
110. Shultz, L.D. et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2Rgammanull mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* **174**, 6477-89 (2005).
111. Ishikawa, F. et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chainnull mice. *Blood* (2005).
112. Andriole, G.L., Mule, J.J., Hansen, C.T., Linehan, W.M. & Rosenberg, S.A. Evidence that lymphokine-activated killer cells and natural killer cells are distinct based on an analysis of congenitally immunodeficient mice. *J Immunol* **135**, 2911-3 (1985).
113. Custer, R.P., Bosma, G.C. & Bosma, M.J. Severe combined immunodeficiency (SCID) in the mouse. Pathology, reconstitution, neoplasms. *Am J Pathol* **120**, 464-77 (1985).
114. Thomsen, M., Yacoub-Youssef, H. & Marcheix, B. Reconstitution of a human immune system in immunodeficient mice: models of human alloreaction in vivo. *Tissue Antigens* **66**, 73-82 (2005).
115. Islas-Ohlmayer, M. et al. Experimental infection of NOD/SCID mice reconstituted with human CD34+ cells with Epstein-Barr virus. *Journal of Virology* **78**, 13891-900 (2004).
116. Aydar, Y., Wu, J., Song, J., Szakal, A.K. & Tew, J.G. FcgammaRII expression on follicular dendritic cells and immunoreceptor tyrosine-based inhibition motif signaling in B cells. *Eur J Immunol* **34**, 98-107 (2004).
117. Heslop, H.E. et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med* **2**, 551-5 (1996).
118. Aldrovandi, G.M. et al. The SCID-hu mouse as a model for HIV-1 infection. *Nature* **363**, 732-6 (1993).
119. Lapidot, T., Fajerman, Y. & Kollet, O. Immune-deficient SCID and NOD/SCID mice models as functional assays for studying normal and malignant human hematopoiesis. *J Mol Med* **75**, 664-73 (1997).
120. Lapidot, T. et al. Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* **255**, 1137-41 (1992).
121. Hamatani, K., Matsuda, Y., Araki, R., Itoh, M. & Abe, M. Cloning and chromosomal mapping of the mouse DNA-dependent protein kinase gene. *Immunogenetics* **45**, 1-5 (1996).
122. Blunt, T. et al. Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the scid mouse. *Proc Natl Acad Sci U S A* **93**, 10285-90 (1996).
123. Dick, J.E. & Lapidot, T. Biology of normal and acute myeloid leukemia stem cells. *Int J Hematol* **82**, 389-96 (2005).
124. Hayashi, K., Teramoto, N. & Akagi, T. Animal in vivo models of EBV-associated lymphoproliferative diseases: special references to rabbit models. *Histol Histopathol* **17**, 1293-310 (2002).
125. Johannessen, I. & Crawford, D.H. In vivo models for Epstein-Barr virus (EBV)-associated B cell lymphoproliferative disease (BLPD). *Rev Med Virol* **9**, 263-77 (1999).

126. Mule, J.J., Jicha, D.L. & Rosenberg, S.A. The use of congenitally immunodeficient mice to study human tumor metastases and immunotherapy. *J Immunother* **12**, 196-8 (1992).
127. Hammarstrom, L., Abedi, M.R., Hassan, M.S. & Smith, C.I. The SCID mouse as a model for autoimmunity. *J Autoimmun* **6**, 667-74 (1993).
128. Jorgensen, C., Bologna, C. & Sany, J. Autoimmunity. Insights provided by the SCID mouse model. *Rev Rhum Engl Ed* **62**, 519-24 (1995).
129. Marmont, A.M. Stem cell transplantation for severe autoimmune diseases: progress and problems. *Haematologica* **83**, 733-43 (1998).
130. Nakano, T. Hematopoietic stem cells: generation and manipulation. *Trends Immunol* **24**, 589-94 (2003).
131. Miyoshi, H., Smith, K.A., Mosier, D.E., Verma, I.M. & Torbett, B.E. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* **283**, 682-6 (1999).
132. Bente, D.A., Melkus, M.W., Garcia, J.V. & Rico-Hesse, R. Dengue fever in humanized NOD/SCID mice. *J Virol* **79**, 13797-9 (2005).
133. Cashman, J., Bockhold, K., Hogge, D.E., Eaves, A.C. & Eaves, C.J. Sustained proliferation, multi-lineage differentiation and maintenance of primitive human haemopoietic cells in NOD/SCID mice transplanted with human cord blood. *Br J Haematol* **98**, 1026-36 (1997).
134. Dick, J.E., Pflumio, F. & Lapidot, T. Mouse models for human hematopoiesis. *Semin Immunol* **3**, 367-78 (1991).
135. Shultz, L.D. Hematopoiesis and models of immunodeficiency. *Semin Immunol* **3**, 397-408 (1991).
136. De Smedt, M., Leclercq, G., Vandekerckhove, B. & Plum, J. Human fetal liver cells differentiate into thymocytes in chimeric mouse fetal thymus organ culture. *Adv Exp Med Biol* **355**, 27-31 (1994).
137. Saito, Y. et al. The in vivo development of human T cells from CD34(+) cells in the murine thymic environment. *Int Immunol* **14**, 1113-24 (2002).
138. Aspinall, R. & Andrew, D. Thymic involution in aging. *J Clin Immunol* **20**, 250-6 (2000).
139. Markert, M.L. et al. Complete DiGeorge syndrome: development of rash, lymphadenopathy, and oligoclonal T cells in 5 cases. *J Allergy Clin Immunol* **113**, 734-41 (2004).
140. Markert, M.L. et al. Successful formation of a chimeric human thymus allograft following transplantation of cultured postnatal human thymus. *J Immunol* **158**, 998-1005 (1997).
141. Markert, M.L. et al. Transplantation of thymus tissue in complete DiGeorge syndrome. *N Engl J Med* **341**, 1180-9 (1999).
142. Weissman, I.L. & Shizuru, J.A. Immune reconstitution. *N Engl J Med* **341**, 1227-9 (1999).
143. Douglas, J., Kelly, P., Evans, J.T. & Garcia, J.V. Efficient transduction of human lymphocytes and CD34+ cells via human immunodeficiency virus-based gene transfer vectors. *Hum Gene Ther* **10**, 935-45 (1999).
144. Jenkinson, E.J., Franchi, L.L., Kingston, R. & Owen, J.J. Effect of deoxyguanosine on lymphopoiesis in the developing thymus rudiment in vitro: application in the production of chimeric thymus rudiments. *Eur J Immunol* **12**, 583-7 (1982).

145. Vandekerckhove, B.A. et al. Clonal analysis of the peripheral T cell compartment of the SCID-hu mouse. *J Immunol* **146**, 4173-9 (1991).
146. An, D.S. et al. High-efficiency transduction of human lymphoid progenitor cells and expression in differentiated T cells. *J Virol* **71**, 1397-404 (1997).
147. Roos, M.T. et al. Changes in the composition of circulating CD8+ T cell subsets during acute epstein-barr and human immunodeficiency virus infections in humans. *J Infect Dis* **182**, 451-8 (2000).
148. Lanier, L.L. NK cell receptors. *Annu Rev Immunol* **16**, 359-93 (1998).
149. Moretta, A., Bottino, C., Mingari, M.C., Biassoni, R. & Moretta, L. What is a natural killer cell? *Nat Immunol* **3**, 6-8 (2002).
150. De Togni, P. et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* **264**, 703-7 (1994).
151. Koni, P.A. et al. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity* **6**, 491-500 (1997).
152. Rennert, P.D., James, D., Mackay, F., Browning, J.L. & Hochman, P.S. Lymph node genesis is induced by signaling through the lymphotoxin beta receptor. *Immunity* **9**, 71-9 (1998).
153. Arbuthnott, J.P., Coleman, D.C. & de Azavedo, J.S. Staphylococcal toxins in human disease. *Soc Appl Bacteriol Symp Ser* **19**, 101S-107S (1990).
154. Iwatsuki, K., Yamasaki, O., Morizane, S. & Oono, T. Staphylococcal cutaneous infections: invasion, evasion and aggression. *J Dermatol Sci* **42**, 203-14 (2006).
155. Kotzin, B.L., Leung, D.Y., Kappler, J. & Marrack, P. Superantigens and their potential role in human disease. *Adv Immunol* **54**, 99-166 (1993).
156. Ohno, T., Furuno, K., Ikeda, K. & Hara, T. [Kawasaki disease (Kawasaki syndrome)]. *Nippon Rinsho* **63 Suppl 5**, 391-9 (2005).
157. Krakauer, T. Immune response to staphylococcal superantigens. *Immunol Res* **20**, 163-73 (1999).
158. Kappler, J. et al. V beta-specific stimulation of human T cells by staphylococcal toxins. *Science* **244**, 811-3 (1989).
159. Jardetzky, T.S. et al. Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature* **368**, 711-8 (1994).
160. Karp, D.R., Teletski, C.L., Scholl, P., Geha, R. & Long, E.O. The alpha 1 domain of the HLA-DR molecule is essential for high-affinity binding of the toxic shock syndrome toxin-1. *Nature* **346**, 474-6 (1990).
161. Choi, Y. et al. Selective expansion of T cells expressing V beta 2 in toxic shock syndrome. *J Exp Med* **172**, 981-4 (1990).
162. Kum, W.W., Cameron, S.B., Hung, R.W., Kalyan, S. & Chow, A.W. Temporal sequence and kinetics of proinflammatory and anti-inflammatory cytokine secretion induced by toxic shock syndrome toxin 1 in human peripheral blood mononuclear cells. *Infect Immun* **69**, 7544-9 (2001).
163. Todd, J., Fishaut, M., Kapral, F. & Welch, T. Toxic-shock syndrome associated with phage-group-I Staphylococci. *Lancet* **2**, 1116-8 (1978).
164. McCormick, J.K., Yarwood, J.M. & Schlievert, P.M. Toxic shock syndrome and bacterial superantigens: an update. *Annu Rev Microbiol* **55**, 77-104 (2001).
165. Kollet, O. et al. beta2 microglobulin-deficient (B2m(null)) NOD/SCID mice are excellent recipients for studying human stem cell function. *Blood* **95**, 3102-5 (2000).

166. Shultz, L.D. et al. NOD/LtSz-Rag1nullPfpnull mice: a new model system with increased levels of human peripheral leukocyte and hematopoietic stem-cell engraftment. *Transplantation* **76**, 1036-42 (2003).
167. Langenkamp, A. et al. T cell priming by dendritic cells: thresholds for proliferation, differentiation and death and intraclonal functional diversification. *Eur J Immunol* **32**, 2046-54 (2002).
168. Kum, W.W., Hung, R.W., Cameron, S.B. & Chow, A.W. Temporal sequence and functional implications of V beta-specific T cell receptor down-regulation and costimulatory molecule expression following in vitro stimulation with the staphylococcal superantigen Toxic shock syndrome toxin-1. *J Infect Dis* **185**, 555-60 (2002).
169. Makida, R., Hofer, M.F., Takase, K., Cambier, J.C. & Leung, D.Y. Bacterial superantigens induce V beta-specific T cell receptor internalization. *Mol Immunol* **33**, 891-900 (1996).
170. Shearer, W.T., Wedner, H.J., Strominger, D.B., Kissane, J. & Hong, R. Successful transplantation of the thymus in Nezelof's syndrome. *Pediatrics* **61**, 619-24 (1978).
171. Dinges, M.M., Orwin, P.M. & Schlievert, P.M. Exotoxins of Staphylococcus aureus. *Clin Microbiol Rev* **13**, 16-34, table of contents (2000).
172. Kaneshima, H. et al. Rapid-high, syncytium-inducing isolates of human immunodeficiency virus type 1 induce cytopathicity in the human thymus of the SCID-hu mouse. *J Virol* **68**, 8188-92 (1994).
173. Stanley, S.K. et al. Human immunodeficiency virus infection of the human thymus and disruption of the thymic microenvironment in the SCID-hu mouse. *J Exp Med* **178**, 1151-63 (1993).
174. Su, L. et al. HIV-1-induced thymocyte depletion is associated with indirect cytopathogenicity and infection of progenitor cells in vivo. *Immunity* **2**, 25-36 (1995).
175. Rabin, L. et al. Use of standardized SCID-hu Thy/Liv mouse model for preclinical efficacy testing of anti-human immunodeficiency virus type 1 compounds. *Antimicrob Agents Chemother* **40**, 755-62 (1996).
176. McCune, J.M., Namikawa, R., Shih, C.C., Rabin, L. & Kaneshima, H. Suppression of HIV infection in AZT-treated SCID-hu mice. *Science* **247**, 564-6 (1990).
177. Shih, C.C. et al. Postexposure prophylaxis with zidovudine suppresses human immunodeficiency virus type 1 infection in SCID-hu mice in a time-dependent manner. *J Infect Dis* **163**, 625-7 (1991).
178. Picchio, G.R. et al. Altered viral fitness of HIV-1 following failure of protease inhibitor-based therapy. *J Acquir Immune Defic Syndr* **25**, 289-95 (2000).
179. Stoddart, C.A. et al. Impaired replication of protease inhibitor-resistant HIV-1 in human thymus. *Nat Med* **7**, 712-8 (2001).
180. Datema, R. et al. Antiviral efficacy in vivo of the anti-human immunodeficiency virus bicyclam SDZ SID 791 (JM 3100), an inhibitor of infectious cell entry. *Antimicrob Agents Chemother* **40**, 750-4 (1996).
181. Shevach, E.M. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* **2**, 389-400 (2002).
182. von Herrath, M.G. & Harrison, L.C. Antigen-induced regulatory T cells in autoimmunity. *Nat Rev Immunol* **3**, 223-32 (2003).
183. Wood, K.J. & Sakaguchi, S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* **3**, 199-210 (2003).

184. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* **6**, 295-307 (2006).
185. Curiel, T.J. et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* **10**, 942-9 (2004).
186. Sakaguchi, S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* **6**, 345-52 (2005).
187. Stephens, L.A., Mottet, C., Mason, D. & Powrie, F. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol* **31**, 1247-54 (2001).
188. Prussin, C. & Metcalfe, D.D. 5. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* **117**, S450-6 (2006).
189. Asahara, T. et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964-7 (1997).
190. Guzick, D.S. et al. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* **345**, 1388-93 (2001).
191. Khakoo, A.Y. & Finkel, T. Endothelial progenitor cells. *Annu Rev Med* **56**, 79-101 (2005).
192. Shaw, J.P., Basch, R. & Shamamian, P. Hematopoietic stem cells and endothelial cell precursors express Tie-2, CD31 and CD45. *Blood Cells Mol Dis* **32**, 168-75 (2004).
193. Asahara, T. et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* **85**, 221-8 (1999).
194. Crisa, L. et al. Human cord blood progenitors sustain thymic T-cell development and a novel form of angiogenesis. *Blood* **94**, 3928-40 (1999).
195. Droetto, S. et al. Vasculogenic potential of long term repopulating cord blood progenitors. *Faseb J* **18**, 1273-5 (2004).

VITAE

Michael W. Melkus was born in Amarillo, Texas, on February 21, 1971 the son of Mary G. and Francis R. Melkus. After graduating from Amarillo High School, Amarillo, Texas, in 1989, he attended Texas Tech University to earn a Bachelor of Science degree in Cell and Molecular Biology with a Minor in Chemistry in 1993. He continued his education studying plant-microbe interactions to earn a Masters of Microbiology in 1995 under the guidance of Dr. Michael J. San Francisco. During the next four years, Dr. Larry Blanton employed him as a research technician at Texas Tech University. He helped develop and taught the laboratory course for Cell and Molecular Biology Techniques and worked with the Howard Hughes High School Outreach Program. In June of 1999, Michael entered the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Dallas. He was awarded the Degree of Doctor of Philosophy in 2006.

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