

Modulation of Immune Cell Niches for Therapeutics in Cancer and Inflammatory Diseases

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Abstract

Immune cell niches are microenvironments that support the survival of specific hematopoietic cells. The size of a given niche is dependent on survival and proliferation signals provided. Modulation of niche size can be a useful therapeutic tool, and a better understanding of the factors that control the size of immune cell niches can lead to more targeted therapies.

Here bone marrow and thymic niches were modulated with tyrosine kinase inhibition to achieve increased engraftment following stem cell transplantation (SCT). SCT resulting in mixed chimerism is curative for several benign blood diseases, but toxicities associated with myeloablative and cytotoxic conditioning regimens limit the application of SCT. Sunitinib inhibits multiple tyrosine kinases including KIT, an essential survival signal within the hematopoietic stem cell and thymic progenitor niches. Sunitinib therapy diminishes hematopoietic and thymic progenitor cells in mice and enhances accessibility of marrow and thymic niches to transplanted bone marrow. This provides a novel, non-cytotoxic approach to accomplish mixed hematopoietic chimerism.

The observation that T cells undergo increased proliferation and accumulate in IL-7R deficient mice compared to other lymphopenic hosts raised questions about the factors that control the size of the T cell niche. Understanding these factors is useful in designing therapeutics to increase T cell responses for treatment of many diseases including cancer. Dendritic cells (DCs) are well known for their ability to modulate T cell responses; however, very little is known about the role of IL-7R signaling on DCs. The data presented here show that bone marrow derived DCs treated with IL-7 were less able to induce T cell proliferation in coculture. In vivo systems using CD11cDTR mice showed a role for IL-7 signaling on CD11c⁺ cells in T cell homeostasis. Together these data suggest that IL-7R signaling on DCs is important for regulating the size of the T cell niche.

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Abbreviations

APC	Antigen Presenting Cell
BM	Bone Marrow
BMDC	Bone Marrow Dendritic Cell
CFSE	Carboxyfluorescein diacetate succinimidyl diester
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
DC	Dendritic Cell
DN	Double Negative (CD4 ⁻ & CD8 ⁻)
DTR	Diphtheria Toxin Receptor
Dtx	Diphtheria Toxin
ETP	Early Thymic Progenitor
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
Flt3	Fms-like tyrosine kinase receptor-3
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HPE	Hematopoietic Peripheral Expansion
HSCT	Hematopoietic Stem Cell Transplant
HSC	Hematopoietic Stem Cell
IV	Intravenous
ILC	Innate Lymphoid Cell
LPL	Lamina Propria Leukocyte
LPS	Lipopolysaccharide
LT-HSC	Long-term Hematopoietic Stem Cell
LTi	Lymphoid Tissue Inducer
MDP	Macrophage and dendritic-cell precursor
NIH	National Institutes of Health
PBS	Phosphate Buffered Saline
RPMI	Media developed at Roswell Park Memorial Institute
SEM	Standard Error of the Mean
SCF	Stem Cell Factor
TCD	T Cell Depleted
TCR	T Cell Receptor
Tg	Transgenic
Treg	T Regulatory Cell
TSLP	Thymic Stromal Lymphopoietin

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1 INTRODUCTION

1.1 OVERVIEW

The immune system is made up of a barrier system of epithelial cells and a network of leukocytes, also known as white blood cells, designed to protect the body from microbial infection. This network of cells is responsible for sensing danger in the form of microbial infection or cell stress and for mounting an appropriate response to kill the pathogen while mitigating collateral damage to healthy tissues. Multiple cell types with different functions work together to achieve this complex goal. The cells communicate with each other through direct cell contact and through excreting proteins called cytokines that serve as extracellular messengers.

The innate immune system is the first to respond to an invading pathogen. Innate cells, such as macrophages and DCs, possess genome-encoded receptors that recognize conserved molecules commonly found on microbes. These pattern recognition receptors (PRR) can sense pathogen-associated molecular patterns (PAMPS)[2]. Activation of a PRR leads to a downstream cascade of events that is designed to rid the body of the pathogen. For example, activation of macrophages leads to induction of the immune response and to recruitment of other cell types such as neutrophils that help clear the infection.

The innate immune system also initiates the antigen-specific or adaptive immune response[3]. B and T lymphocytes are the key players of the adaptive

immune response. During the first exposure to a particular antigen, the immune system will produce a primary response. Innate cells, such as dendritic cells (DCs) or macrophages, take up the antigen, internally process it, and then present it to T lymphocytes on MHC-II[3]. These lymphocytes possess highly specific receptors that will only respond to specific antigens. Since there are very few B and T lymphocytes in the body that can respond to a given antigen, the DC will migrate to the lymph nodes where it has an increased chance of encountering a lymphocyte specific for the antigen it is presenting[4]. The initial encounter of a B or T lymphocyte with its antigen leads to its activation, maturation, and proliferation. The primary response generally consists of the production of antibody molecules specific for the antigen and of the expansion and differentiation of specific helper and effector T cells[3]. This process may take up to 10 days.

After clearing the infection, the body is left with immunologic memory, which is the hallmark of the adaptive immune system. Some of the clonally expanded B and T cells could last for the lifetime of the individual. Memory B cells are long-lived cells that were activated during a primary response, and provide a reactive memory response by quickly responding to a secondary exposure[5]. Some memory B cells differentiate into plasma cells that can provide protective memory. Plasma cells live in the bone marrow and can produce large amounts of specific antibodies that assist in the destruction of pathogens[5, 6]. Similar to memory B cells, memory T cells are long-lived cells that were activated during a primary response. Memory T cells are categorized as either central or effector memory T cells based on expression of CD62L and CCR7, which determine their

ability to move within the body. Effector memory cells can home to non-lymphoid sites and are responsible for producing rapid effector responses involving production of IFN- γ [7]. Central memory cells live only in the lymphoid tissues; they produce IL-2 and are important for producing more effector memory cells[8, 9]. A secondary response to an antigen is much more rapid and more specific than a primary response. It consists of high-affinity antibody production from plasma cells, which are more effective at clearing the infection, and a more rapid and more efficient T cell response[10].

The entire network of different leukocytes derives from a single cell type that resides in the medulla of the bone known as the hematopoietic stem cell (HSC)[11]. Most mature immune cells have short life spans and therefore need to be constantly produced at steady state in order to ensure sufficient supply and maintain homeostasis. Lymphopenia is a state when the number of lymphocytes in the body has become depleted. It can result from a number of different lymphopenic insults such as HIV infection or bone marrow transplant. During lymphopenia there is an increase in lymphocyte growth factors such as interleukin 7 (IL-7) in order to increase the production of lymphocytes and replenish circulating numbers back to normal homeostatic levels[12]. This process is known as lymphopenia driven proliferation or homeostatic peripheral expansion (HPE)[12].

While the immune system is designed to fight foreign and/or harmful pathogens, there are many safeguards in place to protect against the immune system reacting to non-harmful antigens. This system of safeguarding and the fact that

the immune system does not respond to self-antigen are known as immunologic tolerance[13, 14]. Tolerance is a complicated process that involves many mechanisms. Most T cells that are reactive to self-antigens are eliminated in the thymus through negative selection before they even make it into peripheral circulation[13]. Also, there is a subset of T cells known as T regulatory cells (Tregs) that serves a regulatory role by actively suppressing responses against self-antigens[14]. Finally, innate cells are also important in helping determine whether or not an immune stimulus is harmful. T cells require cues from innate cells in order to mount a full response[15]. In addition to MHC/TCR interactions, such cues include co-stimulation usually through ligation of CD28 on T cells with CD80 or CD86 on DCs, and cytokine signals. Self-reactive T cells that do not get these cues may fail to respond[15]. There are also subsets of DCs, such as CD103⁺ DCs, that specialize in maintaining the balance between inflammation and tolerance at mucosal surfaces. In the gut, for example, there is a high exposure to food antigens and commensal bacteria, but also increased exposure to pathogens. CD103⁺ DCs are particularly known for their ability to promote the differentiation of Tregs during homeostasis[16], but they may also play a role in host defense[17].

Sometimes the immune system reacts to non-harmful antigens such as normal self-antigens, commensal bacteria, or food antigens. This overreaction and failure to establish immune tolerance are what cause allergy and autoimmune disease. A better understanding of the factors that control the immune system could help us develop better treatments for inflammatory diseases such as colitis.

1.2 BONE MARROW AND THYMIC NICHES

The bone marrow and thymus are highly specialized regions that provide unique microenvironments for the maturation of hematopoietic cells. Together they are the body's primary lymphoid structures because they are respectively the sites where pre-B and pre-T lymphocytes mature into naïve B and T cells in the absence of foreign antigen.

1.2.1 BONE MARROW HSC NICHE

The medulla of the bone is a highly vascularized, very dynamic region which houses the bone marrow and much of hematopoiesis[18]. Hematopoiesis is the process by which all cellular components of the blood develop from a single cell type known as the hematopoietic stem cell (HSC). HSCs are a self-renewing population that also gives rise to other precursor cell populations such as the multipotent progenitors (MPP). In turn, these progenitors give rise to other more differentiated cell populations, and the process continues like a branching tree until eventually all leukocytes develop from this one stem cell population. Many groups have demonstrated that all hematopoietic lineages can be reconstituted in a lethally irradiated mouse by transfer of a single purified HSC, proving the existence of the HSC[11, 19-22].

Stromal cells within the bone marrow produce growth factors and cytokines such as chemokines and interleukins, which influence hematopoietic cells at certain stages in their differentiation. These cytokines and growth factors create

microenvironments that define cellular niches. Stem cell factor (SCF), for example, is produced by fibroblasts and endothelial cells and is crucial for the HSC niche.

Recent publications using in vivo imaging have brought light to the spatial configuration of hematopoietic and stromal cells within the bone marrow[23, 24]. Hematopoietic cells arrange themselves within the bone marrow according to cell subsets and stage of differentiation[23]. Precursors for macrophages and erythrocytes tend to gather around the blood vessels, while granulocytes can be found at the borders of the bone marrow[23].

The endosteum, a thin layer of connective tissues that lines the medulla of the bone, forms a special zone that normally maintains HSC populations and also promotes their expansion and differentiation after bone marrow damage[24, 25]. The endosteum defines the geographical HSC niche. Fibroblasts and endothelial cells within the endosteum produce SCF and other cytokines necessary for HSC maintenance. When physiological challenges such as irradiation drive either engraftment or expansion, HSCs localize in close proximity to bone, specifically osteoblasts[23]. Molecular crosstalk between HSCs and osteoblasts is thought to regulate HSC self-renewal and differentiation[26]. Specialized endothelium within the bone marrow expresses E-selectin and stromal-cell-derived factor 1 (CXCL12) in discrete discontinuous areas that allow homing of HSC and other bone marrow progenitors[27]. A more recent study has shown that mesenchymal stem cells also highly express HSC maintenance genes and are important for HSC homing and localization[28].

1.2.2 THYMIC NICHE

The thymus is the place of T cell development. Pre-thymocytes leave the bone marrow and seed the thymus, where they undergo differentiation to naïve T cells. It is not exactly clear what cells are responsible for thymic seeding since no cell population has been detected in both the blood and the thymus[29]. However, lymphoid-primed multipotent progenitors (LMPPs) and common lymphoid progenitors (CLPs) injected into the blood can rapidly create thymocytes while HSCs and MPPs cannot[30] (Figure 1.1). Thymic seeding requires expression of CCR7 and CCR9[29, 31-34]. Their ligands CCL19,21,25 are all expressed in the thymus, with CCR7 ligand expression (CCL19,21) being particularly high in the thymic medulla[29, 31].

The thymus is made up of three distinct regions: capsule, cortex, and medulla. Different stages of T cell development take place at different locations within the thymus. The cortex and the medulla are the sites of thymocyte maturation and are separated by a cortico-medullary border. This highly vascularized border is where new thymocytes seed the thymus[35]. The KIT expressing Early Thymic Progenitor (ETP) is the earliest recognized progenitor within the thymus and likely is derived from a thymic seeding progenitor (TSP) that has not yet been identified. The ETP is part of the double negative (DN) population because it does not express CD4 or CD8. Thymocytes develop through four DN stages (DN1-DN4) defined by expression of CD44 and CD25[36, 37]. DN3 cells migrate to the subcapsular zone while rearranging their T cell receptor (TCR) chains.

The enormous unselected T cell repertoire is created through V(D)J recombination[38].

Cells which have successfully rearranged both α and β chains go on to become double positive cells expressing CD4 and CD8. These double positive cells undergo positive selection in the cortex[39, 40]. Thymic stromal and epithelial antigen presenting cells mediate T cell education and selection. They present self-peptides on MHC-II surface molecules. T Cells that are capable of binding to MHC molecules with at least weak affinity are positively selected. Some cells that bind with higher affinity are selected to become regulatory T cells[41] as discussed below in the section dedicated to this cell type. By contrast, non-functional T cells that do not bind MHC are eliminated through death by neglect. Cells that are positively selected become single positive cells expressing either CD4 or CD8. These cells move to the medulla for the final rounds of negative selection and maturation[42].

Negative selection ensures that most self-reactive T cells that bind with high affinity to self-antigens never leave the thymus. This process requires that the T cells be presented not only with local self-antigens but also with peripheral tissue specific self-antigens. Therefore, thymic medullary epithelial cells express genes encoding self-antigens that are otherwise only expressed by peripheral tissues[43, 44]. The protein AIRE controls this expression[45] and enhances elongation of actively transcribing genes. Tissue autophagy allows these self-proteins to be processed and presented on MHC-II[46].

DCs are also thought to play a role in negative selection. Non-activated DCs can bring peripheral self-antigens to the thymus for presentation[47]. Additionally, DCs can cross present antigens expressed by medullary thymic epithelial cells and then directly kill responding cells[48]. Whether presentation by thymic epithelial cells or DCs is primarily responsible for negative selection is an area of active research. Antigen presentation by DCs is discussed in more detail in the section on DCs.

Negative selection in the thymus does not remove all self-reactive T cells and therefore additional mechanisms discussed later in this introduction are necessary to prevent autoimmunity.

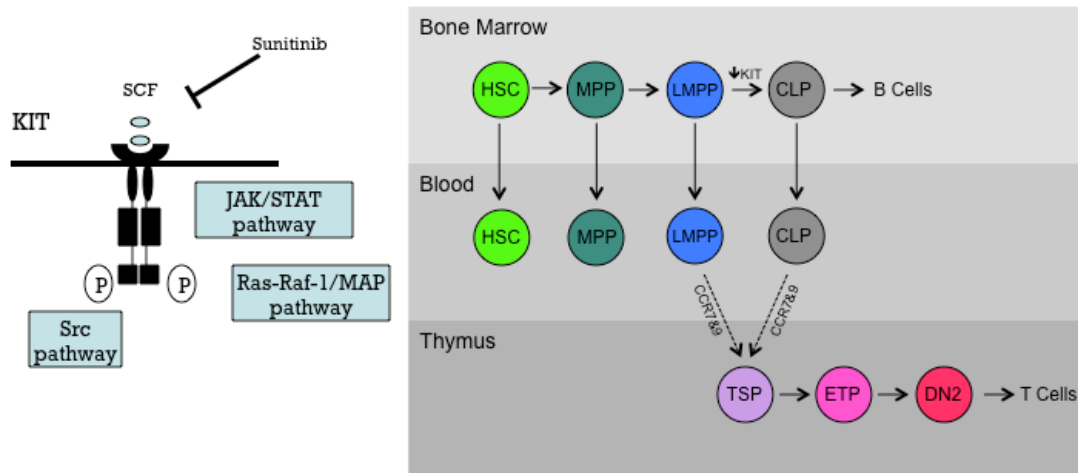


Figure 1.1. Tyrosine kinase Receptor KIT (CD117) is found on many progenitors and is inhibited by sunitinib. KIT is a tyrosine kinase receptor that binds stem cell factor. It can be inhibited by tyrosine kinase inhibitors including sunitinib. KIT is expressed at high levels on BM progenitors including HSC, MPP and LMPP, but expressed at low levels on CLP. KIT is also found on progenitors in the thymus including ETP through DN2. Thymic seeding requires expression of CCR7 and CCR9. It is not clear what cells seed the thymus although there is evidence that it is downstream from LMPP and CLP (dotted lines) since these cells can rapidly create thymocytes when injected into the blood.

1.2.3 KIT

KIT is a receptor tyrosine kinase and a marker of an early progenitor both in the bone marrow and in the thymus. KIT binds to Stem Cell Factor (SCF), also known as KIT ligand. Signals through KIT are important for survival, proliferation, and differentiation of progenitor in the bone marrow and thymus. Early progenitors in the bone marrow express high levels of KIT including HSCs, MPPs, and LMPPs, whereas KIT expression is low on CLPs (Figure 1.1). In the medulla of the bone, SCF is produced by fibroblasts and endothelial cells and is important for HSC maintenance[49]. *Sl/Sl^d* mice, which are SCF deficient, fail to maintain HSCs in vivo[50-52]. KIT is also expressed on thymic progenitors including ETPs through DN2s. However, the role of KIT signaling in the thymus is less well understood.

The importance of KIT signaling on HSCs is exemplified by the fact that animals genetically deficient in KIT are receptive to HSC engraftment without conditioning[53, 54]. The transplanted HSCs with functioning KIT have a competitive advantage over the host KIT deficient HSCs. Additionally, in Rag mice, KIT blockade via molecular antibody treatment created enough space within the HSC niche to permit mixed chimerism following hematopoietic stem cell transplant[55].

Pharmacologic inhibition of KIT is now readily available using a variety of receptor tyrosine kinase inhibitors approved for the treatment of malignancy[56-58]. Sunitinib is a tyrosine kinase inhibitor that has been

approved by the American Food and Drug Administration (FDA) for use in renal cell carcinoma[56-58]. Sunitinib inhibits KIT and multiple other receptor tyrosine kinases, including Flt3, VEGFR1, VEGFR2, VEGFR3, PDGFR α , PDGFR β , RET, and CSF1R[59, 60]. Whether or not KIT inhibition with small molecule inhibitors such as sunitinib would permit mixed chimerism similar to that seen after KIT blockade via molecular antibody treatment had not been tested prior to this thesis.

1.3 HEMATOPOIETIC STEM CELL TRANSPLANT

Hematopoietic stem cell transplantation (HSCT) has experienced great advances since initial clinical trials of this procedure were initiated for leukemia in the 1960s[61]. However, preparative regimen associated toxicities[62, 63] and morbidity related to graft versus host disease remain barriers to progress. Improvements have been made in diminishing the intensity of preparative regimens, but essentially all regimens currently used to accomplish engraftment following HSCT incorporate irradiation and/or cytotoxic agents that carry risks for tissue damage, infection, and second malignancy.

1.3.1 BENIGN BLOOD DISEASES

Benign blood diseases are a heterogeneous group of blood diseases that are grouped together by the simple fact that they are noncancerous. They range from serious diseases such as severe combined immunodeficiency (SCID) and sickle cell anemia to more simple blood disorders such as coagulopathies. HSCT

can be curative for all benign blood diseases but is generally only used in very severe cases. However, if the morbidity and mortality of HSCT were substantially reduced for non-malignant diseases, then this curative treatment may see more widespread use.

Strong cytotoxic preparative regimens for HSCT undertaken to treat malignancy is understandable, since it is often the preparative regimen that rids the body of cancer. Risks associated with cytotoxic preparative regimens are especially pertinent when HSCT is undertaken for benign disease, since short and long-term procedure related morbidity substantially increases the risk/benefit ratio for individual patients. Additionally, for benign blood diseases it is usually not necessary to achieve 100% donor chimerism to cure the disease, and therefore it may not be necessary for strong cytotoxic preparative regimens. In sickle cell anemia, for example, even as little as 11% chimerism has been shown to completely reverse the phenotype[64].

The development of a targeted, non-toxic preparative regimen that could accomplish HSC engraftment would open new possibilities for transplantation of allogeneic or gene modified autologous progenitors for benign diseases.

1.3.2 HSC ENGRAFTMENT REQUIRES SPACE WITHIN THE HSC NICHE

Preparative regimens for HSCT are designed to prevent graft rejection. Since, rejection would not be possible in patients with SCID, it would follow that they would not need such preparative regimens. However, transplantation into SCID

recipients showed less than 1% donor chimerism amongst HSCs[65-68]. Several studies have concluded that HSC engraftment requires space within the HSC niche[55, 69]. Furthermore, Czechowicz et al. reasoned that HSC engraftment was possible simply by creating space within the HSC niche[55].

This reasoning explains why animals genetically deficient in KIT are receptive to HSC engraftment without conditioning since KIT is required for HSC niche maintenance[53, 54]. SCID recipients have a full HSC niche but cannot make B or T cells while KIT deficient recipients have HSC abnormalities.

1.4 T CELLS

T cells are a heterogeneous group of cells that have been subject to a continually evolving classification system. T cells are subdivided into two major groups based on the type of receptor they express. The smaller group expresses receptors made from γ and δ chains (γ/δ T cells) while the larger group expresses antigen-binding receptors made of α and β chains (α/β T cells). α/β T cells are again subdivided into CD4 helper T cells and CD8 cytotoxic T cells based on cell surface markers, differences in how they recognize antigen, and regulatory and effector functions.

CD4 T cells emerge from the thymus as either Foxp3⁺ natural regulatory T cells (nTregs) or as naïve T cells that can differentiate into induced regulatory T cells (iTregs) or helper T cells including T_{H1} cells, T_{H2} cell, or T_{H17} cells. Tregs are a specialized subpopulation of T cells that act to suppress activation of the

immune system and thereby maintain immune system homeostasis and tolerance to self-antigens. Tregs are discussed in more detail later in the introduction under “Immune Regulatory Mechanisms”.

Peripheral differentiation of T cells into the different T cell subsets is induced by cytokines[70] and regulated by transcription factors specific for each subset. T_{H1} cells secrete IFN- γ and lymphotoxin and are important stimulators of the cellular immune system. Their differentiation is regulated by the transcription factor T-bet. T_{H1} cells promote the proliferation of CD8 T cells, induce production of opsonizing antibodies, and increase the killing efficacy of macrophages. T_{H2} cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 and are important stimulators of the humoral immune system. Their differentiation is regulated by the transcription factor Gata-3. T_{H2} cells stimulate B cells to proliferate, undergo class switching, and increase neutralizing antibody production. Both T_{H1} And T_{H2} cells secrete cytokines that serve in positive feedback loops preserving their respective lineages and promoting the differentiation of naïve cells into their respective lineages. Historically, T cells were thought to commit to one subset for the duration of their life. However, more recently it has become clear that there is plasticity between the T cell subsets. T_{H1} cells can be induced to express Gata-3 and produce T_{H2} cytokines, and visa versa[71].

T_{H17} cells secrete IL-17 and are stimulators of the inflammatory system. Their differentiation is induced by TGF- β and IL-23 with IL-6 or IL-21 and is regulated by the transcription factors ROR γ t, ROR α , and STAT3[72]. T_{H17} cells stimulate epithelial cells at barrier surfaces to express CXC chemokines, such as CXCL1 and

CXCL5, resulting in the recruitment of neutrophils[73, 74]. IL-17 also induces the expression of antimicrobial peptides including liboclain-2, Reg3gamma, Beta-defensins, and calprotectin, which control bacterial dissemination of microbes from the gut[73, 75]. The T_{H17} lineage is related to the Treg lineage since both are induced by TGF- β .

CD8 T cells and rarely CD4 T cells can develop into cytotoxic T lymphocytes (CTL). When CTLs encounter their cognate antigen, they can kill the cell expressing the antigen if they receive appropriate co-stimulatory activation. CTLs can produce perforin, which inserts into the membrane of target cells and lysis them, or they can induce apoptosis in target cells through Fas/Fas ligand interactions. CTL's main targets are virus-infected cells.

TCRs can only recognize antigens that are displayed on MHC molecules. In general, CD4 T cells recognized peptides displayed on the MHC-II protein complex of antigen presenting cells (APC). These peptides are derived from extracellular sources that have been acquired by the APC through an endocytic process. CD8 T cells generally recognize peptides displayed on MHC-I. Peptides displayed on MHC-I are derived from internal sources and can come from intracellular pathogens.

1.4.1 THE IDEA OF A T CELL NICHE

The T cell niche is an abstract idea since it does not exist exclusively in one region of the body but rather circulates between multiple lymphoid organs.

However, given that the number of lymphocytes in an adult animal remains stable throughout life, it can easily be thought of as a niche that can be full or depleted. Similar to the bone marrow cells in their niche, T cells respond to signals from both stromal and hematopoietic cells that govern their ability to proliferate and expand. There is a homeostatic equilibrium between the number of T cells produced by the thymus, the number of T cells created in the periphery through cell division, and the number of dying T cells. In this way, the size of the T cell pool and the CD4/CD8 ratio are tightly controlled[76]. Although it is clear that there is a regulated homeostatic balance, all of the mechanisms that control this balance are not known.

1.4.2 LYMPHOPENIA AND HOMEOSTATIC PERIPHERAL EXPANSION

Lymphopenia is the term used to describe the state of individuals with decreased numbers of lymphocytes. There are many disease processes that cause lymphopenia such as infection, malnutrition, stress, sarcoidosis and various autoimmune disorders. Lymphopenia is also commonly caused by medical treatments such as irradiation given as a preparative regimen for bone marrow transplant or cytotoxic therapies given to treat cancer patients. Lymphopenia can be classified as T cell, B cell or NK cell lymphopenia. More often than not, multiple lineages of lymphocytes become depleted at the same time. For simplicity, the body of work presented here focuses on T cell lymphopenia.

During T cell lymphopenia, the body attempts to return the T cell numbers to normal homeostatic levels. The body can recover its T cell numbers through

expansion of peripheral T cells, also known as homeostatic peripheral expansion (HPE)[77] or through increased thymopoiesis. The primary way the body does this is through peripheral T cell expansion, in part because thymic involution occurs early in life and thymic regenerative capacity is limited[78, 79]. HIV studies have shown a clear correlation between thymic output and thymic size[80-84]. Although, thymic rebound is possible until the fifth decade of life in humans[79, 85], even when thymic recovery occurs it is typically delayed for at least 1 year following the onset of lymphopenia. Therefore, early T cell regeneration even in young adults would likely be from peripheral expansion and not from thymic output. Less than half of adults who sustain lymphopenic insults show thymic-dependent T cell regeneration[79].

HPE is very different than antigen driven proliferation. Similar to antigen-driven proliferation, HPE requires MHC-TCR interactions. Historically, HPE was thought to be driven solely by low affinity self-antigens, which normally induce positive selection in the thymus[86-93]. However, recent work has shown the HPE involves proliferation against both self and commensal antigens[94, 95]. The contribution of commensal antigens was demonstrated by the fact the germ-free mice experience greatly diminished HPE[94]. It seems there is still an important role for self-antigens since lymphopenia induced in germ-free mice still yields some T cell proliferation and since in antibiotic treated mice T cell proliferation is decreased in the MLNs but not peripheral lymph nodes[95]. This suggests that the antigen driving the proliferation in the peripheral lymph nodes is likely a self-antigen.

IL-7 is absolutely required for HPE[96] while IL-4, IL-12, and IL-15 can drive HPE but are not required[97]. On the other hand, co-stimulation through CD28 and signals from IL-2 are necessary for antigen driven proliferation but not HPE. There is some data indicating that the result of HPE is a transient memory-like state both phenotypically and functionally. This memory-like state is achieved without going through a state of acute activation and may be short lived although this is still a controversial area[98].

1.4.3 LYMPHOPENIA PREDISPOSES TO AUTOIMMUNE DISEASE

During lymphopenia, T cells show enhanced immune reactivity resulting in increased activation and proliferation in response to self and commensal antigens[94]. As a result, lymphopenia predisposes individuals to autoimmune diseases such as inflammatory bowel disease[99-103].

In mouse models of lymphopenia, thymectomy has led to thyroiditis[104, 105], TCR mutant mice have been shown to develop inflammatory bowel disease[106, 107] and many other lymphopenia inducing mechanisms, such as ionizing radiation[108] and cyclophosphamide[109] treatment, have led to autoimmunity. Additionally, Tregs have been demonstrated to prevent autoimmune colitis during lymphopenia, and Treg-depleted cell infusions induce colitis in lymphopenic[110] but not lymphoreplete settings[99].

While the physiologic response to lymphopenia heightens the risk for autoimmunity, the vast majority of mice and humans who sustain a lymphopenic

insult do not experience autoimmunity or loss of tolerance[103]. How self-tolerance is maintained in the presence of HPE, which should induce substantial T cell activation in response to self-antigens, remains unclear.

1.5 INNATE CELLS AND THEIR INTERACTION WITH THE T CELL NICHE

There is a very important interaction between the innate and adaptive immune systems. The adaptive immune system and T cells in particular are reliant on innate cells for signals that guide development and proliferation. HPE, for example, requires both IL-7 and TCR stimulation by DCs[76]. In this way, the size of the T cell niche is controlled, in part, by signals from innate cells, especially DCs.

1.5.1 DENDRITIC CELLS

As previously discussed T cells recognize antigen that is presented to them on MHC complexes on other cells. The cells that present antigen to T cell are referred to as antigen presenting cells (APCs). The APCs that are most effective at antigen presentation are dendritic cells (DCs), often referred to as professional antigen presenting cells.

The common DC precursor (CDP) develops in the bone marrow and differentiates into plasmacytoid DCs (pDCs) and pre-DCs. Pre-DCs circulate in the blood and eventually take up residence in the tissues where they differentiate into immature DCs in a Flt3L-dependent fashion[111-115]. Under inflammatory

conditions, monocytes may also develop into inflammatory DCs in a GM-CSF-dependent fashion[116].

Immature DCs sample antigens through endocytosis looking for possibly harmful pathogens and damaged tissues[117, 118]. Immature DCs express Fc receptors, receptors for heat shock proteins, and various pattern recognition receptors. Pattern recognition receptors are designed specifically to recognize conserved patterns from many invading pathogens. These conserved patterns are referred to as pathogen associated molecular patterns (PAMPs) and include everything from LPS to flagella. There are various types of pattern recognition receptors such as receptor kinases and C-type lectins, but the best-studied are the Toll-like receptors (TLRs) where each one recognizes different conserved microbial molecules[119]. Lipopolysaccharide (LPS), an endotoxin found on the bacterial cell membrane, is an example of a PAMP that is recognized by TLR4[120]. TLR activation leads to a complex cascade of events that include NF- κ B signaling and the MAP-kinase pathway; this in turn leads to secretion of pro-inflammatory cytokines that can signal to other cells. Pattern recognition receptors may also detect damage associated molecular patterns (DAMPs) released from necrotic cells that can initiate the non-infections inflammatory responses[121].

DC maturation is driven by inflammatory signals from other hematopoietic cells or by danger signals from invading pathogens or dying cells. Such signals include activation of pattern recognition receptors, pro-inflammatory cytokines, and signals from activated T cells such as CD40-ligand. When a DC matures it down regulates pattern recognition receptors and increases expression of MHC

and CD80/86. It acquires the capacity to produce cytokines such as IL-6, IL-12, and IL-23. It also begins to express surface markers such as ICOS and Notch ligand that can aid in T cell polarization. Since most naïve T cells reside in the lymph nodes, upon maturation DCs up regulate expression of chemokine receptors CCR2 and CCR7 which allows them to migrate to the lymph node where there is a greater chance of encountering the T cell specific for the antigen it is displaying on its MHC molecules.

Before APCs can present antigens to T cells, they must process the antigen for presentation. There are two complicated antigen-processing pathways specialized for extracellular and intracellular sources. Briefly, extracellular microbes and proteins are taken up by phagocytosis or endocytosis. The proteins live within endosomes and lysosomes with a very low pH where they are fragmented into peptides and loaded onto MHC-II for presentation. The peptide loading of MHC-II stabilizes its structure so that together they can be transported to the cell surface. Intracellular proteins are fragmented into peptides in the cytosol by the proteases in the proteasome. These peptides are transported to the rough endoplasmic reticulum where they are loaded into MHC-I. Presentation of intracellular proteins by MHC-I is performed by almost all cells and is not specific to APCs.

Although CD4 T cells generally recognize extracellular peptides displayed on MHC-II and CD8 T cells generally recognize intracellular peptides displayed on MHC-I, this is not always the case. Through a process called cross-presentation,

DCs take up other cell types that are infected by intercellular pathogens and display the pathogen-derived peptides on MHC-I.

Like T cells, DCs have been classified and categorized into many subsets. The first major division is between plasmacytoid DCs (pDCs) and conventional DCs (cDCs). pDCs arise directly from the common DC precursor (CDP) while the path to cDCs involves an intermediate precursor, the pre-DC. pDCs are specialized at producing large amounts of type I interferon, an important anti-viral compound[122]. Some pDCs have also been shown to have tolerogenic properties although it is clearly not a universal property of this subset[123, 124].

Lymphoid tissue cDCs have been further subdivided based on expression of CD4, CD8 α , and CD11b. CD8 α ⁺CD11b⁻CD4⁻ DCs are dependent on the transcription factors Batf3 and IRF-8 for their development[125-127]. They are enriched within the T cells zones[128] and are specialized in cross-presentation and are important for inducing peripheral tolerance[129, 130]. CD8 α ⁻CD11b⁺ DCs are dependent on the transcription factors RelB, IRF-2 and IRF-4[131-133]. They reside in the marginal zone[128] where they are important scavengers[127].

Non-lymphoid tissue DCs have also been subdivided into different groups. In particular α E-integrin, CD103, and the fractalkine receptor CX3CR1 have been used to describe distinct DC subsets at mucosal surfaces[134], although more recent studies have shown that CX3CR1⁺ mucosal cells may be better characterized as inflammatory monocytes and not DCs. CD103⁺ DCs have some

tolerogenic properties and are described in more detail below under the section entitled “Immune Regulatory Mechanisms”.

1.5.2 DENDRITIC CELL AND T CELL INTERACTIONS

DCs are important for controlling T cell responses. Antigen presentation by immature DCs, for example, may induce T cells into a state of anergy that is important for maintaining tolerance[135]. This means that when DCs process and present self-antigen from dying apoptotic cells and the DCs do not receive sufficient danger signals to induce DC maturation, they will not be mature enough to elicit complete immune responses from T cells. In this way, the body maintains a state of tolerance toward non-harmful self-antigens.

When DCs receive sufficient danger signals to become mature, they gain the ability to elicit complete immune responses from T cells. Even mature DCs maintain control over the T cell response since T cell differentiation and polarization into effector T cells requires three signals from DCs: MHC/TCR interaction, co-stimulation usually through ligation of CD28 on T cells with CD80 or CD86 on DCs, and cytokine signals. The cytokine signals, in particular, are important for guiding T cell polarization.

DCs take on different maturation phenotypes depending on the microenvironment and specific pathogen signals that induce their maturation. This phenomenon is referred to as DC conditioning. DCs can then elicit tailored pathogen-specific immune responses from T cells that change depending on the

DC's maturation phenotype[136, 137]. In this way, the immune system elicits the appropriate T cell response for the invading pathogen. For example viruses elicit cytotoxic T cell responses while fungal infections elicit polarization toward T_{H17} cells.

1.5.3 INNATE LYMPHOID CELLS

Recent work in immunology has uncovered a heterogeneous group of innate lymphoid cells (ILCs) that play important roles in protective immunity, lymphoid tissue formation, and in the homeostasis and repair of tissue stromal cells[138]. Although this family of cells has diverse functions, they are developmentally related. They all require expression of the transcriptional repressor Id2 as well as signals from one of the common gamma chain cytokines[138, 139]. Analogies have been drawn between these ILCs and T helper cells as many of the ILCs are under the control of the same transcription factor as analogous T cell subsets. The family of ILCs has been referred to in the literature as an “innate version” of T helper cells[138].

The most well studied ILCs are NK cells that derived their name from their ability to lyse and kill other cells. In addition to their ability to lyse cells, they are also efficient producers of IFN- γ [140]. They express activating receptors that allow them to detect and lyse tumors and virally infected cells, as well as Fc receptors that allow them to detect and lyse antibody-coated cells. Importantly, NK cells also express inhibitory receptors for MHC molecules that shut off their lysing abilities[141]. This keeps them from killing normal self-tissues but allows

them to kill virally infected cells that have evaded immune surveillance by down regulating MHC expression.

LTi cells, another well-studied member of the ILC family, are important for the early development of lymph nodes and Peyer's patches[142]. Other members of the ILC family discovered more recently have been referred to as LTi-like cells. Early reports on these other subsets used many names for these cells. For simplicity, in this thesis they will be referred to by the nomenclature described by Spits and Di Santo which emphasizes their analogy to T helper cells[138].

ILC2 cells (including nuocytes and natural helper cells) are similar to T_{H2} cells in that they respond to IL-25 and IL-33 by proliferating and producing T_{H2} cytokines including IL-5 and IL-13. ILC2 cells help mediate the expulsion of infectious parasitic helminthes.

LTi cells, ILC17 cells, and ILC22 cells all express ROR γ t and are dependent on IL-7 signals for their development. Therefore, mice with mutated IL-7R (IL-7R^{-/-} mice) would be expected to be completely deficient in these populations. Since these mice were used extensively in this body of work, it is important to understand the physiological role of these cells and the implications of their absence in IL-7R^{-/-} mice.

LTi cells induce the formation of lymph nodes and Peyer's patches during embryogenesis. Interactions and clustering with stromal cells induce the LTi cells to express adhesion molecules and secrete chemokines that recruit

hematopoietic cells including B cell, T cells and DCs[138, 143, 144]. LTi cells can produce IL-17 and IL-22 suggesting they also play a role in tissue remodeling and host defense[145, 146]. LTi cells are under the transcriptional regulation of Id2 and ROR γ t, and their development is dependent on IL-7R[147]. Thus IL-7R^{-/-} mice have dysregulated lymph node formation and resultant very small lymph nodes.

ILC17 cells are found mostly in the gut. As their name suggests, they produce IL-17. These cells accumulate during inflammation and produce IL-17 in response to IL-23 signals[138, 148].

ILC22 cells reside at mucosal interfaces including the tonsils and the intestinal tract. They express Nkp46 but are different from NK cells because they do not express NK1.1, produce IFN- γ , nor possess the ability to lyse other cells. ILC22 cells secrete large amounts of IL-22 in response to IL-23[149-151]. IL-22 production in the gut is crucial for maintaining the integrity of the gut barrier function[152], suggesting the mice which lack ILC22 cells, such as IL-7R^{-/-} mice, may have decreased gut barrier function.

1.6 IMMUNE REGULATORY MECHANISMS

The immune system, like most biological systems, is subject to intense regulatory control. Broad and complex control mechanisms are necessary to control the timing, specificity, magnitude, and duration of immune responses. Negative selection in the thymus, for example, is one control mechanism that protects

against self-reactive T cells. Negative selection can only protect the body from self-reactive T cells that are specific for self-antigens presented in the thymus. There are numerous self-antigens not presented in the thymus, and other regulatory mechanisms are needed to protect the body from T cells specific for such antigens. Further, since antigens from commensal flora are also not present in the thymus, regulatory mechanisms are necessary to protect the body from immune responses directed towards the body's own commensal microbiota. Once T cells have left the thymus, they are subjected to other regulatory mechanisms including anti-inflammatory cytokines, Tregs, and tolerogenic DCs. Highlighted below are the regulatory mechanisms relevant to the work presented in this thesis.

1.6.1 ANTI-INFLAMMATORY CYTOKINES

There are several anti-inflammatory cytokines that serve to limit and control immune responses. Additionally, there are cytokines that have inflammatory roles in some settings and anti-inflammatory roles in other settings. TGF- β and IL-10 are two of the most well studied anti-inflammatory cytokines and are important for immune regulation.

IL-10 is a type II cytokine produced by many different cells types[153]. Historically, IL-10 was identified as an inhibitor of T_{H1} functions since it was first described in T_{H2} cells where it was produced together with IL-4, IL-5 and IL-13[154, 155]. However, many studies have now shown that IL-10 can also be produced by T_{H1} cells[156, 157]. For both subsets, IL-27 and TGF- β act together

to induce IL-10 production[158, 159]. Subsequently T_{H17} cells have been shown to produce IL-10 in response to TGF- β and IL-6 signals[160]. Myeloid cells also produce IL-10. For example, TLR activation, particularly TLR2, can lead to IL-10 production by DCs. Importantly, in some settings Tregs produce large amounts of IL-10, and it is required for their suppressor function[161, 162]. IL-10 production by Tregs requires TGF- β and is induced by IL-2 and IL-4[163-165].

IL-10 primarily targets leukocytes and limits their secretion of proinflammatory cytokines. This is important for limiting and terminating inflammatory responses[153] and prevents tissue damage caused by infection and inflammation[161]. For example, IL-10 inhibits DC and Macrophage activation[166] and further inhibits their ability to produce IL-12 and IL-23 once they are already activated during inflammation[167]. The role of IL-10 on DCs and macrophages is particularly important for maintaining immune homeostasis in the gut. Since IL-10 plays a central role during the resolution phase of inflammation, it is not surprising that IL-10 deficient mice develop severe autoimmune disease including chronic enterocolitis[168, 169]. The IL-10 pathway has been exploited by pathogens[155, 170-173] and tumors[174] to help them evade immune detection.

TGF- β exists in multiple isoforms that have different functions, and it plays important roles in apoptosis, cell cycle, and immunity. In the immune system, TGF- β is an important modulator of Treg induction and function, such that TGF- β signaling can induce Foxp3 expression and a regulatory phenotype on naïve CD4⁺CD25⁻ T cells[175], and is required for expression of IL-10 by Tregs[163].

1.6.2 REGULATORY T CELLS

Tregs actively suppress immune function and are important for maintaining immune homeostasis. Natural Tregs (nTregs) are selected in the thymus to become Tregs. Their selection requires recognition of self-peptide since Tg mice with TCRs specific for foreign antigens do not make nTregs, but introduction of the foreign cognate ligand in the thymus reestablishes the ability to develop nTregs[176-179]. The strength of the TCR interaction is also important for Treg selection. The TCR repertoire from Tregs is just as diverse as, and partially overlapping with, the repertoire from conventional T cells[180-183] but has increased self-reactivity[180]. Current analysis suggests that Tregs therefore have TCR affinities for self peptide-MHC molecules that are higher than those that mediate positive selection of conventional CD4⁺ T cells. However, retroviral expression of Treg TCR in effector T cells of a defined specificity has demonstrated weak responses to syngeneic APCs compared to robust responses to Tg TCR-recognized foreign ligands[180]. Therefore, current thinking is that the affinities of Tregs for self-antigens are likely below the affinity range of conventional TCRs for foreign antigens[41]. Also, since Foxp3 deficient mice have been shown to have effector cells with the same TCRs as Tregs in normal mice, they must have sufficiently low TCR signal strength to avoid negative selection in the thymus[184].

In addition to TCR signals, nTreg development requires CD28 mediated co-stimulation[185, 186], and cytokine signaling through the common γ -chain.

TGF- β signals are important for Treg survival in the thymus by inhibiting Bim-dependent apoptosis of self-reactive T cells[187]. Also, NF- κ B seems to be important for the development of nTregs, but the exact mechanism has not been determined[41].

iTregs emerge from the thymus as normal naïve T cells but can differentiate into Tregs when they first encounter their cognate antigen with low CD28 co-stimulation in the presence of TGF- β [187]. While differentiation of nTregs requires interactions with self peptide-MHC complexes, the differentiation of iTregs generally occurs in response to non-self-antigens such as commensals and food. In addition to TCR signals, iTreg differentiation is facilitated by high amounts of TGF- β [188-191]. In contrast to nTreg development, CTLA-4 is required for the TGF- β induction of iTregs[192], and CD28 co-stimulation limits their differentiation[193, 194].

Once fully matured in the periphery, it is difficult to distinguish iTregs and nTregs. Importantly, both nTregs and iTregs express the transcription factor Foxp3 that is the master regulator of their function. Bone marrow chimeras with mixed wild-type and Foxp3-deficient bone marrow showed that Tregs only develop from wild-type precursors providing evidence for the critical role of the Foxp3 transcription factor in Treg development[195]. The fact that both humans and mice that lack functional Foxp3 manifest very severe autoimmune phenotypes, testifies to the necessity of Foxp3 in the development of Tregs and their indispensable role in maintaining immune homeostasis[196-200].

It has been well established that T cells, including naïve and memory pools, require IL-7 survival signals for maintenance. Although there is conflicting evidence, Tregs might be the one exception. Tregs have low expression of IL-7R compared to effector T cells[201]. They are dependent on IL-2 for survival signals, and whether or not they require additional signals from IL-7 is controversial. Mice that lack IL-2 signaling have a 50% decrease in Tregs[202, 203] while mice deficient in IL-7 or IL-15 have normal Treg numbers[204]. However, combined loss of all three cytokines or loss of the gamma-c cytokine receptor results in a complete loss of Treg cells[204]. By contrast, one study found the IL-7R signaling was required for the development of Tregs but that IL-7 and TSLP played redundant roles[205]. This was contradicted by others finding that IL-7 was not absolutely required and that there was no role for TSLP[204], as well as the fact the IL-7R^{-/-} mice have been shown to have Tregs[206].

1.6.3 TOLEROGENIC DCs

It is now clear that not only immature DCs but also activated mature DCs can drive protective tolerogenic immunity. pDCs have been shown to regulate peripheral (and recently central) tolerance while CD103⁺ DCs are important for promoting tolerance at mucosal surfaces such as the gut.

pDCs can be powerful immunostimulatory cells. They produce large amounts of type I interferon in response to foreign nucleic acid, and can recruit and/or activate nearly all immune cell types providing an important link between innate and adaptive immunity[124, 207, 208]. However, pDCs have also been shown to

have a tolerogenic role. They can present self-antigens and can prime CD4⁺ T cells to become IL-10 producing Tregs[209-211]. pDCs that express CCR9 have been shown to be able to suppress acute graft versus host disease in vivo[123]. pDCs may also play a role in central tolerance by transporting peripheral antigens to the thymus[212]. A recent review of pDC function has questioned the way that pDCs were defined in these studies and concludes that although pDCs may have some tolerogenic properties in certain situations, it is not a universal quality of all pDCs[124].

Differentiating between harmful and beneficial microbes is particularly important at mucosal surfaces such as the gut where there is high exposure to harmful pathogens but also non-harmful commensal flora and food-antigens. CD103⁺ DCs are known to be specialized in promoting tolerance to non-harmful antigens in this complex and dynamic region. A subset of CD103⁺ DCs in the gut sample antigen at the mucosal surface and then migrate to the MLN, in a CCR7 dependent manner[213, 214], where they can present the antigen to naïve T cells[215].

Originally it was thought that these cells exclusively played a tolerogenic role and were specialized only in the induction of Foxp3⁺ responses in the lymph nodes[17]. Indeed, CD103⁺ DCs show increased expression of CCR6, CCR7, TLR5, TLR9, and co-stimulatory molecules but decreased expression of other TLRs[216]. They also showed decreased phagocytic activity and decreased expression of proinflammatory cytokines compared to CD103⁻ DCs[216]. In the MLN, CD103⁺ DCs induce expression of Foxp3 by naïve T cells and imprint gut

homing marker CCR9 and $\alpha_4\beta_7$ on T cells and B cells, which allows them to migrate to the gut[194, 213, 217, 218]. Additionally, CD103⁺ DCs produce retinoic acid and TGF- β , which is required for the induction and imprinting of Tregs[16, 194, 217].

Although it is not clear how they acquire luminal antigens, CD103⁺ DCs appear to be the only DCs that can present orally administered antigens in the MLN[111, 215]. Since CD103⁺ DCs are the only DC subset known to migrate from the gut mucosa to the MLN[213, 215, 219, 220] in the gut it would also be necessary for them to play a role in immune defense against invading pathogens[17]. Indeed, one study has shown CD103⁺ DCs can produce IL-23 in response to bacterial flagellin[221]. Modulation of the microenvironment during inflammation may change the function of CD103⁺ DCs and needs to be further explored. Perhaps there is a subset of CD103⁺ DCs, such as CD8a⁺CD103⁺ DCs, that is responsible for inflammatory responses[222].

1.7 CANCER IMMUNOTHERAPY

The immune system plays a very important role in preventing tumor formation and growth as evidenced by the fact that immunodeficiency leads to an increase in tumor incidence[223, 224]. The immune system can not only rid the body of cancer causing pathogens, but also detect tumor specific antigens or molecules induced by cellular stress. Through natural selection, tumors develop ways to avoid immune detection and create immunosuppressive microenvironments[225]. For example, tumors have been shown to recruit Tregs

to foster immune privilege[226, 227], secrete anti-inflammatory cytokines such as IL-10[174], down regulate MHC-I[228], increase IDO expression to limit T cell proliferation[229], and even rearrange stromal microenvironments to look like normal lymphoid tissue and promote tolerance[230]. Although lymphocytes can often be found infiltrating tumors, their effector function is limited by immunosuppressive molecules including cytotoxic T lymphocyte antigen 4 (CTLA4), T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), lymphocyte activation gene 3 protein (LAG3), and programmed cell death protein 1 (PD1)[231, 232].

A very large field of cancer immunotherapy has developed around using the immune system to fight tumors. Some of the therapeutic approaches have included blocking the inhibitory pathways of immune evasion or injecting proinflammatory stimulants. Immunotherapy based on adoptive transfer of naturally occurring or genetically engineered T cells has shown some promising results[233].

In certain types of cancers, tumor-infiltrating lymphocytes (TIL) have been associated with better prognosis [234-236]. TILs have been found to be tumor specific[237, 238], and new elaborate but promising therapies have been designed around them. Tumor specific TILs that have been extracted and conditioned ex vivo have shown some promising results when re-introduced into hosts[239]. The ex vivo conditioning removes the cells from the immunosuppressive tumor environments and allows their activation and clonal expansion. While adoptive transfer of autologous TILs has been curative for

some patients, more than 50% of recipients seem to be non-responders[240-242]. The exact mechanisms that determine whether or not a patient will respond to such therapy are not clear. It could have to do with the fact that effector T cell proliferation and function is often limited in the tumor microenvironment[243, 244]. Understanding the mechanisms that control the proliferation of the adoptively transferred cells could lead to more favorable responses. Currently, adoptive cell transfer is not FDA approved, and much work is needed to take this promising technique to a clinical setting.

1.8 INTERLEUKIN-7 RECEPTOR

1.8.1 MECHANISMS OF IL-7R SIGNALING

Historically IL-7 has been thought to signal through a PI3K/Akt or a Jak/STAT pathway. However, recent evidence has shown that at low physiologic levels of IL-7 PI3K is dispensable for IL-7 induced survival of T cells[245] and whether or not the PI3K pathway is important for homeostatic expansion is not clear[246]. The Jak/STAT pathways for both IL-7 and TSLP signaling, however, have been well described. IL-7R serves as part of a heterodimer receptor for both IL-7 and TSLP. IL-7R together with the common gamma chain (γ c) make the receptor for IL-7 while IL-7R together with TSLPR (CRLF2) make the receptor for TSLP (Figure 1.2). IL-7R associates with Jak1, γ c associates with JAK3, and TSLPR associates with JAK2 [74]. As a result, IL-7 signals via JAK1 and JAK3 to mediate phosphorylation of STAT1, STAT3 and STAT5, while TSLP signals via JAK1 and JAK2 to phosphorylate STAT5[247]. The STAT transcription factors translocate to the nucleus where they control expression of target genes important for T

cell survival and proliferation. STAT5 mutant mice as well as mice overexpressing SOCS1, which inactivates the Jak family, have been shown to have decreased peripheral T cell numbers[248, 249].

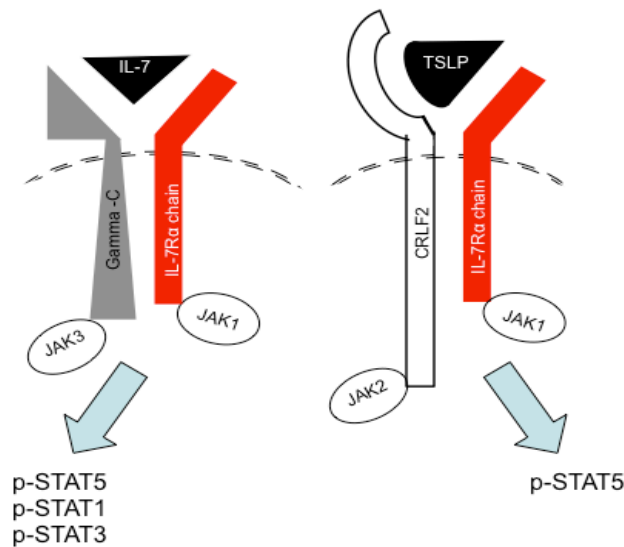


Figure 1.2. IL-7R forms heterodimeric receptors with the common gamma chain to bind IL-7 and with CRLF2 to bind TSLP. IL-7 signals through JAK 1 and JAK3 to yield phosphorylation of STATs 1,3,&5. TSLP signals through JAK1 and JAK2 to yield phosphorylation of STAT5.

1.8.2 IL-7 IS A HOMEOSTATIC CYTOKINE IMPORTANT FOR HPE

IL-7 is produced primarily by stromal and parenchymal cells[250] with lesser amounts produced by DCs; IL-7 is not produced by B cells or T cells. IL-7 production is focused primarily in lymphoid tissues including the thymus, bone marrow, lymph nodes, spleen and gut. In the thymus IL-7 is produced by cortical and medullar epithelial cells[251, 252]. In secondary lymphoid organs the stromal populations that produce IL-7 are Gp38⁺CD31⁻CD35⁻ fibroblastic reticular cells found in the T cell zones[251, 253-255]. IL-7 is a γ c cytokine because like IL-2, -4, -9, -15, and -21 it signals through γ c. Unlike receptors for most γ c cytokines, which are absent on resting cells but upregulated upon

activation, the IL-7R is expressed on most resting lymphocytes, downregulated following T cell activation, then re-expressed on effector cells destined for the memory cell pool[256] and on central memory T cells. Because IL-7 is produced by non-immune cells in the absence of immune activation, is measurable in sera from healthy individuals, and the IL-7R heterodimer is present on most T cells, IL-7 can be conceptualized as a homeostatic cytokine whose signals are continuously available and play an important role in maintaining T cell homeostasis.

The corollary to IL-7's central role in T cell homeostasis is that the availability of IL-7 is increased during lymphopenia[257-259]. Increased IL-7 availability plays a central role in driving immune reconstitution through HPE, which involves exaggerated responses to cognate antigen[260], increased antigen-independent cycling of memory populations and proliferative responses to low affinity antigens comprising both self-antigens and cross-reactive environmental and commensal antigens[86-88, 94]. IL-7 is notable for its immunorestorative properties, its ability to augment both CD4 and CD8 expansion, its tendency to induce cycling of naïve T cells with broad repertoire diversity, and its ability to amplify low affinity, subdominant immune responses such as those required for antitumor immunity. In contrast to the immune activating effects described above, IL-7R signaling on DCs has also been recently implicated in a regulatory axis that controls CD4 homeostatic expansion during lymphopenia[261], an observation that will be explored in more detail here. While IL-7 induces proliferation and survival of naïve T cells, TSLP generally only provides survival signals but does not directly induce their proliferation[262-264]. Although, one

report has shown that TSLP may have a direct effect on the effector function of T_{H2} cells[265].

1.8.3 IL-7R EXPRESSION

The IL-7R is found not only on lymphocytes but also on stromal cells, DCs, and innate cells. On stromal cells IL-7R is thought to play a small role in IL-7 driven feedback inhibition of IL-7 production[246]. The role of IL-7R on DCs is best understood in terms of the function of TSLP signaling on DCs. TSLP signaling induces DC expression of OX40L, which is necessary for DCs to promote a T_{H2} phenotype in T cells[266, 267]. TSLP signaling is particularly important in the gut and other mucosal surfaces where epithelial cells produce TSLP that acts on DCs and causes them to promote a T_{H2} phenotype. TSLP also plays a role in normal gut homeostasis. It is constitutively expressed by gut epithelial cells, and plays a role in tonic signals to DCs[268-271]. TSLP has also been shown to indirectly increase T cell homeostatic proliferation through its tonic signals on DCs[272].

IL-7R is expressed on and required for the development of innate lymphoid cells including LTi cells, ILC-17 cells, and ILC-22 cells. Mice that lack IL-7R signaling including $IL-7R^{-/-}$, $Jak3^{-/-}$, and $\gamma c^{-/-}$ mice all have defects in lymphoid organogenesis. This is because development of secondary lymphoid structures requires LTi cells. Peyer's patches and lymph node development is a three step process: (1) IL-7R+ LTi cells migrate to the lymphoid organ anlagen where they signal resident mesenchymal lymphoid tissue organizer cells to upregulate

expression of VCAM1 and ICAM1 (2) Clustering of IL-7R⁺ LTi cells (3) Recruitment of T and B cells. Lymphoid tissue development is possible even without the final step as demonstrated by the fact that Rag mice still develop normal numbers of lymph nodes and Peyer's patches. IL-7R^{-/-} mice however, do not develop any Peyer's patches and only limited lymph nodes. Interestingly, IL-7^{-/-} mice also do not develop normal secondary tissue but this can be overcome by over expression of TSLP[273].

1.8.4 REGULATION OF IL-7R EXPRESSION

IL-7R expression is down regulated by IL-7 itself. The expression of IL-7R is controlled by both transcriptional and post-translational mechanisms. IL-7R is continuously being endocytosed from the cell surface and then recycled back to the surface. Only a small fraction of what is endocytosed is degraded. The fraction that is degraded is increased by IL-7 stimulation. Ephrins interact directly with IL-7R to stabilize its expression at the cell surface. IL-7R down regulation in response to IL-7 signals is increased in ephrin deficient mice while IL-7R down regulation is delayed in mice with overexpression of ephrins[274, 275].

Gfi-1 is a transcriptional repressor down stream of Cdc42/PAK1 that is well known for its role in IL-7R down regulation in CD8 T cells[276, 277]. IL-7R transcription is also inhibited by recruitment of the histone deacetylase HDAC1 on the IL-7R promoter allowing further recruitment of Gfi-1[278]. While Gfi-1 inhibits IL-7R expression, GABP and Ets-1 are transcription factors that promote

IL-7R expression[279-281]. Foxo 1 is a transcription factor that is inactivated by the PI3K/Akt pathway downstream of TCR and possibly IL-7R itself. T cell specific decreases in the transcription factor Foxo 1 have been shown to lead to decreases in IL-7R expression[282-285].

Both IL-7R and MHC signals are required for T cell homeostasis but how these two signals work together is still an area of active research[246]. Crosstalk between the two receptors is thought to maintain T cell homeostasis. IL-7 has been shown to enhance transcription of CD8, a cofactor for TCR signaling on CD8 T cells[286]. Another possibility that has been suggested is the TCR signaling tunes T cell sensitivity to IL-7. This is supported by the fact that the strength of the TCR affinity to self-pMHC ligands correlates with the efficiency of naïve T cells to undergo HPE. HPE is thought to be driven not only by availability of homeostatic cytokines but also by cell intrinsic factors that affect the cells ability to respond to IL-7 and other homeostatic cytokines. T cell sensitivity to homeostatic cytokines has been shown to correlate with expression levels of lipid rafts[287]. Homeostatic TCR signaling may promote lipid raft formation, which has been shown to enhance IL-7 sensitivity[287]. It has also been shown, however, that homeostatic TCR signaling in specific conditions can decrease IL-7R signaling and thus reducing CD8 expression[286]. Additionally, RASA1 and RasGAP both downstream of TCR are capable of antagonizing IL-7 responsiveness[246]. The interplay between IL-7R and TCR signaling is complex and it appears that each receptor may be responsible for fine-tuning responsiveness of the other.

1.8.5 THE IL-7R^{-/-} MOUSE MODEL

Much of the current understanding of IL-7R has come from experiments using an IL-7R^{-/-} mouse strain. This strain was developed through insertional mutagenesis of the IL-7R gene, thus inhibiting translation of the gene. This mouse model lacks all signals from both IL-7 and TSLP. IL-7R^{-/-} mice are profoundly lymphopenic since IL-7R is required for development and survival of T and B cells in mice. IL-7R^{-/-} mice therefore have decreased splenic and thymic cellularity. T cell development is blocked in the double negative stage IV and B cell development is blocked at pre-pro-B cells. Due to this profound lymphopenia these mice are immunocompromised and extremely susceptible to infection. Immune responses in the IL-7R^{-/-} host are different than normal immune responses for various reasons. First, the mice are T and B cell deficient thus severely limiting any role for the adaptive immune system. Additionally, these animals do not develop normal secondary lymphoid structures since they do not have LT_i cells. The function of IL-7R signaling on innate cells is an area of active research. Innate lymphoid cells including not only LT_i but also ILC-17 and ILC-22 cells would be absent in IL-7R^{-/-} mice[288]. However, it would be expected that much of the innate immune system would be intact in IL-7R^{-/-} mice.

1.9 AIMS

The goal of this study was to determine if immune cell niches could be modified in a way that would be therapeutically beneficial. Specifically, bone marrow and thymic niche modulation was aimed to allow for increased engraftment

following HSCT, while T cell niche modulation was aimed to gain a better understanding of the factors that control the size of the niche.

Given that animals genetically deficient in KIT are receptive to HSC engraftment without conditioning and that KIT blockade via antibody therapy created sufficient space within the HSC niche to permit mixed chimerism following HSCT in immunodeficient hosts, the studies presented here sought to determine if pharmacologic inhibition of KIT would provide a clinically relevant approach to HSCT.

Since it has been reported that IL-7R^{-/-} mice experience increase proliferation and accumulation of T cells, the studies presented here sought to confirm this finding, determine if it provided an advantage in tumor immunotherapy, and determine what factors within the host were controlling the size of the T cells niche.

The specific aims are:

1. To determine if pharmacologic inhibition of KIT using a FDA approved small molecule tyrosine kinase inhibitor would create space in bone marrow and thymic niches to allow for increased engraftment following HSCT.
2. To confirm that IL-7R^{-/-} mice experience increased T cell proliferation and accumulation and explore whether or not the increased T cell proliferation allows for increased cancer immunotherapy.
3. To determine what drives the increased proliferation and accumulation of T cells seen in IL-7R^{-/-} hosts.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 MICE

Mice used at the NIH were all on a C57Bl/6 background unless otherwise specified. C57Bl/6 CD45.1 or CD45.2 (B6, H-2^b) mice were purchased from the Animal Production Unit of the NCI. IL-7R^{-/-}, *Rag1*^{-/-} (CD45.2), B6C3H.SWF1 (H-2^b), and mice bearing non-signaling mutations in the receptor tyrosine kinase KIT (WBB6F1/J-*KIT*^W/*KIT*^{w-v}, referred to here as *KIT*^{W/W^v}) (CD45.2) were purchased from The Jackson Laboratory (Bar Harbor, ME). Bone marrow from TSLPR^{-/-} mice was provided by the Leonard lab (NIH, Bethesda, MD). IL-7R-gfp mice were provided by the Singer lab (NIH, Bethesda, MD). Marilyn transgenic (Tg) mice were provided by the Matzinger lab (NIH, Bethesda, MD), where they were generated using a CD4⁺ T cell clone specific for the male Hy-antigen as previously described[289]. All animals were maintained in a specific pathogen-free animal facility at the NIH. The Animal Care and Use Committee of the National Cancer Institute approved all experiments carried out at the NIH.

All mice used at Oxford were maintained on a C57Bl/6 background. C57Bl/6, *Rag*^{-/-}, IL-7R^{-/-}, IL-7R^{-/-}*Rag*^{-/-}, IL-22^{-/-}*Rag*^{-/-}, IL23R^{-/-}*Rag*^{-/-}, IL-10gfp, OT-II, and CD11cDTR, mice were maintained in micro-isolator cages in a specific pathogen-free accredited animal facility at the University of Oxford. All experiments were performed in accordance with the United Kingdom Animals Scientific Procedures Act (1986).

IL-7R^{-/-}Rag^{-/-} double knockout mice were made both at the NIH and Oxford by crossing IL-7R^{-/-} mice to Rag^{-/-} mice. The F1 generation was entirely heterozygous for both genes: IL-7R^{+/-}Rag^{+/-}. The double knockout pups were selected from the pups of a F1 x F1 cross by standard PCR for both genes and yielded 1 in 16 double knockout mice. These original double knockouts were set up as founding breeders.

2.1.2 ANTIBODIES FOR FLOW CYTOMETRY

The following antibodies were used for flow cytometry staining and were conjugated to FITC, PE, PE-Cy5, Pacific Blue, PerCP-Cy5.5, APC, APC-Alexa Fluor 750, APC-Alexa Fluor 780, and biotin: anti-CD8a(53-6.7), -CD117(2B8), -CD25(7D4), -CD45.1(A20), -CD45.2(104), -CD3e(145-2C11), -CD4(LT34), -Gr-1(RB6-8C5), -Sca-1(Ly6A/E)(7D), -CD11b(M1/70), -CD135(Flt3)(A2F10.1), -CD45R/B220(RA3-6B2), -I-A/I-E(2G9), -Ter119(Ly-76), -NK1.1(PK136), -TCRb(H57-597), -TCRgd(GL3), -CD19(1D3), -CD8b(H35-17.2) (BD Biosciences); anti-CD117(2B8), -CD44(IM7), -CD45.1(A20), -CD45.2(104), -CD3e(145-2C11), -CD11b(M1/70), -Sca-1(Ly6A/E)(7D), -CD4(LT34), -CD135(Flt3)(A2F10.1), -CD45R/B220(RA3-6B2), -TCRb(H57-597), -TCRgd(GL3), -CD8b(H35-17.2), -CD127(IL-7Ra, A7R34), -CD150(SLAM 9D1), -CD11c(N418), -CD103(2E7), (eBioscience); anti-CD45R/B220(RA3-6B2) (Invitrogen). Lineage cocktails were comprised of anti-Ter119, -CD45R/B220(RA3-6B2), -TCRb(H57-597), -CD3e(145-2C11), -TCRgd(GL3) -CD8a(53-6.7), CD8b(H35-17.2), -CD11b(M1/70), -NK1.1(PK136), -CD19(1D3), -CD11c(HL3), -Ly-6G and Gr-1(RB6-8C5). Isotype

controls were used to define background staining. Four-color flow cytometry was performed using a dual laser FACSCalibur (Becton Dickinson, San Jose, CA) and five to nine-color flow cytometry was performed using the FACSaria or the LSR II (Becton Dickinson, San Jose, CA). Fluorescence data were collected from viable cells based on forward and side scatter intensity, and analyzed using FlowJo Software (Tree Star, Inc.).

2.2 CELL ISOLATION

2.2.1 PROCESSING OF SPLEEN, LYMPH NODES, AND THYMUS FOR T CELL ANALYSIS OR FURTHER PURIFICATION

Mice were sacrificed by carbon dioxide asphyxiation and spleens, lymph nodes, and/or thymi were harvested into ice cold PBS with 0.1% BSA. The whole organs were smashed onto a 70 μ m mesh strainer (BD biosciences) and a single cell suspension was made by passing the cells through the strainer. Samples were centrifuged at 1400rpm for 5 minutes at 4°C to pellet cells and supernatant was discarded by decanting. For spleens only, red blood cells were depleted with suspension in ammonium chloride potassium (ACK) lysing buffer (Gibco Life Technologies) for 3 minutes and then the remaining cells were washed in PBS with 0.1% BSA. Cells processed as described here were then resuspended for flow cytometric analysis, further purification or use in other experiments described below.

2.2.2 PURIFICATION OF NAÏVE CD4⁺CD62L⁺ T CELLS

Naïve CD4⁺CD62⁺ T cells selection was performed in a two-step process under sterile conditions. First, after isolation of lymphoid tissue described above, CD4 T cells were negatively selected using an antibody cocktail containing anti-8a(Ly-2), anti-CD45R(B220), anti-CD49b(DX5), anti-CD11b(Mac-1), anti-Ter-119, anti-CD25 and anti-TCR γ/δ followed by immunomagnetic depletion (negative selection) according to the manufacturer's instruction (LD depletion column, Miltenyi). The negative fraction was then used in the second step. CD4⁺CD62L⁺ cells were positively selected from the enriched CD4⁺ fraction by immunomagnetic selection with CD62L (L-selectin) microbeads according to the manufacturer's instructions (MS depletion column, Miltenyi). Cells were resuspended in 1mL PBS and stained with 1 μ L of 5 μ M CFSE (carboxyfluorescein diacetate succinimidyl diester; Molecular Probes) for 5 minutes at 37°C. Ice cold PBS with 0.1% BSA was added to the samples, which were then centrifuged at 1400rpm for 5 minutes at 4°C to pellet cells. The supernatant was discarded by decanting. Cells prepared as described here were used in coculture with DCs or were used in vivo for T cell transfer experiments to analyze peripheral expansion.

2.2.3 PROCESSING OF SPLEEN AND LYMPH NODES FOR DC ANALYSIS AND PURIFICATION

Mice were sacrificed by carbon dioxide asphyxiation and spleens and/or lymph nodes were harvested. Tissues were chopped into small pieces using a sharp scalpel. Tissue pieces were suspended in 4mL digestion media (RPMI 1640, 10% FCS, 7.5mM HEPES, 0.75mg/mL collagenase type VIII (Sigma-Aldrich, UK)) and

incubated at 37°C with 200rpm agitation for 30 minutes. At the end of the digestion 200µL of 0.5M EDTA was added to the cell suspension to quench collagenase activity. A single cell suspension was made by passing the cells through a 70µm mesh strainer (Falcon, BD biosciences). For spleens only, red blood cells were depleted with suspension in ACK lysing buffer (Gibco Life Technologies) for 3 minutes and then the remaining cells were washed in PBS with 0.1% BSA. Cells processed as described here were then resuspended for flow cytometric analysis.

2.2.4 SMALL INTESTINE AND COLONIC LAMINA PROPRIA ISOLATION FOR ANALYSIS

Colon, and small intestine were dissected and adipose tissue was carefully removed. Contents were forced from the colon using forceps and then the colon was cut longitudinally and washed in PBS with 0.1% BSA. The small intestine was cut longitudinally with the contents inside. Then the contents were removed by repeated submersion.

All tissues were blotted on paper towel to removed excess mucous. Tissues were cut laterally into approximately 2cm long pieces. Tissues were placed into 50mL falcon tubes with 25mL pre-warmed (37°C) EDTA media (RPMI 1640, 5% FCS, 100U/mL Penicillin, 100µg/mL streptomycin, 5mM EDTA) and agitated at 200rpm for 30 minutes at 37°C. The supernatant was aspirated off and discarded and the EDTA wash was repeated. Tissues were then transferred into cold wash media (RPMI 1640, 5% FCS, 100U/mL Penicillin, 100µg/mL streptomycin) for 10 minutes at room temperature to remove remaining EDTA.

The tissues were digested in 20mL collagenase media (RPMI 1640, 5% FCS, 100U/mL Penicillin, 100µg/mL streptomycin, 0.5mg/mL collagenase Type VIII from *Clostridium histolyticum* (Sigma-Aldrich, UK) 40µg/mL DNase-I from Bovine pancreas (Roche, Burgess Hill, UK), 1mg/mL Dispase (Gibco)) with agitation at 200rpm for 30 minutes at 37°C. A single cell suspension was made by passing the cells through a 70µm mesh strainer (Falcon, BD biosciences). Cold EDTA media was added to quench the collagenase activity. Supernatant was centrifuged at 1400rpm for 5 minutes to pellet cells and supernatant was discarded aspiration.

Cells were resuspended in 3mL 30% percoll (P30) and layered onto gradients consisting of a 3mL 70% (P70) layer and a 4mL 40% percoll (P40) layer in a 15mL Falcon tube. Gradients were centrifuged at 1800rpm for 20 minutes at 10°C without a brake. Lamina propria leukocytes (LPL) were collected from the P75-P40 interface using a plastic Pasteur pipette. Cells were washed in cold PBS with 0.1% BSA to remove residual percoll prior to flow cytometric analysis.

2.2.5 BONE MARROW ISOLATION FOR ANALYSIS AND TRANSPLANTATION

Donor mice were sacrificed by carbon dioxide asphyxiation and legs were harvested. Bone marrow (BM) was obtained by passage of iced media (RPMI 1640, 10% FCS, 100U/mL Penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 0.05mM beta-mercaptoethanol) through the tibias and femurs, or by crushing vertebrae with a mortar and pestle. A single cell suspension was made by passing the cells through a 70µm mesh strainer (Falcon, BD biosciences). Red

blood cells were depleted with suspension in ACK lysing buffer (Gibco Life Technologies) for 3 minutes and then the remaining cells were washed in PBS with 0.1% BSA. Cells processed as described here were then resuspended for flow cytometric analysis or for injection to make BM chimeras.

2.3 IN VITRO EXPERIMENTS

2.3.1 IMMUNOPRECIPITATION AND WESTERN BLOT

Bone marrow cells were harvested from untreated mice, or where indicated, from mice treated with either sunitinib or vehicle for 4 days. Cells were treated ex vivo with sunitinib (100nM, LC Laboratories, Woburn, MA) or vehicle for 2 hours, and then stimulated with 250ng/mL recombinant mouse SCF (R&D Systems, Minneapolis, MN) for 30 minutes. Cells were then lysed with lysis buffer (40mM Tris.Cl, 280mM NaCl, 20% glycerol, 2% NP-40, 4mM EDTA, 80nM NaF) containing phosphatase inhibitor (0.1µg/mL PMSF) and Protease Inhibitor Cocktail Tablets (Roche Molecular Biochemicals). Protein concentration in lysates was determined using the BCA assay kit according to the manufacturer's instructions (Pierce, Rockford, IL). One mg of protein from each sample was immunoprecipitated overnight at 4°C with anti-KIT antibody (2B8; eBioscience) and protein A-agarose immunoprecipitation Reagent (sc-2001; Santa Cruz Biotechnology, Santa Cruz, CA) Immunocomplexes were washed in lysis buffer containing inhibitors. Proteins were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Membranes were

probed with an anti-phosphotyrosine antibody (sc-508; Santa Cruz Biotechnology), then stripped and reprobed with anti-KIT antibody (2B8).

2.3.2 GROWING BONE MARROW DERIVED DENDRITIC CELLS (BMDCs)

Bone marrow was harvested and prepared into a single cell suspension as described above under sterile conditions. Cells were resuspended in complete media (RPMI 1640, 10% FCS, 100U/mL Penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 0.05mM beta-mercaptoethanol) at 2×10^6 cells/mL with 200ng/mL recombinant Flt3L (Peprotech, London, UK) and plated onto a flat-bottom plate. They were incubated at 37°C for 7 days with no disturbance. After 7 days fresh complete media was added to the plate containing recombinant GM-CSF so that the final concentration of GM-CSF was 20ng/mL. Cells were again incubated at 37°C for 2 additional days with as little disturbance as possible. At the end of the 8th day of culture, where indicated, 1µg/mL LPS from *Escherichia coli* (Sigma-Aldrich, UK) was added to the cultures. Cells were washed and used in experiments starting on day 9.

2.3.3 COCULTURE OF OT-II T CELLS WITH BMDCs

BMDCs were prepared as described above. On the ninth day of the BMDC culture the cells were treated with varying amounts of IL-7 or TSLP as indicated for 24 hours. BMDCs were then washed three times to remove any trace of Cytokines before coculture with T cells. CD4⁺CD62L⁺ OT-II T cells were prepared as described above. Cocultures were prepared on 96-well plates in complete media

(RPMI 1640, 10% FCS, 100U/mL Penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 0.05mM beta-mercaptoethanol) with 2×10^5 T cells, 3×10^4 DCs and 200ng/mL OVA₃₂₃₋₃₃₉ peptide. After 4 days cells were harvested from the plate, counted, and prepared for flow cytometric analysis.

2.4 IN VIVO EXPERIMENTS

2.4.1 BONE MARROW TRANSPLANT AND SUNITINIB ADMINISTRATION

Bone marrow was obtained from donor mice by passage of iced media (RPMI 1640, 10% FCS, 100U/mL Penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 0.05mM beta-mercaptoethanol) through the tibias and femurs, or by crushing vertebrae with a mortar and pestle. Red blood cells were depleted with ACK lysing buffer (Gibco Life Technologies), and where indicated, T-cell depletion was performed using anti-CD4, CD8, and Thy1.2 immunomagnetic selection or lineage depletion was performed using an antibody cocktail containing CD5, CD45R(B220), CD11b, Anti-Gr-1(Ly-6G/C), and Ter-119 followed by immunomagnetic selection according to the manufacturer's instructions (LD depletion column, Miltenyi). Recipient mice received vehicle or sunitinib (60 mg/kg, LC Laboratories, Woburn, MA) suspended in vehicle as previously described[290] (Knowles Apothecary, Kensington, MD) and administered daily by oral gavage for 4 days, with the last dose administered at least 10 hours prior to transplantation. Previous work had established that four consecutive days of sunitinib at 60 mg/kg was well tolerated and effective at inhibiting KIT in murine cancer models[290], and pharmacologic data demonstrated that sunitinib would

be cleared within 10h of the last dose[291] but pharmacodynamic inhibition of KIT would remain at that time point, thus providing a potential advantage to transferred marrow. Where indicated, recipient and control mice were irradiated using gamma-irradiation with the doses indicated at a dose rate of 100-110 cGy/minute prior to transplantation.

2.4.2 SERIAL TRANSPLANTATION

Recipient CD45.2 *Rag1*^{-/-} mice received vehicle or sunitinib for 4 days as described above. BM was harvested from CD45.1 donor mice as described above and FACS sorted as previously described[292]. Donor derived CD45.1 HSCs, defined as Lin⁻Sca-1⁺KIT^{hi}Flt3⁻, were transferred to the recipients via intravenous tail vein injection on day 0. Peripheral blood samples were harvested from these recipients on days 10 and 60 and analyzed by flow cytometry. On day 60, recipients were sacrificed by carbon dioxide asphyxiation and unfractionated BM was harvested as described above, and then transferred into lethally irradiated CD45.2 C57Bl/6 hosts. BM and spleens were harvested from the final recipients and analyzed by flow cytometry.

2.4.3 T CELL TRANSFER FOR ANALYSIS OF PERIPHERAL EXPANSION

Many hosts including IL-7R^{-/-}, Rag^{-/-}, IL-7R^{-/-}Rag^{-/-}, IL-7R^{-/-} chimeras, and CD11cDTR chimeras, received 2x10⁶ naïve T cells by IV injection. For all of these experiments the T cells were prepared in the following way. Spleen and lymph nodes were harvested from untreated donor mice (typically congenic strain

were used as specified). A single cell suspension was made by passing the cells through a 70µm mesh strainer (Falcon, BD biosciences). Naïve CD4⁺CD62⁺ T cells selection was performed in a two-step process. First, CD4 T cell were negatively selected using an antibody cocktail containing anti-8a(Ly-2), anti-CD45R(B220), anti-CD49b(DX5), anti-CD11b(Mac-1), anti-Ter-119, anti-CD25 and anti-TCRγ/δ followed by immunomagnetic depletion (negative selection) according to the manufacturer's instruction (LD depletion column, Miltenyi). The negative fraction was then used in the second step. CD4⁺CD62L⁺ cells were positively selected from the enriched CD4⁺ fraction by immunomagnetic selection with CD62L (L-selectin) microbeads according to the manufacturer's instructions (MS depletion column, Miltenyi). Cells were resuspended in 1mL PBS and stained with 1µL of 5µM CFSE (carboxyfluorescein diacetate succinimidyl diester; Molecular Probes) for 5 minutes at 37°C and then washed. Cells were injected into the tail vein of recipient mice or injected retro-orbitally where indicated. Cells were harvested 7 days later for flow cytometric analysis.

2.4.4 TUMOR INJECTIONS AND ADOPTIVE TRANSFER OF TUMOR SPECIFIC T CELLS

MB49, an H-2^b bladder carcinoma, that expresses the Hy antigen was used for all tumor challenge experiments[293, 294]. The tumor cell line was cultured at 37°C in Iscove's modified Dubecco's medium with 10% FCS, 100U/mL Penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 0.05mM beta-mercaptoethanol. 10⁶ MB49 cells suspended in 200µL PBS were injected subcutaneously into the hind flank of the mice. Tumor size was measured every 2-4 days. The mice were killed according to NCI animal care and use guidelines (maximal tumor

dimensions of 2cm, tumor ulceration, or severe morbidity resulting from tumor growth).

Three days after tumor challenge, where indicated, naïve Hy-specific T cells from Marilyn mice were transferred to tumor bearing mice. Peripheral lymph nodes were harvested from Marilyn Tg mice and naïve CD4⁺ CD62L⁺ T cell were isolated as described above. 2x10⁶ T cells were suspended in 400µl PBS and injected into the tail vein.

2.4.5 BONE MARROW CHIMERAS

Bone marrow chimeras were made for four different purposes: (1) To determine if stromal or hematopoietic cells were responsible for the phenotype of IL-7R^{-/-} animals. (2) To make CD11cDTR chimers which only expressed the diphtheria toxin receptor on their hematopoietic cells so that they could be treated with diphtheria toxin for an extended period of time. (3) To make competitive chimeras to determine if IL-7R is necessary for the development of DCs. (4) To analyze immune reconstitution post bone marrow transplant. In all four cases the BM chimeras were made in the following way. Recipient mice received gamma-irradiation of 1100 cGy in 2 doses unless otherwise specified. Bone marrow cells were harvested from untreated mice as described above and then counted. 5x10⁶ unfractionated BM cells were suspended in PBS and injected into the tail vein of the recipient mice after the second dose of irradiation.

2.4.6 DC CHIMERAS IN CD11c⁺DTR RECIPIENTS

Bone marrow chimeras were made with CD11cDTR marrow in the manner described above. After recovering for 90 days the recipient mice received 500 cGy of gamma-irradiation to induce lymphopenia on day 0. Diphtheria toxin was used to deplete CD11c⁺ cells in these hosts as previously described[295]. They were treated with 200ng diphtheria toxin (Dtx; Sigma Aldrich) by intra-peritoneal injection every other day for 8 days starting on day 0. On day 1 the mice received 5×10^6 BMDCs from either IL-7R^{-/-} or wild-type donors by retro-orbital IV injection. BMDCs were prepared in the manner described under in vitro methods. On day two mice were given 2×10^6 naïve T cells (prepared as described above) by retro-orbital IV injection. On day 8 the mice were sacrificed by carbon dioxide asphyxiation and spleens, MLN, and colonic LPL were analyzed. A schematic outline of the experimental methods is shown in Figure 5.10A.

2.4.7 ANALYSIS OF IMMUNE RECONSTITUTION AFTER BONE MARROW TRANSPLANT

Bone marrow chimeras were generated as described above on day zero. Where indicated, they were treated with 200ng diphtheria toxin (Dtx; Sigma Aldrich) by intra-peritoneal injection every other for the duration of the experiment starting on day 1. On day 12 the mice were sacrificed by carbon dioxide asphyxiation and spleens were harvested for analysis. A schematic outline of the experimental methods is shown in Figure 5.11A.

2.5 STATISTICS

Data were analyzed using Prism (Graphpad Software, San Diego, CA). Groups were compared using Mann-Whitney with p-values < 0.05 considered significant. Percentage donor and recipient chimerism was determined by expression of CD45.1 (donor) vs. CD45.2 (recipient) on at least 50,000 cells evaluated by flow cytometry.

3 TYROSINE KINASE INHIBITION OF THYMIC AND BONE MARROW NICHES

3.1 INTRODUCTION

Preparative regimens currently used for HSCT incorporate irradiation and/or cytotoxic agents that carry risks for tissue damage, infection and second malignancies. The high risk:benefit ration means that HSCT although curative for benign diseases, it is generally only used in very severe circumstances. If the morbidity and mortality associated with HSCT could be dramatically reduced HSCT could be used, for example, to increase the quality of life for patients with sickle cell anemia and other benign blood diseases.

Since it is not necessary to achieve 100% donor chimerism to cure benign diseases, strong cytotoxic preparative regimens for HSCT are not necessary when HSCT is undertaken for benign diseases. In sickle cell anemia, for example, even as little as 11% chimerism has been shown to completely reverse the phenotype[64]. The development of a targeted, non-toxic preparative regimen that could accomplish HSC engraftment would open new possibilities for transplantation of allogeneic or gene modified autologous progenitors for benign disease.

Extensive work has demonstrated an essential role for KIT signaling within the HSC niche. Animals genetically deficient in KIT are receptive to HSC engraftment without conditioning,[53, 54] and several studies have concluded that HSC

engraftment requires space within the HSC niche[55, 69]. KIT blockade via moAb therapy created sufficient “space” within the HSC niche to permit mixed chimerism following HSCT in immunodeficient hosts[55].

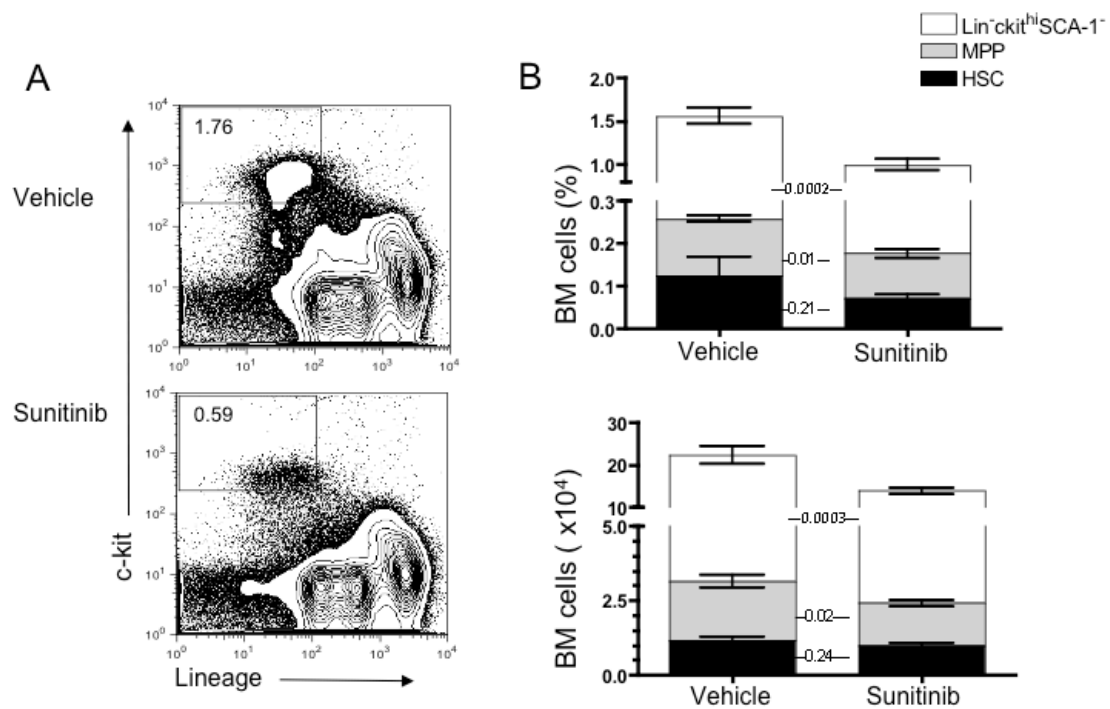
KIT blockade is particularly attractive as a preparative regimen since KIT is also expressed on ETPs, the subset believed to represent the earliest T cell progenitor within the thymus[296]. In HSCT thymic engraftment is thought to be particularly important for establishing graft tolerance and limiting graft vs. host disease. Since the thymus is the place where the naïve T cell repertoire is selected, antigens that are expressed in the thymus are generally regarded as self and therefore not attacked by T cell that have matured and been educated within the thymus. Thymic engraftment therefore can allow donor T cells to see self-antigens during selection and result in tolerance toward these antigens.

Pharmacologic inhibition of KIT is now readily available using a variety of FDA approved receptor tyrosine kinase inhibitors[56-58]. The experiments described in this chapter were designed to determine whether engraftment following HSCT could be accomplished in mice by modulating HSC and thymic progenitor niche accessibility via pharmacologic inhibition of KIT, in lieu of a cytotoxic preparative regimen.

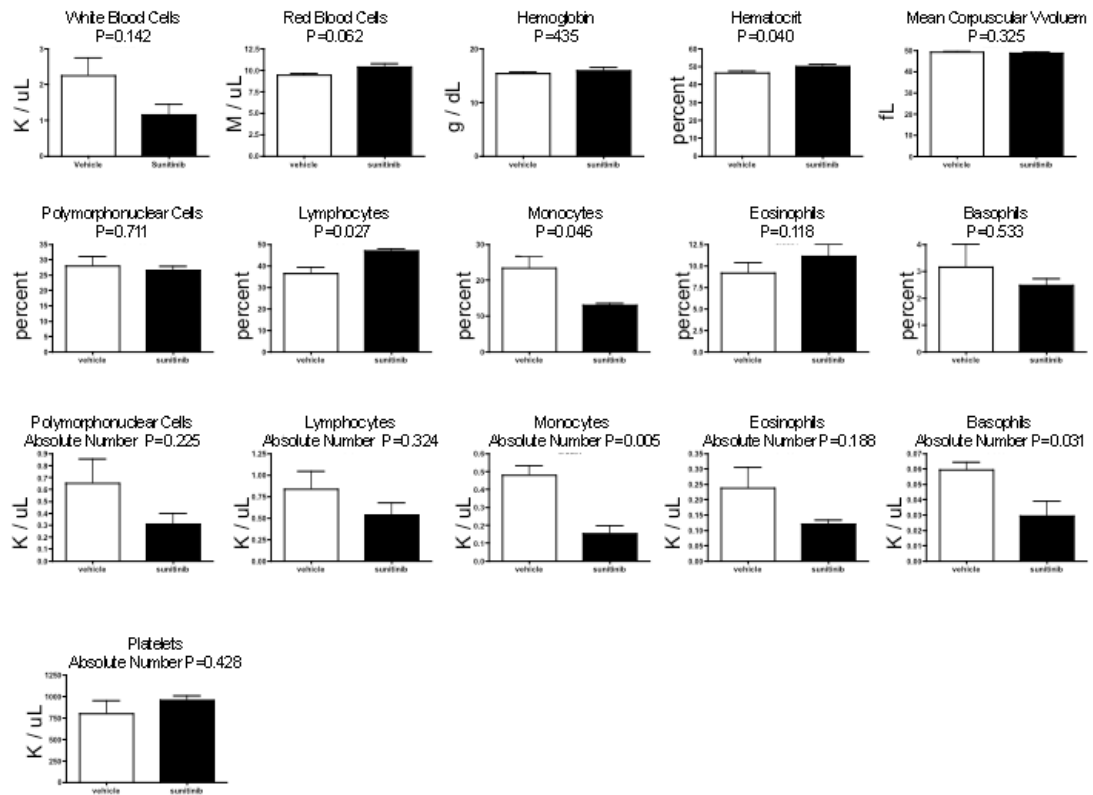
3.2 RESULTS

3.2.1 SUNITINIB TREATMENT DECREASES BONE MARROW PROGENITORS

Sunitinib inhibits several receptor tyrosine kinases, including Flt3, VEGFR1, VEGFR2, VEGFR3, PDGFR α , PDGFR β , RET, CSF1R, and KIT[59, 60]. KIT signaling plays a primary role in maintaining the HSC niche and is required for stem cell renewal[297]. KIT is also expressed on ETPs, believed to be the earliest intrathymic T cell progenitors[296]. Previous work had established that 60 mg/kg of sunitinib was well tolerated and effective at inhibiting KIT in murine models[290]. The experiments described here were designed to determine the effects of sunitinib therapy on early hematopoietic and thymic progenitors, as well as on marrow and thymic engraftment after BMT in mice. After 4 daily doses of sunitinib monotherapy the percentages and absolute numbers of Lin⁻KIT^{hi} bone marrow progenitors were significantly reduced, (dot plots from a representative mouse in Figure 3.1A and the sum total of the stacked bar graphs in Figure 3.1B, top p<0.001, bottom p<0.05), predominantly due to reductions in committed Lin⁻KIT^{hi}Sca-1⁻ progenitors and SCA-1^{hi}MPPs. The percentages and absolute numbers of HSCs (LSKFlt3⁻) were also decreased but these changes were not statistically significant (Figure 3.1B). Despite the fact that sunitinib inhibits Flt3 signaling, similar reductions in Flt3⁺ vs. Flt3⁻ subgroups within the SCA-1⁻ population were observed, and although sunitinib recipients trended towards slightly lower peripheral blood leukocyte numbers, only peripheral blood monocytes and basophils were significantly reduced after 4 days of therapy (Figure 3.2).



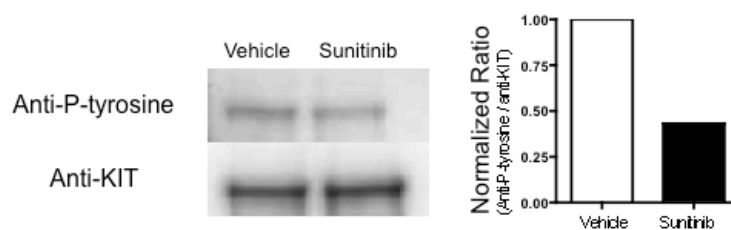
3.1. Sunitinib treatment decreases bone marrow progenitors. (A) Representative flow cytometry plots showing diminished frequencies of Lin-KIT^{hi} marrow progenitors in WT mice 4h following the 4th daily dose of vehicle (top panel) or sunitinib (bottom panel). (B) Frequency (top panel) and absolute numbers (bottom panel) of total marrow Lin-KIT^{hi} cells (represented by the total of all three stacked bar graphs) are reduced following sunitinib therapy administered as in A (mean percent \pm SEM 1.55 \pm 0.1 after sunitinib vs. 1.03 \pm 0.08 after vehicle, $p=0.0004$, and mean absolute number \pm SEM 19.2 \pm .75 $\times 10^4$ after sunitinib vs 11.42 \pm 0.74 $\times 10^4$ after vehicle, $p=0.0002$). Within the Lin-KIT^{hi} population, frequency and absolute numbers of MPPs and Lin-KIT^{hi}SCA-1⁻ progenitors are significantly reduced by sunitinib, whereas changes in HSC frequency and absolute numbers are not significant (p values shown). Data represent pooled results from four independent experiments, $n= 18-20$ /group.



3.2. CBC from sunitinib treated mice. Peripheral blood was taken from WT mice 4h following the 4th daily dose of sunitinib or vehicle. Results from a complete blood counts are shown. Bargraphs show the mean +/- Standard Error of the mean. n = 3-4 mice / group.

3.2.2 EX VIVO SUNITINIB TREATMENT BLOCKS PHOSPHORYLATION OF KIT ON BONE MARROW CELLS

The capacity for marrow to respond to stem cell factor, the ligand for KIT, following sunitinib therapy was also evaluated. Consistent with known effects of this agent, sunitinib treatment of unfractionated bone marrow ex vivo diminished KIT phosphorylation following exposure to stem cell factor (Figure 3.3). Interestingly, the data did not show complete inhibition of KIT phosphorylation following ex vivo exposure to sunitinib, nor could diminished KIT phosphorylation be detected after in vivo exposure to stem cell factor in bone marrow cells harvested from sunitinib treated animals (data not shown).



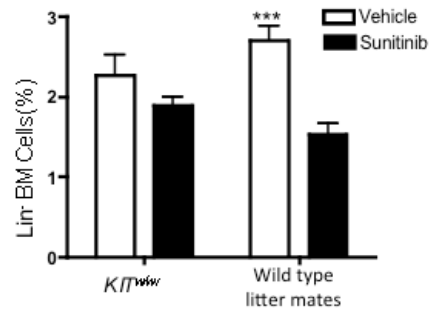
3.3. Sunitinib treatment inhibits KIT signaling in the bone marrow. BM cells were harvested from untreated WT mice, then treated with sunitinib (100nM) or vehicle for 2 hours followed by rmSCF (250ng/mL) for 30 minutes, then immunoprecipitation for KIT was performed and blotted with anti-phosphotyrosine (left). Densitometric normalized ratios of phospho-tyrosine/KIT is shown (right). This experiment was repeated 3 times with similar results.

3.2.3 LIN- BONE MARROW PROGENITORS WERE NOT AFFECTED BY SUNITINIB THERAPY IN $KIT^{W/Wv}$ MICE.

In order to determine whether the effects of sunitinib on BM progenitors were KIT dependent, and since sunitinib did not completely inhibit KIT phosphorylation ex vivo, the effects of sunitinib on KIT deficient were analyzed. $KIT^{W/Wv}$ (WBB6F1/J)- KIT^W/KIT^{w-v}) are compound heterozygotes for the KIT gene. The molecular basis of both mutations has been previously described[298]. The W mutation is a loss of function mutation while the Wv mutation is a missense mutation in the kinase domain of the KIT coding sequence. The compound heterozygote has been used in many studies as a KIT deficient model, since the W mutation is lethal when homozygous while the Wv mutation leads to incomplete loss of KIT function when homozygous. $KIT^{W/Wv}$ mice possess pleiotropic defects in germ cells, pigment-forming cells, red blood cells, and mast cells. They lack intermediate cells, resulting in hearing impairment. They are mast cells deficient and have impaired resistance to parasitic infection[299]. They have an intrinsic progenitor cell defect and are therefore sterile. Lack of germ cells in these mice also leads to the development of some ovarian tumors associated with an overproduction of pituitary gonadotropic hormone[300, 301]. Most important for this project $KIT^{W/Wv}$ mice cannot signal through KIT and are receptive to HSC engraftment without conditioning[53, 54].

Since $KIT^{W/Wv}$ mice are KIT deficient, it was not possible to enumerate HSC, LSK, and MPP specific subsets in these mice before and after sunitinib therapy since HSCs, LSKs, and MPPs are all defined as being KIT^{hi} . However, the effects of

sunitinib were clearly observed on Lin⁻ BM progenitors in wild-type mice ($p < 0.0001$), whereas no significant effects on this population were observed in *KIT^{W/W^v}* mice (Figure 3.4). Together, the data confirm that sunitinib therapy inhibits KIT signaling in BM progenitor cells, and reduces marrow progenitors in animals dependent upon KIT signaling for hematopoiesis. Further, they implicate KIT as the primary target of sunitinib responsible for the hematopoietic effects observed since no significant effect was seen in animals genetically deficient in KIT signaling.

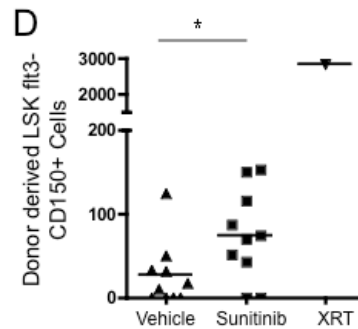
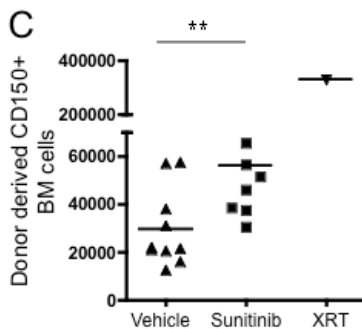
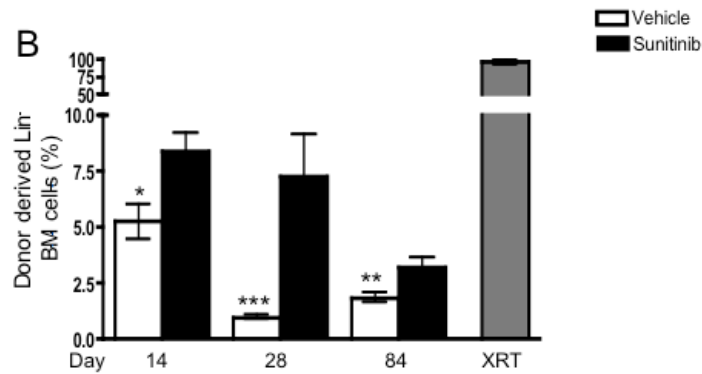
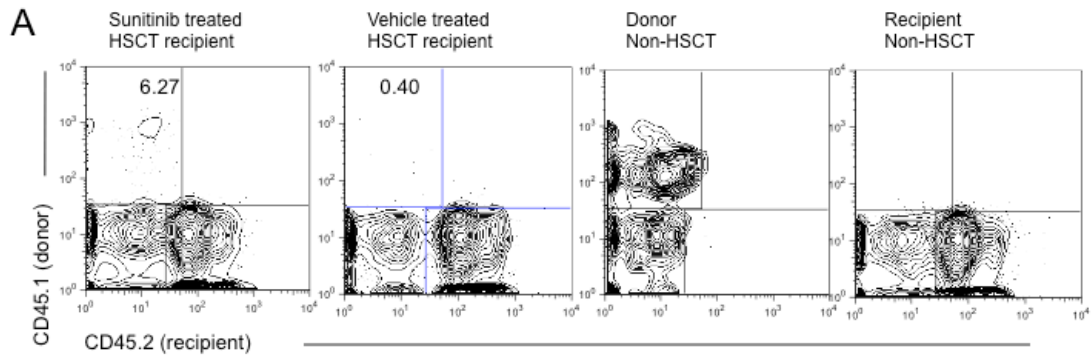


3.4. Sunitinib treatment does not decrease bone marrow progenitors in KIT deficient mice *KIT^{W/W}* recipients and WT littermates received sunitinib or vehicle for four consecutive days. Differences between sunitinib and vehicle treated *KIT^{W/W}* mice were insignificant ($p = 0.18$) while sunitinib significantly decreased the number of LinBM progenitors in wild type littermate controls ($p < 0.0001$), $n = 10$ mice per group. Similar results were observed in two different experiments.

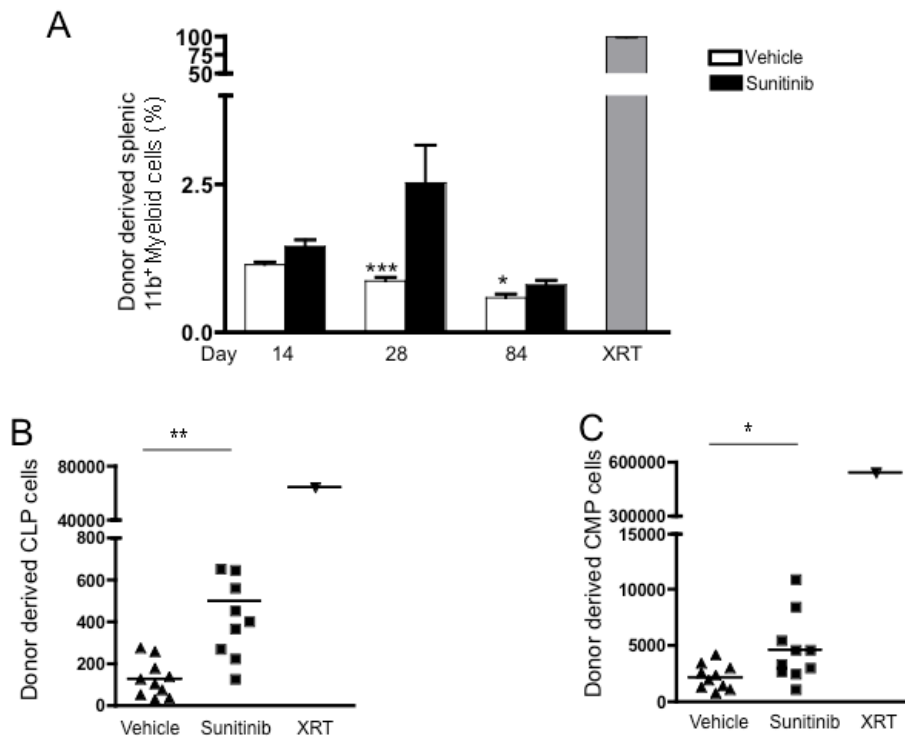
3.2.4 SUNITINIB ENHANCES HSC ENGRAFTMENT IN IMMUNODEFICIENT MICE

Previous work demonstrated that mice with mutations in KIT signaling are receptive to engraftment[53] and that moAb mediated blockade of KIT enhanced engraftment following HSCT in *Rag1*^{-/-} recipients[55]. The next set of experiments therefore sought to determine whether sunitinib induced changes within the marrow stem cell and progenitor pool enhance engraftment following BMT. *Rag1*^{-/-} mice received sunitinib or vehicle prior to administration of T cell depleted congenic bone marrow. As early as day 14 after BMT, sunitinib treated *Rag1*^{-/-} recipients showed increased donor chimerism in the Lin⁻ BM compartment, compared to vehicle treated controls. The magnitude of the differences observed diminished with time, but the differences between sunitinib and vehicle treated animals remained significant as late as day 84 post-BMT (Figure 3.5A,B).

Self-renewing long-term HSCs (LT-HSCs) have been recently defined using SLAM family markers[302] such as CD150. The effects of sunitinib therapy were therefore evaluated on day 28 engraftment of total CD150⁺ BM cells (Figure 3.5C) and LSK Flt3⁻ CD150⁺ LT-HSCs and showed similar increases in engraftment as observed in other HSC subsets (Figure 3.5D). Sunitinib recipients also showed increased levels of splenic myeloid reconstitution on days 28 and 84 post-BMT, a peripheral cell subset demonstrated to be a surrogate for BM HSC chimerism[303] (Figure 3.6A), and increased levels of common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) on day 28 post-BMT (Figures 3.6B,C).



3.5. Sunitinib enhances marrow engraftment following BMT into *Rag1*^{-/-} recipients. CD45.2⁺*Rag1*^{-/-} recipients received daily doses of sunitinib or vehicle on days -3 through 0, then 5×10^6 TCD BM cells from WT CD45.1⁺C57Bl/6 mice on days 0 and 1. **(A)** Representative flow cytometry plots of gated Lin⁻ marrow on day 28 show increased donor derived progenitors in sunitinib recipients. **(B)** Mean \pm SEM donor derived marrow Lin⁻ cell frequencies from groups of *Rag1*^{-/-} recipients treated as in A and studied at serial time points. Sunitinib treated recipients showed significantly increased proportions of donor derived Lin⁻ marrow progenitors compared to vehicle controls at all time points and significantly increased CD150⁺ BM cells **(C)** and LSK Flt3⁻ CD150⁺ BM cells **(D)**. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0001$. XRT comprised *Rag1*^{-/-} recipients conditioned with 1000 cGy prior to BMT as controls. For Figure B Day 14, n=4-5 mice/group; day 28, n=8-13 mice/group; day 84, n=4-15 mice/group. Data represent pooled results from two independent experiments. Experiments were repeated 2-5 times with similar results. For figures C and D, n=10 mice/group. Only day 28 data are available for the cell subsets shown in C and D.

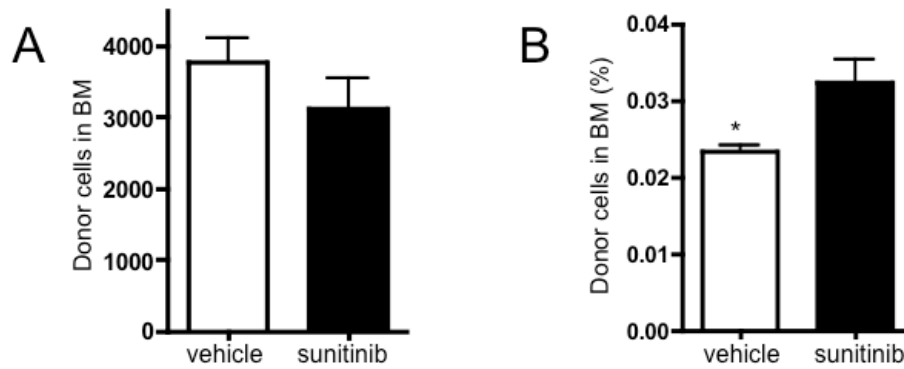


3.6. Sunitinib enhances splenic myeloid engraftment following BMT into *Rag1*^{-/-} recipients.

CD45.2⁺*Rag1*^{-/-} recipients received daily doses of sunitinib or vehicle on days -3 through 0, then 5×10^6 TCD BM cells from WT CD45.1⁺C57Bl/6 mice on days 0 and 1. **(A)** Mean \pm SEM donor derived splenic myeloid cell frequencies studied at serial time points. Sunitinib treated recipients showed significantly increased donor derived CD3⁺B220⁻CD11b⁺ myeloid splenocytes on day 28 and 84 and significantly increased donor derived CLPs **(B)** CMPs **(C)** on day 28. * = $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$. XRT comprised *Rag1*^{-/-} recipients conditioned with 1000cGy prior to BMT as controls. For figure A Day 14, $n = 4-5$ mice/group; day 28, $n = 8-13$ mice/group; day 84, $n = 4-15$ mice/group. Data represent pooled results from two independent experiments. Experiments were repeated 2-5 times with similar results. For figures B, and C, $n = 10$ mice/group. Only day 28 data are available for the cell subsets shown in B and C.

3.2.5 SUNITINIB TREATMENT DOES NOT AFFECT HOMING OF BONE MARROW CELLS

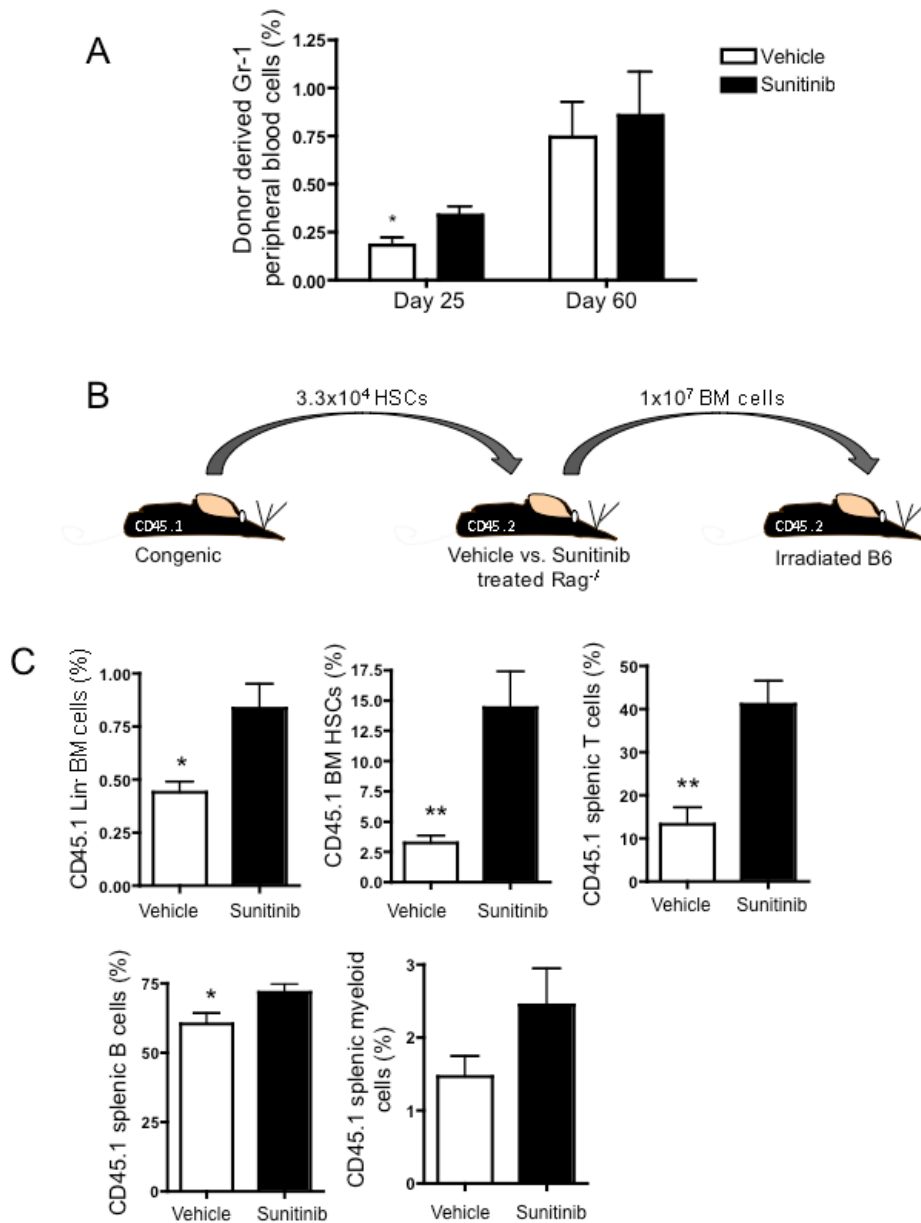
To investigate whether sunitinib mediated effects on marrow homing could be responsible for the findings observed, the following experiment was designed to test the ability of cells transferred from sunitinib vs. vehicle treated recipients to home to the bone marrow. 7×10^5 Lin⁻ CD45.1 BM cells were transferred to CD45.2⁺ *Rag1*^{-/-} recipients; fifteen hours after the transfer the recipients were sacrificed and their BM was harvested for flow cytometric analysis. No difference was seen in the absolute number of donor cells homing to the recipient marrow (Figure 3.7A), however, the percent of donor BM cells was higher in sunitinib treated recipients (Figure 3.7B), consistent with sunitinib mediated decreases in the size of the recipient marrow progenitor pool (Figure 3.1). Thus the data showed no evidence for modulation of marrow homing by sunitinib therapy.



3.7. Sunitinib therapy does not affect the ability of transferred cells to home to the bone marrow. (A and B) *Rag1^{-/-}* CD45.2 recipients received 4 daily doses of sunitinib or vehicle followed by transfer of 7×10^5 Lin⁻CD45.1 BM cells. 15 hours after transfer, recipients were sacrificed and BM was analyzed. (A) There was no difference in the absolute number of donor cells, but the percentage (B) of donor derived BM cells is significantly higher in sunitinib treated recipients. * = $p < 0.05$, n = 10 mice / group.

3.2.6 ENHANCED ENGRAFTMENT BY SUNITINIB IS TRANSFERABLE TO SECONDARY RECIPIENTS

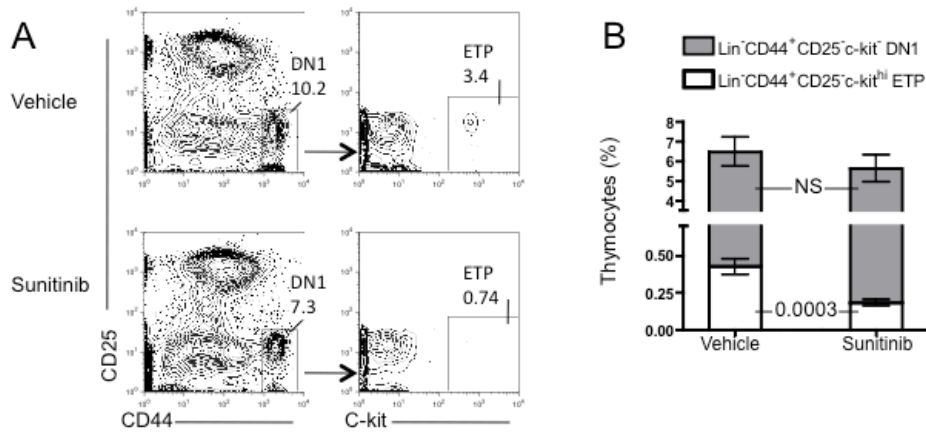
Together the results supported sunitinib-mediated enhancement of HSC engraftment, since engraftment involved multiple lineages and was long lasting, but the following experiments were designed to further address this issue by administering FACS sorted populations of congenic HSCs (KIT⁺Sca-1⁺Lin⁻Flt3⁻) to *Rag1*^{-/-} mice ± sunitinib therapy. Donor Gr-1⁺ peripheral blood cells were used as a marker to follow engraftment over time via peripheral blood and avoid sacrificing the animals so that they could be used as marrow donors in a later experiment. Analysis of Gr-1⁺ peripheral blood cells on day 25 demonstrated significantly increased engraftment in recipients treated with sunitinib as compared to those receiving vehicle (Figure 3.8A). The difference was non-significant at Day 60, but nonetheless subsequent serial transfer of marrow from animals receiving the purified HSCs described above into lethally irradiated CD45.2 C57Bl/6 hosts, demonstrated that marrow from sunitinib treated recipients mediated enhanced engraftment of CD45.1 cells derived from the original HSC inoculum. Indeed, serial transfer of marrow harvested from sunitinib vs. vehicle treated hosts showed significantly increased engraftment and/or reconstitution of bone marrow progenitors, HSCs, splenic B and T cells, but no significant increase in splenic myeloid reconstitution in secondary recipients (Figure 3.8C). Together, these data demonstrate that sunitinib monotherapy administered to immunodeficient *Rag1*^{-/-} mice prior to HSCT enhances bone marrow engraftment, with the greatest effect on short-term marrow progenitors and significant, but lesser effects, on HSC engraftment.



3.8. Enhanced engraftment by sunitinib is transferable to secondary recipients. CD45.1⁻CD45.2⁺ *Rag1*^{-/-} recipients received sunitinib or vehicle as described in Figure 1A, followed by transfer of 3.3×10^4 FACS sorted HSCs from CD45.1⁺CD45.2⁻C57Bl/6 mice on day 0. **(A)** Sunitinib recipients showed an increased percentage of donor derived Gr-1 peripheral blood cells compared to vehicle recipients on Day 25, N=4 mice/group. **(B)** Sixty days following BMT, whole BM from recipients described in A was transferred to lethally irradiated CD45.1⁻CD45.2⁺C57Bl/6 mice. **(C)** Frequencies of CD45.1⁺ cells in the recipients of the secondary transplant are shown 7 months following the secondary transplant. Bar graphs show the mean \pm SEM. n=8 for sunitinib group, n=5 for vehicle group. Similar results were seen on day 32 and day 63 following the secondary transplant. * = $p < 0.05$, ** $p < 0.001$.

3.2.7 SUNITINIB TREATMENT DECREASES THYMIC PROGENITORS

In addition to effects on early marrow progenitors, both KIT and Flt3 also provide essential survival signals during early thymopoiesis. Therefore, the effects of sunitinib therapy on thymic progenitor populations were specifically investigated. Thymic size was not affected by short term sunitinib therapy as administered here, but percentages of thymic DN1 cells (Lin⁻CD44⁺CD25⁻) were reduced by sunitinib, with preferential reductions in KIT^{hi} ETPs within this population (Figure 3.9A,B). This finding could result from decreased input from marrow progenitors, and/or from direct effects on the KIT^{hi} thymocytes, but would be expected to enhance access to thymic niches and facilitate thymic engraftment following BMT.

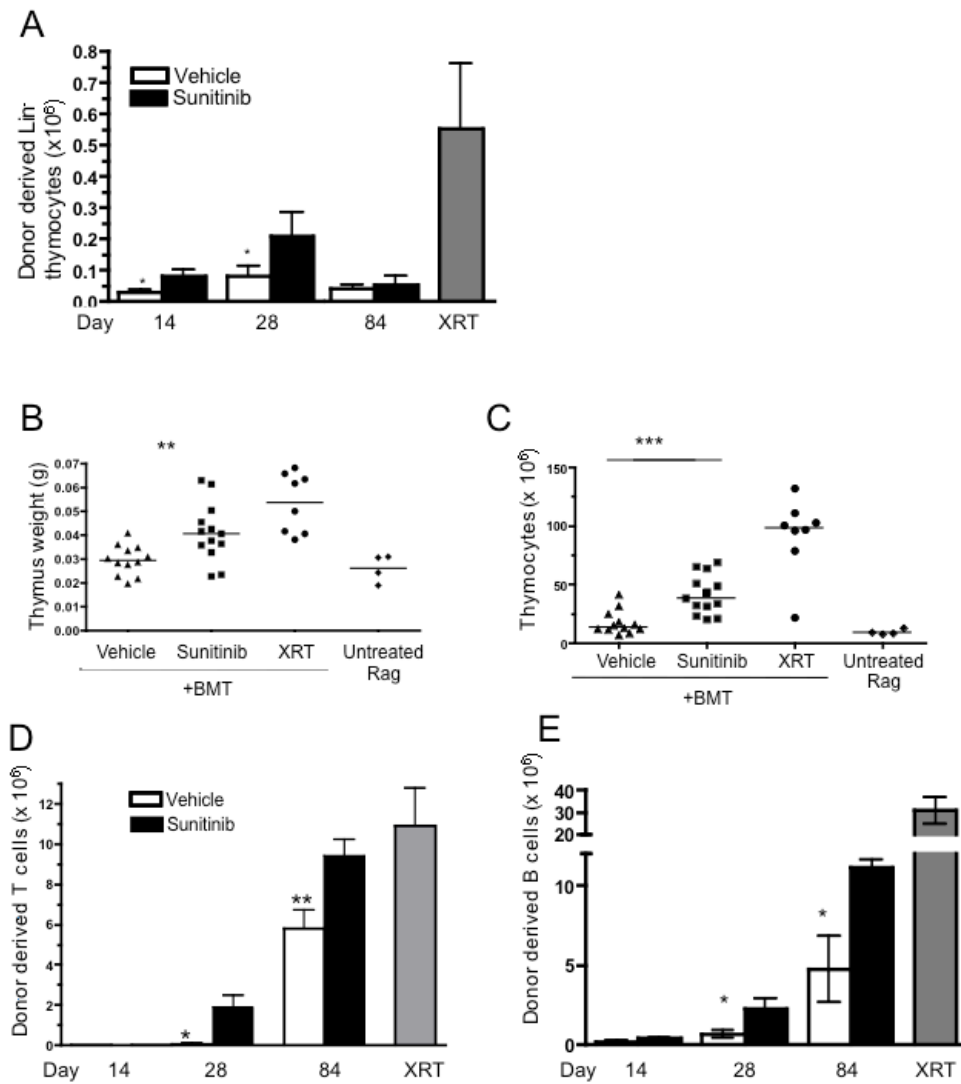


3.9. Sunitinib treatment decreases thymic progenitors. WT mice received sunitinib or vehicle as described in figure 1A and thymi were analyzed on day 4. **(A)** Representative flow cytometry plot showing diminished frequencies of DN1 thymocytes and thymic ETPs in sunitinib (bottom panel) vs. vehicle (top panel) treated mice. **(B)** Sunitinib did not significantly decrease the percentage of $\text{CD44}^+\text{CD25}^-\text{DN1}$ thymocytes (represented by total of stacked bar graphs) but it did induce a selective, significant decline of the KIT^+ ETP (white stacked bar graph) within the DN1 population. Bar graphs show the mean \pm SEM. Data represent pooled results from four independent experiments, with $n=20$ mice/group. * = $p<0.05$, ** $p<0.005$, *** $p<0.0001$.

3.2.8 SUNITINIB TREATMENT ENHANCES THYMIC AND PERIPHERAL LYMPHOID DONOR CHIMERISM AFTER BONE MARROW TRANSPLANT

To further investigate effects of sunitinib on T cell development in the thymus, thymic engraftment was analyzed in the thymi of sunitinib vs. vehicle treated *Rag1*^{-/-} recipient mice after BMT. As early as day 14 post-BMT, sunitinib recipients showed higher absolute numbers of donor-derived Lin⁻ thymocytes than vehicle treated recipients (Figure 3.10A), and on day 28 post-BMT, thymi from sunitinib treated recipients showed increased weight (Figure 3.10B), cellularity (Figure 3.10C) and numbers of donor derived thymocytes (84.22 ± 2.37 vs. $54.37 \pm 7.25 \times 10^6$ in sunitinib vs. vehicle controls; $p=0.0008$). Enhanced thymic engraftment was not maintained long term however as the differences observed on Day 84 were not significant. In the periphery, sunitinib recipients showed enhanced donor lymphoid chimerism in the spleen and higher numbers of donor derived mature B and T lymphocytes than animals treated with vehicle prior to transplant (Figure 3.10D,E). Interestingly, donor derived peripheral T cell reconstitution following sunitinib monotherapy was essentially equal to that accomplished following ablative radiotherapy. In contrast, the effects on B cell reconstitution were not as substantial as on T cell reconstitution since they did not reach the same numbers as irradiated controls. The effects on both peripheral B and T cells were long lasting with continued significant differences on Day 84. Thus, sunitinib dramatically enhances T cell regeneration following BMT for immunodeficiency and the degree of donor T cell chimerism accomplished with sunitinib monotherapy is substantially greater than the degree of sunitinib induced myeloid chimerism or B cell chimerism. However,

the immunodeficient mice used in these experiments provide a potent selection advantage for reconstitution of T cell populations that confounds the ability to determine whether these findings relate to more marked effects of sunitinib therapy on thymic engraftment compared to HSC engraftment and/or is a result of the immunodeficient model system used here.

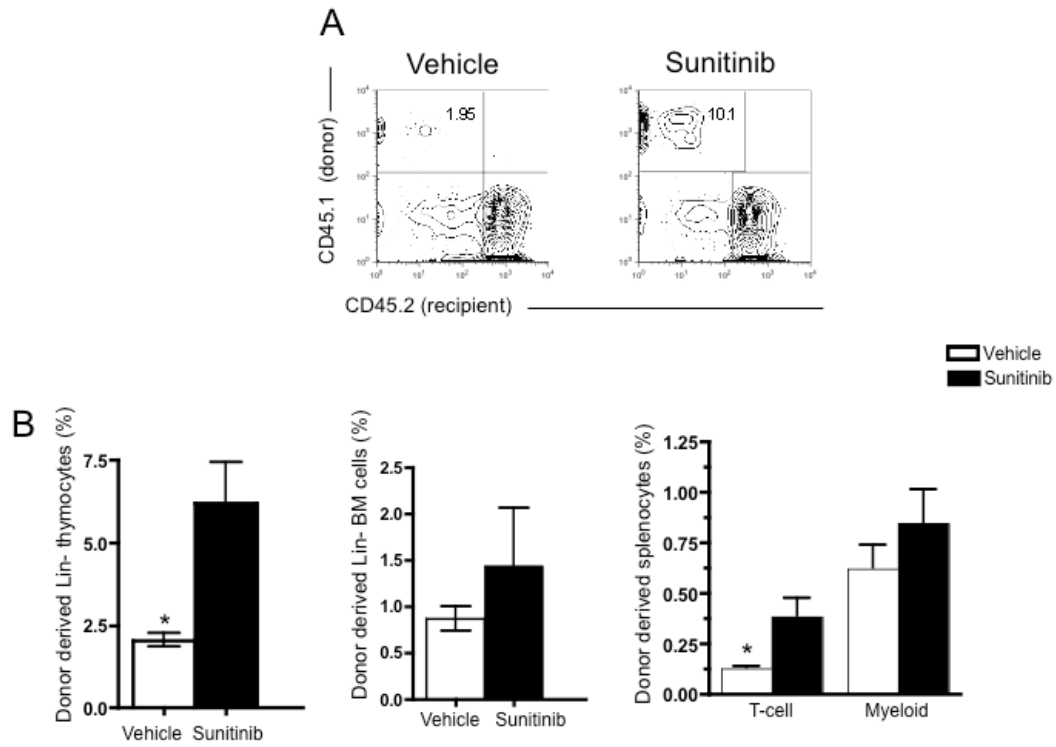


3.10. Sunitinib treatment decreases thymic progenitors and enhances thymic and peripheral lymphoid donor chimerism following BMT. *Rag1*^{-/-} recipients received sunitinib or vehicle for 4 days, followed by transfer of 5x10⁶ TCD congenic CBM cells from WT C57Bl/6 mice on days 0 and 1. On day 28 following BMT, sunitinib recipients showed increased numbers of donor derived Lin⁻ thymocytes (A), increased thymic weight (B), increased total thymocyte numbers (C), increased numbers of donor-derived splenic CD3⁺ (D) and splenic B cells (CD3⁺B220⁺MHCI⁺) (E) compared to vehicle controls. XRT comprised *Rag1*^{-/-} recipients conditioned with 1000cGY prior to BMT as controls. Bar graphs show the mean ± SEM. Data represent pooled results from three independent experiments. In scatterplots, each shape represents a mouse from day 28 of figures B and C. Day 14, n=4-5 mice/group; day 28, n= 8-13 mice/group; day 84, n=4-15 mice/group. Data represent pooled results from two independent experiments. Experiments were repeated 2-5 times with similar results. * = p<0.05, ** p<0.005, *** p< 0.0001.

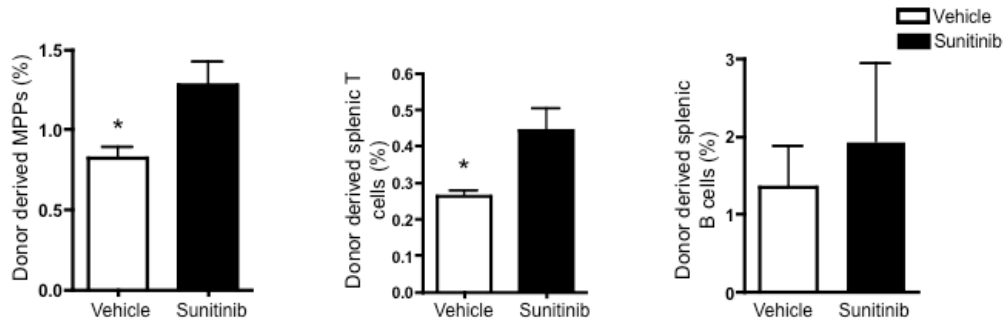
3.2.9 SUNITINIB ENHANCES ENGRAFTMENT IN IMMUNOCOMPETENT HOSTS

To determine if sunitinib enhanced myeloid and/or lymphoid chimerism following BMT in immunocompetent recipients, where donor lymphocytes would not experience a competitive advantage, marrow from CD45.1 C57Bl/6 donors was transferred into vehicle or sunitinib treated CD45.2 C57Bl/6 recipients. No difference was observed in thymic size between groups in these experiments. However, sunitinib treated recipients showed significantly increased proportions of donor derived Lin⁻ thymocytes (Figure 3.11A,B), and donor derived peripheral T-cells, with trends toward increased BM engraftment and peripheral myeloid reconstitution that did not reach statistical significance (Figure 3.11B). Similarly, sunitinib monotherapy significantly increased BM multipotent progenitor (p = 0.03) engraftment and peripheral T-cell (p = 0.03) reconstitution following transfer of parental C57BL/6 (H-2^b) bone marrow into C57BL/6 x C3H.SW (H-2^b) (F1) recipients mismatched for minor histocompatibility antigens (Figure 3.12), but did not enhance myeloid or B cell reconstitution in this setting. Importantly, sunitinib does not enhance engraftment in parent into F1 transplants with a major mismatch (C57BL/6 (H-2^b) marrow into C57BL/6 x C3H.HeN (H-2^b x H-2^k) F1 recipients) presumably due to hybrid resistance, an NK cell mediated rejection of parental cells by a hybrid recipient[304]. Sunitinib also did not mediate engraftment across minor histocompatibility barriers C57BL/6 (H-2^b) marrow into C3H.SW (H-2^b) recipients. Thus, sunitinib does not overcome immunologic barriers to engraftment after HSCT.

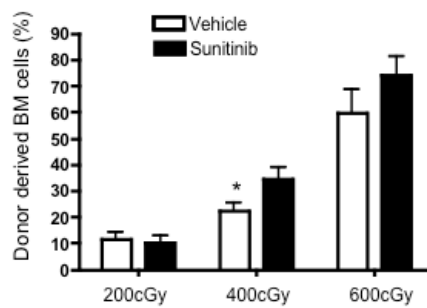
Sunitinib monotherapy enhanced myeloid reconstitution and more potently enhanced lymphoid reconstitution in immunodeficient hosts, and enhanced lymphoid reconstitution in immunocompetent hosts, but chimerism levels remained low. To determine whether sunitinib could enhance myeloid and/or lymphoid constitution in conjunction with low dose irradiation, chimerism rates were compared in animals receiving congenic marrow following treatment with 200, 400 or 600 cGy of irradiation \pm sunitinib. Significant increases were observed in marrow ($p=0.047$), peripheral T cells ($p=0.002$), peripheral B cells ($p=0.012$) and splenic myeloid cells ($p=0.012$) reconstitution when sunitinib was combined with 400 cGy (Figure 3.13). Interestingly, even 200 cGy of irradiation alone induced substantial thymic engraftment but these effects were not further enhanced by sunitinib (data not shown).



3.11. Sunitinib enhances engraftment in immunocompetent hosts. C57Bl/6 CD45.2 recipients received 4 daily doses of sunitinib or vehicle followed by transfer of 5×10^6 congenic CD45.1 TCD BM cells on days 0 and 1. Organs were harvested and analyzed by FACS on day 28. **(A)** Representative flow cytometry plots of Lin⁻-gated thymocytes from vehicle (left) vs sunitinib (right) treated recipients are shown. **(B)** Sunitinib recipients showed significantly increased Lin⁻ thymic and splenic T-cell engraftment compared to vehicle controls. Bar graphs show the mean \pm SEM. * = $p < 0.05$



3.12. Sunitinib enhances engraftment in immunocompetent hosts with mismatched minor histocompatibility antigens. C57BL/6 x C3H.SW (H-2^b) (F1) recipients received 4 daily doses of sunitinib or vehicle followed by transfer of 5×10^6 congenic CD45.1 TCD BM cells on days 0 and 1. Organs were harvested and analyzed by FACS on day 28. Sunitinib significantly increased BM multipotent progenitor ($p = 0.03$) and peripheral T-cell ($p = 0.03$) engraftment. Bar graphs show the mean \pm SEM. $n = 4$ mice per group. * = $p < 0.05$.

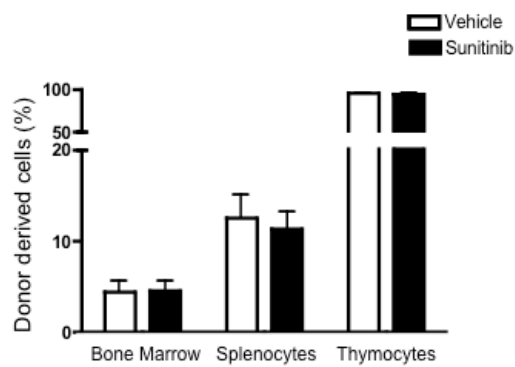


3.13. Sunitinib enhances engraftment in combination with sub-lethal total body irradiation.

C57Bl/6 CD45.2 recipients received 4 daily doses of sunitinib or vehicle followed by total body irradiation with the doses indicated on day 0 and transfer of 5×10^6 congenic CD45.1 TCD BM cells on days 0 and 1. On day 28 sunitinib treated mice show increased BM engraftment compared to vehicle controls. This difference was significant at 400 cGy. Bar graphs show the mean \pm SEM. n=5 mice/group. * = $p < 0.05$.

3.2.10 SUNITINIB DOES NOT ENHANCE ENGRAFTMENT IN KIT^{W/W^v} MICE.

To investigate the mechanism by which sunitinib enhances engraftment following BMT, TCD BM from CD45.1 C57B1/6 donors was transferred into vehicle or sunitinib treated CD45.2 KIT^{W/W^v} recipients. Previous work has shown that KIT^{W/W^v} mice are receptive to HSC engraftment without conditioning due to mutations in KIT signaling[53, 54]. KIT^{W/W^v} show incomplete HSC engraftment following transfer of TCD marrow, which is not increased by sunitinib therapy (Figure 3.14). Interestingly however, these mice experienced almost complete donor chimerism in the thymus, despite less than 10% donor chimerism in the bone marrow. Therefore, genetic mutations in KIT signaling induce results similar to that seen with sunitinib therapy, namely substantial thymic engraftment and significant but more modest effects on HSC engraftment. Together, these results suggest that the effects of sunitinib are primarily related to blockade of KIT signaling, as opposed to the numerous other tyrosine kinases inhibited by this agent, since no further increase in engraftment was observed when sunitinib was administered to KIT^{W/W^v} hosts. Furthermore, the fact that increased thymic as compared to marrow engraftment is observed both with mutant KIT and sunitinib is consistent with a model wherein KIT mediated survival signals play essential roles within the thymic niche.



3.14. Sunitinib does not enhance engraftment in *KIT^{fl/w}* mice. *KIT^{fl/w}* recipients received 4 daily doses of sunitinib or vehicle followed by transfer of 5×10^6 CD45.1⁺ CD BM cells on days 0 and 1. Sunitinib did not significantly increase the frequency of BM cells ($p=0.93$) splenocytes ($p=0.70$) or thymocytes ($p=0.69$) 28 days following BMT. Data represent pooled results from two independent experiments. Bar graphs show the mean \pm SEM. $n=13-14$ mice/group.

3.3 DISCUSSION

This work provides proof-of-principle that administration of a tyrosine kinase inhibitor that inhibits KIT signaling for a period exceeding its pharmacokinetic half life can provide an advantage to transplanted bone marrow, resulting in enhanced engraftment. The data shown here demonstrate that sunitinib mediated tyrosine kinase inhibition decreases hematopoietic and thymic progenitors, allowing for dramatic increases in lymphoid reconstitution and significant increases in myeloid reconstitution in *Rag1*^{-/-} immunodeficient recipients. T cell reconstitution was also enhanced by sunitinib following transfer of congenic marrow and parental marrow into F1 immunocompetent recipients, although the effects were less potent for bone marrow progenitor engraftment and peripheral T cell reconstitution and non-significant for myeloid reconstitution. It was further demonstrated that these effects of sunitinib are likely due to KIT inhibition since mice deficient in KIT do not show increased engraftment when treated with sunitinib. Given that current models implicate an essential role for KIT signaling in the HSC niche[55, 305], these results are not unexpected.

However, these studies provide the unexpected observation that sunitinib enhances thymic engraftment and T cell reconstitution to a greater extent than myeloid reconstitution. This enhancement is not likely due to differential effects on marrow myeloid vs. lymphoid progenitors since short-term studies show similar reconstitution rates for CLPs and CMPs with sunitinib. Rather, the enhanced effects of sunitinib on T cell reconstitution likely reflects potent

modulation of thymic niches by KIT inhibition, thus giving donor thymic progenitors a competitive advantage during early thymopoiesis, and allowing donor derived T cells to effectively seed the periphery. The lesser effects of sunitinib in enhancing peripheral B cell compared to T cell reconstitution are also consistent with a potent effect of sunitinib on thymic engraftment. Given that sunitinib also inhibits Flt3 signaling, and Flt3L plays a role in early thymopoiesis, the specific TK inhibition profile of sunitinib could contribute to these results. However, only a small subset of ETPs express Flt3 and Flt3^{-/-} mice undergo normal thymopoiesis[296]. Rather, this may illustrate the essential role that KIT plays in early thymopoiesis. KIT expression is a hallmark of the ETP thymic subset[296] and the data shown here that sunitinib therapy selectively and efficiently depletes ETPs are consistent with KIT providing an essential survival signal for this population. ETPs are proposed to be the critical T cell progenitor population within the thymus[306], and the potent decrease in ETP numbers induced by sunitinib (Figure 3.9A) would be expected to preferentially enhance T cell, as compared to myeloid and B cell, reconstitution. Although the high level T cell reconstitution observed in *Rag1*^{-/-} hosts treated with sunitinib is largely related to the void within the T cell compartment, increased T cell reconstitution compared to B cell or myeloid reconstitution was also seen in immunocompetent hosts treated with sunitinib (Figure 3.11B, right panel). Furthermore, the studies in *KIT*^{W/W^v mice also showed a similar pattern of increased thymic engraftment and peripheral T cell reconstitution compared to myeloid reconstitution(Figure 3.14), despite the fact that *KIT*^{W/W^v presumably have normal Flt3 signaling. Together, the data are consistent with ETP KIT signaling playing an essential role within the thymic niche, and suggest that}}

preparative regimens that selectively target KIT signals will preferentially enhance T cell reconstitution to a greater extent than myeloid or B cell reconstitution.

Potent effects on progenitor engraftment with lesser effects on HSC engraftment would explain the relatively low levels of myeloid reconstitution observed and the diminished effects observed over time. Some data demonstrating increased engraftment of CD150⁺ LT-HSCs (Figures 3.5C,D), combined with the fact that Lin⁻ BM engraftment in sunitinib vs. vehicle treated animals remained significant even at late time points such as 84 days post-BMT (Figure 3.5B) and the enhanced engraftment seen in the serial transplant (Figures 3.8B,C) are consistent with sunitinib mediated enhancement of true self-renewing HSC engraftment. However, engraftment percentages were diminished at later compared to earlier time points (Figure 3.5B), consistent with a model wherein hematopoietic progenitors efficiently engraft early following sunitinib monotherapy but their relative contributions diminish over the weeks following engraftment. Indeed, the data demonstrating that sunitinib depletes MPPs more efficiently than HSCs (Figure 3.1B), are consistent with more effective engraftment of MPPs than HSCs with this approach. KIT^{W/W^v} mice show increased engraftment over time, as the competitive disadvantage is permanent in this system whereas it is transient with sunitinib and thus increased engraftment over time would not be expected.

Therefore it appears that KIT based targeting does not efficiently modulate the LT-HSC niche, which is comprised of a non-synchronized, largely quiescent

population of cells. Rather, transient inhibition of KIT dependent cycling and/or KIT mediated trophic signals likely disadvantages only a fraction of the quiescent HSC pool at any given time. This is in contrast to a tumor population, such as gastrointestinal stromal tumors with mutated KIT, where constitutive KIT activation in all cells results in oncogene addiction[307] and substantial cell death following transient exposure to a KIT inhibitor such as sunitinib[308].

In summary, benign diseases favor less intense conditioning regimens in the context of HSCT, since stable mixed chimerism is often sufficient for clinical benefit and the risks associated with cytotoxic preparative regimens are more difficult to justify. It has been demonstrated here that an entirely non-cytotoxic approach, which reversibly inhibits KIT signaling, can achieve HSC engraftment by modulating accessibility of bone marrow and thymic niches. Future studies are needed to evaluate the effectiveness of this approach as a prelude to genetically modified autologous HSCs or in combination with antirejection therapy in a minor mismatch setting.

4 IL-7R DEFICIENT MICE EXPERIENCE ENHANCED T CELL PROLIFERATION: AN AXIS THAT MAY BE USEFUL IN CANCER IMMUNOTHERAPY

4.1 INTRODUCTION

The size of the T cell niche is kept relatively constant throughout life. The rate of cell death is equal to the rate at which cells are created either from dividing peripheral cells or from thymically derived cells[76-79]. During lymphopenia the body up regulates the rate at which peripheral T cells divide in a process called homeostatic peripheral expansion (HPE). Many factors that drive HPE have been rigorously studied including IL-7[96] and MHC-TCR interactions involving self and commensal antigens[86-93]. However, it would be expected that there must also be some regulatory mechanism that limits uncontrolled T cell proliferation during lymphopenia. All of the checks and balances that control this system have not been clearly defined. Much research is needed to gain a better understanding of the mechanisms that limit HPE and to paint a clearer picture of all of the factors that control the size of the T cell niche.

Understanding the factors that control the size of the T cell niche would be very useful in designing therapeutics to increase T cell responses for treatment of infectious diseases and cancers or decrease T cell responses for treatment of autoimmune and inflammatory diseases. In particular, tumor microenvironments are known to be immunosuppressive[225]. In an attempt to evade immune detection tumors have been shown to recruit Tregs to foster

immune privilege[226, 227]. Furthermore, effector T cell proliferation and function is often limited in the tumor microenvironment[243, 244]. Increased IDO expression is one way that tumors can limit T cells proliferation since increased IDO depletes available local tryptophan and T cells are particularly sensitive to this change[229]. Understanding additional mechanism that limit T cell proliferation in this setting could yield more effective targeted therapies.

There are many mouse models that have been engineered to study cancer immunotherapy. One such model used in this chapter utilizes a bladder cancer cell line, MB49, engineered to express the male Hy-antigen[293]. Cell lines are not as physiological as spontaneous models, however, they do offer advantages in terms of experimental manipulation. Thus, this model can be used in female mice where the antigen is foreign or in male mice where the antigen is a self-antigen. Since the body sometimes recognizes tumor antigens as foreign and sometimes as self this is a useful property[309]. Additionally, there are strains of Tg mice that have been developed to primarily express T cells that are specific for the Hy-antigen. The Marilyn mouse, for example, is a Tg mouse that expresses an Hy specific TCR on CD4 T cells that only recognize the Hy-antigen when it is presented on MHC-II[289]. The Matahari Tg mouse expresses an Hy specific TCR CD8 T cells that recognize the Hy-antigen presented on MHC-I[310]. Together these models allow for analysis of CD4 and CD8 tumor responses.

IL-7R^{-/-} mice are severely lymphopenic mice and lack IL-7 and TSLP signaling because of insertional mutagenesis of the IL-7R gene[311]. A previous publication has shown that CD4⁺ T cells undergo increased homeostatic

proliferation in IL-7R deficient hosts[261] but there are still many unanswered questions about the nature of this proliferation. The questions include: i) whether or not Tregs are amongst the CD4⁺ T cells that experience increased proliferation; ii) whether or not the increased T cell proliferation signifies a break in self-tolerance; iii) whether this increased proliferation is applicable to a disease model such as tumor immunotherapy; and iv) finally, does the increased proliferation only apply to homeostatic proliferation or do IL-7R^{-/-} hosts also support increased antigen-driven proliferation.

The following body of work will attempt to answer some of these questions and generally explore the nature of T cell proliferation in IL-7R^{-/-} hosts as a possible tool that can be used in cancer immunotherapy.

4.2 RESULTS

4.2.1 T CELLS TRANSFERRED INTO IL-7R DEFICIENT MICE UNDERGO INCREASED PROLIFERATION AND ACCUMULATION COMPARED TO RAG^{-/-} CONTROLS.

It has been well documented that T cells transferred into lymphopenic hosts undergo HPE [76, 92, 93, 312, 313]. Since both IL-7R^{-/-} and Rag^{-/-} mice are severely lymphopenic, as shown in Figure 4.1A, it would be expected that naïve T cells transferred into these mice would undergo homeostatic proliferation.

The IL-7R^{-/-} mice used in the following experiment are on a B6 background, however the B6 mouse is not a suitable control because it is not lymphopenic.

Given lymphopenia itself induces T cell proliferation the control host for an experiment looking at homeostatic proliferation in IL-7R^{-/-} mice would also need to be lymphopenic. To address this IL-7R^{-/-} mice were compared to Rag^{-/-} controls. This is not an ideal comparison because the two strains are lymphopenic for different reasons. The Rag^{-/-} host lacks the recombination-activating gene required to undergo V(D)J recombination and therefore cannot make any mature T or B cells, while the IL-7R^{-/-} host also cannot make most mature T or B cells because IL-7 is required for their development. Additionally, the lymphopenia is more severe in Rag^{-/-} compared to the IL-7R^{-/-} hosts (Figure 4.1A), and it might therefore be expected that the Rag^{-/-} host would experience increased T cell proliferation.

CD45.1⁺ congenic T cells were harvested from the spleen and peripheral lymph nodes of wild-type congenic mice. Naïve CD4⁺CD62L⁺ T cells were isolated using a Miltenyi MACS Isolation Kit according to the manufacturer's instructions. IL-7R^{-/-} mice and Rag1^{-/-} controls, both CD45.2⁺, received 2x10⁶ CFSE labeled CD45.1⁺ wild-type naïve T cells by IV injection. Seven days later the mice were sacrificed and splenocytes were harvested and analyzed by flow cytometry. T cell proliferation was evaluated by gating on live CD45.1⁺CD4⁺ donor derived cells and then looking at the CFSE pattern as shown in Figure 4.1B.

Surprisingly, even though the Rag^{-/-} hosts are more severely lymphopenic, the data show that donor derived cells from IL-7R^{-/-} recipients had undergone more divisions (Figure 4.1B) and show increased accumulation (Figure 4.1C) compared to Rag^{-/-} controls.

Since IL-7R^{-/-} mice are lymphopenic, it is not surprising that T cells undergo expansion in IL-7R^{-/-} hosts. What was surprising, however, was that T cells undergo dramatically increased expansion compared to other hosts that are also lymphopenic.

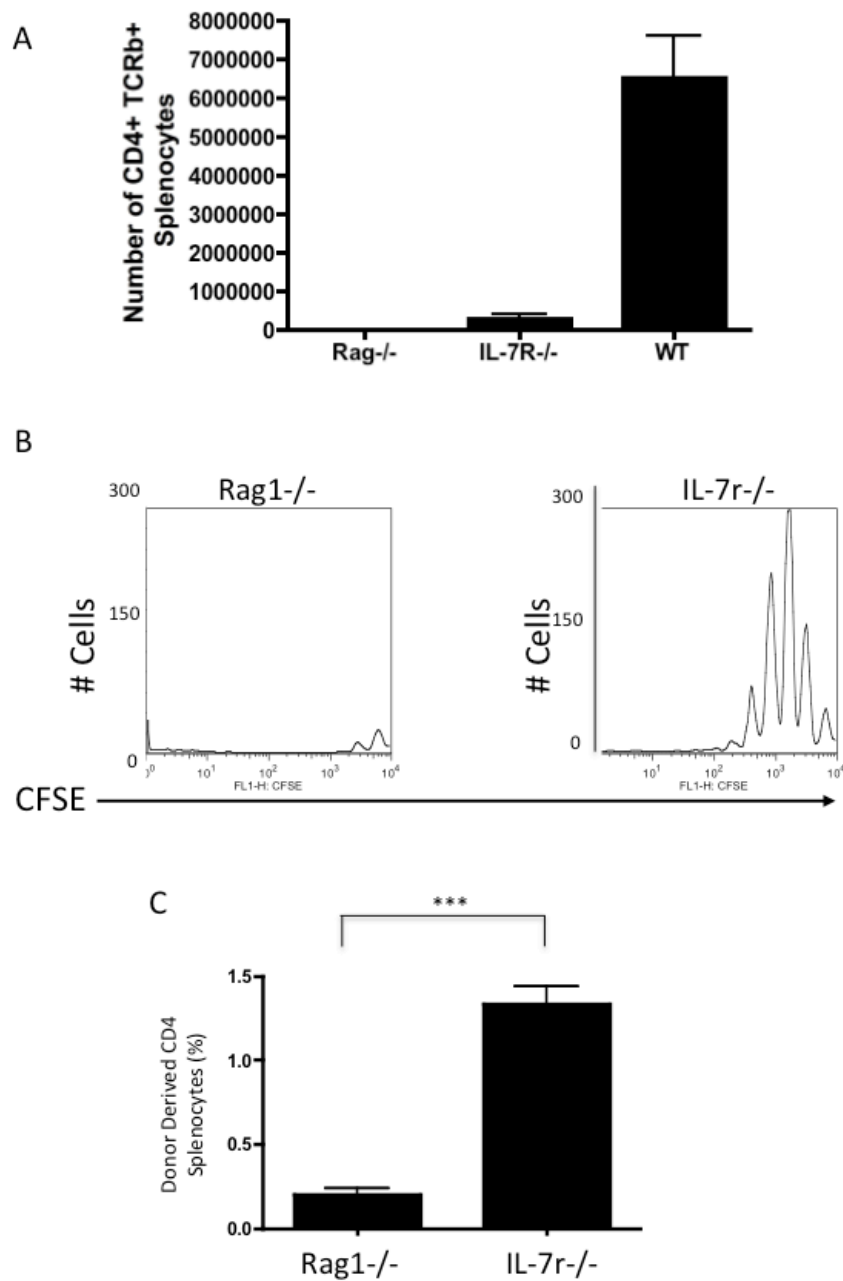


Figure 4.1. Lymphopenic IL-7R^{-/-} mice show increased proliferation and accumulation of transferred T cells compared to similarly lymphopenic Rag1^{-/-} controls. CD45.2⁺IL-7R^{-/-} or Rag1^{-/-} recipient mice received 2x10⁶ CFSE labeled CD45.1⁺ wild-type naïve T cells by intravenous injection. Seven days later mice were sacrificed and splenocytes were analyzed by flow cytometry. **(A)** Baseline measurement of mean ± SEM CD4⁺TCRβ⁺ splenocytes from stock mice. **(B)** Representative histograms of CFSE dilution of CD45.1⁺CD4⁺T cells **(C)** Bar graphs showing the mean percent ± SEM of CD4 T cells recovered from the spleen. n=10 mice per group. Results are representative of 3 separate experiments. *** = P < 0.0001.

4.2.2 IL-7R^{-/-} MICE DO NOT SUPPORT CANCER IMMUNOTHERAPY

The impressive T cell expansion seen in IL-7R^{-/-} mice led to the hypothesis that IL-7R^{-/-} mice might experience increased cancer immunity. The immune system is known to suppress the development and progression of cancer and studies have shown that the adoptive transfer of tumor specific T cells can stop tumor growth and lead to tumor regression in mice[293, 314] and humans[315, 316]. However, since tumors can have strong immunosuppressive activity it would be advantageous to have a better understanding of the factors that drive proliferation of tumor specific T cells.

In order to determine if IL-7R^{-/-} mice exhibit increased anti-tumor immunity the following experiment was designed using MB49 tumor cells, which express the male Hy antigen, and Marilyn Rag/TCR Tg CD4⁺ T cells which are specific for the Hy antigen. Traditionally cytotoxic CD8⁺ T cell have been thought to play an important role in cancer immunity. However, CD4⁺ cells have also been shown to have the capability to induce tumor regression in mice[293]. A schematic outline of the experimental methods can be seen in Figure 4.2A. Recipient IL-7R^{-/-} or Rag1^{-/-} mice received 1x10⁶ MB49 tumor cells by subcutaneous injection in the hind flank. Three days later they received 2x10⁶ naïve Marilyn tumor-specific T cells by IV injection. Tumor burden and survival was followed over time.

Rag^{-/-} hosts show increased tumor burden over time. The Rag^{-/-} group that received the adoptive transfer of tumor specific Marilyn T cells did significantly

better than the group that did not receive any T cells (Figure 4.2B top panel). Tumor and T cell doses were based on previous experiments and were titrated here so that approximately 50% of the Rag control group eventually succumbed to the tumor. IL-7R^{-/-} host also showed increased tumor burden over time. However, in these hosts there was no advantage of an adoptive transfer of tumor specific T cells (Figure 4.2B bottom panel) suggesting that IL-7R^{-/-} hosts do not support tumor immunotherapy.

This result was very surprising given that IL-7R^{-/-} hosts experience increased proliferation of transferred T cells, and it has been shown that transferred Marilyn T cells are capable of limiting tumor burden of MB49 tumors (Figure 4.2B top panel). It was expected that IL-7R^{-/-} hosts would have an increased number of tumor specific T cells and therefore be better equipped to reject the tumor. By contrast, the results observed were exactly the opposite of this.

This new data raised two important questions that could lead to a better understanding of HPE and tumor immunotherapy. Were the tumor specific T cells indeed able to proliferate in the IL-7R^{-/-} hosts or was there something inherently different about these Tg T cells that limited their proliferation in this setting? Did the presence of the tumor itself limit T cell proliferation or function in IL-7R^{-/-} hosts but not Rag^{-/-} hosts?

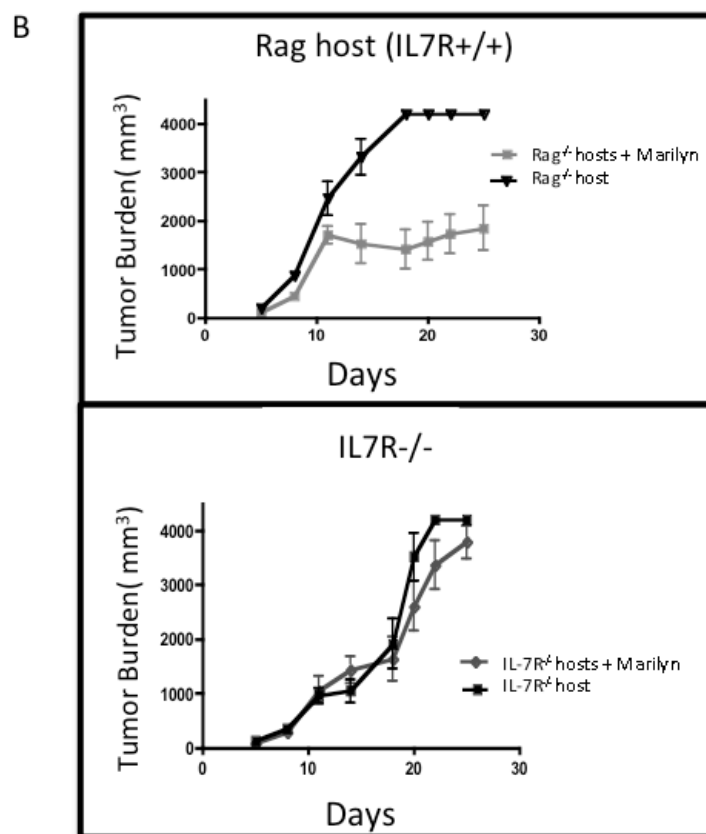
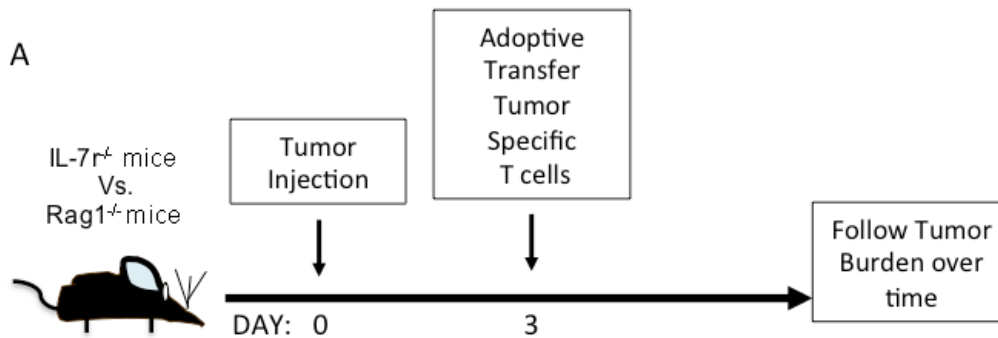


Figure 4.2. IL-7R deficient hosts do not support cancer immunotherapy. Recipient Rag1^{-/-} or IL-7R^{-/-} mice received 1×10^6 MB49 Tumor cells, which express the male Hy antigen, by subcutaneous injection in the hind flank. Three days later they received 2×10^6 naive Marilyn Hy-specific T cells by IV injection. Tumor burden and survival was followed over time. **(A)** Visual representation of the methods described above. **(B)** Graph showing tumor burden over time comparing mice that received the tumor-antigen specific T cells to mice that did not receive any T cells in Rag⁺ hosts (top panel) or IL-7R^{-/-} host (Bottom panel). Each dot represents the mean tumor volume \pm SEM. n = 7-10 mice/group. Results are representative of 2 separate experiments.

4.2.3 IL-7R^{-/-} MICE SHOW INCREASED HOMEOSTATIC BUT NOT TUMOR ANTIGEN DRIVEN T CELL PROLIFERATION.

In order to determine if Tg T cells experienced increased proliferation in IL-7R^{-/-} hosts the experiment from Figure 1 was repeated using Marilyn Rag/TCR Tg CD4⁺ Hy-specific T cells instead of wild-type T cells. Here the CD45 congenic marker could not be used to distinguish host and donor cells. Instead, since the TCR clone used to make the Tg mice was a V β 6 TCR, V β 6 staining was used to identify donor derived T cells. V β 6 TCR arrangements can occur naturally, therefore not all of the V β 6⁺ T cells recovered would necessarily be donor derived. However, considering that the host is lymphopenic, and naturally occurring V β 6⁺ cells are rare, most V β 6⁺CD4⁺ T cells recovered would likely be of donor origin. Again, the T cells showed increased proliferation and accumulation in IL-7R^{-/-} hosts (Figure 4.3 left panel).

It could be concluded that Rag/TCR Tg Hy-specific T cells in addition to wild-type T cells undergo increased homeostatic proliferation in IL-7R^{-/-} hosts. There was not something inherently different about Rag/TCR Tg Hy-specific T cells that limited their proliferation in this setting.

Next it was assessed whether the presence of the antigen-bearing tumor itself limited T cell proliferation or function in IL-7R^{-/-} hosts but not Rag^{-/-} hosts. Recipient Rag1^{-/-} or IL-7R^{-/-} mice received 1x10⁶ MB49 tumor cells, which express the male Hy antigen, by subcutaneous injection in the hind flank on day 0. They then received 2x10⁶ CFSE labeled naïve Marilyn Rag/TCR Tg CD4⁺ Hy-

specific T cells by IV injection on day 3. Then on day 10 the mice were sacrificed and splenocytes were analyzed by flow cytometry. Not surprisingly, the tumor bearing mice experienced dramatically increased T cell proliferation compared to the non-tumor bearing mice (Figure 4.3). However, in the presence of the tumor there was no longer increased proliferation in IL-7R^{-/-} hosts compared to Rag^{-/-} hosts. In fact, although the data was not significant it trended in the opposite direction (Figure 4.3 right panel). The increased proliferation in tumor bearing mice could have been caused by the presence of the tumor or by the presence of the antigen on the tumor. A control group with non-Hy expressing tumors and/or a control group that receives Hy antigen without tumor would help determine exactly why the proliferation was increased in tumor bearing mice. Although, since there was a dramatic increase in proliferation and since the donor cells are on a Rag background it is likely that the proliferation was antigen driven. This dramatically increased proliferation in the presence of the tumor may have masked the increased homeostatic T cell proliferation seen previously in IL-7R^{-/-} mice. The possibility that IL-7R^{-/-} hosts experience increased homeostatic but not antigen driven proliferation is interesting and needs to be further investigated. The data shown in Figures 4.2 and 4.3 combined raise questions about the presence of Tregs within this model.

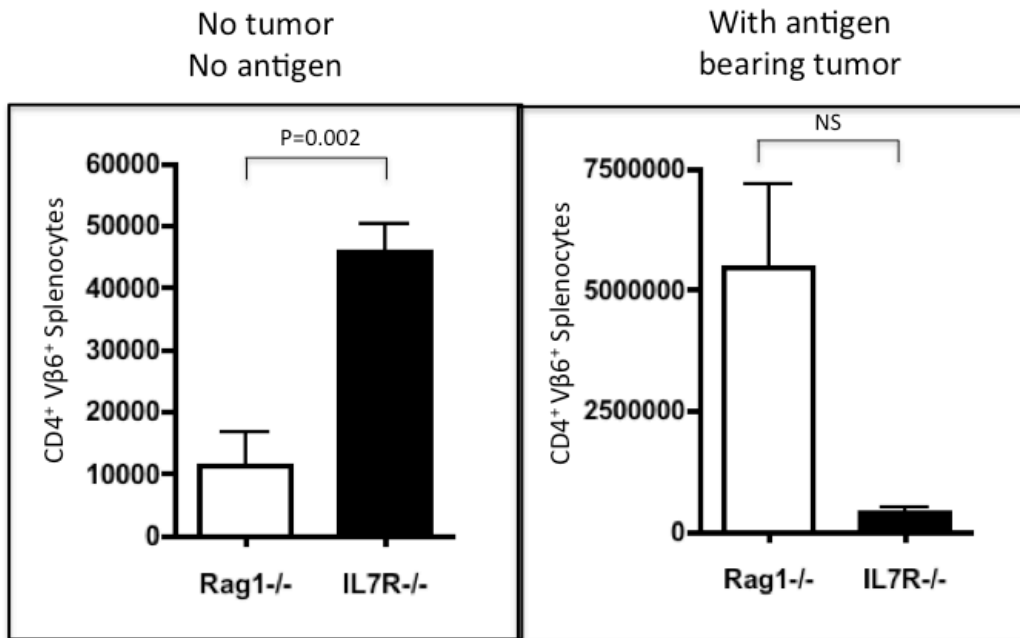


Figure 4.3. Homeostatic but not tumor antigen driven proliferation is increased in IL-7R deficient hosts. Rag1^{-/-} or IL-7R^{-/-} recipient mice received 1x10⁶ MB49 tumor cells expressing the male Hy antigen by subcutaneous injection in the hind flank. After 5 days, they were intravenously injected with 2x10⁶ CFSE-labeled naïve Marilyn Rag/TCR TgHy-specific T cells. Mice were sacrificed on day 12 and splenocytes were analyzed by flow-cytometry. Bar graphs show the mean number ± SEM of Vβ6⁺CD4⁺T cells recovered from the spleen. n=7 mice per group. Results are representative of 2 separate experiments.

4.2.4 IL-7R^{-/-} MICE HAVE AN INCREASED NUMBER OF TREGS AND PREFERENTIALLY DRIVE TREG EXPANSION

Tregs are known regulators of the T cell niche. They are essential for self-tolerance and are negative regulators of the immune response[317]. They control T cell proliferation and activation by direct cell-to-cell contact[318] and by secreting anti-inflammatory cytokines such as IL-10 and TGF- β [319-321]. In the case of the IL-7R deficient mice a decreased presence of Tregs could be responsible for the increased T cell proliferation. Conversely an increased presence of Tregs could be what is limiting tumor immunotherapy. The following experiments were designed to investigate these possibilities. First IL-7R^{-/-}, Rag^{-/-}, and wild-type stock mice were analyzed for expression of Foxp3⁺ Tregs (Figure 4.4A). There are fewer Tregs (Figure 4.4B) but the Tregs make up a larger percent of the CD4⁺ T cell pool (Figure 4.4C) in IL-7R^{-/-} compared to wild-type mice, and Rag^{-/-} mice have no Tregs. As Tregs have been suggested to be less dependent on IL-7 it was a possibility that IL-7R^{-/-} mice had an increased percent of Foxp3⁺CD4⁺ cells. Indeed, analysis of stock mice showed an increased percent of Foxp3⁺ cells although the total number was reduced compared to wild-type mice. Second, recipient IL-7R^{-/-} or Rag^{-/-} control mice, both CD45.2⁺, were given 2x10⁶ CD45.1⁺ wild-type congenic naïve T cells by injection in the tail vein. Seven days later mice were sacrificed and splenocytes were analyzed by flow cytometry to look at the proliferation of Tregs. Again there was an increased number of total donor derived CD4⁺ splenocytes in IL-7R^{-/-} hosts (Figure 4.4D). Additionally, there was an increased number of donor Foxp3⁺CD4⁺ splenocytes in IL-7R^{-/-} compared to Rag^{-/-} hosts (Figure 4.4, black bar). Interestingly, there

was preferential expansion of Tregs within the IL-7R^{-/-} hosts as the Tregs made up a great percent of the total CD4⁺ pool in these hosts. (Figure 4.4E).

Although this finding is of interest, it was not followed up. Instead the experiments presented here continue with the investigation of the increased T cell proliferation seen in IL-7R deficient host. Given the differential presence of Tregs in IL-7R^{-/-} mice compared to Rag^{-/-} mice a IL-7R^{-/-}Rag^{-/-} strain was needed to directly compare to the Rag^{-/-}.

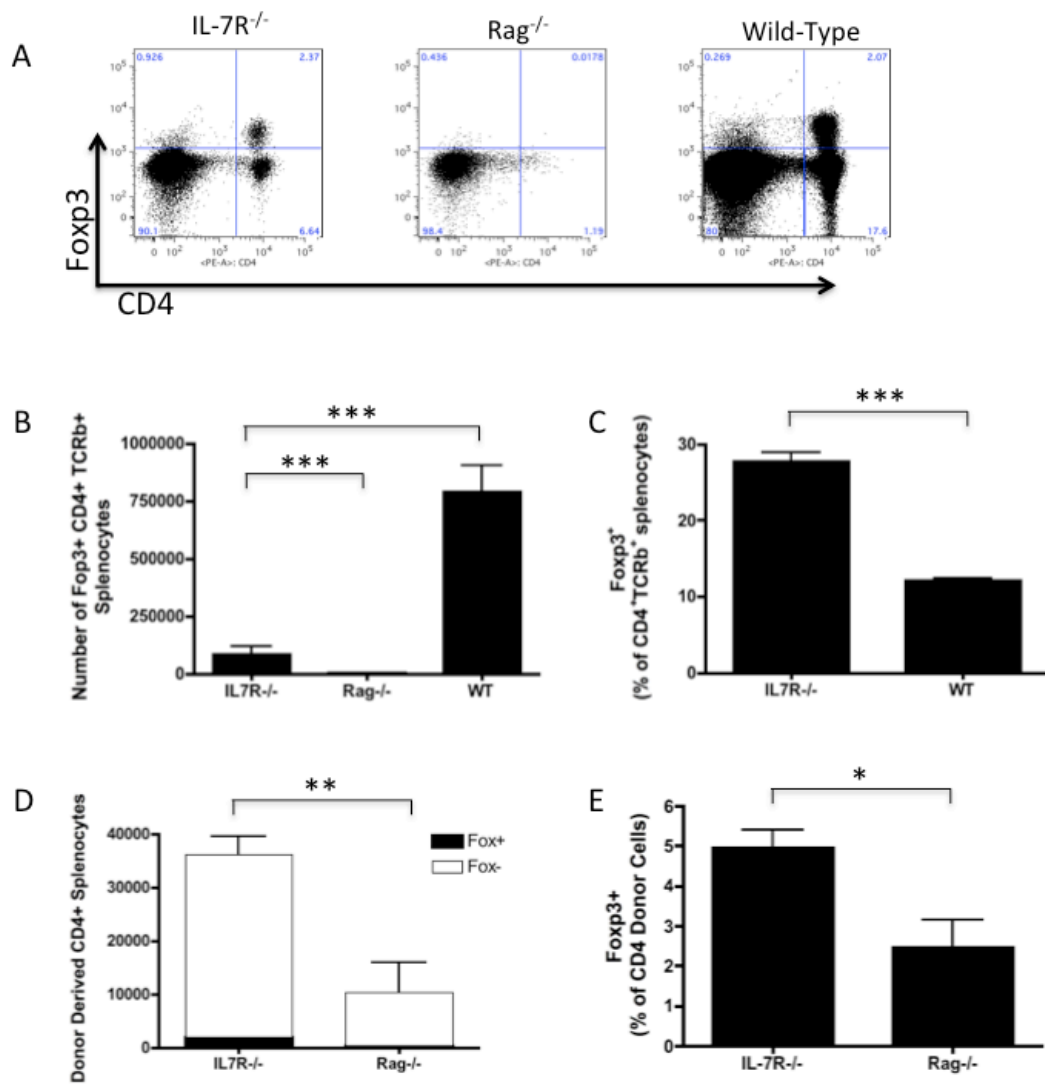


Figure 4.4. IL-7R^{-/-} mice have increased numbers of Tregs and preferential Treg expansion. Fop3⁺ Tregs frequency and number were analyzed in Stock IL-7R^{-/-}, Rag^{-/-}, and wild-type mice (**A**) Representative FACS plots showing CD4⁺ and Fop3⁺ expression on splenocytes. (**B**) Mean number ± SEM of Fop3⁺CD4⁺TCRβ⁺ splenocytes (**C**) Mean percent ± SEM of CD4⁺TCRβ⁺ splenocytes that express Fop3. (**D and E**) CD45.2⁺IL-7R^{-/-} or Rag1^{-/-} recipient mice received 2x10⁶ CD45.1⁺ wild-type naïve T cells by intravenous injection. Seven days later, mice were sacrificed and splenocytes were analyzed by flow-cytometry. (**D**) Mean total number ± SEM of donor derived CD45.1⁺CD4⁺ splenocytes. White and black shaded parts indicate the mean total ± SEM of fop3⁺ and fop3⁻ donor derived cells, respectively. (**E**) Mean percent ± SEM of CD45.1⁺CD4⁺ donor splenocytes that express Fop3. N=3-5 mice/group. Representative of 3 experiments. * = p<0.05, ** = p<0.001, *** = p<0.0001.

4.2.5 IL-7R^{-/-}RAG^{-/-} DOUBLE KNOCKOUT MICE SHOW INCREASED T CELL EXPANSION WITHOUT EXPANSION OF TREGS

Since Rag^{-/-} mice are not a suitable control for IL-7R^{-/-} mice a double knockout strain was developed by crossing the two strains together. 1 in every 16 pups from the F2 generation was knocked out for both genes and was used as founders to establish an IL-7R^{-/-}Rag^{-/-} double knockout colony. Now IL-7R^{-/-}Rag^{-/-} mice can be compared to Rag^{-/-} mice and the only difference between the two groups will be IL-7R signaling.

Recipient IL-7R^{-/-}Rag^{-/-} or Rag^{-/-} mice, both CD45.2⁺, received 2x10⁶ CFSE labeled CD45.1⁺ wild-type congenic naïve T cells by IV injection in the tail vein. Seven days later the mice were sacrificed and splenocytes were analyzed by flow cytometry. Again T cells showed increased proliferation (Figure 4.5A) and accumulation (Figure 4.5B) in IL-7R^{-/-}Rag^{-/-} mice compared to Rag^{-/-} controls. From this better controlled experiment it can be concluded that the increased proliferation seen in IL-7R deficient mice is actually due to a lack of IL-7R and not just an artifact of the IL-7R^{-/-} host.

Additionally, there was no enhanced expansion of Tregs in the IL-7R^{-/-}Rag^{-/-} double knockout host as seen in the IL-7R^{-/-} single knockout (Figure 4.5C). Since the host cells are now Rag deficient and therefore cannot make Tregs[322], there would have been no Tregs present at baseline.

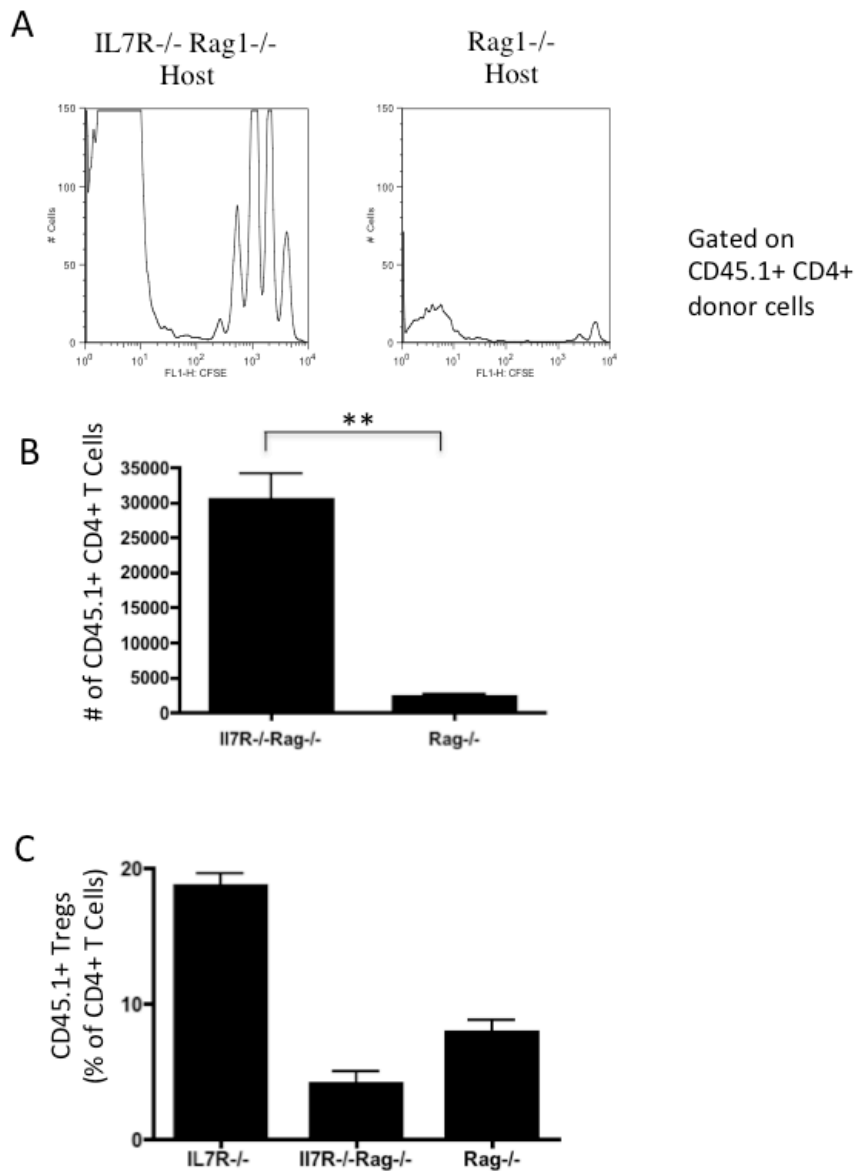


Figure 4.5. IL-7R^{-/-}Rag^{-/-} double knockout mice show increase T cell proliferation and accumulation without expansion of Tregs. CD45.2⁺IL-7R^{-/-}Rag1^{-/-} or Rag^{-/-} recipient mice received 2×10^6 CFSE labeled CD45.1⁺wild-type naïve T cells by i.v injection. After 7 days, mice were sacrificed and splenocytes were analyzed by flow-cytometry. **(A)** CFSE dilution showing the proliferation pattern of CD45.1⁺CD4⁺ donor-derived T cells using. **(B)** Mean number \pm SEM of CD45.1⁺CD4⁺ donor-derived T cells recovered from the spleen. **(C)** Mean proportion \pm SEM of Foxp3⁺ relative to CD4⁺T cells recovered from the spleen. N = 7 mice per group. Results are representative of 2 separate experiments. ** = $p < 0.001$

4.2.6 IL-7R^{-/-}RAG^{-/-} DOUBLE KNOCKOUT MICE SUPPORT CANCER IMMUNOTHERAPY BUT ANY ADVANTAGE AT TUMOR REJECTION HAS NOT YET BEEN DETERMINED.

With this new system using double knockout hosts it was possible to go back to the tumor model and further investigate the possibility that IL-7R deficient mice might experience increased cancer immunotherapy in a setting without enhanced Treg expansion. The experiment was designed along similar lines to the experiment shown in Figure 4.2 using MB49 tumor cells, which express the male Hy antigen, and Marilyn Tg T cells which are specific for the Hy antigen. Due to a limited number of double knockout hosts available in the lab the entire experiment was carried out in chimeric hosts. Bone marrow chimeras were made in Rag^{-/-} hosts by lethally irradiating the hosts and then transferring either IL-7R^{-/-}Rag^{-/-} or Rag^{-/-} bone marrow. After waiting for 6 weeks the following experiment was carried out in these chimeric hosts.

Recipient IL-7R^{-/-}Rag^{-/-} or Rag^{-/-} chimeric mice received 1×10^6 MB49 tumor cells by subcutaneous injection in the hind flank. Three days later, where indicated, they received 2×10^6 naïve Marilyn tumor-specific T cells by IV injection. The tumor burden and survival was followed over time. Both Rag^{-/-} and IL-7R^{-/-}Rag^{-/-} hosts showed complete tumor regression in mice that received an adoptive transfer of Marilyn tumor-specific T cells (Figure 4.6). This was in contrast to increasing tumor burden and the ultimate death of mice that did not receive any T cells (Figure 4.6).

Notably the tumor doses and T cell doses were exactly the same as the doses used in Figure 4.2. However, since the mice had previously received a bone marrow transplant this may have led to heightened immune activity[323]. The tumor and T cells doses should have been optimized so that the Rag^{-/-} control arm of the experiment again showed approximately 50% survival. Unfortunately, in this experiment the control arm showed complete tumor regression and 100% survival. The original hypothesis predicting that the IL-7R^{-/-}Rag^{-/-} double knockout hosts would show increased tumor immunity that could not have been detected in this experiment. The experiment is therefore inconclusive. Unfortunately, due to time constraints follow-up experiments with titrated tumor and T cell doses have not been completed. In order to make any final conclusion about tumor immunity in IL-7R^{-/-}Rag^{-/-} mice this experiment will need to be repeated with an increased tumor dose and/or a decreased T cell dose.

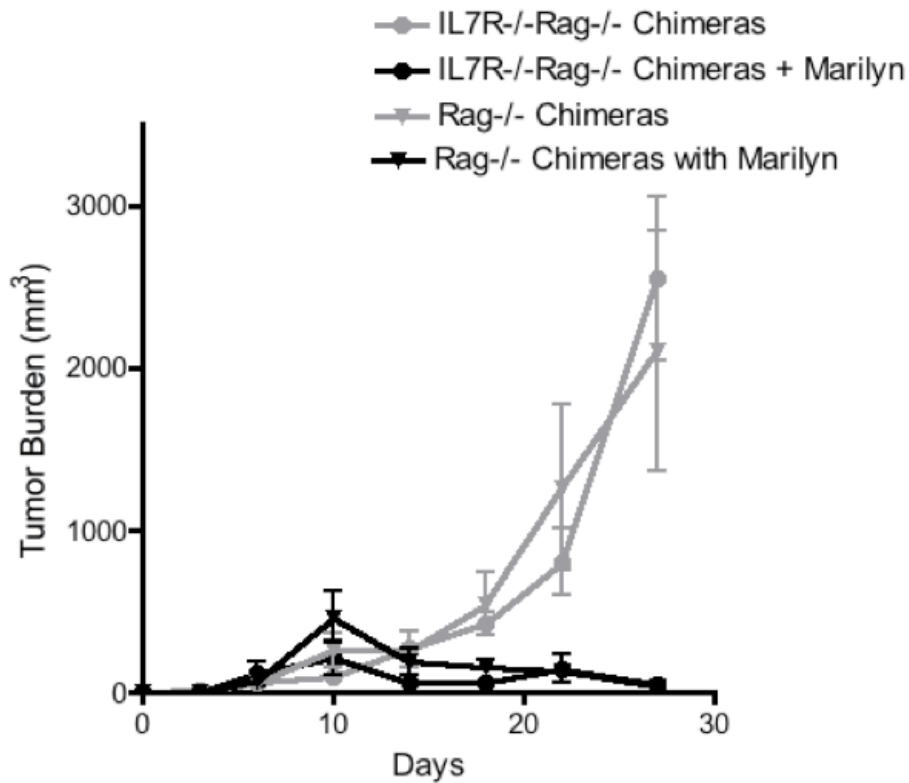


Figure 4.6. IL-7R^{-/-}Rag^{-/-} hosts support cancer immunotherapy but differences between IL-7R^{-/-}Rag^{-/-} hosts and the Rag hosts cannot be determined from this experiment. In order to expand the number of mice available for this experiment bone marrow chimeras were made in Rag^{-/-} hosts using Rag^{-/-} or IL-7R^{-/-}Rag^{-/-} bone marrow. Recipient Rag^{-/-} or IL-7R^{-/-}Rag^{-/-} chimeric mice received 1x10⁶ MB49 Tumor cells, which express the male Hy antigen, by subcutaneous injection in the hind flank. Three days later they received 2x10⁶ naive Marilyn Hy-specific T cells by IV injection. Tumor burden and survival was followed over time. Graph shows tumor burden over time comparing mice that received the tumor-antigen specific T cells to mice that did not receive any T cells in Rag^{-/-} hosts and IL-7R^{-/-}Rag^{-/-} hosts. Each dot represents the mean tumor volume ± SEM. N = 5-7 mice per group. This experiment was not repeated.

4.3 DISCUSSION

The immune system employs a diverse set of inhibitory pathways that are important for maintaining self-tolerance and controlling immune responses. Tumors are known to co-opt these inhibitory pathways as a major mechanism of escaping immune surveillance particularly against T cells that are specific for tumor antigens[324]. Therefore blocking inhibitory pathways has been proposed as the most promising way to activate therapeutic antitumor immunity[324]. The increased proliferation of T cell seen in IL-7R deficient mice could be an indication of a blocked inhibitory pathway that normally limits HPE or other types of proliferation and is worthy of further investigation.

The data presented here confirm the previous finding[261] that although IL-7R^{-/-} mice and Rag^{-/-} mice are both lymphopenic, naïve T cells undergo increased proliferation and accumulation in IL-7R^{-/-} hosts compared to Rag^{-/-} hosts (Figure 4.1). This data led to the hypothesis that IL-7R^{-/-} mice might therefore experience increased ability to support cancer immunotherapy because of an increased ability to expand tumor specific T cell, which turned out not to be the case. In fact, while Rag^{-/-} mice were able to reject an Hy expressing tumor when transferred Hy-specific T cells, IL-7R^{-/-} were not able to reject the same tumor under the same circumstances (Figure 4.2). Although the IL-7R^{-/-} mice experience increased homeostatic proliferation of tumor specific T cells they did not experience increased proliferation in the presence of the tumor compared to Rag^{-/-} mice (Figure 4.3), possibly because of the increased number of Tregs seen

in IL-7R^{-/-} mice upon adoptive transfer of T cells (Figure 4.4D,E). Tregs are known to suppress tumor immunity[325, 326]. It has been shown that Tregs congregate in tumor microenvironments, provide immune evasion to tumor cells[327], and correlate with worse tumor grades[328].

The increased presence of Tregs in IL-7R^{-/-} mice (Figure 4.4A) was not surprising given that most T cells are highly dependent on IL-7 signals for survival and proliferation, but Tregs are the rare exception. Tregs do not require IL-7 for ontogeny, function, or peripheral homeostasis[329], and in fact, there is an inverse correlation between IL-7R expression and Foxp3 expression on CD4⁺ T cells[330]. However, it has also been reported that signaling through IL-7R by either IL-7 or TSLP is required for the development of Tregs but that the two cytokines play a redundant role[205]. This is directly contradicted by the presence of Tregs in IL-7R^{-/-} mice demonstrated here (Figure 4.4A) and in published work[206], where they further demonstrate that IL-7R plays a redundant role with IL-2R since double knockout mice lack Tregs but reconstitution of IL-2R signaling was sufficient for restoring Treg development[206].

The unique ability of IL-7R^{-/-} mice to experience endogenous Treg proliferation when all other T cells are severely limited by lack of IL-7R inhibited our ability to study the increased proliferation in this host since there was not a suitable control. It was therefore necessary to develop a better-controlled model. Thus the IL-7R^{-/-}Rag^{-/-} strain was developed so that it could be compared to Rag^{-/-} mice in future experiments. IL-7R^{-/-}Rag^{-/-} mice, similar to IL-7R^{-/-} mice, showed

increased homeostatic proliferation compared to Rag^{-/-} mice confirming that the lack of IL-7R was indeed driving the increased proliferation (Figure 4.5). When the Hy expressing tumor model was repeated in the IL-7R^{-/-}Rag^{-/-} the results were inconclusive, and the experiment needs to be repeated with a larger tumor dose.

Figure 4.3 shows that IL-7R^{-/-} mice experience increased homeostatic proliferation but did not experience increased proliferation of tumor specific T cells in the presence of tumor. All of the tumor experiments shown here were performed in female mice where the Hy antigen is foreign, but the body may see tumor-antigens as self-antigens [309, 331]. Since homeostatic proliferation, the type of proliferation increased in IL-7R deficient hosts, usually involves proliferation against self-antigens[86-93] it would be interesting to explore this tumor model in a male host where the Hy tumor antigen would be seen as self. MB49 has been shown to grow faster in male mice than in female mice indicating that there is a natural response to the foreign Hy antigen by the female hosts[332], which may render this model less clinically relevant when used in female hosts. Whether male IL-7R deficient mice would experience increased Hy tumor-antigen driven proliferation remains to be determined.

Future experiments will also include determining if IL-7R^{-/-}Rag^{-/-} hosts experience increased tumor-antigen driving proliferation or increased cancer immunotherapy compared to Rag^{-/-} hosts using an increased tumor dose. It would also be interesting to complete similar experiments using Matahari Hy-specific CD8⁺ cells instead of Marilyn cells to gain a better understanding of

whether or not the increased proliferation is specific to CD4⁺ T cells or if it also applies to CD8⁺ T cells. Finally the MB49 Hy-expressing tumor only represents one possible tumor model. It would also be interesting to explore other tumor models such as the B16 melanoma model that utilizes CD8⁺ tumor specific cells from Tg Pmel mice.

5 AN EXPLORATION OF THE CAUSE OF THE INCREASED T CELL EXPANSION IN IL-7R DEFICIENT MICE

5.1 INTRODUCTION

It has been previously published that wild-type T cells undergo increased proliferation and accumulation in IL-7R deficient hosts, which was attributed to the influence of IL-7 on DCs[261]. Others have suggested that the increased proliferation reflects increased availability of IL-7 in IL-7R^{-/-} hosts[333], since IL-7R^{-/-} hosts have been shown to have increased serum levels of IL-7[261]. However, if increased IL-7 levels are what is driving the increased proliferation, then it would be expected that injection of IL-7 into lymphopenic hosts would have a similar effect. Indeed, administration of IL-7 to lymphopenic hosts did result in increased proliferation of CD4⁺ T cells[25]. However, in that study there was a relative decrease in Tregs, unlike the preferential increase in Tregs observed in IL-7R^{-/-} hosts[25]. Given these uncertainties, the work in this chapter was designed to test the hypothesis that cellular IL-7R signaling restrains T cell proliferation in lymphopenia.

It is important to note that since both TSLP and IL-7 signal through the IL-7R, IL-7R^{-/-} mice fail to respond to either cytokine. Therefore, the increased T cell proliferation in IL-7R^{-/-} mice could be caused by deficiency in signals from TSLP, IL-7, or both.

IL-7R is expressed on a wide variety of cells. In order to determine which cell type might be responsible for the increased T cell proliferation in IL-7R^{-/-} hosts and how, it is important to consider all of the cells within the body that normally express IL-7R and are therefore affected by the mutation present in the IL-7R^{-/-} mice. This includes epithelia and stromal cells[261], DCs[271, 334], and ILCs[138]. B and T cells, of course, also express IL-7R. However, since the increased proliferation is seen in lymphopenic mice that lack B and T cell function, they can be ruled out as possible candidates.

Stromal and epithelial cells express IL-7R and are the main producers of IL-7[247]. The role of IL-7R on stromal cells is not well studied, but the current understanding is that IL-7R present on stromal cells plays a role in feedback inhibition of IL-7 production. Splenic stromal cells from lymphopenic SCID or Rag^{-/-} mice with high serum IL-7 showed lower expression of IL-7 mRNA than splenic stromal cells from non-lymphopenic wild-type mice with lower serum IL-7 levels[261]. These data suggest that IL-7R signaling on the stroma leads to decreased production of IL-7 by the stromal cells. The data also suggest that increased IL-7 during lymphopenia results from decreased consumption rather than increased production. Although it is unlikely that IL-7R signaling on stromal cells leads to increased T cell proliferation, it is worthy of further investigation.

The most well studied role of the IL-7R on DCs is in TSLP signaling, where TSLP causes DCs to promote a T_{H2} phenotype in T cells[266, 267]. The role of IL-7 itself on DCs is a very understudied area with only two publications in recent

years. The first paper described the increased proliferation seen in IL-7R^{-/-} mice, and suggested that IL-7 signaling on DCs controls the size of the T cell niche[261]. They demonstrated that pDCs in lymphopenic mice, such as Rag^{-/-} mice, showed decreased expression of MHC-II, but that when IL-7R signaling was inhibited, the pDCs returned to normal expression levels of MHC-II. They concluded that IL-7 signaling on DCs mediates down regulation of MHC-II[261]. The second paper used competitive bone marrow chimeras to show that IL-7 signals are intrinsically required for the development of cDCs and pDCs from the spleen and peripheral lymph nodes, but these findings were only barely statistically significant[334]. Since they also saw that IL-7 was required for the development of CLPs and not CMPs they proposed that DCs derive from lymphoid precursors[334], a theory which has since been disproved[111, 112, 335]. Since the findings in the first paper have been disputed[333] and the proposed conclusions of the second paper may also be flawed, further studies are required to establish the role of IL-7R on DCs.

IL-7 signals are also required for the production of some ILCs including lymphoid tissues inducer (LTi) cells and ILC-22[138]. LTi cells are important for the development of lymphoid tissues during embryogenesis. Mice that lack LTi cells, such as the IL-7R^{-/-} mice, have abnormal and small secondary lymphoid structures[143, 336, 337]. ILC-22 in the gut respond to IL-23 by producing IL-22, which is important for gut barrier functions. Although it has not yet been directly studied, mice that lack ILC-22, such as IL-7R^{-/-} mice, might be expected to have decreased gut barrier function. Decreased barrier function in these mice could lead to translocation of commensals, which could then lead to increased

proliferation of T cells. Therefore, the lack of ILC-22 in the gut could be the cause of the increased T cell proliferation in IL-7R^{-/-} hosts and is worthy of investigation.

5.2 RESULTS

5.2.1 DEFICIENT IL-7R SIGNALING ON HEMATOPOIETIC CELLS IS SUFFICIENT FOR T CELL EXPANSION IN IL-7R DEFICIENT MICE

Bone marrow chimeras were generated for two purposes: (1) to determine if IL-7R signaling on non-hematopoietic cells is necessary for the increased T cell proliferation seen in IL-7R^{-/-} mice, and (2) to determine if the absence of LT_i cells during early development is necessary for the increased proliferation seen in IL-7R^{-/-} mice. LT_i cells depend on IL-7 signals for their development and therefore are not present in IL-7R^{-/-} mice. Since LT_i cells are necessary for the development of secondary lymphoid structures, IL-7R^{-/-} mice lack normal secondary lymphoid structures. This lack of structure itself could be responsible for the increased T cell proliferation seen in this model. Although Rag^{-/-} mice also have abnormal secondary lymphoid structure, they do have normal LT_i function and were chosen as hosts for this experiment since they were used in the control arm of the previous experiments.

To assess this, Rag^{-/-} mice were given 1300 cGy of total body irradiation in 2 doses and then transferred with 5x10⁶ IL-7R^{-/-} or Rag^{-/-} bone marrow by IV injection. After waiting for immune reconstitution post-bone marrow transplant,

T cell proliferation was analyzed as described in Figure 4.1. IL-7R^{-/-} chimeric mice and Rag^{-/-} chimeric controls, both CD45.2⁺, received 2x10⁶ CD45.1⁺ wild-type congenic naïve T cells by retro-orbital IV injection. Seven days later the mice were sacrificed and splenocytes were analyzed by flow cytometry. Again T cells showed increased accumulation in IL-7R deficient chimeras compared to Rag^{-/-} chimera controls (data not shown). This proves that IL-7R signals on hematopoietic cells are sufficient for the increased proliferation seen in IL-7R^{-/-} mice and that IL7R deficiency on stromal and epithelial cells is not required. Additionally, differences in secondary lymphoid structure due to lack of LTi cells during early development are not required for the increased proliferation seen in IL-7R^{-/-} mice, since both groups here should have similar secondary lymphoid structure.

5.2.2 IL-23 DRIVEN IL-22 PRODUCING ILCs ARE NOT RESPONSIBLE FOR THE INCREASED PROLIFERATION SEEN IN IL-7R^{-/-} MICE

Recent work has shown that IL-7 is important for the development of innate lymphoid cell (ILC) subsets including not only Lymphoid Tissue Inducer (LTi) cells, but also ILC-22, which are dependent on ROR γ t[338]. ILC-22 are known producers of IL-22, which is produced in response to IL-23 signals[148, 339], and is important for barrier function and wound healing in the gut[340]. Since ILC-22 are important for barrier function, it is logical that IL-7R^{-/-} mice, which lack ILC-22, may have decreased barrier function and could potentially experience translocation of commensals which would yield increased T cell proliferation. In order to further investigate this possibility, T cell proliferation

and accumulation was evaluated in other mouse strains that also lack ILC-22 function.

First, T cell proliferation was evaluated in IL23R^{-/-}Rag^{-/-} mice. IL23R^{-/-}Rag^{-/-} mice or Rag^{-/-} controls were given an IV injection of naïve T cells. These mice do not lack ILC-22, but since they cannot produce IL-23, which would normally induce production of IL-22 by ILC-22, they may therefore have a similar decreased barrier function as IL-7R^{-/-} may have. For this experiment, due to availability of donor strain, the CD90 Thy1 congenic marker was used to track donor cells instead of the CD45 marker. There was no significant difference in the expansion of T cell in the two hosts as seen in representative histograms (Figure 5.1A) or bar graphs showing the mean number of Thy1.1⁺CD4⁺ donor derived T cells recovered from the spleen (Figure 5.1B).

Second, T cell proliferation was evaluated in IL-22^{-/-}Rag^{-/-} mice. Again, these mice do not lack ILC-22, but they cannot produce IL-22 and therefore have impaired barrier function[152, 341]. Recipient IL22^{-/-}Rag^{-/-} hosts or Rag^{-/-} control, both Thy1.2⁺, were given 2x10⁶ wild-type Thy1.1⁺ T cells by IV injection. The mice were sacrificed 7 days later and their spleens were harvested for analysis by flow cytometry. There was no difference in T cell proliferation observed between the two hosts. (Figure 5.2). Together these two lines of investigation suggest that lack of IL-23 dependent IL-22 production from ILCs is not a primary cause of increased T cell proliferation in IL-7R deficient mice. A caveat is that these strains are not perfectly comparable to IL-7R^{-/-} mice, as they still have ILC-22 populations. A more definitive experiment would be to look at T

cell proliferation in ROR γ t^{-/-}Rag^{-/-} hosts, which would actually lack a similar set of ILCs as IL-7R^{-/-} hosts.

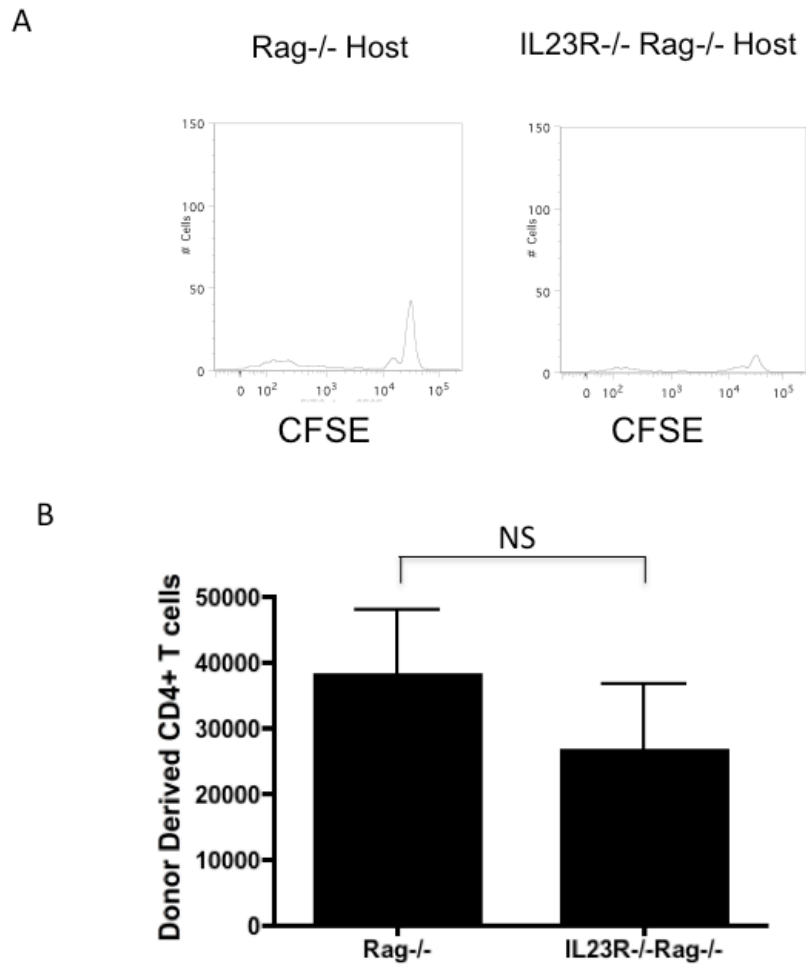


Figure 5.1. T cell transfer into IL-23R^{-/-}Rag^{-/-} hosts does not lead to increased proliferation.

Thy1.2⁺ IL23R^{-/-}Rag^{-/-} or Rag^{-/-} recipient mice received 2×10^6 CFSE labeled Thy1.1⁺ wild-type naïve T cells by intravenous injection. After 7 days, mice were sacrificed and splenocytes were analyzed by flow-cytometry. **(A)** Representative histograms show the proliferation pattern of Thy1.1⁺CD4⁺ donor-derived T cells. **(B)** Mean number \pm SEM of Thy1.1⁺CD4⁺ donor-derived T cells recovered from the spleen. N = 5 mice per group. Results are representative of 2 independent experiments.

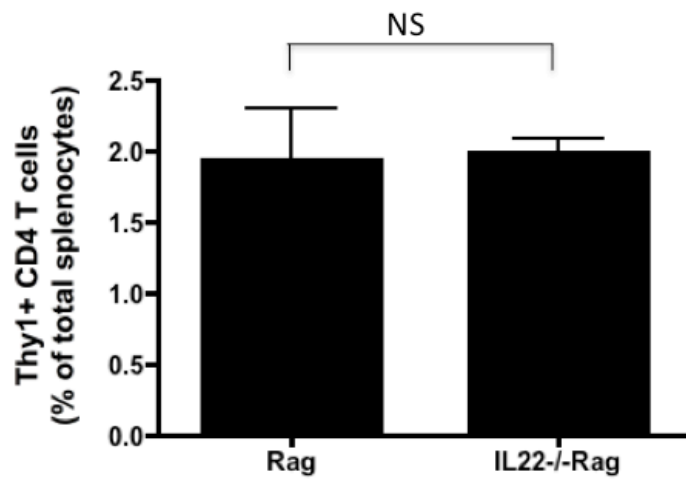


Figure 5.2. T cell transfer into IL22^{-/-}Rag⁺ hosts does not lead to increased proliferation.

Thy1.2⁺IL22^{-/-}Rag⁺ or Rag⁺ recipient mice received 2×10^6 CFSE labeled Thy1.1⁺ wild-type congenic naïve T cells by intravenous injection. After 7 days, mice were sacrificed and splenocytes were analyzed by flow-cytometry. Bar graphs show the mean percent \pm SEM of Thy1.1⁺CD4⁺ donor derived T cells recovered from the spleen. N = 5 mice per group. This experiment was not repeated.

5.2.3 TOLEROGENIC SUBSETS OF DCs SHOW INCREASED EXPRESSION OF THE IL-7R

Since stromal cells and ILCs did not seem to be important for the increased T cell proliferation seen in IL-7R deficient mice, the next set of experiments focus on DCs. Previous studies in the Powrie lab demonstrated that a subset of tolerogenic mucosal DCs that express the integrin CD103 and promote the differentiation of Tregs showed increased expression of IL-7R in a microarray (Figure 5.3A) and by qPCR (Figure 5.3B) comparing CD103⁺ and CD103⁻ DCs from the MLN. These results suggested that IL-7R signaling might be important for the development or function of tolerogenic DCs. Flow cytometric analysis of MLN cells confirmed increased expression of IL-7R by CD103⁺ compared to CD103⁻ DCs (Figure 5.3C).

A: microarray data courtesy of Janine Coombes

Log2 Fold Change	Probability	Gene
5.41	3.01E-04	Interleukin 7 receptor

B: qPCR data courtesy of Janine Coombes

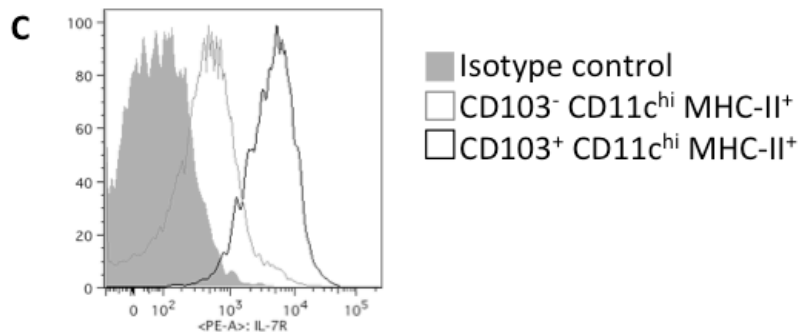
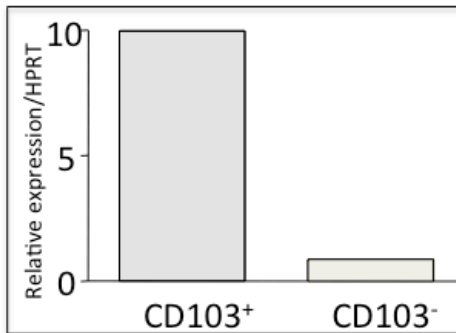


Figure 5.3. CD103⁺ DCs show increased expression of IL-7R. The data shown in parts A and B of this figure were produced by Janine Coombes. MLNs were harvested from wild-type mice and CD103⁺CD11c^{hi}CD3e⁻ vs. CD103⁻CD11c^{hi}CD3e⁻ DCs were FACS sorted for **(A)** microarray and **(B)** qPCR analysis. **(C)** IL-7R expression of cell from the MLN of wild-type mice. Results in part (C) are representative of 2 independent experiments.

5.2.4 HOMEOSTATIC DC SUBSETS CAN BE PRODUCED FROM BONE MARROW CELL IN VITRO AND EXPRESS THE IL-7R

To study the role of IL-7R in T cell / DC interactions, an in vitro assay was developed. For many years BMDCs have been developed in vitro with the use of GM-CSF, and it is true that under inflammatory conditions DCs can develop in vivo from monocytes in a GM-CSF dependent manner. However, recent work has demonstrated that under homeostatic conditions DCs normally develop from pre-DCs in a Flt3L dependent fashion[111-115]. Since the increased proliferation in IL-7R^{-/-} hosts seems to be a homeostatic mechanism and not an inflammatory one (Figure 3.3), it seemed most reasonable to investigate the effects of IL-7R signaling on homeostatic rather than inflammatory DCs.

Recent studies in the Powrie lab have developed a method of growing BMDCs in vitro that took advantage of the new data implicating Flt3L in homeostatic DC development. Bone marrow was harvested from wild-type mice and cultured in complete media with 200ng/mL Flt3L for the first seven days. On the seventh day of culture, 20ng/mL GM-CSF was added, and on the eighth day of culture 1ug/mL of LPS was added to mature the DCs. Figure 5.4A is a schematic of the methods used. The cells isolated from the cultures on day nine showed high expression of CD11c and MHC-II (Figure 5.4B), and CD103 and CD11b subsets could be identified (Figure 5.4B).

Flt3L developed BMDCs were evaluated for expression of IL-7R. IL-7R expression could be easily distinguished compared to the Isotype controls

(Figure 5.5A) with increased expression amongst the CD103⁺ group (Figure 5.5B). This technique of deriving DCs from BM cells can be utilized for future experiments to look at the effects of IL-7R signaling on DCs isolated in culture.

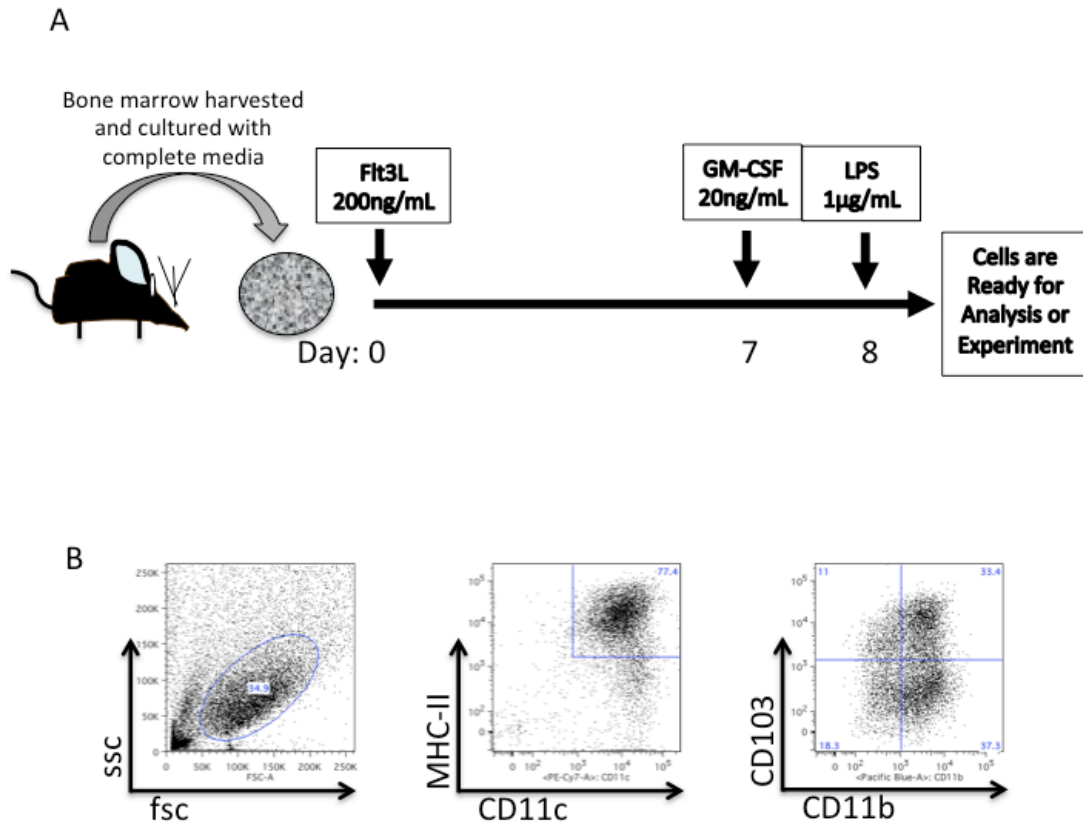


Figure 5.4. DC subsets can be produced from bone marrow cells. Bone marrow was harvested from wild-type B6 mice and cultured with Flt3 Ligand for 8 days, along with GMCSF during the last 48 hours and LPS during the last 24 hours. **(A)** Visual representation of the methods. **(B)** Representative FACS plots showing DC subsets based on CD103 and CD11b expression. Data are representative of 6 different experiments.

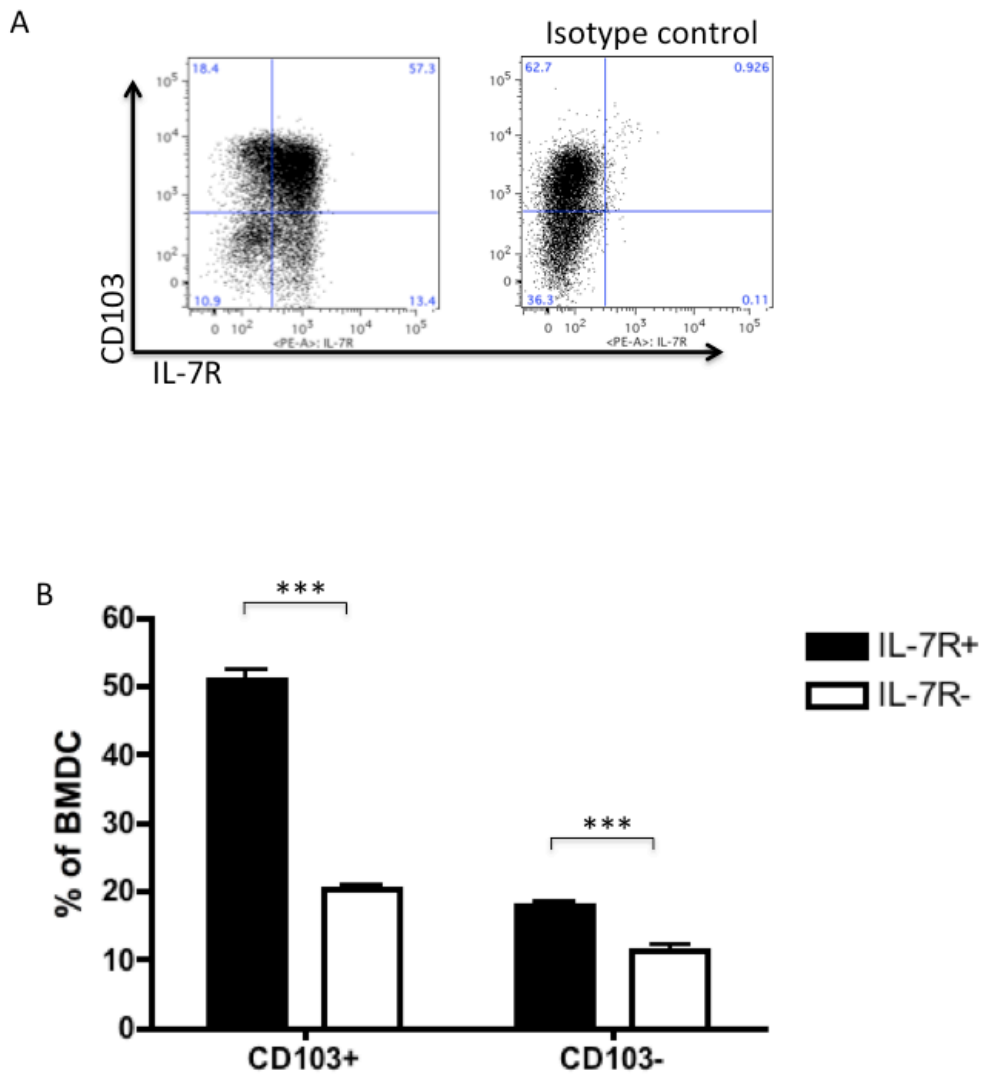


Figure 5.5. BMDCs express the IL-7R with increased expression amongst the CD103⁺ fraction. Bone marrow was harvested from wild-type B6 mice and cultured with Flt3 Ligand for 8 days, along with GM-CSF during the last 48 hours and LPS during the last 24 hours. On day 9 the cells were analyzed for expression of IL-7R. **(A)** Representative FACS plots showing IL-7R expression on BMDCs cells (left panel) compared to an isotype control (right panel). **(B)** Mean percent \pm SEM of cells that express the IL-7R amongst CD103⁺ and CD103⁻ groups. N = 10 samples per group. Results representative of 2 separate experiments. *** = $p < 0.0001$.

5.2.5 BMDCs TREATED WITH IL-7 SHOW NO PHENOTYPIC CHANGES

Flt3L generated BMDCs were treated with IL-7 to test whether there is a direct effect of IL-7 on the phenotype of the BMDCs. Bone marrow was harvested from wild-type mice and cultured in complete media with 200ng/mL Flt3L for the first seven days. On the seventh day of culture, 20ng/mL GM-CSF was added, and on the eighth day of culture 1ug/mL of LPS was added to mature the DCs. BMDCs were then washed and treated with varying amounts of IL-7 or TSLP as indicated. Representative FACS plots (Figure 5.6A) show the gating strategy used to define viable DCs using forward and side scatter and CD11c⁺MHC-II⁺ double positive populations. Surprisingly, even very high concentrations of IL-7 showed negligible changes in expression of CD11c and MHC-II (Figure 5.6B).

Since CD103⁺ DCs showed increased expression of the IL-7R (Figure 5.3), IL-7 and TSLP treated BMDC were analyzed to determine if IL-7R signaling had an effect on the proportion of BMDCs that express CD103⁺. Treated cells were analyzed by flow cytometry and gated on forward and side scatter; CD11c⁺MHC-II⁺ DCs were then categorized by expression of CD11b and CD103 (Figure 5.7A). DCs treated with 20ng/mL IL-7 showed increased expression of CD103 (Figure 5.7B). However, when the experiment was repeated with more controls and more IL-7 concentrations the results were not significant (Figure 5.7C).

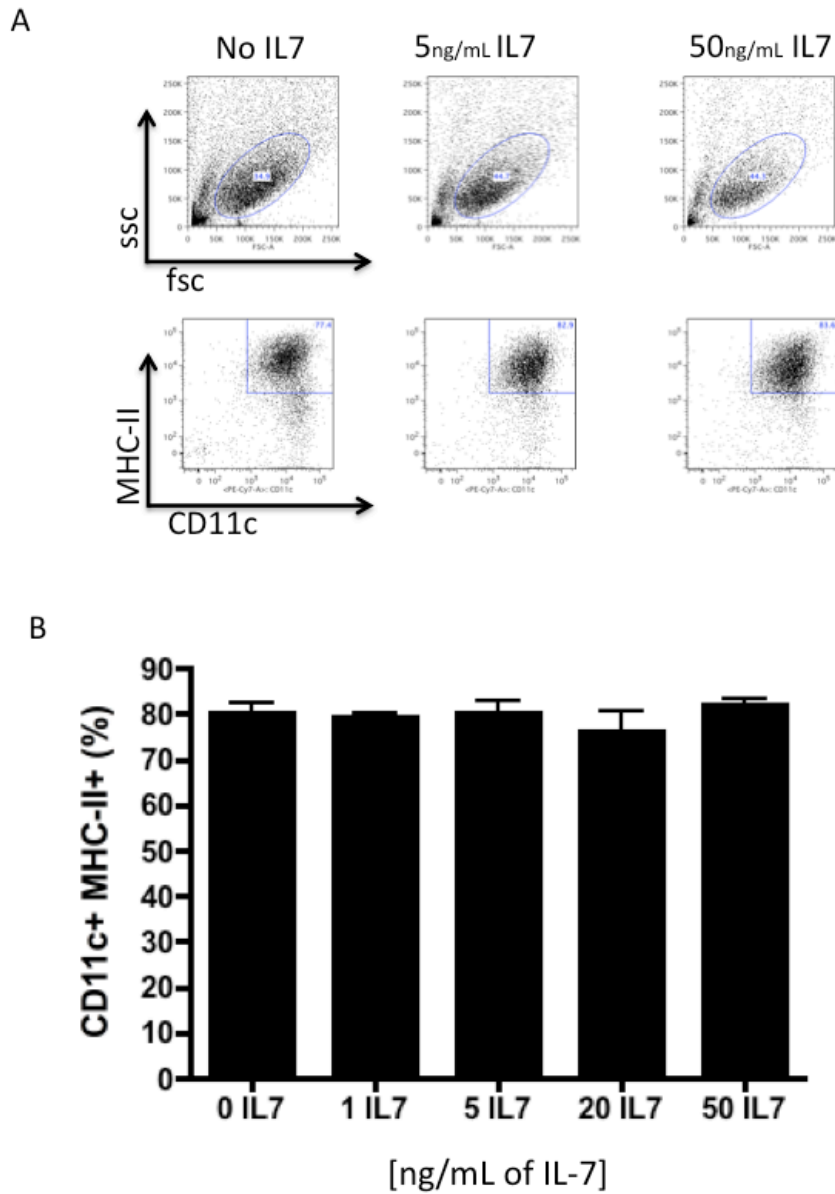


Figure 5.6. IL-7 treatment of BMDCs yields no change in expression of CD11c & MHC-II. Flt3 generated BMDCs treated with indicated amounts of IL-7. After 24 hours, cells were subjected to flow cytometric analysis. **(A)** Representative FACS plots showing forward vs. side scatter and CD11c vs. MHC-II expression after treatment with different concentrations of IL-7. **(B)** Mean percent \pm SEM of cells that expressed CD11c and MHC-II after treatment with different concentrations of IL-7. N = 4-5 different culture wells per group.

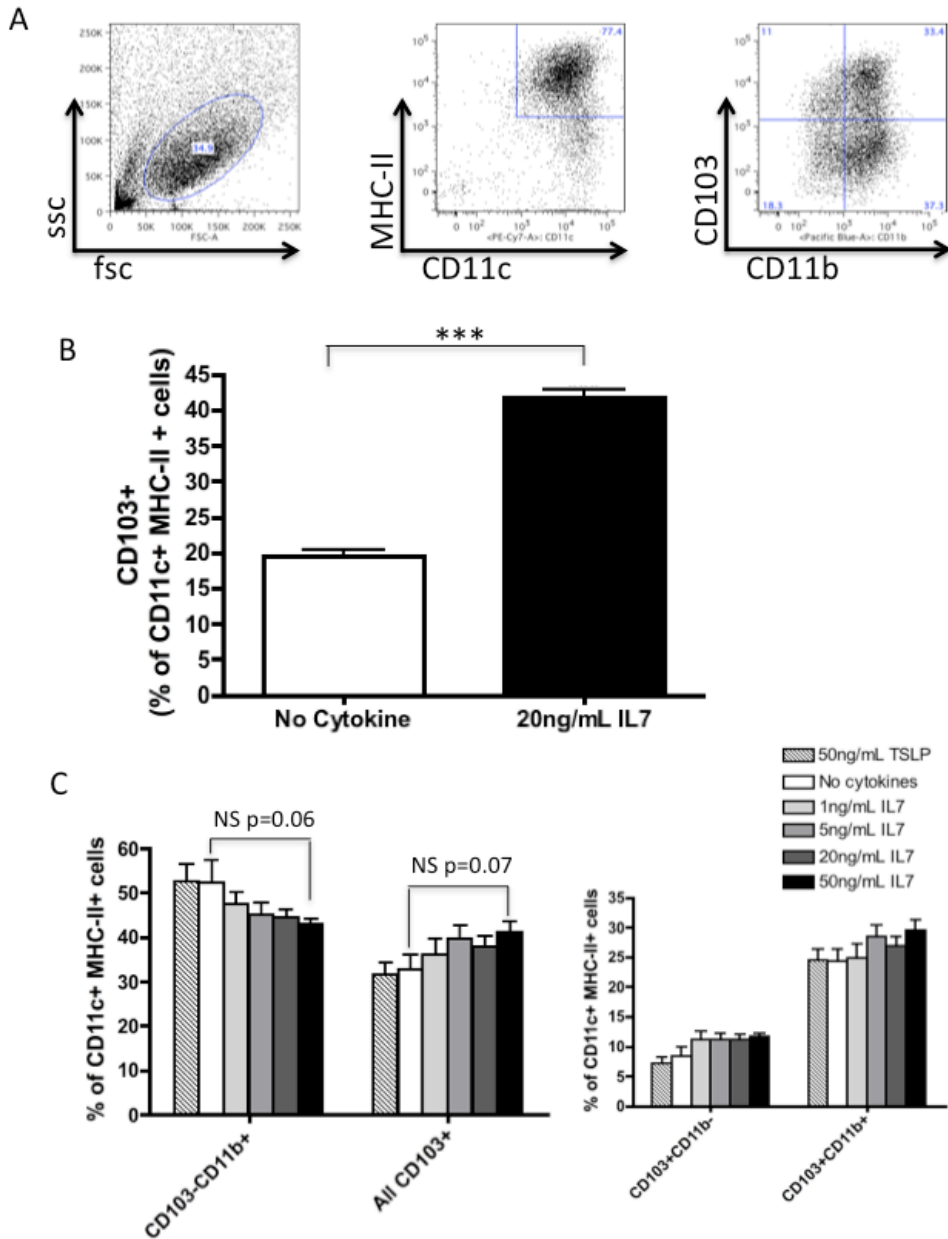


Figure 5.7. BMDCs treated with IL-7 show increased expression of CD103. Flt3L generated BMDCs were washed and treated with the indicated amounts of IL-7 or TSLP for 24 hours. **(A)** Gating strategy used to define dendritic cell subsets. **(B)** Bar graphs showing mean percent \pm SEM of CD103⁺ cells within the CD11c⁺MHC-II⁺ gate. N = 9 culture wells per group. **(C)** The experiment from (A) was repeated with more variation in cytokine dose, including a control group treated with TSLP. Bar graphs show the mean percent \pm SEM of CD11c⁺MHC-II⁺ cells that were positive for CD11b and/or CD103. N = 4-5 culture wells per group. Results are representative of 2 independent experiments. *** = p < 0.0001

5.2.6 TREATMENT OF BMDCs WITH IL-7 SHOWS A FUNCTIONAL CHANGE IN THEIR ABILITY TO INDUCE T CELLS PROLIFERATION

As there was no big change in phenotype following IL-7 treatment, the following cocultures of DCs and T cells were designed to test whether there was a change in function. Bone marrow was harvested from wild-type B6 mice and cultured with Flt3L, then GM-CSF, and then stimulated with LPS. The BMDCs were washed and then treated with varying amounts of IL-7 or TSLP for 24 hours. The BMDCs were washed 3 times to remove any trace of the cytokine before T cells were added for coculture. Finally, CFSE labeled OT-II T cells and ova peptide (200ng/mL) were added to the BMDCs for a 4-day coculture. On the fourth day of coculture, the T cells were counted and analyzed by flow cytometry. A schematic of the methods is shown in Figure 5.8A. BMDCs treated with 50ng/mL IL-7 looked phenotypically the same as (Figure 5.6), but supported less proliferation of T cells than, BMDCs treated with TSLP or not treated (Figure 5.8B). T cells cocultured with BMDCs treated with increasing amounts of IL-7 showed decreased accumulation (Figure 5.8C) and fewer cells that had undergone 4 or more divisions by CFSE gating (Figure 5.8D). This data shows that high-dose IL-7 treatment of BMDCs inhibits their ability to induce proliferation of naïve CD4 T cells.

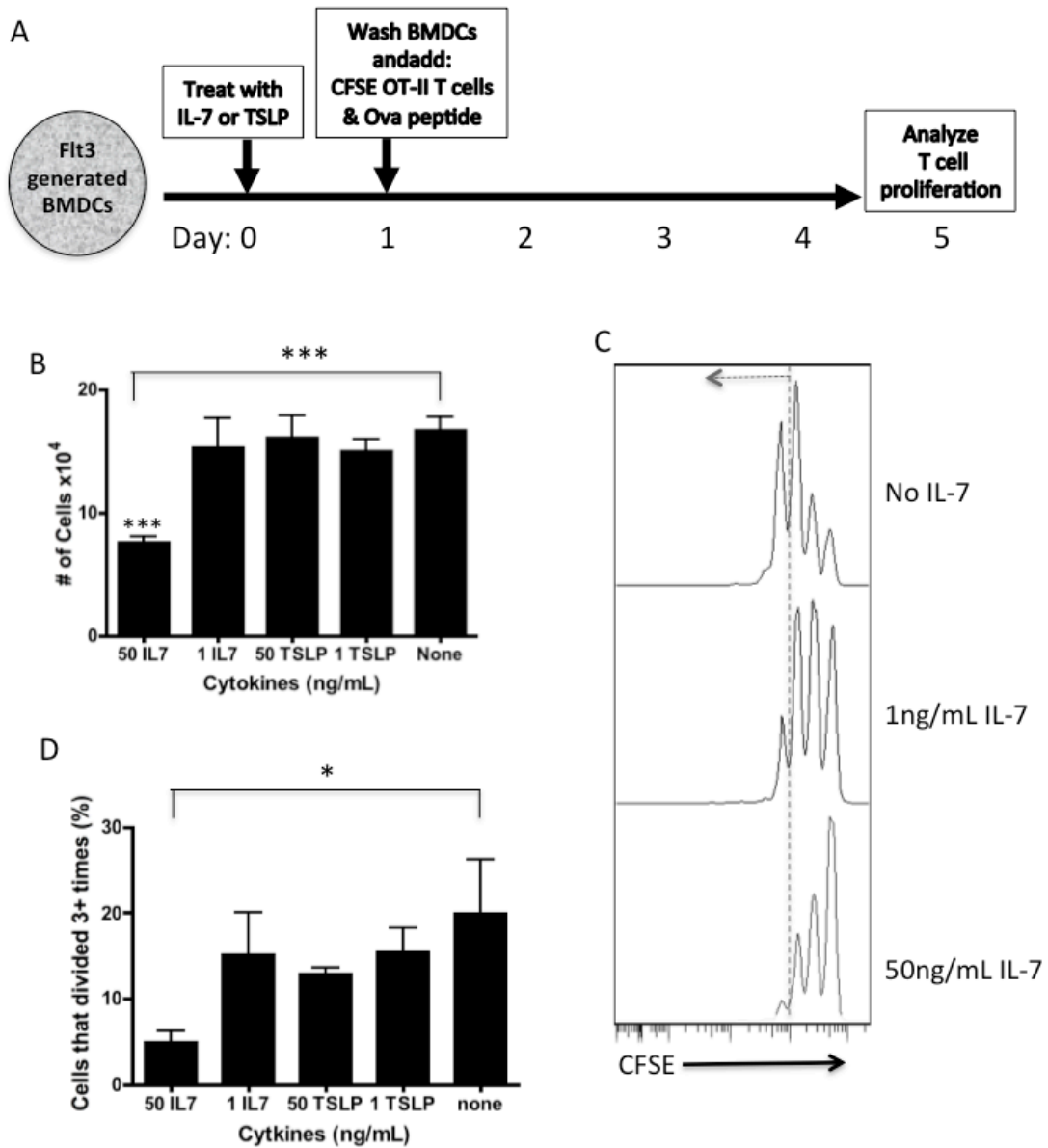


Figure 5.8. BMDCs treated with IL-7 are less effective at inducing proliferation of naïve T cells. Flt3L generated BMDCs were treated with the indicated amounts of IL-7 or TSLP for 24 hours and washed 3 times to remove cytokine traces. BMDCs were subsequently co-cultured with CFSE-labeled OT-II T cells and OVA peptide. After 4 days, cells were analyzed by flow-cytometry. **(A)** Visual representation of methods **(B)** Bar graph showing the mean number \pm SEM of T cells on the last day of co-culture. **(C)** Representative CFSE proliferation pattern of OT-II T cells on the last day of co-culture. **(D)** Bar graph showing the mean percent \pm SEM of T cells that had undergone 3 or more division by the last day of co-culture. N = 4-5 wells per group. Results are representative of 3 independent experiments. ***=p<0.0001, * = p<0.05.

5.2.7 IL-7 IS NOT NECESSARY FOR THE DEVELOPMENT OF DENDRITIC CELLS

Recent publications report that DCs with distinct functions in the gut have different developmental origins[111, 112]. These DC subsets share a common precursor called the macrophage and dendritic-cell precursor (MDP). The MDP gives rise to pre-DCs and monocytes. Pre-DCs are dependent on growth factor Flt3 and can develop into CD103⁺ DCs. Monocytes develop into CD103⁻ DCs in the gut through an M-CSF-driven pathway. The upstream signaling molecules that determine whether an MDP will develop into a pre-DC or a monocyte are unknown. Here competitive chimeras were used to determine if IL-7 was important for the development of different DC subsets *in vivo*, as outlined in Figure 5.9A. Wild-type recipient mice were lethally irradiated with 1300 cGy in two doses and then transferred with 5x10⁶ bone marrow cells by IV injection. 50% of the donor bone marrow cells were from CD45.2⁺ IL-7R^{-/-} mice, and 50% were from CD45.1⁺ wild-type mice. Control groups include mice that received 50% TSLPR^{-/-} bone marrow and 50% wild-type bone marrow, 100% WT bone marrow, 100% IL-7R^{-/-} bone marrow, or 100% TSLPR^{-/-}. Recipient mice were sacrificed 28 days later and spleens, MLNs, and small intestine lamina propria cells were harvested and analyzed by flow cytometry. Conventional DCs from the spleen (Figure 5.9B) and small intestine LPL (Figure 5.9C) developed evenly from IL-7R^{-/-} (CD45.1) and wild-type (CD45.2) precursors, indicating that IL-7 is not required for the development of these DCs. DC subsets from the small intestine LPL defined using CD103 and CD11b were gated as shown in Figure 5.9D and also showed equal proportions developed from knockout and wild-type bone marrow (Figure 5.9E). Similar results were seen for DCs from the MLN

(Figures 5.9F,G). These results suggest that ontogeny of DCs is not directly affected by IL-7 or TSLP signaling.

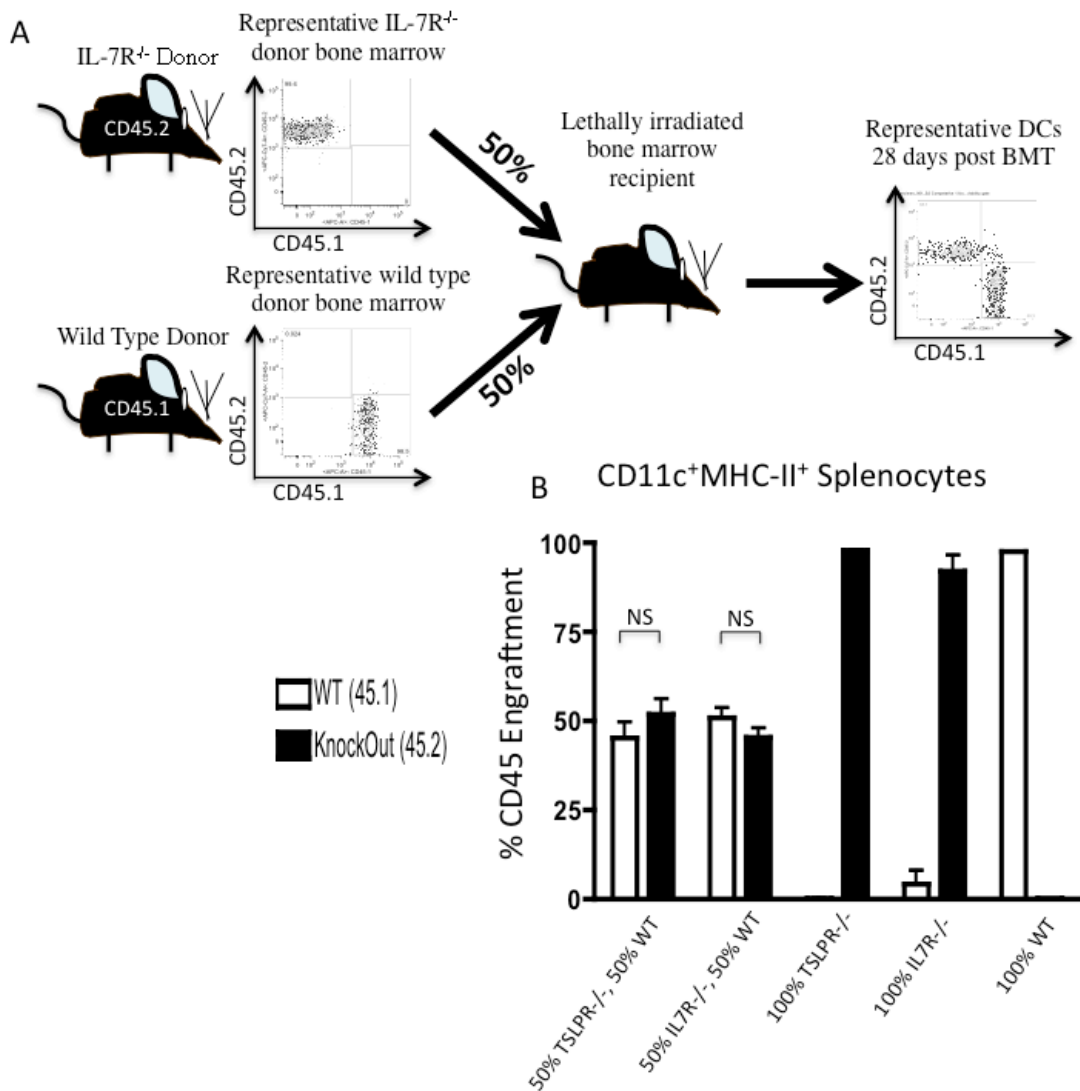


Figure 5.9. IL-7R is not required for the development of Dendritic Cells. CD45.1⁺ wild-type recipient mice were irradiated with 1300 RADS in two doses and intravenously injected with 5x10⁶ bone marrow cells. 50% of the donor cells derived from 45.2⁺IL-7R^{-/-} mice and 50% from 45.1⁺ wild-type mice. Controls shown include mice that received 50% TSLPR^{-/-} bone marrow and 50% wild-type bone marrow, 100% WT bone marrow, 100% IL-7R^{-/-} bone marrow, or 100% TSLPR^{-/-}. Recipient mice were sacrificed 28 days later and spleen, MLN, and small intestine lamina propria cells were subjected to flow-cytometric analysis. X-axis labels indicate the genotype of bone marrow given for all bar graphs. **(A)** Visual representation of the methods described above. **(B)** Bar graph showing mean percent ± SEM CD45 chimerism of CD11c⁺MHC-II⁺ cells from the spleen and **(C)** from the small intestine lamina propria. **(D)** Representative gating strategy used to define DC subsets within the CD11c⁺MHC-II⁺ gate of the small intestine lamina propria **(E)** Bar graph showing mean percent ± SEM CD45 chimerism of CD103⁺ and CD11b⁺ cells as specified within the CD11c⁺MHC-II⁺ gate from the small intestine lamina propria. **(F)** Bar graph showing mean percent ± SEM CD45 chimerism of CD11c⁺MHC-II⁺ cells from the MLN **(G)** Bar graph showing mean percent ± SEM CD45 chimerism of cells gated on CD103 and Cd11b as specified within the CD11c⁺MHC-II⁺ gate from the MLN. N = 5-6 mice per group. Results are representative of 2 independent experiments.

Figure 5.9 continued:

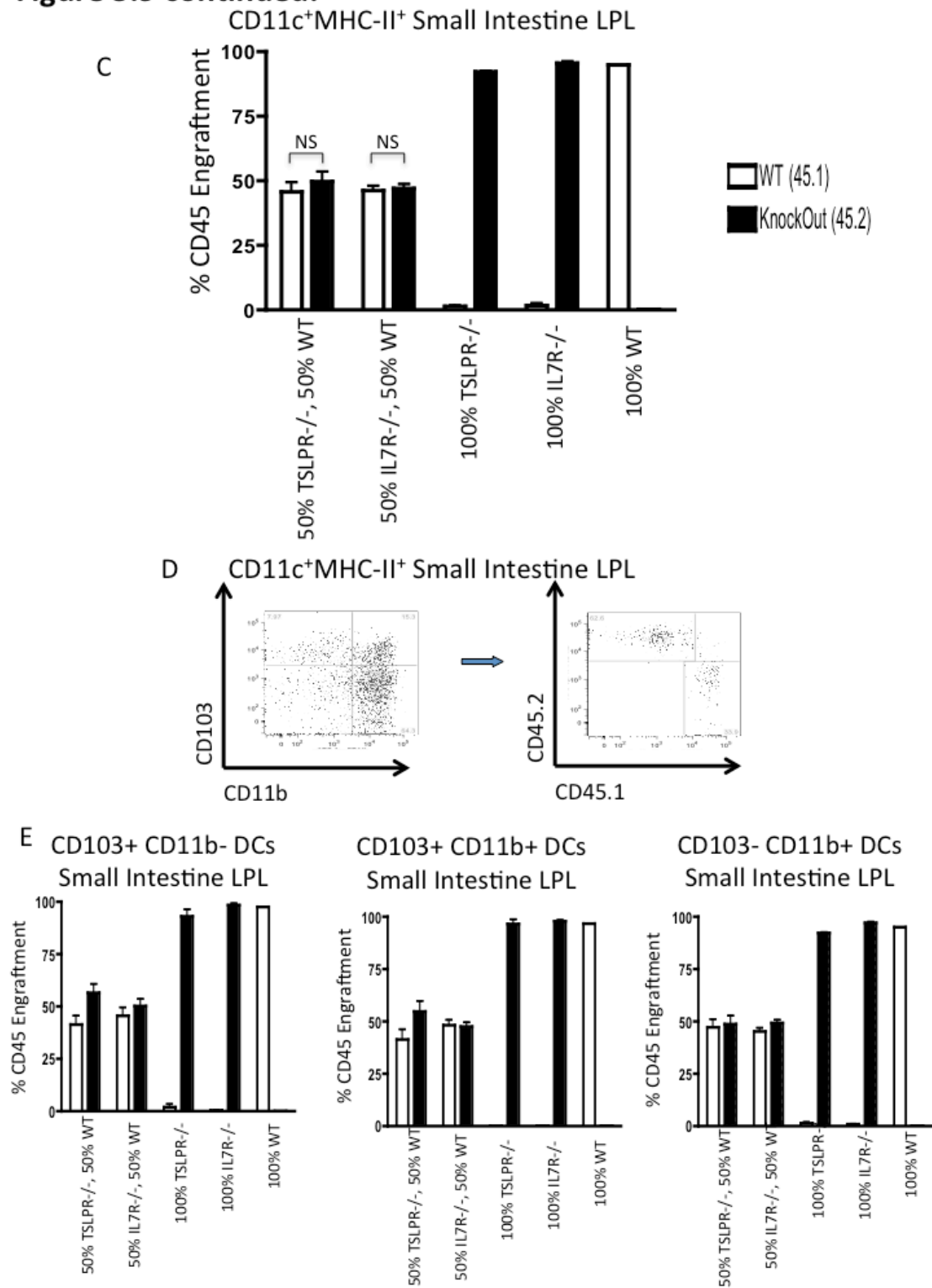
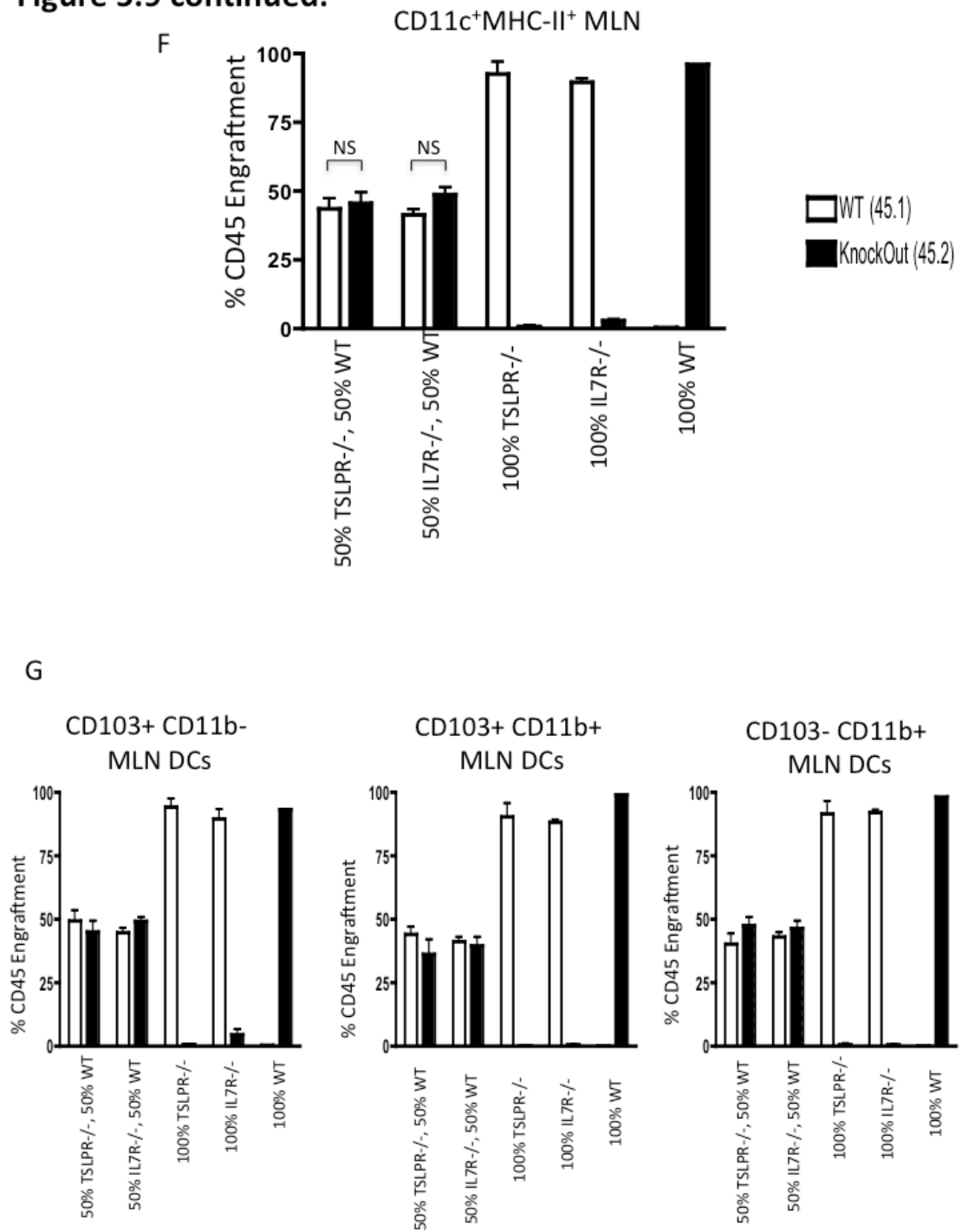


Figure 5.9 continued:



5.2.8 ANALYSIS OF IL-7R SIGNALING ON CD11c⁺ CELLS IN VIVO USING CD11c⁻DTR MICE

To further investigate if IL-7R signaling on DCs was indeed responsible for the increased accumulation of T cells seen in IL-7R deficient mice, two CD11cDTR systems were designed to create animals that were only deficient in IL-7R on DCs.

In the first system bone marrow chimeras were made in wild-type recipients using CD11cDTR bone marrow and left to recover for 90 days. Making chimeras was a necessary step to allow for multiple doses of diphtheria toxin to be given to deplete CD11cDTR cells over an extended period of time. CD11cDTR chimeric mice were given sub-lethal irradiation (500 cGy) to induce lymphopenia. They were then treated with diphtheria toxin every other day for 8 days starting on day 0. They were transferred with IL-7R^{-/-} or wild-type (IL-7R^{+/+}) Flt3L-derived BMDCs on day 1 by IV injection. Then, on day 2, the mice were given congenic CFSE labeled T cells, also by IV injection. On day 9, the mice were sacrificed and spleens, MLNs and Colon LPLs were harvested for flow cytometric analysis. A schematic outline of the experimental methods is shown in Figure 5.10A.

At the end of the experiment mice that received diphtheria toxin showed decreased spleen size and decreased absolute number of splenic DCs (Figure 5.10B). However, there was no difference in the number of DCs recovered from mice that received diphtheria toxin (Dtx) and were then given BMDCs compared to mice that were given diphtheria toxin but no BMDCs (Figure 5.10B). It was

therefore not possible to find the DCs that had been injected. Nonetheless, there was an increased frequency of T cells in the spleens of mice that were restored with IL-7R^{-/-} DCs (Figure 5.10C). T cells recovered from the MLN and colonic LPL were not significantly different possibly due to decreased ability of the DCs to home to these distal areas. These preliminary data show that IL-7R^{-/-} BMDCs support increased T cell expansion in vivo, and suggest that the increased proliferation and accumulation of T cells in IL-7R^{-/-} hosts is at least partially due to a lack of IL-7R signaling on CD11c⁺ cells. This experiment needs to be repeated to confirm these preliminary findings with additional controls looking at T cell proliferation in mice that were not treated with diphtheria toxin and in mice that were treated with diphtheria toxin but did not receive an injection of BMDCs.

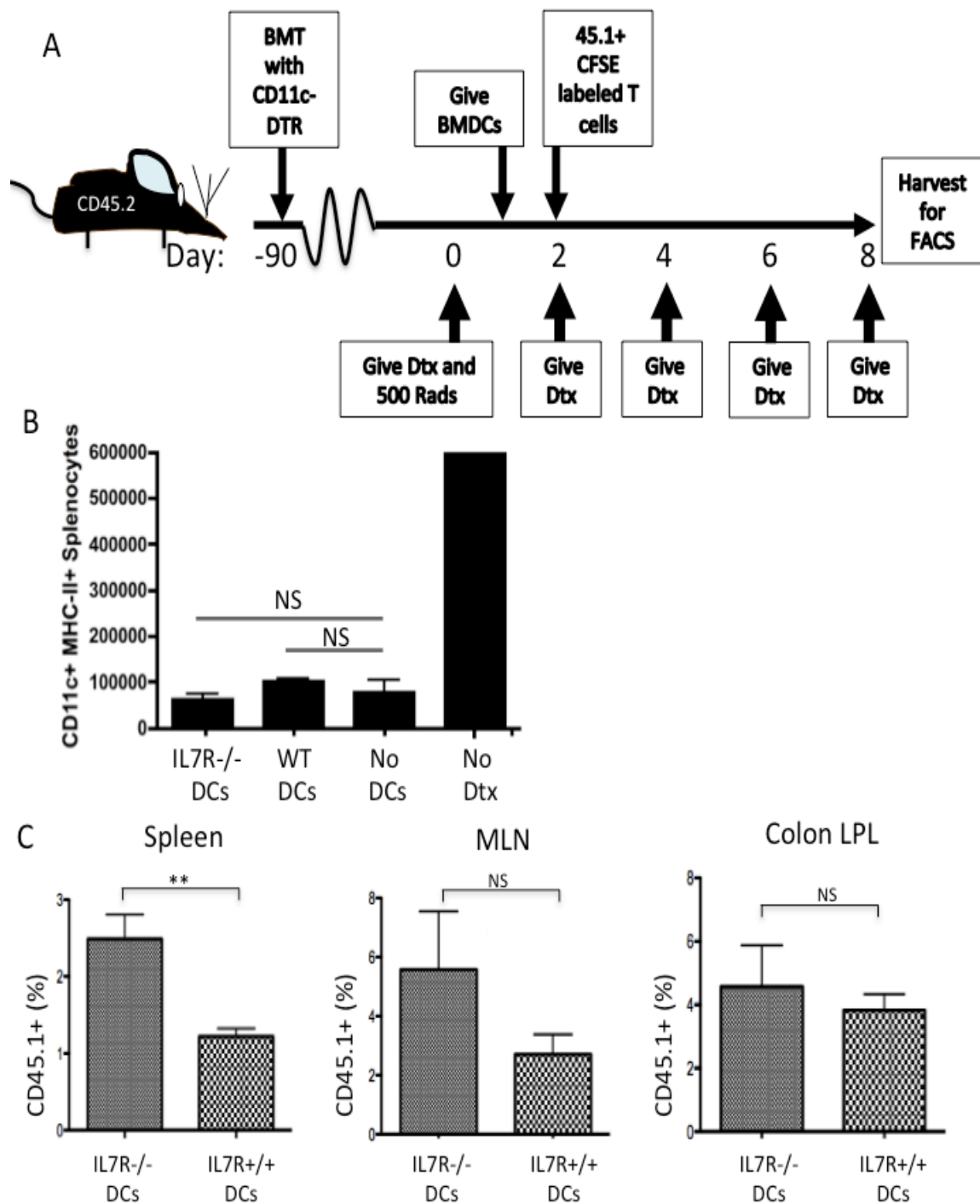


Figure 5.10. Lymphopenic mice that lack IL-7R exclusively on dendritic cells show increased proliferation and accumulation of T cells compared to wild-type lymphopenic mice. Bone Marrow chimeras were made in wild-type recipients using CD11cDTR bone marrow and left to recover for 90 days. On day 0, CD11cDTR chimeric mice were given sub-lethal irradiation (500 Rads) to induce lymphopenia and subsequently treated with diphtheria toxin every other day for 8 days. Mice were intravenously injected with IL-7R^{-/-} or wild-type BMDCs on day 1, as well as congenic CFSE-labeled T cells on day 2. All mice were sacrificed on day 9 and spleens, MLNs and colons were harvested for flow-cytometric analysis. **(A)** Visual representation of the methods described above. **(B)** Mean number ± SEM of CD11c⁺MHC-II⁺ dendritic cells recovered from the spleen at the end of the experiment. **(C)** Mean frequency ± SEM of donor-derived T cells indicated tissues. N = 4-5 mice per group, except the control that did not receive diphtheria toxin n = 1. This experiment was only completed once and is therefore preliminary data. **= $p < 0.005$, NS = not significant.

are high as the body vigorously tries to recover from profound lymphopenia. HPE is crucial to the reconstitution of the T cell pool. In the next experiment CD11cDTR mice were utilized to create a system to evaluate the effects of IL-7R signaling on CD11c⁺ cells during HPE associated with immune reconstitution after bone marrow transplant.

Again, the goal was to create a model that only had deficient IL-7R signaling on CD11c⁺ cells. Since a Cre-floxed system was not yet available, the CD11cDTR model was used. Age-matched Rag^{-/-} hosts were lethally irradiated and then given back whole bone marrow that was 50% CD11cDTR⁺, with the other 50% either IL-7R^{-/-} or wild-type. Where indicated, groups were given diphtheria toxin every other day for the duration of the experiment starting on the day after the transplant. In this experimental setup, mice that received 50% IL-7R^{-/-} bone marrow never had CD11c⁺ cells with functioning IL-7R while the mice that received 50% wild-type bone marrow had CD11c⁺ cells with normal IL-7R signaling. The methods are outline in Figure 5.11A. One advantage to this DTR experiment over the previous DTR experiment is that the mice already had the DCs of interest throughout their bodies and thus did not require an injection of BMDCs and subsequent DC homing. Mice with deficient IL-7R signaling on CD11c⁺ cells showed increased recovery of their CD4⁺ T cell pool compared to mice with normal IL-7R signaling on CD11c⁺ cells (Figures 5.11B,C).

One possible caveat to this experiment might be that the IL-7R deficient cells existed in all lineages of hematopoietic cells within the group that received 50% IL-7R^{-/-} bone marrow. Hypothetically, just the presence of the other CD11c⁻ IL-

7R deficient cells could be driving the increased proliferation. A control arm of the experiment was set up to control for this scenario. The control arm included the same groups as described above but without the diphtheria toxin administered. In this way, if it was simply the presence of some other cell type deficient in IL-7R signaling that was driving the T cell proliferation, it would be expected that between the groups that did not receive the diphtheria toxin there would be increased immune reconstitution in the group that got 50% IL-7R^{-/-} bone marrow. Since there was no significant difference between groups that did not receive diphtheria toxin (Figure 5.11C), the increased proliferation could not have been caused by deficient IL-7R signaling on some CD11c⁺ group of cells.

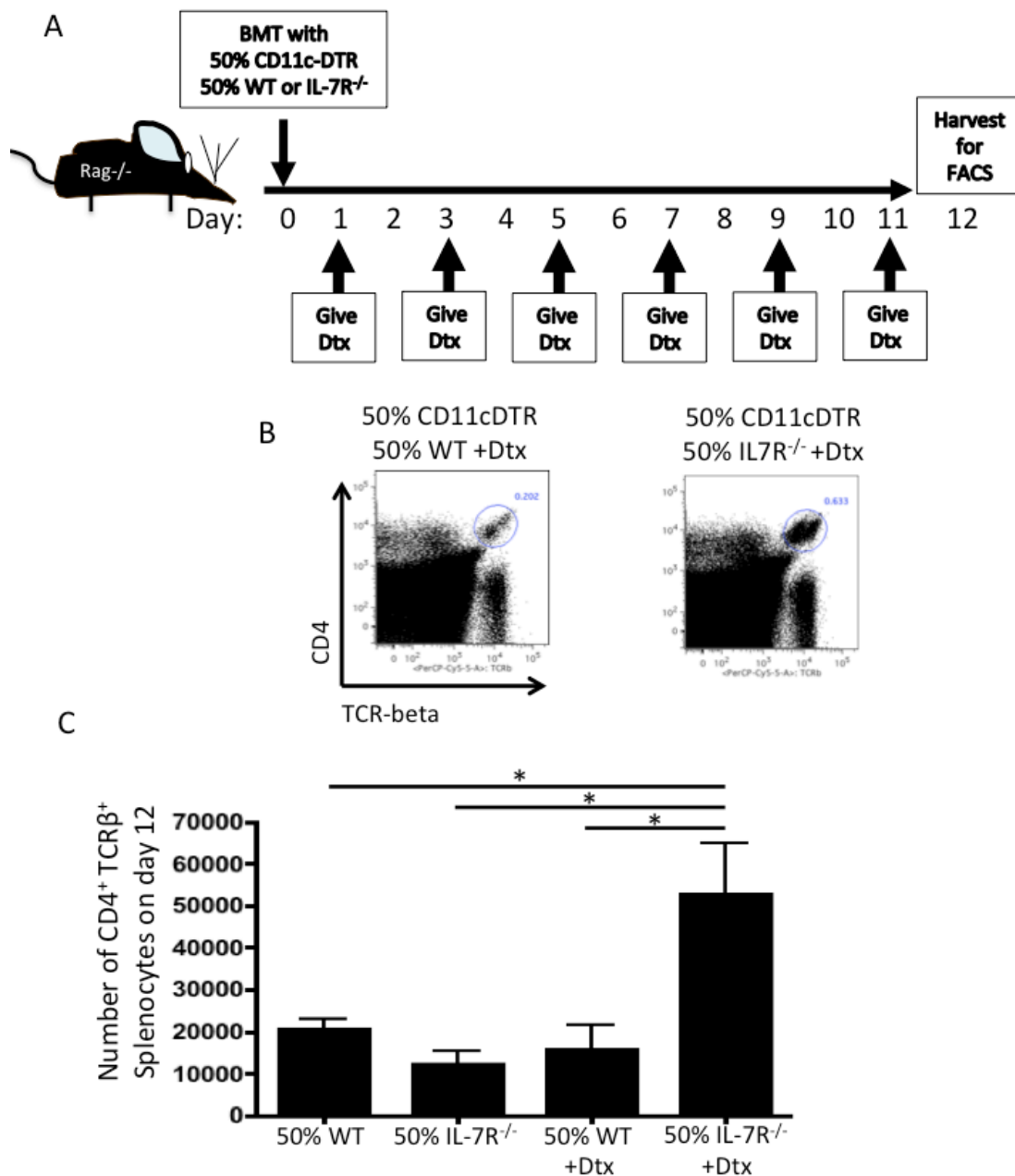


Figure 5.11. Dendritic-cell specific IL-7 signaling deficiency ameliorates immune reconstitution of CD4⁺T cell after bone marrow transplant. *Rag*^{-/-} hosts were lethally irradiated and reconstituted with 50% CD11cDTR and 50% wild-type or IL-7R^{-/-} bone marrow cells. Where indicated, mice were subsequently treated with diphtheria toxin every other day starting on day 1. All animals were sacrificed on day 12 and spleens were analyzed for immune reconstitution. **(A)** Visual representation of the methods described above. **(B)** Representative dot-plots showing increased CD4 T cell reconstitution in mice with 50% IL-7R^{-/-} bone marrow. **(C)** Mean number ± SEM of CD4⁺TCRβ⁺T cells recovered from the spleen. N = 3-5 mice per group. **p*<0.05. Results are representative of 2 independent experiments.

5.3 DISCUSSION

The increased T cell proliferation seen in IL-7R^{-/-} hosts could be explained by increased IL-7 availability or by some cell population within the host that drives T cell proliferation as a result of a lack of IL-7 or TSLP signals. Although the data presented here do not provide a conclusive answer, they do rule out stromal and epithelial cells, lack of IL-23 driven production of IL-22 by ILC-22, and lack of LTi cells during early development as being necessary for increased proliferation seen in IL-7R^{-/-}. It is likely that the increased availability of IL-7 contributes to the increased proliferation but is not the sole driving factor, since IL-7 administered to lymphopenic hosts does not drive Treg proliferation[25] as seen in IL-7R^{-/-} mice (Figure 4.4). However, further evidence is provided here that suggest a role for IL-7 signaling on DCs in T cell proliferation in IL-7R^{-/-} mice.

Lack of IL-7 or TSLP signaling on stromal or epithelial cells is not necessary for increased T cell proliferation in IL-7R^{-/-} hosts. This is clear since the proliferation was also present in bone marrow chimeras that did not lack IL-7 or TSLP signals on stromal or epithelial cells. IL-7R signaling on stromal cells plays a role in a feedback inhibition loop and has been shown to lead to decreased production of IL-7 by stromal cells[261]. This data has been used to explain part of the reason why IL-7R^{-/-} mice have increased serum levels of IL-7[261]. Therefore, IL-7R^{-/-} bone marrow chimeras and Rag^{-/-} bone marrow chimeras would be expected to have more similar serum levels of IL-7 than IL-7R^{-/-} and Rag^{-/-} mice. Of course, decreased consumption of IL-7 by IL-7R^{-/-} hematopoietic

cells means that even the chimeras will not have exactly the same serum levels of IL-7. Due to the difficulty of measuring IL-7 serum levels in mice, this was not analyzed in these experiments. However, since the theoretically more similar levels of IL-7 in the serum did not change the increased proliferation, it further supports rejecting the hypothesis that the increased proliferation is driven solely by increased IL-7 availability.

Since IL-7R^{-/-} hosts lack LTi cells, they lack normal peripheral lymphoid structures[138, 336]. The bone marrow chimeras also rule this out as being necessary for the increased T cell proliferation, since chimeras were made in mice that had normal functioning LTi cells during embryogenesis, and both arms of the experiment were completed in hosts that had matching secondary lymphoid structures.

Commensal antigens are important drivers of lymphopenia-induced proliferation[94]. Therefore, any decrease in gut barrier function would be expected to lead to increased proliferation of T cells. It is surprising that IL-22R^{-/-}Rag^{-/-} mice that are known to have decreased barrier function[152, 340, 341], did not experience increased proliferation of T cells compared to Rag^{-/-} hosts (Figure 5.1). Since IL-23 driven production of IL-22 by ILC-22 is important for gut barrier function[152, 339, 340], T cell proliferation was also examined in IL-23R^{-/-}Rag^{-/-} hosts, who also did not show increased T cells proliferation compared to Rag^{-/-} hosts (Figure 5.2). Either the decreased barrier function itself or the extent of barrier breakdown present on these strains is not sufficient to cause the increased proliferation of transferred T cells. It was not directly tested

here, but presumably IL-7R^{-/-} mice have decreased barrier function since they lack ILC-22 cells. However, based on the results seen in Figures 5.1 & 5.2 the increased T cell proliferation observed in IL-7R^{-/-} mice is not likely due to decreased barrier function from lack of IL-23 induced IL-22 production from ILC-22.

Since recent publications have shown that under homeostatic conditions DCs develop in a Flt3L dependent manner, Flt3L was used to develop homeostatic DCs that could be divided into subsets based on CD11b and CD103 expression (Figure 5.4). Flt3 generated BMDCs were treated with varying amounts of IL-7 and TSLP but showed very little change in phenotype (Figure 5.6). Even if the data looking at the size of the CD103⁺ subset had been reproducible (Figure 5.7), based on the data seen in the competitive chimeras, IL-7R signals are not important for the development of the CD103⁺ subset or any other DC subset in vivo. Although the phenotype of BMDCs was not affected by IL-7 treatment, there seemed to be a functional change. BMDCs treated with large amounts of IL-7 were not able to induce proliferation of OT-II T cells in the presence of ova peptide as well as BMDCs that were not treated with IL-7 (Figure 5.8). This implies that IL-7 signaling on BMDCs limits their ability to induce T cell proliferation.

Competitive bone marrow chimeras were used to determine if IL-7 or TSLP signaling is required for the development of DCs. Since every subset analyzed showed approximately 50% contribution from the wild-type bone marrow and 50% contribution from the knockout bone marrow, it can be concluded that IL-7

and TSLP are not intrinsically required for the development of cDCs or the various subsets of cDCs based on CD103 and CD11b expression (Figure 5.8). This directly contradicts published work which showed an increased number of DCs derived from wild-type compared to IL-7R^{-/-} precursors[334]. The different results cannot be easily explained since the experiments were almost identical. Deciphering chimerism requires having very clear staining for the congenic markers, in this case CD45.1 and CD45.2. In the previous publication, they show a large number of the resulting CD11c⁺ cells are double positive for CD45.1 and CD45.2, indicating that either the antibodies were not properly titrated or they did not gate out doublets[334]. Unfortunately, the data looking at pDCs were inconclusive (data not shown) and the experiment needs to be repeated. It is still possible that IL-7 or TSLP signaling may be required for the development of pDCs.

Based on the in vitro functional assay with BMDCs, it seems plausible that the lack of IL-7R signaling on DCs was what was causing the increased T cell proliferation in IL-7R^{-/-} mice. CD11cDTR mice were utilized to develop two model systems to look at the effects of IL-7R signaling on DCs in vivo. In the first experiment, radiation was given to induce lymphopenia in CD11cDTR bone marrow chimeras. CD11c⁺ cells were depleted using diphtheria toxin as previously described[295], and then BMDCs that were either IL-7R^{-/-} or wild-type (IL-7R^{+/+}) were given back. Subsequently, T cell proliferation was analyzed by transfer of naïve, wild-type, congenic T cells, in a similar way to how it was analyzed in previous experiments. Increased T cell proliferation was seen in the group that received IL-7R^{-/-} BMDCs (Figure 5.9), indicating that blocking IL-7R

signals on BMDCs allows them to support increased T cell proliferation. It would be expected that the two groups would have similar serum levels of IL-7 in this experiment since the hosts do not have deficient IL-7R signaling on stromal cells and most other cell populations are the same between the two groups. This further contributes to rejection of the hypothesis that the increased proliferation in IL-7R^{-/-} hosts is caused solely by increased IL-7 availability. This experiment was completed at the NIH and could not be repeated there due to time constraints. It could also not be repeated at Oxford due to differences in animal protocol licensing. Therefore, this data should be considered preliminary until it can be repeated.

The second experiment that utilized the CD11cDTR strain was designed to look at immune reconstitution after bone marrow transplant. This is a recovery period of time when HPE is crucial for driving the reconstitution of T cells[342]. Mice with deficient IL-7R signaling on CD11c⁺ cells showed increased immune reconstitution of CD4⁺ T cells compared to mice with normal IL-7R signaling on CD11c⁺ cells (Figure 5.10). Immune reconstitution after bone marrow transplant happens through both thymic-dependent and thymic-independent routes depending on host age[77, 79]. Since the two groups presented here where age matched thymic output should have been equal for both groups. Additionally, early responses to transplant conditioning regimens are dominated by HPE of mature T cell populations, including those contained in T cell-replete donor grafts[79, 343-346]. Since the bone marrow has a rich supply of T cells[347], thymic-independent routes likely predominated in this experiment. The data presented here is even more impressive considering that the group with 50%

wild-type bone marrow likely received twice as many T cells from the bone marrow transplant as the group that received 50% IL-7R^{-/-} bone marrow. This discrepancy can be controlled for in future experiments by T cell depleting both the IL-7R^{-/-} and wild-type bone marrow given. Also, additional wild-type T cells could be added to the system by adoptive transfer to skew the system toward a more thymic-independent immune reconstitution.

One possibility that was not explored here is that there may be differences in the flora of IL-7R^{-/-} hosts and other lymphopenic hosts. HPE is known to be driven not only by self-antigens but also commensal antigens, evidenced by the fact that germ-free mice experience very little T cell proliferation [94, 95]. A recent publication has highlighted the importance of gut flora in the proliferation of T cells especially during lymphopenia [95]. Differences in the flora of IL-7R^{-/-} and Rag^{-/-} mice have not yet been explored but could be important driving factors in the increased T cell proliferation seen in IL-7R^{-/-} hosts. Future experiments will explore this possibility.

More work is still needed to determine definitively if DCs are responsible for the increased T cell proliferation in IL-7R^{-/-} mice. Additional future experiments include using CD11c-Cre IL-7R-floxed hosts. This strain is currently breeding in the lab and will be crossed to a Rag^{-/-} host so that IL-7R signaling on CD11c⁺ cells can be analyzed during lymphopenia. More work is also needed to determine how CD11c⁺ cells cause the increased proliferation. It seems that blocking IL-7R signaling on DCs can drive increased T cell proliferation both in vitro (Figure 5.7) and in vivo (Figures 5.9 & 5.10), but the mechanism has not been described. It

may be a change in the production of cytokines or a change in MHC-II expression that has not been detected by the experiments described here.

6 GENERAL DISCUSSION

Biomedical research has seen an explosion in the number of newly available targeted therapies, largely driven by the development of new drugs for cancer. This opens new opportunities to precisely modulate a variety of essential physiologic pathways for treatment of benign disease as well. Promising results have been demonstrated using targeted therapies for autoimmune diseases[348], and for the treatment of benign tumors[349], and even more new applications for targeted therapies in benign disease will result from for the plethora of small molecules becoming available.

As our understanding of the immune system and immune cell niches grows, our ability to apply targeted therapies also grows. The path to developing targeted immunotherapies that are ready for use in the clinic is a multi-step pathway. First, it involves understanding the disease pathology, understanding the immune system and how it might be directed to fight the disease, and finally developing a targeted therapy to accomplish that goal. The projects presented in this thesis are at different points along this pathway. The sunitinib project is much closer to clinical application than the IL-7R project, which is still in the discovery phase to gain a better understanding of the factors that control the size of the T cell niche.

6.1 TAKING PHARMACOLOGIC KIT INHIBITION FOR HSCT TO THE CLINIC

Recent models have shown an important role for KIT signaling in the HSC niche, and increased HSC engraftment following HSCT has been demonstrated using an antibody blockade of KIT signaling[55]. Thus, it is not surprising that pharmacologic tyrosine kinase inhibition with sunitinib also allowed for increased engraftment following HSCT, and it is an important principle that will be useful in future clinical application. It was also demonstrated here that the effects of sunitinib were likely due to its effects on KIT, since KIT deficient mice did not experience increased engraftment with sunitinib treatment.

With regard to the potential for clinical translation of this approach for augmenting engraftment after HSCT, several points should be noted. Most importantly, while sunitinib mediated TK inhibition enhanced HSC, myeloid and thymic engraftment of histocompatible bone marrow in immunodeficient hosts, myeloid reconstitution was generally low and immunocompetent hosts did not demonstrate enhanced myeloid reconstitution with sunitinib therapy. These results are likely due to weak effects of sunitinib on HSC engraftment, and the reasons for this remain unclear. The results could be due to incomplete HSC KIT inhibition with sunitinib as administered here, since only approximately 50% inhibition of KIT signaling was observed in bone marrow treated with sunitinib *ex vivo* (Figure 3.3); furthermore, the degree of KIT inhibition observed in marrow progenitors after sunitinib therapy *in vivo* was below detection levels (data not shown). If incomplete KIT inhibition is the cause, then regimens incorporating alternative doses and schedules of this or other KIT inhibitors

could increase the efficiency of both KIT inhibition, HSC engraftment, and myeloid reconstitution beyond that observed here. However, myelosuppression is not a major dose limiting toxicity of any of the KIT TK inhibitors currently on the market,[57, 58, 308] suggesting that these drugs in bioactive doses do not completely inhibit stem cell function over a prolonged period. Furthermore, similar low levels of engraftment were observed with anti-KIT antibody therapy[55], suggesting that low level engraftment may be a general property of preparative regimens based solely upon KIT inhibition.

KIT inhibition does not efficiently modulate the whole LT-HSC niche. Rather, KIT inhibition seems to disadvantage only a fraction of the quiescent HSC pool at any given time, possible due to HSC cycling. However, since there is some chimerism of LT-HSC and given that short-term pharmacologic inhibition of KIT with tyrosine kinase inhibitors is essentially devoid of toxicity, multiple rounds of KIT inhibition followed by HSCT could potentially overcome this limitation.

Despite the limitations on HSC engraftment observed, even low-level chimerism, as accomplished here may be clinically significant, since mixed chimerism following HSCT of allogeneic or genetically modified autologous cells is potentially curative for some benign diseases. This is especially true if the underlying defect results in a competitive disadvantage to resident hematopoietic or lymphoid populations. This is clearly evidenced when HSCT is undertaken for severe combined immunodeficiency both in the clinical setting and in the models presented here, where near normalization of the peripheral lymphoid pool is observed[350]. Indeed, the potent effects of sunitinib on T cell

reconstitution are likely to persist in the long-term, since homeostatic cycling of mature T cells provides the primary source for peripheral T cell renewal in vivo. This is clinically relevant to transplantation for immunodeficiency, where replacement of the T cell pool with minimal toxicity is desired. Similarly, transplantation for systemic metabolic disorders[351], hemoglobinopathies, such as thalassemia[352], and sickle cell anemia[353] can also reap substantial benefits even in the face of low-level HSC chimerism. Furthermore, immunomodulatory effects of low-level engraftment are significant since mixed chimerism appears to decrease the risk for graft versus host disease[354] and microchimerism achieved through HSCT can induce immune tolerance when used as a prelude to solid organ transplantation[355],[356, 357]. For conditions where higher levels of chimerism are necessary for clinical benefit, the data presented here also demonstrate that administration of a targeted TK inhibitor may allow one to lower the dose of a cytotoxic agent, such as irradiation, and still accomplish significant engraftment, an approach which could diminish toxicity.

Finally, it is important to note that sunitinib, as administered here, did not overcome immunologic rejection either in a minor mismatch model or hybrid resistance in the parent into F1 model. Thus, while this approach provides a mechanism to increase “marrow” and “thymic” space, clinical application across allogeneic barriers in an outbred population would require incorporation of anti-rejection therapies to accomplish engraftment. However, this could potentially also be accomplished with non-cytotoxic therapies such as rapamycin, which has been shown to prevent graft rejection in non-myeloablative model systems for benign disease[358, 359].

In summary, much work is needed before tyrosine kinase inhibitors are ready for clinical use as a preparative regimen for HSCT, but the potential benefits of a non-cytotoxic approach to HSCT are great. First, alternative doses and schedules for sunitinib and other KIT inhibitors could be investigated to optimize efficiency of KIT inhibition. Additionally, KIT inhibition could be explored in conjunction with anti-rejection treatments in immunocompetent hosts. Although the chimerism achieved with sunitinib therapy is low, it may still be clinically relevant for benign diseases, and additional engraftment may be possible by giving multiple rounds of sunitinib therapy followed by HSCT.

6.2 POSSIBLE MECHANISMS OF A BLOCKED INHIBITORY PATHWAY

The discovery that transferred T cells undergo increased proliferation in IL-7R^{-/-} mice may be an important indication of a blocked inhibitory pathway. IL-7 levels are high during lymphopenia to drive HPE[257], but no system in the body is without checks and balances. Therefore, it would not be surprising if evolution had developed an inhibitory pathway that prevents uncontrolled T cell proliferation during lymphopenia. Since IL-7 levels are directly correlated with lymphopenia[257], what better molecular signal to drive this inhibitory pathway than IL-7 itself?

The data show that IL-7R^{-/-} mice experience increased proliferation of transferred T cells compared to Rag^{-/-} mice. Since the proliferation involved

preferential expansion of Tregs (Figure 4.4), it is likely that this increased proliferation was due to some blocked inhibitory pathway in IL-7R^{-/-} hosts and not just due to an increase in availability of IL-7, which would not be expected to drive proliferation of Tregs[25]. It appears that IL-7R signaling on DCs may be an inhibitory pathway that limits T cell proliferation and that this inhibitory pathway is blocked in IL-7R^{-/-} mice. IL-7 treatment of Flt3L generated BMDCs inhibits their ability to support T cell proliferation in vitro (Figure 5.8), while IL-7R^{-/-} Flt3L generated BMDCs support more T cell proliferation than wild-type Flt3L generated BMDCs in vivo (Figure 5.10). Finally, mice that lack IL-7 function on all CD11c⁺ cells show increased proliferation of T cells compared to mice that lack IL-7R function on only half of the CD11c⁺ cells and compared to mice with normal IL-7R function (Figure 5.11).

The mechanism that might allow IL-7R signaling on DCs to inhibit T cell proliferation was not described here. Since T cell proliferation requires MHC/TCR interactions and co-stimulation signals usually provided by CD80 or CD86, it is important to consider MHC, CD80, and CD86 expression by DCs. One possibility that has been previously described is that IL-7 signaling on DCs leads to increased expression of MHC-II by DCs[261]. This possibility was explored here in BMDCs, but IL-7 showed no effect on MHC-II expression on BMDCs (Figure 5.6B). Inability to demonstrate this mechanism on BMDCs in vitro does not rule out the possibility that the pathway might exist on naturally occurring DCs in vivo. The effect of IL-7 on CD80 and CD86 expression by DCs has not yet been analyzed. Further investigation is needed to explore these possibilities and other possible mechanisms.

Another hypothesis is that IL-7 might affect IDO expression by DCs, since DCs are known to express IDO, which can limit T cell proliferation[360-363]. Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the rate-limiting step of the degradation of tryptophan. Therefore, increased expression of IDO depletes available local tryptophan. Since T cells rely on tryptophan to make proteins necessary for proliferation, they are particularly sensitive to this change[229, 361, 362]. It would be interesting to explore whether or not IL-7 treatment affects IDO expression by DCs. If IL-7 treatment increased expression of IDO by dendritic cells, then that could explain why IL-7 treated BMDCs have decreased ability to induce T cell proliferation (Figure 5.8) and why IL-7R^{-/-} mice experience increased T cells proliferation (Figures 4.1 & 4.5).

Additionally, supernatants from DCs cultures could be analyzed for cytokines. DCs could be excreting cytokines that limit CD4 proliferation such as IL-10 or TGF- β [364, 365]. Retinoic acid production by DCs can induce the expression of Foxp3⁺ T cells and is also worth exploring[366, 367].

6.3 CLINICAL APPLICATION OF LESSONS FROM IL-7R^{-/-} MICE

Before the IL-7R project can even be considered for clinical translation, the mechanism must be worked out. If the mechanism can be worked out however, then highly specialized targeted therapies are becoming more of a reality everyday. It is now possible to target specific pathways even on rare cell

populations. Since DCs are phagocytic cells, they are already taking up things from the environment. Targeting phagocytic cells with small molecule inhibitors could be easier than targeting other cells.

Even if IL-7R signaling on DCs can be absolutely confirmed as the cause of the increased T cell proliferation in IL-7R^{-/-} mice, the clinical application of this knowledge is difficult since the preferential proliferation of Tregs might limit the ability to produce effective immune stimulation. However, the preferentially expansion of Tregs seen in IL-7R^{-/-} hosts seems to be negated in IL-7R^{-/-}Rag^{-/-} hosts. This implies that the preferentially expansion of Tregs specifically was not necessarily a result of the blocked inhibitory pathway, which also exists in the IL-7R^{-/-}Rag^{-/-} hosts, but more likely a result of the tolerogenic milieu present in IL-7R^{-/-} hosts. Since these hosts can make Tregs and have a relatively large pool of them at baseline (Figure 4.4A), the presence of Tregs could be polarizing the naïve T cells in the direction of becoming Tregs themselves. Tregs are known producers of TGF- β , and TGF- β drives Treg polarization[175]. Therefore, taking advantage of blocking the IL-7R related inhibitory pathway in a clinical setting will not necessary result in increased Treg production, but this needs to be further explored.

Tumors are known to take advantage of inhibitory pathways as a major mechanism of escaping immune surveillance[324]. Therefore, blocking inhibitory pathways has been proposed as the most promising way to activate therapeutic antitumor immunity[324]. Since it appears that IL-7R signaling DCs

may be inhibiting T cell proliferation, this could be a potential new target in cancer immunotherapy.

It would be interesting to go back to the tumor immunotherapy model to determine if the increased proliferation is relevant in a disease setting. First, the tumor experiment in IL-7R^{-/-}Rag^{-/-} mice from Figure 4.6 needs to be repeated with an increased tumor dose. If the results show increased tumor immunotherapy for the IL-7R^{-/-}Rag^{-/-} group, then it would be necessary to determine if the DCs were responsible. The Hy-tumor model could be applied to an experiment similar to the one presented in Figure 5.11 where there is a group that has mixed chimerism in the hematopoietic compartment but DCs that are exclusively IL-7R^{-/-}. This experiment could implicate IL-7R signaling on DC as being an important factor in tumor immunotherapy.

The increased proliferation in IL-7R^{-/-} mice has implications about the role of IL-7R signaling on DCs in tolerance. Thus, it would be interesting to evaluate T cell transfer colitis in IL-7R^{-/-}Rag^{-/-} hosts. The data presented here would suggest that IL-7R^{-/-}Rag^{-/-} hosts would experience increased T cell proliferation and possibly worse colitis than Rag^{-/-} controls. A recent publication has shown a trend toward worse colitis in IL-7R^{-/-}Rag^{-/-} hosts, but the experiment was not powered to show if there was a real difference[368]. Treatment of autoimmune and inflammatory diseases would obviously not target this inhibitory pathway to block it, as would be the goal for cancer immunotherapy, but instead may target this inhibitory pathway to promote it and to further limit T cell proliferation.

6.4 FINAL CONCLUSIONS

Immune cell niches are interesting therapeutic targets since their modulation can expand or contract the number of cells in the niche. Here it has been shown that modulation of bone marrow and thymic niches can create space within the niche to allow for increased engraftment following HSCT. Additionally, expansion of other niches, such as the T cell niche, may have profound implications for the treatment of cancer and is worthy of further investigation.

7 REFERENCES

1. Fewkes, N.M., et al., *Pharmacologic modulation of niche accessibility via tyrosine kinase inhibition enhances marrow and thymic engraftment after hematopoietic stem cell transplantation*. Blood, 2010. **115**(20): p. 4120-9.
2. Bianchi, M.E., *DAMPs, PAMPs and alarmins: all we need to know about danger*. J Leukoc Biol, 2007. **81**(1): p. 1-5.
3. Paul, W.E., *Fundamental Immunology*. Sixth ed 2010: Lippincott Williams & Wilkins.
4. Martín-Fontecha, A., et al., *Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming*. J Exp Med, 2003. **198**(4): p. 615-21.
5. Bernasconi, N.L., E. Traggiai, and A. Lanzavecchia, *Maintenance of serological memory by polyclonal activation of human memory B cells*. Science, 2002. **298**(5601): p. 2199-202.
6. Yoshida, T., et al., *Memory B and memory plasma cells*. Immunol Rev, 2010. **237**(1): p. 117-39.
7. Reinhardt, R.L., et al., *Visualizing the generation of memory CD4 T cells in the whole body*. Nature, 2001. **410**(6824): p. 101-5.
8. Sallusto, F., J. Geginat, and A. Lanzavecchia, *Central memory and effector memory T cell subsets: function, generation, and maintenance*. Annu Rev Immunol, 2004. **22**: p. 745-63.
9. Cyster, J.G., *Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs*. Annu Rev Immunol, 2005. **23**: p. 127-59.
10. Raff, M.C., *Role of thymus-derived lymphocytes in the secondary humoral immune response in mice*. Nature, 1970. **226**(5252): p. 1257-8.
11. Spangrude, G.J., S. Heimfeld, and I.L. Weissman, *Purification and characterization of mouse hematopoietic stem cells*. Science, 1988. **241**(4861): p. 58-62.
12. Fry, T.J. and C.L. Mackall, *The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance*. J Immunol, 2005. **174**(11): p. 6571-6.
13. Kappler, J.W., N. Roehm, and P. Marrack, *T cell tolerance by clonal elimination in the thymus*. Cell, 1987. **49**(2): p. 273-80.
14. Piccirillo, C.A. and E.M. Shevach, *Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance*. Semin Immunol, 2004. **16**(2): p. 81-8.

15. Schwartz, R.H., *T cell anergy*. Annu Rev Immunol, 2003. **21**: p. 305-34.
16. Coombes, J.L., et al., *A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism*. J Exp Med, 2007. **204**(8): p. 1757-64.
17. Scott, C.L., A.M. Aumeunier, and A.M. Mowat, *Intestinal CD103+ dendritic cells: master regulators of tolerance?* Trends Immunol, 2011. **32**(9): p. 412-9.
18. Nagasawa, T., *Microenvironmental niches in the bone marrow required for B-cell development*. Nat Rev Immunol, 2006. **6**(2): p. 107-16.
19. Osawa, M., et al., *Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell*. Science, 1996. **273**(5272): p. 242-5.
20. Wagers, A.J., et al., *Little evidence for developmental plasticity of adult hematopoietic stem cells*. Science, 2002. **297**(5590): p. 2256-9.
21. Matsuzaki, Y., et al., *Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells*. Immunity, 2004. **20**(1): p. 87-93.
22. Kiel, M.J., et al., *SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells*. Cell, 2005. **121**(7): p. 1109-21.
23. Lo Celso, C., et al., *Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche*. Nature, 2009. **457**(7225): p. 92-6.
24. Xie, Y., et al., *Detection of functional haematopoietic stem cell niche using real-time imaging*. Nature, 2009. **457**(7225): p. 97-101.
25. Rosenberg, S.A., et al., *IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells*. J Immunother, 2006. **29**(3): p. 313-9.
26. Taichman, R.S., *Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche*. Blood, 2005. **105**(7): p. 2631-9.
27. Sipkins, D.A., et al., *In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment*. Nature, 2005. **435**(7044): p. 969-73.
28. Mendez-Ferrer, S., et al., *Mesenchymal and haematopoietic stem cells form a unique bone marrow niche*. Nature, 2010. **466**(7308): p. 829-34.
29. Love, P.E. and A. Bhandoola, *Signal integration and crosstalk during thymocyte migration and emigration*. Nat Rev Immunol, 2011. **11**(7): p. 469-77.

30. Schwarz, B.A., et al., *Selective thymus settling regulated by cytokine and chemokine receptors*. J Immunol, 2007. **178**(4): p. 2008-17.
31. Zlotoff, D.A., et al., *CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus*. Blood, 2010. **115**(10): p. 1897-905.
32. Wurbel, M.A., B. Malissen, and J.J. Campbell, *Complex regulation of CCR9 at multiple discrete stages of T cell development*. Eur J Immunol, 2006. **36**(1): p. 73-81.
33. Krueger, A., et al., *CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus*. Blood, 2010. **115**(10): p. 1906-12.
34. Liu, C., et al., *Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization*. Blood, 2006. **108**(8): p. 2531-9.
35. Lind, E.F., et al., *Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development*. J Exp Med, 2001. **194**(2): p. 127-34.
36. Pearse, M., et al., *A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor*. Proc Natl Acad Sci U S A, 1989. **86**(5): p. 1614-8.
37. Shinkai, Y., et al., *RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement*. Cell, 1992. **68**(5): p. 855-67.
38. Goldrath, A.W. and M.J. Bevan, *Selecting and maintaining a diverse T-cell repertoire*. Nature, 1999. **402**(6759): p. 255-62.
39. Kisielow, P., et al., *Positive selection of antigen-specific T cells in thymus by restricting MHC molecules*. Nature, 1988. **335**(6192): p. 730-3.
40. Jameson, S.C., K.A. Hogquist, and M.J. Bevan, *Positive selection of thymocytes*. Annu Rev Immunol, 1995. **13**: p. 93-126.
41. Josefowicz, S.Z., L.F. Lu, and A.Y. Rudensky, *Regulatory T cells: mechanisms of differentiation and function*. Annu Rev Immunol, 2012. **30**: p. 531-64.
42. Peterson, P., T. Org, and A. Rebane, *Transcriptional regulation by AIRE: molecular mechanisms of central tolerance*. Nat Rev Immunol, 2008. **8**(12): p. 948-57.
43. Kyewski, B. and L. Klein, *A central role for central tolerance*. Annu Rev Immunol, 2006. **24**: p. 571-606.

44. Derbinski, J., et al., *Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self*. Nat Immunol, 2001. **2**(11): p. 1032-9.
45. Anderson, M.S., et al., *Projection of an immunological self shadow within the thymus by the aire protein*. Science, 2002. **298**(5597): p. 1395-401.
46. Abramson, J., et al., *Aire's partners in the molecular control of immunological tolerance*. Cell, 2010. **140**(1): p. 123-35.
47. Bonasio, R., et al., *Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus*. Nat Immunol, 2006. **7**(10): p. 1092-100.
48. Derbinski, J. and B. Kyewski, *How thymic antigen presenting cells sample the body's self-antigens*. Curr Opin Immunol, 2010. **22**(5): p. 592-600.
49. Linenberger, M.L., et al., *Stem cell factor production by human marrow stromal fibroblasts*. Exp Hematol, 1995. **23**(10): p. 1104-14.
50. McCulloch, E.A., et al., *The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype Sl-Sld*. Blood, 1965. **26**(4): p. 399-410.
51. Barker, J.E., *Early transplantation to a normal microenvironment prevents the development of Steel hematopoietic stem cell defects*. Exp Hematol, 1997. **25**(6): p. 542-7.
52. Barker, J.E., *Sl/Sld hematopoietic progenitors are deficient in situ*. Exp Hematol, 1994. **22**(2): p. 174-7.
53. Wang, Z. and K.D. Bunting, *Hematopoietic stem cell transplant into non-myeloablated W/Wv mice to detect steady-state engraftment defects*. Methods Mol Biol, 2008. **430**: p. 171-81.
54. Waskow, C., et al., *Hematopoietic stem cell transplantation without irradiation*. Nat Methods, 2009. **6**(4): p. 267-9.
55. Czechowicz, A., et al., *Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches*. Science, 2007. **318**(5854): p. 1296-9.
56. Heinrich, M.C., et al., *Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor*. Blood, 2000. **96**(3): p. 925-32.
57. Ahmad, T. and T. Eisen, *Kinase inhibition with BAY 43-9006 in renal cell carcinoma*. Clin Cancer Res, 2004. **10**(18 Pt 2): p. 6388S-92S.
58. Abrams, T.J., et al., *SU11248 inhibits KIT and platelet-derived growth factor receptor beta in preclinical models of human small cell lung cancer*. Mol Cancer Ther, 2003. **2**(5): p. 471-8.

59. Fabian, M.A., et al., *A small molecule-kinase interaction map for clinical kinase inhibitors*. Nat Biotechnol, 2005. **23**(3): p. 329-36.
60. Faivre, S., et al., *Molecular basis for sunitinib efficacy and future clinical development*. Nat Rev Drug Discov, 2007. **6**(9): p. 734-45.
61. Urso, P. and C.C. Congdon, *The effect of the amount of isologous bone marrow injected on the recovery of hematopoietic organs, survival and body weight after lethal irradiation injury in mice*. Blood, 1957. **12**(3): p. 251-60.
62. Copelan, E.A., *Hematopoietic stem-cell transplantation*. N Engl J Med, 2006. **354**(17): p. 1813-26.
63. Slavin, S., et al., *Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases*. Blood, 1998. **91**(3): p. 756-63.
64. Walters, M.C., et al., *Stable mixed hematopoietic chimerism after bone marrow transplantation for sickle cell anemia*. Biol Blood Marrow Transplant, 2001. **7**(12): p. 665-73.
65. Muller, S.M., et al., *Similar pattern of thymic-dependent T-cell reconstitution in infants with severe combined immunodeficiency after human leukocyte antigen (HLA)-identical and HLA-nonidentical stem cell transplantation*. Blood, 2000. **96**(13): p. 4344-9.
66. Tjonnfjord, G.E., et al., *Evidence for engraftment of donor-type multipotent CD34+ cells in a patient with selective T-lymphocyte reconstitution after bone marrow transplantation for B-SCID*. Blood, 1994. **84**(10): p. 3584-9.
67. Bhattacharya, D., et al., *Purified hematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning*. J Exp Med, 2006. **203**(1): p. 73-85.
68. Cavazzana-Calvo, M., et al., *Long-term T-cell reconstitution after hematopoietic stem-cell transplantation in primary T-cell-immunodeficient patients is associated with myeloid chimerism and possibly the primary disease phenotype*. Blood, 2007. **109**(10): p. 4575-81.
69. Micklem, H.S., et al., *Fate of chromosome-marked mouse bone marrow cells transfused into normal syngeneic recipients*. Transplantation, 1968. **6**(2): p. 299-302.
70. O'Garra, A., *Cytokines induce the development of functionally heterogeneous T helper cell subsets*. Immunity, 1998. **8**(3): p. 275-83.

71. Bluestone, J.A., et al., *The functional plasticity of T cell subsets*. Nat Rev Immunol, 2009. **9**(11): p. 811-6.
72. Korn, T., et al., *IL-17 and Th17 Cells*. Annu Rev Immunol, 2009. **27**: p. 485-517.
73. Ishigame, H., et al., *Differential roles of interleukin-17A and -17F in host defense against mucoc epithelial bacterial infection and allergic responses*. Immunity, 2009. **30**(1): p. 108-19.
74. Liang, S.C., et al., *An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment*. J Immunol, 2007. **179**(11): p. 7791-9.
75. Raffatellu, M., et al., *Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine*. Cell Host Microbe, 2009. **5**(5): p. 476-86.
76. Rocha, B., N. Dautigny, and P. Pereira, *Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo*. Eur J Immunol, 1989. **19**(5): p. 905-11.
77. Mackall, C.L., et al., *Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy*. N Engl J Med, 1995. **332**(3): p. 143-9.
78. Mackall, C.L. and R.E. Gress, *Thymic aging and T-cell regeneration*. Immunol Rev, 1997. **160**: p. 91-102.
79. Hakim, F.T., et al., *Age-dependent incidence, time course, and consequences of thymic renewal in adults*. J Clin Invest, 2005. **115**(4): p. 930-9.
80. Kolte, L., et al., *Association between larger thymic size and higher thymic output in human immunodeficiency virus-infected patients receiving highly active antiretroviral therapy*. J Infect Dis, 2002. **185**(11): p. 1578-85.
81. McCune, J.M., et al., *High prevalence of thymic tissue in adults with human immunodeficiency virus-1 infection*. J Clin Invest, 1998. **101**(11): p. 2301-8.
82. Smith, K.Y., et al., *Thymic size and lymphocyte restoration in patients with human immunodeficiency virus infection after 48 weeks of zidovudine, lamivudine, and zalcitabine therapy*. J Infect Dis, 2000. **181**(1): p. 141-7.
83. Rubio, A., et al., *Changes in thymus volume in adult HIV-infected patients under HAART: correlation with the T-cell repopulation*. Clin Exp Immunol, 2002. **130**(1): p. 121-6.

84. Ruiz-Mateos, E., et al., *Comparison of thymic function-related markers to predict early CD4 T-cell repopulation in adult HIV-infected patients on HAART*. *Antivir Ther*, 2003. **8**(4): p. 289-94.
85. Sfikakis, P.P., et al., *Age-related thymic activity in adults following chemotherapy-induced lymphopenia*. *Eur J Clin Invest*, 2005. **35**(6): p. 380-7.
86. Viret, C., F.S. Wong, and C.A. Janeway, Jr., *Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition*. *Immunity*, 1999. **10**(5): p. 559-68.
87. Ernst, B., et al., *The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery*. *Immunity*, 1999. **11**(2): p. 173-81.
88. Goldrath, A.W. and M.J. Bevan, *Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts*. *Immunity*, 1999. **11**(2): p. 183-90.
89. Bender, J., et al., *CD4+ T cell division in irradiated mice requires peptides distinct from those responsible for thymic selection*. *J Exp Med*, 1999. **190**(3): p. 367-74.
90. Murali-Krishna, K. and R. Ahmed, *Cutting edge: naive T cells masquerading as memory cells*. *J Immunol*, 2000. **165**(4): p. 1733-7.
91. Cho, B.K., et al., *Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells*. *J Exp Med*, 2000. **192**(4): p. 549-56.
92. Kieper, W.C. and S.C. Jameson, *Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands*. *Proc Natl Acad Sci U S A*, 1999. **96**(23): p. 13306-11.
93. Ge, Q., et al., *Dependence of lymphopenia-induced T cell proliferation on the abundance of peptide/ MHC epitopes and strength of their interaction with T cell receptors*. *Proc Natl Acad Sci U S A*, 2001. **98**(4): p. 1728-33.
94. Kieper, W.C., et al., *Recent immune status determines the source of antigens that drive homeostatic T cell expansion*. *J Immunol*, 2005. **174**(6): p. 3158-63.
95. Do, J.S., et al., *Both exogenous commensal and endogenous self antigens stimulate T cell proliferation under lymphopenic conditions*. *Cell Immunol*, 2012. **272**(2): p. 117-23.
96. Schluns, K.S., et al., *Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo*. *Nat Immunol*, 2000. **1**(5): p. 426-32.

97. Kieper, W.C., et al., *Il-12 enhances CD8 T cell homeostatic expansion*. J Immunol, 2001. **166**(9): p. 5515-21.
98. Goldrath, A.W., L.Y. Bogatzki, and M.J. Bevan, *Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation*. J Exp Med, 2000. **192**(4): p. 557-64.
99. McHugh, R.S. and E.M. Shevach, *Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease*. J Immunol, 2002. **168**(12): p. 5979-83.
100. Khoruts, A. and J.M. Fraser, *A causal link between lymphopenia and autoimmunity*. Immunol Lett, 2005. **98**(1): p. 23-31.
101. Baccala, R. and A.N. Theofilopoulos, *The new paradigm of T-cell homeostatic proliferation-induced autoimmunity*. Trends Immunol, 2005. **26**(1): p. 5-8.
102. Schulze-Koops, H., *Lymphopenia and autoimmune diseases*. Arthritis Res Ther, 2004. **6**(4): p. 178-80.
103. Krupica, T., Jr., T.J. Fry, and C.L. Mackall, *Autoimmunity during lymphopenia: a two-hit model*. Clin Immunol, 2006. **120**(2): p. 121-8.
104. Penhale, W.J., et al., *Spontaneous thyroiditis in thymectomized and irradiated Wistar rats*. Clin Exp Immunol, 1973. **15**(2): p. 225-36.
105. Nishizuka, Y., et al., *Murine thyroiditis induced by neonatal thymectomy*. Experientia, 1973. **29**(11): p. 1396-8.
106. Sakaguchi, S., et al., *Induction of autoimmune disease in mice by germline alteration of the T cell receptor gene expression*. J Immunol, 1994. **152**(3): p. 1471-84.
107. Mombaerts, P., et al., *Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice*. Cell, 1993. **75**(2): p. 274-82.
108. Sakaguchi, N., K. Miyai, and S. Sakaguchi, *Ionizing radiation and autoimmunity. Induction of autoimmune disease in mice by high dose fractionated total lymphoid irradiation and its prevention by inoculating normal T cells*. J Immunol, 1994. **152**(5): p. 2586-95.
109. Barrett, S.P., et al., *Organ-specific autoimmunity induced by adult thymectomy and cyclophosphamide-induced lymphopenia*. Eur J Immunol, 1995. **25**(1): p. 238-44.
110. Powrie, F., et al., *Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice*. Int Immunol, 1993. **5**(11): p. 1461-71.

111. Bogunovic, M., et al., *Origin of the lamina propria dendritic cell network*. Immunity, 2009. **31**(3): p. 513-25.
112. Varol, C., et al., *Intestinal lamina propria dendritic cell subsets have different origin and functions*. Immunity, 2009. **31**(3): p. 502-12.
113. Sathe, P. and K. Shortman, *The steady-state development of splenic dendritic cells*. Mucosal Immunol, 2008. **1**(6): p. 425-31.
114. Fogg, D.K., et al., *A clonogenic bone marrow progenitor specific for macrophages and dendritic cells*. Science, 2006. **311**(5757): p. 83-7.
115. Liu, K., et al., *In vivo analysis of dendritic cell development and homeostasis*. Science, 2009. **324**(5925): p. 392-7.
116. Waskow, C., et al., *The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues*. Nat Immunol, 2008. **9**(6): p. 676-83.
117. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity*. Nature, 1998. **392**(6673): p. 245-52.
118. Merad, M. and M.G. Manz, *Dendritic cell homeostasis*. Blood, 2009. **113**(15): p. 3418-27.
119. Kawai, T. and S. Akira, *TLR signaling*. Semin Immunol, 2007. **19**(1): p. 24-32.
120. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene*. Science, 1998. **282**(5396): p. 2085-8.
121. Kono, H. and K.L. Rock, *How dying cells alert the immune system to danger*. Nat Rev Immunol, 2008. **8**(4): p. 279-89.
122. Siegal, F.P., et al., *The nature of the principal type 1 interferon-producing cells in human blood*. Science, 1999. **284**(5421): p. 1835-7.
123. Hadeiba, H., et al., *CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease*. Nat Immunol, 2008. **9**(11): p. 1253-60.
124. Reizis, B., et al., *Plasmacytoid dendritic cells: recent progress and open questions*. Annu Rev Immunol, 2011. **29**: p. 163-83.
125. Hildner, K., et al., *Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity*. Science, 2008. **322**(5904): p. 1097-100.
126. Tamura, T., et al., *IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity*. J Immunol, 2005. **174**(5): p. 2573-81.

127. Aliberti, J., et al., *Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells*. *Blood*, 2003. **101**(1): p. 305-10.
128. Pulendran, B., et al., *Developmental pathways of dendritic cells in vivo: distinct function, phenotype, and localization of dendritic cell subsets in FLT3 ligand-treated mice*. *J Immunol*, 1997. **159**(5): p. 2222-31.
129. Liu, K., et al., *Immune tolerance after delivery of dying cells to dendritic cells in situ*. *J Exp Med*, 2002. **196**(8): p. 1091-7.
130. Belz, G.T., et al., *The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens*. *J Exp Med*, 2002. **196**(8): p. 1099-104.
131. Burkly, L., et al., *Expression of relB is required for the development of thymic medulla and dendritic cells*. *Nature*, 1995. **373**(6514): p. 531-6.
132. Ichikawa, E., et al., *Defective development of splenic and epidermal CD4+ dendritic cells in mice deficient for IFN regulatory factor-2*. *Proc Natl Acad Sci U S A*, 2004. **101**(11): p. 3909-14.
133. Suzuki, S., et al., *Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha-dendritic cell development*. *Proc Natl Acad Sci U S A*, 2004. **101**(24): p. 8981-6.
134. Heath, W.R. and F.R. Carbone, *Dendritic cell subsets in primary and secondary T cell responses at body surfaces*. *Nat Immunol*, 2009. **10**(12): p. 1237-44.
135. Mahnke, K., et al., *Immature, but not inactive: the tolerogenic function of immature dendritic cells*. *Immunol Cell Biol*, 2002. **80**(5): p. 477-83.
136. Huang, Q., et al., *The plasticity of dendritic cell responses to pathogens and their components*. *Science*, 2001. **294**(5543): p. 870-5.
137. Napolitani, G., et al., *Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells*. *Nat Immunol*, 2005. **6**(8): p. 769-76.
138. Spits, H. and J.P. Di Santo, *The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling*. *Nat Immunol*, 2011. **12**(1): p. 21-7.
139. Satoh-Takayama, N., et al., *IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors*. *J Exp Med*, 2010. **207**(2): p. 273-80.

140. Arase, H., N. Arase, and T. Saito, *Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-PI cross-linking*. *J Exp Med*, 1996. **183**(5): p. 2391-6.
141. Anfossi, N., et al., *Human NK cell education by inhibitory receptors for MHC class I*. *Immunity*, 2006. **25**(2): p. 331-42.
142. Eberl, G., et al., *An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells*. *Nat Immunol*, 2004. **5**(1): p. 64-73.
143. Mebius, R.E., *Organogenesis of lymphoid tissues*. *Nat Rev Immunol*, 2003. **3**(4): p. 292-303.
144. van de Pavert, S.A. and R.E. Mebius, *New insights into the development of lymphoid tissues*. *Nat Rev Immunol*, 2010. **10**(9): p. 664-74.
145. Ouyang, W., J.K. Kolls, and Y. Zheng, *The biological functions of T helper 17 cell effector cytokines in inflammation*. *Immunity*, 2008. **28**(4): p. 454-67.
146. Wolk, K., et al., *Biology of interleukin-22*. *Semin Immunopathol*, 2010. **32**(1): p. 17-31.
147. Yoshida, H., et al., *Different cytokines induce surface lymphotoxin-alpha on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches*. *Immunity*, 2002. **17**(6): p. 823-33.
148. Buonocore, S., et al., *Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology*. *Nature*, 2010. **464**(7293): p. 1371-5.
149. Luci, C., et al., *Influence of the transcription factor RORgamma(t) on the development of NKp46+ cell populations in gut and skin*. *Nat Immunol*, 2009. **10**(1): p. 75-82.
150. Sanos, S.L., et al., *RORgamma(t) and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells*. *Nat Immunol*, 2009. **10**(1): p. 83-91.
151. Satoh-Takayama, N., et al., *Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense*. *Immunity*, 2008. **29**(6): p. 958-70.
152. Sonnenberg, G.F., L.A. Fouser, and D. Artis, *Functional biology of the IL-22-IL-22R pathway in regulating immunity and inflammation at barrier surfaces*. *Adv Immunol*, 2010. **107**: p. 1-29.
153. Moore, K.W., et al., *Interleukin-10 and the interleukin-10 receptor*. *Annu Rev Immunol*, 2001. **19**: p. 683-765.

154. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann, *Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones*. J Exp Med, 1989. **170**(6): p. 2081-95.
155. Moore, K.W., et al., *Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRF1*. Science, 1990. **248**(4960): p. 1230-4.
156. Assenmacher, M., J. Schmitz, and A. Radbruch, *Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-gamma and in interleukin-4-expressing cells*. Eur J Immunol, 1994. **24**(5): p. 1097-101.
157. Simonelli, C., et al., *Both CD8+ and CD16+ human T cell clones can provide B cell help for immunoglobulin production*. Int J Clin Lab Res, 1992. **22**(1): p. 36-9.
158. Stumhofer, J.S., et al., *Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10*. Nat Immunol, 2007. **8**(12): p. 1363-71.
159. Awasthi, A., et al., *A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells*. Nat Immunol, 2007. **8**(12): p. 1380-9.
160. McGeachy, M.J., et al., *TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology*. Nat Immunol, 2007. **8**(12): p. 1390-7.
161. Ouyang, W., et al., *Regulation and functions of the IL-10 family of cytokines in inflammation and disease*. Annu Rev Immunol, 2011. **29**: p. 71-109.
162. Josefowicz, S.Z. and A. Rudensky, *Control of regulatory T cell lineage commitment and maintenance*. Immunity, 2009. **30**(5): p. 616-25.
163. Maynard, C.L., et al., *Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10*. Nat Immunol, 2007. **8**(9): p. 931-41.
164. de la Rosa, M., et al., *Interleukin-2 is essential for CD4+CD25+ regulatory T cell function*. Eur J Immunol, 2004. **34**(9): p. 2480-8.
165. Barthlott, T., et al., *CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production*. Int Immunol, 2005. **17**(3): p. 279-88.
166. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells*. Immunity, 2006. **24**(2): p. 179-89.
167. Aste-Amezaga, M., et al., *Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10*. J Immunol, 1998. **160**(12): p. 5936-44.

168. Berg, D.J., et al., *Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses*. J Clin Invest, 1996. **98**(4): p. 1010-20.
169. Powrie, F. and M.W. Leach, *Genetic and spontaneous models of inflammatory bowel disease in rodents: evidence for abnormalities in mucosal immune regulation*. Ther Immunol, 1995. **2**(2): p. 115-23.
170. Netea, M.G., et al., *Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells*. J Immunol, 2004. **172**(6): p. 3712-8.
171. Ejrnaes, M., et al., *Resolution of a chronic viral infection after interleukin-10 receptor blockade*. J Exp Med, 2006. **203**(11): p. 2461-72.
172. van der Kleij, D., et al., *A novel host-parasite lipid cross-talk. Schistosomal lysophosphatidylserine activates toll-like receptor 2 and affects immune polarization*. J Biol Chem, 2002. **277**(50): p. 48122-9.
173. Jang, S., et al., *IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on TLR2-mediated recognition*. J Immunol, 2004. **173**(5): p. 3392-7.
174. Mocellin, S., et al., *Kinetics of cytokine expression in melanoma metastases classifies immune responsiveness*. Int J Cancer, 2001. **93**(2): p. 236-42.
175. Fantini, M.C., et al., *Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7*. J Immunol, 2004. **172**(9): p. 5149-53.
176. Kawahata, K., et al., *Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression*. J Immunol, 2002. **168**(9): p. 4399-405.
177. Apostolou, I., et al., *Origin of regulatory T cells with known specificity for antigen*. Nat Immunol, 2002. **3**(8): p. 756-63.
178. Walker, L.S., et al., *Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo*. J Exp Med, 2003. **198**(2): p. 249-58.
179. Jordan, M.S., et al., *Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide*. Nat Immunol, 2001. **2**(4): p. 301-6.

180. Hsieh, C.S., et al., *Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors*. *Immunity*, 2004. **21**(2): p. 267-77.
181. Pacholczyk, R. and J. Kern, *The T-cell receptor repertoire of regulatory T cells*. *Immunology*, 2008. **125**(4): p. 450-8.
182. Pacholczyk, R., et al., *Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells*. *Immunity*, 2006. **25**(2): p. 249-59.
183. Wong, J., et al., *Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4+ T cells*. *J Immunol*, 2007. **178**(11): p. 7032-41.
184. Hsieh, C.S., et al., *An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires*. *Nat Immunol*, 2006. **7**(4): p. 401-10.
185. Salomon, B., et al., *B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes*. *Immunity*, 2000. **12**(4): p. 431-40.
186. Tai, X., et al., *CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2*. *Nat Immunol*, 2005. **6**(2): p. 152-62.
187. Ouyang, W., et al., *Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development*. *Immunity*, 2010. **32**(5): p. 642-53.
188. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3*. *J Exp Med*, 2003. **198**(12): p. 1875-86.
189. Selvaraj, R.K. and T.L. Geiger, *A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-beta*. *J Immunol*, 2007. **179**(2): p. 11 p following 1390.
190. Kretschmer, K., et al., *Inducing and expanding regulatory T cell populations by foreign antigen*. *Nat Immunol*, 2005. **6**(12): p. 1219-27.
191. Zheng, S.G., et al., *Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10*. *J Immunol*, 2004. **172**(9): p. 5213-21.
192. Zheng, S.G., et al., *TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells*. *J Immunol*, 2006. **176**(6): p. 3321-9.

193. Kim, J.M. and A. Rudensky, *The role of the transcription factor Foxp3 in the development of regulatory T cells*. Immunol Rev, 2006. **212**: p. 86-98.
194. Benson, M.J., et al., *All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation*. J Exp Med, 2007. **204**(8): p. 1765-74.
195. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells*. Nat Immunol, 2003. **4**(4): p. 330-6.
196. Brunkow, M.E., et al., *Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse*. Nat Genet, 2001. **27**(1): p. 68-73.
197. Chatila, T.A., et al., *JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome*. J Clin Invest, 2000. **106**(12): p. R75-81.
198. Bennett, C.L., et al., *The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3*. Nat Genet, 2001. **27**(1): p. 20-1.
199. Wildin, R.S., et al., *X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy*. Nat Genet, 2001. **27**(1): p. 18-20.
200. Gambineri, E., T.R. Torgerson, and H.D. Ochs, *Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis*. Curr Opin Rheumatol, 2003. **15**(4): p. 430-5.
201. Seddiki, N., et al., *Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells*. J Exp Med, 2006. **203**(7): p. 1693-700.
202. Fontenot, J.D., et al., *A function for interleukin 2 in Foxp3-expressing regulatory T cells*. Nat Immunol, 2005. **6**(11): p. 1142-51.
203. Burchill, M.A., et al., *Interleukin-2 receptor signaling in regulatory T cell development and homeostasis*. Immunol Lett, 2007. **114**(1): p. 1-8.
204. Vang, K.B., et al., *IL-2, -7, and -15, but not thymic stromal lymphopoietin, redundantly govern CD4+Foxp3+ regulatory T cell development*. J Immunol, 2008. **181**(5): p. 3285-90.
205. Mazzucchelli, R., et al., *Development of regulatory T cells requires IL-7/Ralpha stimulation by IL-7 or TSLP*. Blood, 2008. **112**(8): p. 3283-92.

206. Bayer, A.L., et al., *A function for IL-7R for CD4+CD25+Foxp3+ T regulatory cells*. J Immunol, 2008. **181**(1): p. 225-34.
207. Jego, G., et al., *Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6*. Immunity, 2003. **19**(2): p. 225-34.
208. Krug, A., et al., *TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function*. Immunity, 2004. **21**(1): p. 107-19.
209. Kuwana, M., et al., *Induction of antigen-specific human CD4(+) T cell anergy by peripheral blood DC2 precursors*. Eur J Immunol, 2001. **31**(9): p. 2547-57.
210. Martin, P., et al., *Characterization of a new subpopulation of mouse CD8alpha+ B220+ dendritic cells endowed with type I interferon production capacity and tolerogenic potential*. Blood, 2002. **100**(2): p. 383-90.
211. Billsborough, J., et al., *Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties*. Immunology, 2003. **108**(4): p. 481-92.
212. Hadeiba, H., et al., *Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance*. Immunity, 2012. **36**(3): p. 438-50.
213. Johansson-Lindbom, B., et al., *Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing*. J Exp Med, 2005. **202**(8): p. 1063-73.
214. Jang, M.H., et al., *CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes*. J Immunol, 2006. **176**(2): p. 803-10.
215. Schulz, O., et al., *Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions*. J Exp Med, 2009. **206**(13): p. 3101-14.
216. del Rio, M.L., et al., *CX3CR1+ c-kit+ bone marrow cells give rise to CD103+ and CD103- dendritic cells with distinct functional properties*. J Immunol, 2008. **181**(9): p. 6178-88.
217. Kang, S.G., et al., *Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells*. J Immunol, 2007. **179**(6): p. 3724-33.
218. Johansson-Lindbom, B. and W.W. Agace, *Generation of gut-homing T cells and their localization to the small intestinal mucosa*. Immunol Rev, 2007. **215**: p. 226-42.

219. Persson, E.K., E. Jaansson, and W.W. Agace, *The diverse ontogeny and function of murine small intestinal dendritic cell/macrophage subsets*. Immunobiology, 2010. **215**(9-10): p. 692-7.
220. Varol, C., E. Zigmund, and S. Jung, *Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria*. Nat Rev Immunol, 2010. **10**(6): p. 415-26.
221. Kinnebrew, M.A., et al., *Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense*. Immunity, 2012. **36**(2): p. 276-87.
222. Fujimoto, K., et al., *A new subset of CD103+CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity*. J Immunol, 2011. **186**(11): p. 6287-95.
223. Kersey, J.H., B.D. Spector, and R.A. Good, *Primary immunodeficiency diseases and cancer: the immunodeficiency-cancer registry*. Int J Cancer, 1973. **12**(2): p. 333-47.
224. Gallagher, B., et al., *Cancer incidence in New York State acquired immunodeficiency syndrome patients*. Am J Epidemiol, 2001. **154**(6): p. 544-56.
225. Gajewski, T.F., Y. Meng, and H. Harlin, *Immune suppression in the tumor microenvironment*. J Immunother, 2006. **29**(3): p. 233-40.
226. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival*. Nat Med, 2004. **10**(9): p. 942-9.
227. Liyanage, U.K., et al., *Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma*. J Immunol, 2002. **169**(5): p. 2756-61.
228. Meissner, M., et al., *Defects in the human leukocyte antigen class I antigen processing machinery in head and neck squamous cell carcinoma: association with clinical outcome*. Clin Cancer Res, 2005. **11**(7): p. 2552-60.
229. Uyttenhove, C., et al., *Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase*. Nat Med, 2003. **9**(10): p. 1269-74.
230. Shields, J.D., et al., *Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21*. Science, 2010. **328**(5979): p. 749-52.

231. Baitsch, L., et al., *Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients*. J Clin Invest, 2011. **121**(6): p. 2350-60.
232. Ahmadzadeh, M., et al., *Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired*. Blood, 2009. **114**(8): p. 1537-44.
233. Restifo, N.P., M.E. Dudley, and S.A. Rosenberg, *Adoptive immunotherapy for cancer: harnessing the T cell response*. Nat Rev Immunol, 2012. **12**(4): p. 269-81.
234. Clark, W.H., Jr., et al., *Model predicting survival in stage I melanoma based on tumor progression*. J Natl Cancer Inst, 1989. **81**(24): p. 1893-904.
235. Clemente, C.G., et al., *Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma*. Cancer, 1996. **77**(7): p. 1303-10.
236. Scanlan, M.J., A.J. Simpson, and L.J. Old, *The cancer/testis genes: review, standardization, and commentary*. Cancer Immun, 2004. **4**: p. 1.
237. Kawakami, Y., et al., *Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes*. J Exp Med, 1994. **180**(1): p. 347-52.
238. Bakker, A.B., et al., *Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes*. J Exp Med, 1994. **179**(3): p. 1005-9.
239. Dudley, M.E., et al., *Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma*. J Clin Oncol, 2005. **23**(10): p. 2346-57.
240. Rosenberg, S.A., et al., *Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy*. Clin Cancer Res, 2011. **17**(13): p. 4550-7.
241. Itzhaki, O., et al., *Establishment and large-scale expansion of minimally cultured "young" tumor infiltrating lymphocytes for adoptive transfer therapy*. J Immunother, 2011. **34**(2): p. 212-20.
242. Besser, M.J., et al., *Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients*. Clin Cancer Res, 2010. **16**(9): p. 2646-55.

243. Tada, T., et al., *Transforming growth factor-beta-induced inhibition of T cell function. Susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state.* J Immunol, 1991. **146**(3): p. 1077-82.
244. Young, M.R., et al., *Suppression of T cell proliferation by tumor-induced granulocyte-macrophage progenitor cells producing transforming growth factor-beta and nitric oxide.* J Immunol, 1996. **156**(5): p. 1916-22.
245. Palmer, M.J., et al., *Signaling thresholds govern heterogeneity in IL-7-receptor-mediated responses of naive CD8(+) T cells.* Immunol Cell Biol, 2011. **89**(5): p. 581-94.
246. Carrette, F. and C.D. Surh, *IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis.* Semin Immunol, 2012. **24**(3): p. 209-17.
247. Lundstrom, W., N.M. Fewkes, and C.L. Mackall, *IL-7 in human health and disease.* Semin Immunol, 2012. **24**(3): p. 218-24.
248. Yao, Z., et al., *Stat5a/b are essential for normal lymphoid development and differentiation.* Proc Natl Acad Sci U S A, 2006. **103**(4): p. 1000-5.
249. Seki, Y., et al., *IL-7/STAT5 cytokine signaling pathway is essential but insufficient for maintenance of naive CD4 T cell survival in peripheral lymphoid organs.* J Immunol, 2007. **178**(1): p. 262-70.
250. Mazzucchelli, R. and S.K. Durum, *Interleukin-7 receptor expression: intelligent design.* Nat Rev Immunol, 2007. **7**(2): p. 144-54.
251. Mazzucchelli, R.I., et al., *Visualization and identification of IL-7 producing cells in reporter mice.* PLoS One, 2009. **4**(11): p. e7637.
252. Alves, N.L., et al., *Characterization of the thymic IL-7 niche in vivo.* Proc Natl Acad Sci U S A, 2009. **106**(5): p. 1512-7.
253. Kim, G.Y., C. Hong, and J.H. Park, *Seeing is believing: illuminating the source of in vivo interleukin-7.* Immune Netw, 2011. **11**(1): p. 1-10.
254. Link, A., et al., *Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells.* Nat Immunol, 2007. **8**(11): p. 1255-65.
255. Repass, J.F., et al., *IL7-hCD25 and IL7-Cre BAC transgenic mouse lines: new tools for analysis of IL-7 expressing cells.* Genesis, 2009. **47**(4): p. 281-7.

256. Kaech, S.M., et al., *Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells*. Nat Immunol, 2003. **4**(12): p. 1191-8.
257. Fry, T.J., et al., *A potential role for interleukin-7 in T-cell homeostasis*. Blood, 2001. **97**(10): p. 2983-90.
258. Bolotin, E., et al., *Serum levels of IL-7 in bone marrow transplant recipients: relationship to clinical characteristics and lymphocyte count*. Bone Marrow Transplant, 1999. **23**(8): p. 783-8.
259. Napolitano, L.A., et al., *Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis*. Nat Med, 2001. **7**(1): p. 73-9.
260. Mackall, C.L., et al., *Thymic-independent T cell regeneration occurs via antigen driven expansion of peripheral T cells resulting in a repertoire that is limited in diversity and prone to skewing*. Journal of Immunology, 1996. **156**: p. 4609-4616.
261. Guimond, M., et al., *Interleukin 7 signaling in dendritic cells regulates the homeostatic proliferation and niche size of CD4+ T cells*. Nat Immunol, 2009. **10**(2): p. 149-57.
262. Al-Shami, A., et al., *A role for thymic stromal lymphopoietin in CD4(+) T cell development*. J Exp Med, 2004. **200**(2): p. 159-68.
263. Rochman, Y. and W.J. Leonard, *The role of thymic stromal lymphopoietin in CD8+ T cell homeostasis*. J Immunol, 2008. **181**(11): p. 7699-705.
264. Isaksen, D.E., et al., *Uncoupling of proliferation and Stat5 activation in thymic stromal lymphopoietin-mediated signal transduction*. J Immunol, 2002. **168**(7): p. 3288-94.
265. Omori, M. and S. Ziegler, *Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin*. J Immunol, 2007. **178**(3): p. 1396-404.
266. Ito, T., et al., *TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand*. J Exp Med, 2005. **202**(9): p. 1213-23.
267. Soumelis, V., et al., *Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP*. Nat Immunol, 2002. **3**(7): p. 673-80.
268. Zaph, C., et al., *Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis*. Nature, 2007. **446**(7135): p. 552-6.
269. Taylor, B.C., et al., *TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis*. J Exp Med, 2009. **206**(3): p. 655-67.

270. Iliev, I.D., et al., *Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells*. Gut, 2009. **58**(11): p. 1481-9.
271. Rimoldi, M., et al., *Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells*. Nat Immunol, 2005. **6**(5): p. 507-14.
272. Watanabe, N., et al., *Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4+ T cell homeostatic expansion*. Nat Immunol, 2004. **5**(4): p. 426-34.
273. Chappaz, S. and D. Finke, *The IL-7 signaling pathway regulates lymph node development independent of peripheral lymphocytes*. J Immunol, 2010. **184**(7): p. 3562-9.
274. Luo, H., et al., *Efnb1 and Efnb2 proteins regulate thymocyte development, peripheral T cell differentiation, and antiviral immune responses and are essential for interleukin-6 (IL-6) signaling*. J Biol Chem, 2011. **286**(48): p. 41135-52.
275. Luo, H., et al., *Ephrinb1 and Ephrinb2 are associated with interleukin-7 receptor alpha and retard its internalization from the cell surface*. J Biol Chem, 2011. **286**(52): p. 44976-87.
276. Park, J.H., et al., *Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival*. Immunity, 2004. **21**(2): p. 289-302.
277. Guo, F., et al., *Coordination of IL-7 receptor and T-cell receptor signaling by cell-division cycle 42 in T-cell homeostasis*. Proc Natl Acad Sci U S A, 2010. **107**(43): p. 18505-10.
278. Chandele, A., et al., *Formation of IL-7Ralphahigh and IL-7Ralphalow CD8 T cells during infection is regulated by the opposing functions of GABPalpha and Gfi-1*. J Immunol, 2008. **180**(8): p. 5309-19.
279. Xue, H.H., et al., *GA binding protein regulates interleukin 7 receptor alpha-chain gene expression in T cells*. Nat Immunol, 2004. **5**(10): p. 1036-44.
280. Clements, J.L., S.A. John, and L.A. Garrett-Sinha, *Impaired generation of CD8+ thymocytes in Ets-1-deficient mice*. J Immunol, 2006. **177**(2): p. 905-12.
281. Grenningloh, R., et al., *Ets-1 maintains IL-7 receptor expression in peripheral T cells*. J Immunol, 2011. **186**(2): p. 969-76.
282. Kerdiles, Y.M., et al., *Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor*. Nat Immunol, 2009. **10**(2): p. 176-84.

283. Ouyang, W., et al., *An essential role of the Forkhead-box transcription factor Foxo1 in control of T cell homeostasis and tolerance*. *Immunity*, 2009. **30**(3): p. 358-71.
284. Gubbels Bupp, M.R., et al., *T cells require Foxo1 to populate the peripheral lymphoid organs*. *Eur J Immunol*, 2009. **39**(11): p. 2991-9.
285. Fabre, S., et al., *FOXO1 regulates L-Selectin and a network of human T cell homing molecules downstream of phosphatidylinositol 3-kinase*. *J Immunol*, 2008. **181**(5): p. 2980-9.
286. Park, J.H., et al., *'Coreceptor tuning': cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR*. *Nat Immunol*, 2007. **8**(10): p. 1049-59.
287. Cho, J.H., et al., *T cell receptor-dependent regulation of lipid rafts controls naive CD8+ T cell homeostasis*. *Immunity*, 2010. **32**(2): p. 214-26.
288. Vonarbourg, C. and A. Diefenbach, *Multifaceted roles of interleukin-7 signaling for the development and function of innate lymphoid cells*. *Semin Immunol*, 2012. **24**(3): p. 165-74.
289. Grandjean, I., et al., *Are major histocompatibility complex molecules involved in the survival of naive CD4+ T cells?* *J Exp Med*, 2003. **198**(7): p. 1089-102.
290. Ebos, J.M., et al., *Multiple circulating proangiogenic factors induced by sunitinib malate are tumor-independent and correlate with antitumor efficacy*. *Proc Natl Acad Sci U S A*, 2007. **104**(43): p. 17069-74.
291. Mendel, D.B., et al., *In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship*. *Clin Cancer Res*, 2003. **9**(1): p. 327-37.
292. Weissman, I.L. and J.A. Shizuru, *The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases*. *Blood*, 2008. **112**(9): p. 3543-53.
293. Perez-Diez, A., et al., *CD4 cells can be more efficient at tumor rejection than CD8 cells*. *Blood*, 2007. **109**(12): p. 5346-54.
294. Summerhayes, I.C. and L.M. Franks, *Effects of donor age on neoplastic transformation of adult mouse bladder epithelium in vitro*. *J Natl Cancer Inst*, 1979. **62**(4): p. 1017-23.
295. Jung, S., et al., *In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens*. *Immunity*, 2002. **17**(2): p. 211-20.

296. Bhandoola, A., et al., *Early T lineage progenitors: new insights, but old questions remain.* J Immunol, 2003. **171**(11): p. 5653-8.
297. Ogawa, M., et al., *Expression and function of c-kit in hemopoietic progenitor cells.* J Exp Med, 1991. **174**(1): p. 63-71.
298. Nocka, K., et al., *Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: W37, Wv, W41 and W.* EMBO J, 1990. **9**(6): p. 1805-13.
299. Cara, D.C., K.V. Ebbert, and D.M. McCafferty, *Mast cell-independent mechanisms of immediate hypersensitivity: a role for platelets.* J Immunol, 2004. **172**(8): p. 4964-71.
300. Arguello, F., et al., *Incidence and distribution of experimental metastases in mutant mice with defective organ microenvironments (genotypes Sl/Sld and W/Wv).* Cancer Res, 1992. **52**(8): p. 2304-9.
301. Murphy, E.D., *Hyperplastic and early neoplastic changes in the ovaries of mice after genic deletion of germ cells.* J Natl Cancer Inst, 1972. **48**(5): p. 1283-95.
302. Kiel, M.J., et al., *SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells.* Cell, 2005. **121**(7): p. 1109-21.
303. Wright, D.E., et al., *Physiological migration of hematopoietic stem and progenitor cells.* Science, 2001. **294**(5548): p. 1933-6.
304. Bennett, M., *Biology and genetics of hybrid resistance.* Adv Immunol, 1987. **41**: p. 333-445.
305. Kent, D., et al., *Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway.* Clin Cancer Res, 2008. **14**(7): p. 1926-30.
306. Zlotoff, D.A., B.A. Schwarz, and A. Bhandoola, *The long road to the thymus: the generation, mobilization, and circulation of T-cell progenitors in mouse and man.* Semin Immunopathol, 2008.
307. Weinstein, I.B. and A. Joe, *Oncogene addiction.* Cancer Res, 2008. **68**(9): p. 3077-80; discussion 3080.
308. George, S., *Sunitinib, a multitargeted tyrosine kinase inhibitor, in the management of gastrointestinal stromal tumor.* Curr Oncol Rep, 2007. **9**(4): p. 323-7.
309. Pardoll, D., *Does the immune system see tumors as foreign or self?* Annu Rev Immunol, 2003. **21**: p. 807-39.

310. Valujskikh, A., et al., *Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway*. Nat Immunol, 2002. **3**(9): p. 844-51.
311. Osborne, L.C., et al., *Impaired CD8 T cell memory and CD4 T cell primary responses in IL-7R alpha mutant mice*. J Exp Med, 2007. **204**(3): p. 619-31.
312. Moses, C.T., et al., *Competition for self ligands restrains homeostatic proliferation of naive CD4 T cells*. Proc Natl Acad Sci U S A, 2003. **100**(3): p. 1185-90.
313. Kassiotis, G., R. Zamoyska, and B. Stockinger, *Involvement of avidity for major histocompatibility complex in homeostasis of naive and memory T cells*. J Exp Med, 2003. **197**(8): p. 1007-16.
314. Cui, Y., et al., *Harnessing the physiology of lymphopenia to support adoptive immunotherapy in lymphoreplete hosts*. Blood, 2009. **114**(18): p. 3831-40.
315. Rosenberg, S.A., et al., *Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report*. N Engl J Med, 1988. **319**(25): p. 1676-80.
316. Robbins, P.F., et al., *Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1*. J Clin Oncol, 2011. **29**(7): p. 917-24.
317. Sakaguchi, S., *Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses*. Annu Rev Immunol, 2004. **22**: p. 531-62.
318. Nakamura, K., A. Kitani, and W. Strober, *Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta*. J Exp Med, 2001. **194**(5): p. 629-44.
319. Buer, J., et al., *Interleukin 10 secretion and impaired effector function of major histocompatibility complex class II-restricted T cells anergized in vivo*. J Exp Med, 1998. **187**(2): p. 177-83.
320. Sundstedt, A., et al., *Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo*. J Immunol, 2003. **170**(3): p. 1240-8.
321. O'Garra, A., et al., *IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage*. J Clin Invest, 2004. **114**(10): p. 1372-8.

322. Mombaerts, P., et al., *RAG-1-deficient mice have no mature B and T lymphocytes*. Cell, 1992. **68**(5): p. 869-77.
323. Kline, J., et al., *Autologous graft-versus-host disease: harnessing anti-tumor immunity through impaired self-tolerance*. Bone Marrow Transplant, 2008. **41**(6): p. 505-13.
324. Pardoll, D.M., *The blockade of immune checkpoints in cancer immunotherapy*. Nat Rev Cancer, 2012. **12**(4): p. 252-64.
325. Nishikawa, H. and S. Sakaguchi, *Regulatory T cells in tumor immunity*. Int J Cancer, 2010. **127**(4): p. 759-67.
326. Beyer, M. and J.L. Schultze, *Regulatory T cells in cancer*. Blood, 2006. **108**(3): p. 804-11.
327. Roux, S., et al., *CD4+CD25+ Tregs control the TRAIL-dependent cytotoxicity of tumor-infiltrating DCs in rodent models of colon cancer*. J Clin Invest, 2008. **118**(11): p. 3751-61.
328. El Andaloussi, A. and M.S. Lesniak, *CD4+ CD25+ FoxP3+ T-cell infiltration and heme oxygenase-1 expression correlate with tumor grade in human gliomas*. J Neurooncol, 2007. **83**(2): p. 145-52.
329. Peffault de Latour, R., et al., *Ontogeny, function, and peripheral homeostasis of regulatory T cells in the absence of interleukin-7*. Blood, 2006. **108**(7): p. 2300-6.
330. Liu, W., et al., *CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells*. J Exp Med, 2006. **203**(7): p. 1701-11.
331. Houghton, A.N., *Cancer antigens: immune recognition of self and altered self*. J Exp Med, 1994. **180**(1): p. 1-4.
332. Melchionda, F., et al., *Escape from immune surveillance does not result in tolerance to tumor-associated antigens*. J Immunother, 2004. **27**(5): p. 329-38.
333. Martin, C.E., et al., *Is IL-7 from dendritic cells essential for the homeostasis of CD4+ T cells?* Nat Immunol, 2010. **11**(7): p. 547-8; author reply 548.
334. Vogt, T.K., et al., *Novel function for interleukin-7 in dendritic cell development*. Blood, 2009. **113**(17): p. 3961-8.
335. Laffont, S. and F. Powrie, *Immunology: Dendritic-cell genealogy*. Nature, 2009. **462**(7274): p. 732-3.
336. Schmutz, S., et al., *Cutting edge: IL-7 regulates the peripheral pool of adult ROR gamma+ lymphoid tissue inducer cells*. J Immunol, 2009. **183**(4): p. 2217-21.

337. Yokota, Y., et al., *Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2*. Nature, 1999. **397**(6721): p. 702-6.
338. Sawa, S., et al., *Lineage relationship analysis of ROR γ mat⁺ innate lymphoid cells*. Science, 2010. **330**(6004): p. 665-9.
339. Geremia, A., et al., *IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease*. J Exp Med, 2011. **208**(6): p. 1127-33.
340. Pickert, G., et al., *STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing*. J Exp Med, 2009. **206**(7): p. 1465-72.
341. Aujla, S.J., et al., *IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia*. Nat Med, 2008. **14**(3): p. 275-81.
342. Mackall, C.L., et al., *IL-7 increases both thymic-dependent and thymic-independent T-cell regeneration after bone marrow transplantation*. Blood, 2001. **97**(5): p. 1491-7.
343. Bahceci, E., et al., *Early reconstitution of the T-cell repertoire after non-myeloablative peripheral blood stem cell transplantation is from post-thymic T-cell expansion and is unaffected by graft-versus-host disease or mixed chimaerism*. Br J Haematol, 2003. **122**(6): p. 934-43.
344. Fallen, P.R., et al., *Factors affecting reconstitution of the T cell compartment in allogeneic haematopoietic cell transplant recipients*. Bone Marrow Transplant, 2003. **32**(10): p. 1001-14.
345. Hakim, F.T., et al., *Constraints on CD4 recovery postchemotherapy in adults: thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells*. Blood, 1997. **90**(9): p. 3789-98.
346. Mackall, C.L., et al., *T-cell regeneration after bone marrow transplantation: differential CD45 isoform expression on thymic-derived versus thymic-independent progeny*. Blood, 1993. **82**(8): p. 2585-94.
347. Price, P.W. and J. Cerny, *Characterization of CD4⁺ T cells in mouse bone marrow. I. Increased activated/memory phenotype and altered TCR Vbeta repertoire*. Eur J Immunol, 1999. **29**(3): p. 1051-6.
348. Nanda, S. and J.M. Bathon, *Etanercept: a clinical review of current and emerging indications*. Expert Opin Pharmacother, 2004. **5**(5): p. 1175-86.

349. Bissler, J.J., et al., *Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangiomyomatosis*. N Engl J Med, 2008. **358**(2): p. 140-51.
350. Qasim, W., et al., *Allogeneic hematopoietic stem-cell transplantation for leukocyte adhesion deficiency*. Pediatrics, 2009. **123**(3): p. 836-40.
351. Antin, J.H., et al., *Establishment of complete and mixed donor chimerism after allogeneic lymphohematopoietic transplantation: recommendations from a workshop at the 2001 Tandem Meetings of the International Bone Marrow Transplant Registry and the American Society of Blood and Marrow Transplantation*. Biol Blood Marrow Transplant, 2001. **7**(9): p. 473-85.
352. Andreani, M., et al., *Long-term survival of ex-thalassemic patients with persistent mixed chimerism after bone marrow transplantation*. Bone Marrow Transplant, 2000. **25**(4): p. 401-4.
353. Kean, L.S., et al., *Chimerism and cure: hematologic and pathologic correction of murine sickle cell disease*. Blood, 2003. **102**(13): p. 4582-93.
354. Sykes, M., M.A. Sheard, and D.H. Sachs, *Graft-versus-host-related immunosuppression is induced in mixed chimeras by alloresponses against either host or donor lymphohematopoietic cells*. J Exp Med, 1988. **168**(6): p. 2391-6.
355. Lisini, D., et al., *Donor/recipient mixed chimerism does not predict graft failure in children with beta-thalassemia given an allogeneic cord blood transplant from an HLA-identical sibling*. Haematologica, 2008. **93**(12): p. 1859-67.
356. Sykes, M., *Mixed chimerism and transplant tolerance*. Immunity, 2001. **14**(4): p. 417-24.
357. Scandling, J.D., et al., *Tolerance and chimerism after renal and hematopoietic-cell transplantation*. N Engl J Med, 2008. **358**(4): p. 362-8.
358. Powell, J.D., et al., *Low-dose radiation plus rapamycin promotes long-term bone marrow chimerism*. Transplantation, 2005. **80**(11): p. 1541-5.
359. Hsieh, M.M., et al., *Allogeneic hematopoietic stem-cell transplantation for sickle cell disease*. N Engl J Med, 2009. **361**(24): p. 2309-17.
360. Hwu, P., et al., *Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation*. J Immunol, 2000. **164**(7): p. 3596-9.

361. Frumento, G., et al., *T cell proliferation is blocked by indoleamine 2,3-dioxygenase*. *Transplant Proc*, 2001. **33**(1-2): p. 428-30.
362. Fallarino, F., et al., *Functional expression of indoleamine 2,3-dioxygenase by murine CD8 alpha(+) dendritic cells*. *Int Immunol*, 2002. **14**(1): p. 65-8.
363. Munn, D.H., et al., *Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase*. *Science*, 2002. **297**(5588): p. 1867-70.
364. de Waal Malefyt, R., et al., *Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression*. *J Exp Med*, 1991. **174**(4): p. 915-24.
365. Jutel, M., et al., *IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy*. *Eur J Immunol*, 2003. **33**(5): p. 1205-14.
366. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid*. *J Exp Med*, 2007. **204**(8): p. 1775-85.
367. Elias, K.M., et al., *Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway*. *Blood*, 2008. **111**(3): p. 1013-20.
368. Shinohara, T., et al., *Upregulated IL-7 receptor alpha expression on colitogenic memory CD4+ T cells may participate in the development and persistence of chronic colitis*. *J Immunol*, 2011. **186**(4): p. 2623-32.