ST2/MYD88 SIGNALING IS A THERAPEUTIC TARGET
ALLEVIATING MURINE ACUTE GRAFT-VERSUS-HOST
DISEASE SPARING T REGULATORY CELL FUNCTION

Brad Griesenauer

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Doctoral Committee

______________________________
Sophie Paczesny, M.D., Ph.D., Chair

______________________________
Alexander L. Dent, Ph.D.

January 10, 2018

______________________________
Mark H. Kaplan, Ph.D.

______________________________
Reuben Kapur, Ph.D.
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Acute graft-versus-host disease (aGVHD) hinders the efficacy of allogeneic hematopoietic cell transplantation (HCT). Plasma levels of soluble serum stimulation-2 (sST2) are elevated during human and murine aGVHD and are correlated to a type 1 T cell response. Membrane-bound ST2 (ST2) on donor T cells has been shown to be protective against aGVHD. ST2 signals through the adapter protein myeloid differentiation primary response 88 (MyD88). The role of MyD88 signaling in donor T cells during aGVHD remains unknown. We found that knocking out MyD88 in the donor T cells protected against aGVHD independent of interleukin 1 receptor (IL-1R) and toll-like receptor 4 (TLR4) signaling, both of which also signal through MyD88, in two murine HCT models. This protection was entirely driven by MyD88−/− CD4 T cells, leading to a decreased type 1 response without affecting T cell proliferation, apoptosis, or migration. In our aGVHD models, loss of intrinsic MyD88 signaling is not responsible for the observed protection. However, transplanting donor MyD88−/− T conventional cells (Tcons) with wild type (WT) or MyD88−/− T regulatory cells (Tregs) ameliorated aGVHD severity and lowered aGVHD mortality. Transcriptome analysis of sorted MyD88−/− CD4 T cells from the intestine ten days
post-HCT showed lower levels of Il1rl1 (gene of ST2), Ifng, Csf2, Stat5, and Jak2, among others. Decreased sST2 was confirmed at the protein level with less secretion of sST2 and more expression of ST2 compared to WT T cells.

Transplanting donor ST2−/− Tcons with WT or ST2−/− Tregs mirrored observations when using donor MyD88−/− Tcons. This suggests that Treg suppression from lack of MyD88 signaling in Tcons during alloreactivity uses the ST2 but not the IL-1R or TLR4 pathways. The results of our study confirm that ST2 represents an aGVHD therapeutic target that spares Treg function.

Sophie Paczesny, M.D., Ph.D., Chair
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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>aGVHD</td>
<td>Acute Graft-versus-Host Disease</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BATF</td>
<td>Basic leucine zipper transcription factor, ATF-like</td>
</tr>
<tr>
<td>BCL6</td>
<td>B cell lymphoma 6</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma-X large</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>CCL</td>
<td>C-C motif chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C motif chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<tr>
<td>cGVHD</td>
<td>chronic Graft-versus-Host Disease</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>CXCL</td>
<td>C-X-C motif chemokine ligand</td>
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<tr>
<td>CXCR</td>
<td>C-X-C motif chemokine receptor</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOCK8</td>
<td>Dedicator of cytokinesis 8</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sulfate sodium</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
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<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
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<td>Foxp3</td>
<td>Forkhead box P3</td>
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<tr>
<td>FVD</td>
<td>Fixable viability dye</td>
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<tr>
<td>GATA</td>
<td>GATA binding protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GRK2</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HCT</td>
<td>Hematopoietic cell transplant</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intracellular adhesion molecule 1</td>
</tr>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
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<td>IL-1R</td>
<td>Interleukin 1 receptor</td>
</tr>
<tr>
<td>IL-1RAP</td>
<td>Interleukin 1 receptor accessory protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>IL-15R</td>
<td>Interleukin 15 receptor</td>
</tr>
<tr>
<td>IL-6R</td>
<td>Interleukin 6 receptor</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant natural killer T cell</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MCMV</td>
<td>Mouse cytomegalovirus</td>
</tr>
<tr>
<td>MD2</td>
<td>Lymphocyte antigen 96</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>miHA</td>
<td>Minor histocompatibility antigen</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed leukocyte reaction</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>ns</td>
<td>Not significant</td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural T regulatory cell</td>
</tr>
<tr>
<td>OX40L</td>
<td>Tumor necrosis factor superfamily member 4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>pTreg</td>
<td>Peripheral T regulator cell</td>
</tr>
<tr>
<td>PU.1</td>
<td>Purine-rich box 1</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination-activating genes</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RORγt</td>
<td>Retinoic acid receptor-related orphan receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>Single Ig IL-1-related receptor</td>
</tr>
<tr>
<td>sST2</td>
<td>Soluble serum stimulation-2</td>
</tr>
<tr>
<td>ST2</td>
<td>Serum stimulation-2</td>
</tr>
<tr>
<td>ST2LV</td>
<td>Serum stimulation-2 long variant</td>
</tr>
<tr>
<td>ST2V</td>
<td>Serum stimulation-2 variant</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Syn</td>
<td>Syngeneic</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box transcription factor TBX 21</td>
</tr>
<tr>
<td>Tc</td>
<td>T cytotoxic</td>
</tr>
<tr>
<td>TCD</td>
<td>T cell depleted</td>
</tr>
<tr>
<td>Tcon</td>
<td>T conventional cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
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</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-Trinitrobenzenesulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor associated factors</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
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</table>
CHAPTER 1: INTRODUCTION

1.1: Immune Response

The immune system is composed of various effector cells and molecules that respond to and protect the body from bacterial, fungal, and viral infections as well as toxins, proteins, and other macromolecules that are recognized as foreign and could cause damage to the body. An effective immune response is composed of four components: 1) immunological recognition, 2) effector function, 3) immune regulation, and 4) immunological memory. Immunological recognition requires that the immune system detect the invading microbe or foreign macromolecule. Once recognized, the immune system begins to upregulate effector molecules to contain and destroy the foreign entity. While the response is ongoing, the immune system must be able to self-regulate both during and after the infection. Failure at self-regulation leads to autoimmune disease. Once an infection or foreign entity is cleared, the immune system can develop memory cells, which respond quickly to any recurring foreign antigen and clear it.

1.1.1: Innate Immune System

In humans the immune system is composed of two cooperative arms: the innate immune system and the adaptive immune system. The innate immune system rapidly responds to control and remove any infection. Activation and response of the innate immune system to infection relies on pattern recognition receptors (PRRs) either on the cell surface, in the cytoplasm, or secreted by innate immune
These PRRs recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), leading to effector response. The innate immune system is also responsible for the activation and modulation of the adaptive immune response through antigen presentation on major histocompatibility complexes (MHC) and secretion of various cytokines.\textsuperscript{3}

Innate immunity provides an early line of defense for the body and is composed of multiple components. An infection or foreign antigen must first pass the physical barriers of the body. These include the skin, gastrointestinal tract, respiratory tract, and urogenital tract. The skin, in addition to being a physical barrier, produces antimicrobial peptides, which can kill microbes through disruption of the cytoplasmic membrane, DNA and protein synthesis, and protein folding.\textsuperscript{4} The epithelia in the gastrointestinal, respiratory, and urogenital tracts are coated in mucus to prevent adherence of microbes. Cilia on these cells expel mucus and the microbes trapped in the mucus. Microbes which pass through the mucus then have to get through the epithelial barrier, which is held together with tight junctions. These tight junctions prevent easy passage of microbes between epithelial cells. Breaking of these barriers leads to microbes entering the body. When this happens innate immune cells are recruited to destroy the invading microbes. This cell-mediated immunity is facilitated by neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages, dendritic cells (DCs), and natural killer cells (NK cells).\textsuperscript{5}
Each innate immune cell type has a specific function in promoting an effective immune response. Neutrophils are the most abundant immune cells and are one of the first to migrate toward a site of infection. These cells follow chemical signals such as interleukin (IL)-8, N-formylmethionyl-leucyl-phenylalanine, Leukotriene B4, and H$_2$O$_2$. Once recruited, neutrophils can phagocytose microbes or macromolecules that have been opsonized by antibodies. As well, neutrophils can release cytokines and other cytotoxic proteins through degranulation, which may also recruit other immune cells to amplify the inflammatory response. Finally, neutrophils have recently been shown to create neutrophil extracellular traps to help kill microbes.\textsuperscript{6} Eosinophils are granulocytes which release various chemical mediators such as peroxidase, neurotoxin, ribonuclease, and major basic protein; growth factors; and cytokines.\textsuperscript{7} Basophils are granulocytes which release histamine, proteoglycans, and elastase and secrete various cytokines and lipid mediators.\textsuperscript{8} Mast cells are very similar to basophils, except are found in tissues rather than circulating in the blood.

Monocytes are recruited to the site of infection and differentiate into either macrophages or DCs. They can also act as a professional antigen presenting cells (APC) through phagocytosis of infected cells and presentation of antigen to T cells. Macrophages phagocytose dying or dead cells and cellular debris containing microbes, which can then be presented to other immune cells. They can also secrete both pro- and anti-inflammatory cytokines, depending on the immune environment. DCs phagocytose microbes and present antigen to T and B cells in the lymph nodes.
1.1.2: Adaptive Immune System

The innate immune system recognizes microbes through PRRs, which are not specific for a single antigen but rather a class of macromolecules. PRRs are unable to change or adapt to specific invaders, making the innate immune system fixed in what it can recognize. Unlike the innate immune system, the adaptive immune system can express a diverse repertoire of antigen specific receptors. T cells and B cells, which comprise the adaptive immune response, develop antigen specificity through somatic recombination in their T cell receptor (TCR) and B cell receptor (BCR) genes followed by two processes called positive and negative selection. Somatic recombination occurs during early lymphocyte development mediated by recombination activating genes 1 and 2. A single T cell or B cell can undergo multiple somatic recombination events of their TCR or BCR, respectively. After a recombination event, T cells undergo positive selection. Positive selection of T cells checks to be sure that these cells can recognize self-peptide on self-MHC, or, to put another way, these cells are MHC-restricted to recognize only self-MHC. This process occurs in the thymus and is dependent on thymic stromal cells. Failure to pass the positive selection checkpoint leads to either another recombination event or death of the immature cell through lack of survival signals received. Following positive selection, these cells must pass another checkpoint called negative selection. Negative selection checks for the affinity between the TCR with self-peptide on self-MHC. This process also happens in the thymus, but is mediated mostly by bone marrow derived dendritic cells and macrophages. Thymic epithelial cells play less of a
role in negative selection. Those cells determined to have too high an affinity to self-antigen are induced to die in normal physiology. However, too high an affinity could lead to damaging autoreactivity. Cells that pass both positive and negative selection are then released into the periphery. Selection for B cells is different than that for T cells. Development and selection for B cells mostly occurs in the bone marrow. Positive selection for B cells does not require MHC at all but rather is dependent solely on the B cell itself. The BCR of B cells consists of two chains: a heavy chain and a light chain. In short, positive selection occurs when these two chains assemble a complex leading to signaling and proliferation. Negative selection against autoreactive BCRs is necessary as the recombination of the heavy and light chains are random. However, unlike with T cells, B cells that do not initially pass negative selection are not necessarily killed. Some of these autoreactive B cells can undergo a process called receptor editing in which the light chain is able to continue recombination until either a new, non-autoreactive receptor is made or until recombination events are exhausted. If the B cells are still autoreactive once recombination events are exhausted, most stop receiving survival signals and die. The autoreactive B cells that do not immediately stop receiving survival signals either become anergic or become clonally ignorant. Both of these processes begin in the bone marrow but finish in the periphery. Anergic B cells are no longer able to respond to antigen. Although these anergic cells are released from the bone marrow, they die in the periphery from lack of survival signals. Clonally ignorant B cells are self-reactive; however, they interact so weakly that little or no signally actually occurs. Alternatively, these clonally
ignorant B cells may not be encountering antigen due to the antigen not being available in the bone marrow or spleen.¹,¹⁰

There are two main subpopulations of T cells: CD4⁺ and CD8⁺ T cells. CD4⁺ T cells, or T helper cells (Th cells), help other immune cells primarily through release of various cytokines. These cytokines can either promote or suppress the immune response, depending on which cytokines are being produced. Naïve CD4⁺ T cells are activated through interaction with a peptide bound on MHC class II on APCs and an antigen non-specific co-stimulatory signal from the APC. These APCs also produce cytokines which help differentiate the CD4⁺ T cells towards a specific subtype, each with their own signature cytokine and transcription factor profile: type 1 T helper (Th1), Th2, Th9, Th17, regulatory T helper (Treg), and follicular T helper (Tfh) (Figure 1).¹¹ CD8⁺ T cells, also referred to as cytotoxic T cells (CTLs), are specialized to recognize and kill tumor cells and cells infected by intracellular pathogens. CTLs kill through release of granules containing granzymes and perforin. To prevent non-specific killing, CTLs only release these cytotoxic granules after binding to the cell through creation of an immune synapse.¹² Naïve CTLs are activated by TCR recognition of peptide antigen presented on MHC class I by APCs along with co-stimulatory signaling. As all nucleated cells in the body contain MHC class I, CTLs can recognize most cells in the body and kill infected cells as long as the peptide antigen is present on MHC class I.
Th1 cells are present during intracellular infections and during tumor growth. They are generated in the presence of IL-12. IL-12 signaling activates signal transducer and activator of transcription 4 (STAT4), which induces the Th1-master transcription factor T-box transcription factor TBX21 (T-bet). Both STAT4 and T-bet are required for optimal Th1 responses.\textsuperscript{13} Th1 cells produce both interferon gamma (IFN-\gamma) and tumor necrosis factor alpha (TNF-\alpha) for clearance of intracellular pathogens. If left uncontrolled, Th1 cells can cause autoimmune diseases and chronic inflammation.

Th2 cells are essential in the resolution of extracellular pathogens. IL-4 signaling induces STAT6 activation, which promotes GATA binding protein 3 (GATA3) expression, the master transcription factor for Th2 cells. As well, thymic stromal lymphopoietin (TSLP) can help with Th2 differentiation through increased IL-4 production.\textsuperscript{14} TSLP is not necessary for Th2 cell differentiation or function in already mature Th2 cells,\textsuperscript{15} but is important for their generation/maintenance \textit{in vivo}.\textsuperscript{16} Th2 cells secrete IL-4, IL-5, and IL-13 as effector cytokines for elimination of extracellular pathogens. An uncontrolled Th2 response leads to allergic diseases.

Th9 cells, like Th2 cells, help with eradication of extracellular pathogens, including helminths and parasites. Unlike Th2 cells, Th9 cells have been shown to be beneficial in preventing melanoma growth.\textsuperscript{17} These cells require transforming growth factor beta (TGF-\beta) and IL-4 for differentiation. This leads to
PU.1, interferon regulatory factor 4 (IRF4), and STAT6 activation and the subsequent production of predominately IL-9 and some IL-10 and IL-2. Like Th2 cells, Th9 cells contribute to allergic diseases if left unchecked.

Th17 cells help with pathogen clearance at mucosal surfaces and with anti-fungal response. They require TGF-β, IL-6, and IL-23 for their differentiation. These cytokines activate STAT3 signaling, leading to the expression of the master transcription factor retinoic acid receptor-related orphan receptor gamma t (RORγt). Once differentiated toward Th17, these cells begin to produce IL-17A, IL-17F, IL-21, and IL-22. Th17 cells also can contribute to various autoimmune diseases and glioma.

Tregs are critical for suppressing the immune response after clearance of pathogens and for maintaining tolerance to self-antigens. Tregs can be classified into two groups, depending on where they originate: natural or thymic Tregs (nTregs or tTregs) and peripheral or induced Tregs (pTregs or iTregs). nTregs develop in the thymus while pTregs develop extrathymically at peripheral sites. While pTregs require the cytokine TGF-β for their differentiation, nTregs do not require TGF-β but seem to require IL-2/IL-15 signaling through CD122. IL-2 is also involved in maintenance of pTregs. Both require the master transcription factor for Tregs Forkhead box P3 (Foxp3). The regulatory function of Tregs is multifaceted; they use secretion of the cytokines IL-10 and TGF-β, cell contact-dependent modulation and suppression, and cytolytic killing through granzyme or
perforin.\textsuperscript{23} Loss of Treg activity leads to fatal autoimmune disease while too much Treg activity leads to failure to clear pathogens and prevention of tumor clearance.

Tfh cells help with humoral immunity through activating follicular B cells in secondary lymphoid organs. After follicular B cell and Tfh cell interaction, germinal centers are formed and maintained. CD40L on Tfh cells interacting with CD40 on follicular B cells and secretion of IL-4 and IL-21 by the Tfh cells help follicular B cells expand and differentiate into both plasma cells and memory B cells. Tfh cells require IL-6 and IL-21 for their differentiation, leading to the activation of multiple STAT proteins with STAT3 being the most important.\textsuperscript{24} Activation of these STAT proteins leads to B cell lymphoma 6 (Bcl6) expression, the master transcription factor for Tfh cells. An abnormal Tfh response can cause B cells to produce autoreactive antibodies leading to autoimmune disease.

The B cell component of the adaptive immune system is responsible for the vast majority of the humoral immune response. Naïve B cells are activated upon antigen binding to BCR. While both T and B cells can recognize peptides, B cells can also recognize unprocessed proteins, glycoproteins, and polysaccharides. Also like T cells, B cells require a secondary signal to become fully activated. The secondary signal can come from T cells through CD40L/CD40 interaction and is called T cell-dependent activation. The secondary signal can also come from the antigen itself through recognition of a common microbial constituent or through
cross-linking of multiple BCR to repeating epitopes. This is called T cell-independent activation. BCR binding and secondary signaling leads to B cell proliferation and differentiation. After activation some B cells undergo immunoglobulin (Ig) class switching. Naïve mature B cells express IgM and IgD subclasses at first, but after activation can switch to expressing IgG, IgE, or IgA subclasses, each with a distinct effector function. Which Ig subclass the B cell will switch to depends on the cytokine environment in which the B cell is located. The variable regions of the antibody do not change, only the constant region of the Ig heavy chain, ensuring antigen specificity remains. Along with Ig class switching, activation of B cells causes them to undergo affinity maturation. This involves mutations in the variable regions of IgM, IgG, IgA, and IgE antibodies to increase affinity toward an antigen in processes called somatic hypermutation and clonal selection. This ensures that only B cells with the highest affinity toward an antigen survive. Those that do survive produce highly efficient antibodies that bind a specific antigen for neutralization and elimination.25
Figure 1. Th cell differentiation

After TCR activation naïve CD4⁺ T cells begin differentiation based on the cytokine milieu encountered. These cytokine milieus activate specific transcription factors leading to the differentiation of various Th cells subsets. Each subset has specific transcription factors, chemokine receptors, and cytokines produced leading to unique immune function for each subset to help against microbe invasion. Immune pathologies occur when a specific subset is allowed uncontrolled differentiation.
1.2: ST2

In 1989, the \( il1rl1 \) gene product, given the name ST2 and later defined as the IL-33 receptor, was discovered.\(^{26,27} \) It belongs to the IL-1-receptor superfamily. Some literature misnamed ST2 as “suppressor of tumorigenicity 2”, when in fact the original name was “growth stimulation expressed gene 2”.\(^{27} \) ST2 has recently been renamed by the original discoverer, Shin-ichi Tominaga, as “serum stimulation-2”,\(^{28} \) as it was first discovered to function as a mediator of type 2 inflammatory responses.\(^{29} \) \( IL1RL1 \) is located on chromosome 2q12.1 in humans, while the gene “suppressor of tumorigenicity 2”, also called ST2, is located on chromosome 11p14.3-p12 in humans.

ST2 has two main splice variants due to differential promoter usage: a membrane bound form (ST2), which promotes NF-\( \kappa B \) signaling, and a soluble form (sST2), which prevents its signaling. It was not until 2005 that the ligand for ST2, the cytokine IL-33, was identified through database searching for genes homologous to other IL-1 superfamily members.\(^{30,31} \) IL-33 has been identified as a mediator of various inflammatory diseases such as asthma, cardiovascular diseases, and allergic diseases.\(^{31} \) Besides being secreted, IL-33 can be found in the nucleus of human high endothelial venules,\(^{32} \) lung airway epithelium, keratinocytes, fibroblastic reticular cells, and some epithelial cells of the stomach and salivary glands.\(^{33} \) Due to the presence of a N-terminal domain nuclear localization sequence and a homeodomain-like helix-turn-helix motif, IL-33 is
able to bind heterochromatin, potentially giving IL-33 transcriptional regulatory capacity.\textsuperscript{32}

Dysregulation of IL-33/ST2 signaling and sST2 production have been implicated in a variety of inflammatory diseases such as cardiac disease,\textsuperscript{34-37} intestinal bowel disease (IBD),\textsuperscript{38-41} graft-versus-host disease (GVHD),\textsuperscript{42-49} small bowel transplant rejection,\textsuperscript{50} and type-2 diabetes.\textsuperscript{47,51-53}

1.2.1: Two main isoforms of ST2

The ST2 gene is located on human chromosome 2q12.1 and is approximately 40 kb long. Homologues of ST2 are found in the genomes of mouse, rat, and fruit fly. ST2 has four splice isoforms from a single transcript dependent on the promoter being used: ST2, a membrane receptor; sST2, a soluble factor; ST2V, a variant form of ST2, and ST2LV, another variant form of ST2, which are differentially regulated through alternative promoter usage.\textsuperscript{54-56} Little is known about ST2V other than it is expressed highly in gastrointestinal organs.\textsuperscript{57} ST2LV lacks the transmembrane domain found in ST2, is secreted by eye, heart, lung, and liver tissues, and is found during later stages of embryogenesis.\textsuperscript{58} Other information on ST2LV is currently lacking.

By cloning the \textit{Il1rl1} gene in rat and sequencing sST2 and ST2 cDNAs, it was found that sST2 and ST2 have different exon 1 sequences.\textsuperscript{54} Mapping the promoter regions for \textit{Il1rl1} showed that the transcription start site for sST2 is in a
proximal promoter region while the transcription start site for ST2 is in a distal promoter region, 15kb upstream from the sST2 proximal promoter (Figure 2).\textsuperscript{54} Three to four GATA transcription factors have been identified at the distal promoter region within 1001 bp, two of which were conserved between human and mouse \textit{Il1rl1} genes.\textsuperscript{56,59} These GATA elements binding to the distal promoter lead to ST2 expression. The transcription factor PU.1 also binds to the distal promoter near the GATA elements in both human mast cells and basophils.\textsuperscript{60} PU.1 and GATA2 cooperatively transactivate the distal ST2 promoter inducing expression of ST2, but not sST2.\textsuperscript{60} Loss of PU.1 significantly decreased ST2 expression.\textsuperscript{60} Conversely, a PMA-responsive element has been found near the proximal promoter region of ST2 in the mouse fibroblast line NIH 3T3.\textsuperscript{61} Similarly, activating the human fibroblast line TM12, which only uses the proximal promoter for \textit{Il1rl1} transcription, led to sST2 expression.\textsuperscript{56} These data further suggest that the distal promoter is used to transcribe ST2 and the proximal promoter is used to transcribe sST2. These results indicate key transcription factors important in ST2 or sST2 expression; however, ChIP-seq experiments have yet to be performed.
1.2.2: Membrane ST2

ST2 was first found in serum-stimulated BALB/c-3T3 cells in the presence of cycloheximide.\textsuperscript{62} It contains an extracellular domain, which binds IL-33 with the help of IL-1 receptor accessory protein (IL-1RAP), a transmembrane domain, and an intercellular domain called a Toll/Interleukin-1 receptor (TIR) domain. Due to the presence of the TIR domain, ST2 has been classified as a member of the IL-1 receptor superfamily. ST2 is expressed on cardiomyocytes\textsuperscript{63} and a large variety of immune cells, including T conventional cells, particularly type 2,\textsuperscript{64} T regulatory cells (Tregs),\textsuperscript{65} innate helper 2 cells (ILC2),\textsuperscript{66} M2 polarized macrophages,\textsuperscript{67} mast cells,\textsuperscript{68} eosinophils,\textsuperscript{69} basophils,\textsuperscript{70} neutrophils,\textsuperscript{70} NK,\textsuperscript{71} and iNKT cells.\textsuperscript{71} Signaling through ST2 in immune cells induces type-2 and Treg immune responses, IgE production, and eosinophilia.\textsuperscript{30,64-66,72}

1.2.3: Soluble ST2

sST2 protein lacks the transmembrane and cytoplasmic domains contained on ST2 and contains a unique nine amino-acid C-terminal sequence.\textsuperscript{59} In vitro, sST2 production has been shown to be enhanced by proinflammatory cytokines (IL-1β, TNF-α) in human lung epithelial cells and cardiac myocytes. In humans, sST2 can be produced spontaneously by cells in the lung, kidney, heart, small intestine,\textsuperscript{73} but can also be produced after activation with IL-33 in mast cells\textsuperscript{74} or anti-CD3/anti-CD28 in both CD4 and CD8 conventional T cells.\textsuperscript{75} In a murine aGVHD model, it has recently been shown that intestinal Th17 and Tc17 cells produced large amounts of sST2 following alloreactivity.\textsuperscript{75} This enhanced sST2
presence has been shown to inhibit the production of the type 2 cytokines IL-4 and IL-5 but not the type 1 cytokine IFN-γ.
Figure 2. Different promoter usage dictates ST2 and sST2 expression

ST2 consists of two main isoforms: ST2 and sST2. These isoforms are splice variants of each other regulated by alternative promoter bindings, the distal promoter for ST2 and the proximal promoter for sST2. Exon 1 varies between ST2 and sST2 depending on the promoter being bound. In immune cells GATA1, GATA2, and PU.1 have been shown to bind to the distal promoter. The proximal promoter has not been as well studied; however, a PMA-responsive element has been shown to induce sST2 transcription.
1.3: IL-33/ST2 signaling

1.3.1: The membrane bound form of ST2 signals through MyD88/NF-κB

Upon IL-33 binding, the membrane-anchored ST2 forms a heterodimer along with IL-1RAP\(^{77,78}\) leading to the dimerization of the TIR domain. This leads to the recruitment of the TIR domain binding protein MyD88 and subsequent IL-1R-associated kinase (IRAK) activation, which can activate MAP kinases and NF-κB pathways (Figure 3).\(^{30,31}\) In regards to IL-33/ST2 signaling, how IL-33/ST2 signals specifically to either the MAPK or NF-κB is currently unclear. However, downstream events of ST2 do seem to occur differentially, as TRAF6 is required for NF-κB activation and induction of type 2 cytokines but TRAF6 is not needed for IL-33 induced ERK (a MAPK protein) activation.\(^{79}\) How TRAF6 independent activation of ERK occurs after IL-33 binding ST2 is currently unknown.

A recent report has shown that signaling through IL-33/ST2 in colonic Tregs helps to promote Foxp3 and GATA3 expression while also promoting Treg function through enhancing TGF-β1-mediated differentiation.\(^{65}\) This enhancement is caused by phosphorylation of GATA3, which leads to more GATA3 and RNA polymerase II binding to the Foxp3 promoter.\(^{65}\) GATA3 binds to and activates the ST2 promoter, enhancing ST2 on the surface of both Th2 cells\(^{80,81}\) and Tregs.\(^{65,81}\) IL-33 has been shown to drive NF-κB and p38 signaling in Tregs, leading to the selective expansion of ST2\(^+\) Tregs.\(^{82}\) As this effect is observed in Tregs in a non-diseased setting, independent of outside inflammatory responses, we believe the IL-33/ST2-GATA3-Foxp3 pathway to be
canonical. Conversely, in a non-canonical MyD88 dependent pathway, IRF1 signaling can inhibit Tregs by binding to the Foxp3 promoter and preventing Foxp3 transcription in murine T cells; however, this signaling leading to IRF1 activation through MyD88 has only been shown to be induced using CpG-B, a TLR9 agonist and a pathway independent from IL-33/ST2. Whether IL-33/ST2 can activate IRF1 in a MyD88-dependent pathway and whether this IL-33/ST2-IRF1 activation can affect Treg function is currently unknown.

Unlike IL-1RAP, the single immunoglobulin domain IL-1R-related molecule (SIGIRR or TIR8) SIGIRR can form a complex with ST2 upon IL-33 stimulation and can inhibit IL-33/ST2-mediated signaling both in vitro and in vivo. IL-33 binding to ST2 has also been shown to negatively regulate ST2 through protein polyubiquitination, internalization, and degradation.

1.3.2: The soluble form, (s)ST2, is a decoy receptor and does not signal

sST2 acts as a decoy receptor to sequester free IL-33, preventing IL-33/ST2 signaling. This was shown using a thymoma cell line transfected to express ST2, but not sST2, in the presence of added IL-33. When these thymoma cells were pre-treated with sST2, they showed suppressed NF-κB activity. Another group used IL-33-treated cardiomyocytes and observed blocked pro-hypertrophic effects of angiotensin II or phenylephrines in the presence of sST2. Blocking NF-κB signaling in lung alveolar epithelial cells and cardiac myocytes with the specific NF-κB inhibitor CAPE prevented sST2 production by these cells. In a
human endotoxin model, healthy donors injected with LPS (2 ng/kg) had increased sST2 in their plasma within 24 hours of injection. Fibroblast growth factor 2 enhanced sST2 production in the human breast adenocarcinoma cell line MCF-7 through MEK/ERK signaling. Lysophosphatidic acid has also been shown to increase sST2 production by human bronchial epithelial cells in an NF-κB or JNK-dependent manner. Enhanced sST2 plasma circulation has been correlated with pulmonary fibrosis, acute myocardial infarction, subclinical brain injury and stroke, celiac disease, gastric cancer, HBV-related acute-on-chronic liver failure, HIV progression, and GVHD.

1.3.3: IL-33 regulation and release

IL-33 is expressed mainly by nonhematopoietic cells, including endothelial cells, adipocytes, fibroblasts, and intestinal and bronchial epithelial cells; however, some hematopoietic cells like dendritic cells have also been shown to express IL-33 when activated. In many nonhematopoietic tissues, IL-33 is constitutively expressed. Constitutive expression of IL-33 in epithelial cells suggests that IL-33 is used as an alarmin in response to infection or injury. An alarmin is an endogenous molecule that is constitutively available and released when tissue is damaged. Upon release, an alarmin helps activate the immune system. Further suggesting IL-33 is an alarmin, IL-33 is released by damaged or necrotic cells, leading to activation of the immune system through IL-33/ST2 signaling.
During homeostasis IL-33 is found primarily in the nucleus due to a nuclear localization sequence in the N-terminus, leading to binding of heterochromatin in the nucleus.\textsuperscript{32} Nuclear IL-33 can bind directly to NF-κB, sequestering it and preventing NF-κB signaling in HEK293RI cells, causing a downregulation of proinflammatory signaling.\textsuperscript{100} Further evidence of IL-33 having the ability to repress gene transcription is described because there is a structural similarity between a part of the IL-33 protein and the Kaposi sarcoma herpes virus motif latency-associated nuclear antigen.\textsuperscript{100} This homology suggests that IL-33 can bind to the H2A-H2B chromatin dimer and regulate the compaction of chromatin through nucleosome-nucleosome interactions. Recent discoveries have shown that nuclear IL-33 can bind to multiple sites in the promoter regions of ST2 in human endothelial cells and that knockdown of IL-33 increased sST2 levels.\textsuperscript{101} Loss of the nuclear localization domain of IL-33 led to non-resolving lethal inflammation.\textsuperscript{102} However, IL-33\textsuperscript{-/-} mice fail to develop autoimmune disease, and no one has shown whether nuclear IL-33 has been found in immune cells. These results indicate that nuclear IL-33 could act as a moderator of inflammation, but more evidence is needed to confirm the extent of the ability of nuclear IL-33 to moderate inflammation.

During cell stress or damage, IL-33 is passively released from the nucleus in full-length form and can bind to ST2, leading to activation of the IL-33/ST2 pathway. Like other IL-1 superfamily members, IL-33 can be cleaved at the N-terminus to enhance its biological activity. Unlike other IL-1 superfamily members, however,
IL-33 is not cleaved via caspases. Surprisingly, caspase-1, caspase-3, or caspase-7 processing actually leads to IL-33 inactivation. Inactivation of IL-33 via caspases is therefore thought to alleviate the immune response, rather than enhance it. Other proteins are able to cleave released IL-33, such as the neutrophil serine proteases cathepsin G and elastase, mast cell derived serine proteases, tryptase, and chymase. These proteins, unlike caspases, increase the biological activity of IL-33 by 10 to 30 times compared to that of full length IL-33.
Figure 3. IL-33/ST2 signaling pathway

IL-33 either binds to the ST2/IL1RAP heterodimer, recruiting MyD88 to its intracellular domain, or the sST2 decoy receptor, which does not signal. MyD88 binding recruits IRAK and TRAF6, leading to either the NF-κB or AP-1 pathways being activated. NF-κB and AP-1 activation promote inflammatory cytokine expression. On Tregs, IL-33/ST2 signaling has been shown to promote the expression of Foxp3 and GATA3 while also promoting Treg function and expansion through enhancing TGF-β1-mediated differentiation though a p38-dependent mechanism.
1.4: ST2 signaling in lymphoid cells

1.4.1: Th2 cells

ST2 was first shown both in vitro and ex vivo to be preferentially expressed on murine Th2 cells (Figure 4) expressing predominantly IL-4, IL-5, or IL-10, but not IFN-γ or IL-2. Its expression is independent of IL-4, IL-5, and IL-10, as loss of any of these cytokines does not affect ST2 expression on Th2 cells. ST2 expression on Th2 cells is dependent on GATA3 signaling and is enhanced by IL-6, IL-1, TNF-α, and IL-5. Given that ST2 expression in Th2 cells is independent of IL-4 and dependent on GATA3 signaling, it makes sense that ST2 expression occurs late during Th2 differentiation. IL-33 stimulation of Th2 cells in vitro increased the amount of IL-5 and IL-13 produced. Antigen-specific ST2+ Th2 cells were shown to produce more IL-5 and IL-13 compared to non-antigen-specific Th cells and ST2−/− Th2 cells. Interestingly, IL-33 polarization of antigen stimulated murine and human naïve CD4+ T cells leads to high IL-5 production but no IL-4 production, independent of GATA3 and STAT6 induction but dependent on MAPK and NF-κB signaling. Adoptive transfer of these cells into naïve IL-4−/− mice still triggered airway inflammation. In vivo administration of IL-33 led to an increase in the number of lymphocytes circulating in the blood and increased type 2 cytokine secretion in the thymus, spleen, liver, and lung. IL-33 has also been shown to be a chemoattractant for Th2 cells, as adoptive transfer of Th2 cells into Il1rl1−/− mice followed by IL-33 administration into the footpad of these mice led to the accumulation of the transferred Th2 cells. Loss of ST2 on Th2 during infection with the helminthic
parasite *Nippostrongylus brasiliensis* did not affect Th2-mediated clearance of the infection, nor was recruitment of Th2 cells in a murine model of asthma dependent on ST2 indicating that ST2 is not necessary for Th2 function. Recently, it was shown that human and murine Th2 cells do not produce sST2 *in vitro*.75

### 1.4.2: Th9 cells

IL-9-producing Th9 cells are the newest T cell subset to be described, polarized through TGF-β and IL-4 signaling.115,116 When used separately on naïve T cells, TGF-β alone would cause Treg development, while IL-4 would induce Th2 cell differentiation. It has been found that the PU.1 gene is a Th9-specific transcription factor, which could induce IL-9 production in cells under Th2- or Th9-stimulating condition *in vitro*.117 Human or mouse PU.1-deficient T cells have diminished IL-9 production. Furthermore, IRF4 binds directly to the IL-9 promoter, and is required for the development of Th9 cells, similar to PU.1.118 However, unlike PU.1, IRF4 is also required for the development of other Th cell subsets, including Th2 and Th17 cells.119,120 Studies have shown that Th9 cells primarily secrete IL-9 to mediate the immune response in several diseases, such as asthma, autoimmune diseases, and parasitic infections,121 and IL-9 is associated with impaired Th1 immune response in patients with tuberculosis.122 Treatment of *in vitro* polarized human Th2 cells with TGF-β and IL-33 increases expression of IL-9 and ST2.123,124
1.4.3: Tregs

IL-33/ST2 signaling in Tregs was first suggested to enhance their protective ability in an experimental colitis model in which IL-33 treatment ameliorated colonic tissue injury and colitis symptoms. IL-33 was shown to increase both ST2 and Foxp3 levels and expand Tregs in mice with colitis. IL-33/ST2 signaling in Tregs has also been shown to increase Treg frequency and decrease IL-17 and IFN-γ production in an EAE model. ST2+ Treg expansion is helped by IL-33 signaling in dendritic cells, as IL-33 has been shown to stimulate dendritic cell production of IL-2 which selectively expands ST2+ Tregs. In a model of aGVHD, treatment of mice daily with IL-33 from 10 days pre-transplantation to day 4 post-transplantation enhanced their frequency of ST2+ Tregs, which persisted after irradiation, leading to disease amelioration through prevention of T conventional cell accumulation in target aGVHD organs. Treatment of mice receiving a heart transplant with IL-33 prolonged graft survival through increased Treg and myeloid derived-suppressor cell numbers. Similarly, mice treated with IL-33 after skin transplantation had increased Treg numbers in the graft, decreased IFN-γ and IL-17 production, increased IL-10 production, and increased skin graft survival. This group also showed that IL-33/ST2 signaling can convert Foxp3− CD4 cells into Foxp3+ CD4 Tregs in the periphery. We have shown that in a murine model of allogeneic hematopoietic stem cell transplantation HCT, transplanting Il1rl1−/− Tregs with WT T conventional cells worsens aGVHD compared to mice receiving WT T conventional cells and Tregs, further indicating the enhanced suppressive effect of ST2+ Tregs.
Conversely to the enhanced protective effect of Tregs through IL-33/ST2, it has been reported that IFN regulatory factor 1 (IRF1) is downstream of MyD88\textsuperscript{130} and negatively regulates Foxp3 transcription\textsuperscript{84,130} although whether or not IL-33/ST2 signaling increases IRF1 expression, leading to decreased Treg function, has yet to be studied. These data show that IL-33 signaling on Tregs increases their immunomodulatory function and could be further studied for their potential clinical benefits in a variety of diseases.

1.4.4: Innate lymphoid cells type 2 (ILC2s)

ILC2 cells were first discovered in the mouse and human fat-associated lymphoid clusters located in the mesentery. These cells were found to be lineage marker negative, c-Kit positive, Sca-1 positive, IL-7Rα positive, and ST2 positive\textsuperscript{66,131}. These cells have been shown to play a protective role against helminth infection and regulate metabolic homeostasis\textsuperscript{132}. In humans ST2\textsuperscript{+} ILC2s were later found in the lung and gut\textsuperscript{133} and these ILC2s produced IL-5 and IL-13. During ILC2 activation ST2 is upregulated in a GATA3 and Gfi1-dependent manner\textsuperscript{134,135}. Treatment of Rag2 KO mice with IL-33 induced IL-5 and IL-13 production, whereas Rag2 and common gamma chain double KOs, which still have mast cells and basophils (both of which express ST2 and secrete type 2 cytokines), did not increase IL-5 or IL-13 production, indicating that this increase is due to ILC2 stimulation with IL-33\textsuperscript{66}. IL-33/ST2 signaling enhancement was shown to expand ILC2s \textit{in vivo}\textsuperscript{66,136}. This group also found that ILC2s are major producers of type 2 cytokines after \textit{Nippostrongylus brasiliensis} infection. It was also shown
using the *N. brasiliensis* infection model that loss of both IL-33 and IL-25 signaling on ILC2s completely abrogated the early response against this infection due to impaired expansion of ILC2s and lack of IL-13 production and adoptive transfer of WT ILC2s rescued this phenotype.66 During lung inflammation ILC2s produce IL-9,137 and IL-33 can promote cytokine production by ILC2s.138 Recently, it was shown that in a murine eosinophilic airway inflammation model that T-bet regulates IL-9 production by IL-33-stimulated ILC2s.139 IL-33/ST2 signaling in ILC2s is also important for protection against lung infection, as blocking ST2 signaling during influenza infection in mice lowered ILC2 frequency and number in the lung, and resulted in diminished lung function, loss of airway epithelial integrity, and impaired respiratory tissue remodeling.140 Histological examination of influenza-infected lungs from anti-ST2 treated mice showed severe damage similar to that seen in a similar experiment where ILC2s were depleted.140 ILC2s have been recently reported to home to the skin in humans, where activation induces upregulation of ST2.138 IL-33/ST2 signaling of ILC2s in the murine skin has been shown to promote atopic dermatitis-like inflammation,138,141 but also promote skin wound repair.142 However, overstimulation of ILC2s with IL-33 during tissue remodeling of the liver after chemical injury promoted liver fibrosis.143 Also, signaling through IL-33/ST2 on ILC2s during breast cancer has been shown to promote breast cancer growth and metastasis.144 These data indicate that beneficial or harmful IL-33/ST2 stimulation in ILC2s is dependent on certain disease states.
1.4.5: CD8 T cells

CD8 T cells have been shown to either express ST2 or produce sST2. Although CD8 T cells express low levels of ST2, loss of either IL-33 or ST2 impaired the CD8 T cell response to lymphocytic choriomeningitis virus (LCMV) infection. IL-33/ST2 signaling has also been shown to enhance CD8 T cell antitumor activity. During aGVHD, however, IL-33 treatment during peak inflammation significantly increased aGVHD severity and mortality in part through increased expansion of Tc1 cells. Given that IL-33 can increase type 1 responses when IL-12 levels are high, IL-33 treatment during peak inflammation was deleterious in this case.

1.4.6: B cells

ST2 has been shown to be expressed on B1 B cells but not B2 B cells, leading to enhanced proliferation capacity and IgM, IL-5, and IL-13 production both in vitro and in vivo; neutralizing IL-5 almost completely abolished this effect. Recent studies have also shown that IL-33 treatment in mice increases circulating IL-10-producing B cells that are neither conventional B1 or B2 B cells. Adoptive transfer of these IL-33-treated, IL-10 producing B cells prevented spontaneous colitis in IL-10−/− mice without affecting Treg frequency.

1.4.7: iNKT cells and NK cells

IL-33/ST2 signaling in murine iNKT cells causes their expansion and activation. Mice treated with IL-33 had twice as many iNKT cells in the spleen
and liver compared to untreated mice. Unexpectedly, ST2 signaling in iNKT cells induced IFN-γ instead of IL-4 upon TCR engagement, which synergized in the presence of IL-12. This effect was also seen in Vα24+ human iNKT cells. NK cells constitutively express ST2 and IL-33/ST2 signaling increases IFN-γ levels synergistically with IL-12. Loss of ST2 in Ly49H+ NK cells did not affect their development but did impair their ability to expand and protect against MCMV. These data have not yet been translated to human disease.
Figure 4. IL-33 signaling in immune cells

Tissue damage and mechanical stress to epithelial, endothelial, and stromal cells leads to the release of IL-33 from these cells. IL-33 then signals through many different immune cells, enhancing their function.
1.5: sST2 expression in lymphoid cells

1.5.1: Th1 and Th17 cells

Although much of the research on IL-33/ST2 signaling in T conventional cells has been devoted to type 2 signaling, recent studies have come out on IL-33/ST2 signaling in type 1 and type 17-mediated diseases. Blockade of IL-33 with 200 μg anti-IL-33 every other day from day 0 until day 18 post-MOG$_{35-55}$ injection during MOG-induced experimental autoimmune encephalomyelitis (EAE) ameliorated the disease in part through decreased IL-17 and IFN-γ production, and treatment of 50 μg/kg IL-33 during this same time course enhanced IL-17 and IFN-γ production.$^{24}$ However, the amount of IL-33 given here is not physiological, so caution must be advised when interpreting this data. Conversely, another group using the same EAE model found that treatment with 1 μg IL-33 daily from day 12 to day 20 after immunization reduced IL-17 and IFN-γ production and alleviated the disease.$^{125}$ Seemingly, the timing of IL-33/ST2 treatment affects response, perhaps through differing environments. In a murine model of collagen-induced arthritis, treatment with anti-ST2 antibody reduced both IFN-γ and IL-17 production.$^{152}$ In a murine model of rheumatoid arthritis, treatment with an sST2-Fc fusion protein attenuated disease and decreased production of IFN-γ, TNF-α, and IL-6.$^{153}$ Recently, we were the first to show that both murine and human Th1 and Th17 cells produce sST2 \textit{in vitro} and \textit{in vivo} after HCT.$^{75}$ Blocking ST2 with a blocking antibody \textit{in vivo} decreased sST2 production in intestinal T cells 10 days after HCT while maintaining ST2. Recipients of ST2$^{-/-}$ T cells, compared to WT T cells, showed lower frequencies of Th1 and Th17 cells.
and higher frequencies of Th2 and Treg cells. Importantly, anti-ST2 treatment did not lead to loss of immunomodulatory ST2+ Tregs but rather maintained them in the intestine. Based on our findings, we have suggested that increased sST2 production affects the normal balance of pathogenic Th1/Th17 cells and immunomodulatory Th2/Treg cells by promoting the Th1/Th17 response and dampening the ST2-mediated Th2/Treg response through sequestering IL-33.

1.5.2: Tc1 and Tc17 cells

We were also the first to demonstrate that CD8 T cells, particularly Tc1 and Tc17 cells but not Tc2 cells, produce significant amounts of sST2 \textit{in vitro} and after HCT due to alloreactivity. sST2 secretion by donor T cells significantly increased as aGVHD progressed. Similarly to CD4 T cells, blocking ST2 with a blocking antibody decreased sST2 production by Tc1 and Tc17 cells \textit{in vivo} after HCT. Our data indicates that sST2 secretion by Tc1 and Tc17 cells sequesters free IL-33, preventing IL-33/ST2-mediated Th2/Treg responses. In patients with early HIV infection, sST2 levels were strongly correlated with CD8 T cell count and their expression of the activation markers HLA-DR and CD38. However, it is not known if sST2 was produced from the CD8 T cells themselves or if sST2 is only a marker of gut damage and disease progression. While our study was the first to show that preventing sST2 secretion from CD8 T cells prevented disease pathogenesis, further studies are warranted to determine their role in other disease pathogeneses.
1.6: Myeloid cells

1.6.1: Macrophages

Macrophages, mast cells, basophils, eosinophils, and dendritic cells all have been shown to express ST2.\textsuperscript{67-70,154} IL-33 amplifies the expression of M2 markers on murine macrophages.\textsuperscript{67,155} Bone derived human macrophages have been shown to constitutively express both ST2 and sST2; however, skewing these macrophages toward an M2 phenotype using IL-4 and IL-13 increased the expression of ST2 while not affecting sST2 expression.\textsuperscript{156} IL-33/ST2 signaling has been shown to enhance the activation of macrophages by upregulating the LPS receptor components TLR4 and MD2, soluble CD14, and MyD88.\textsuperscript{155}

1.6.2: Mast cells

IL-33/ST2 signaling on both murine and human mast cells has been shown to promote their survival in the peritoneum through upregulation of B-cell lymphoma-X large (Bcl-xL).\textsuperscript{157} IL-33/ST2 signaling also promotes mast cell activation and maturation, as IL-33 treatment of CD34\textsuperscript{+} mast cell precursors accelerated their maturation \textit{in vitro} and induced GM-CSF, IL-5, IL-13, CXCL8, CCL17, CCL22, and CCL2 secretion.\textsuperscript{158,159} These cytokine and chemokine secretions may be NFAT and AP-1 signaling-dependent.\textsuperscript{160} It is well documented that mast cells can produce a variety of type 2 cytokines after ST2 signaling,\textsuperscript{24,161,162} however, IL-33/ST2 signaling on mast cells during airway inflammation has also been shown to promote a Th17 response.\textsuperscript{60}
1.6.3: Basophils and Eosinophils

IL-33/ST2 signaling in basophils promotes not only type 2 cytokine secretion such as IL-4 and IL-13 but also IL-8 in synergy with IL-3 or Fcε receptor activation. Basophils can also release sST2 after activation via IL-3 and C5a or anti-FcεRIα antibody, while IL-33 prevents sST2 release. IL-33 induces the degranulation of eosinophils and production of superoxide, controls their responsiveness to Siglec 8, and increases IL-13, TGF-β, CCL3, CCL17, and CCL24 in the lungs during airway inflammation. Treatment with anti-ST2 antibodies prevented the upregulation of CD11b and also decreased the survival of eosinophils.

1.6.4: Dendritic cells

Dendritic cells (DCs) express low basal levels of ST2 on their cell surface; however, activation of DCs with rapamycin strongly upregulates ST2 through autocrine IL-1β signaling. Treatment of DCs with IL-33 has been shown to increase surface levels of MHC-II, CD40, CD80, CD86, OX40L, and CCR7. IL-33/ST2 signaling in DCs also increases their production of IL-4, IL-5, IL-13, CCL17, TNF-α, and IL-1β. In the presence of naïve CD4+ T cells, IL-33-activated DCs induce IL-5 and IL-13 but not IL-4 and IFN-γ from the T cells. Interestingly, sST2 has also been shown to be internalized by DCs, suggesting a non-canonical method of action for sST2 besides sequestering free IL-33. It is currently unknown what internalization of sST2 by DCs means and whether sST2 can be internalized by other immune cells. IL-33-activated murine
DCs have recently been shown to be required for in vitro and in vivo expansion of ST2+ Tregs through DC IL-2 production, which could be used for therapeutic benefit against inflammatory diseases through expansion of Tregs both in vitro and in vivo. ST2 expression on host hematopoietic cells, including DCs, and non-hematopoietic cells was not implicated in the severity of aGVHD as recipient ST2 knockout (KO) bone marrow chimera did not modify aGVHD severity.

1.6.5: Neutrophils

While ST2 has been shown to be present on neutrophils, not much is known about the role of ST2 on neutrophils. Activation of TLR4 on neutrophils leads to downregulation of CXCR2, which is important for their recruitment to sites of infection; however, IL-33-treated murine and human neutrophils do not downregulate CXCR2 after TLR4 activation by inhibiting GRK2. IL-33 injected into the ears of mice induced neutrophil recruitment to the skin; however, it is not clear if IL-33/ST2 signaling on the neutrophils directly led to their migration.
1.7: IL-33/ST2 in intestinal diseases

1.7.1: Inflammatory bowel disease

It is believed that inflammatory bowel disease (IBD) starts with a dysregulated immune response to either food or commensal gut bacteria, leading to the production of proinflammatory cytokines such as TNF-α, IL-6, IL-1, and IL-8. Expression of these cytokines along with chemokine release leads to attraction of T cells, specifically type 1 T cells, to the intestines. Continual damage of the gut mucosa by these type 1 cells and other immune cells such as macrophages, neutrophils, and dendritic cells leads to the release of various alarmins and other proteins. sST2 was found to be significantly increased in both the gut mucosa and serum in both patients and experimental models of IBD. However, in IBD patients, ST2 expression in the colonic mucosa remained similar to that of healthy patients. In the lamina propria of active ulcerative colitis (UC) patients, ST2 predominately came from CD11b+ and CD4+ cells. These findings suggest that increased sST2 production by lymphocytes or the gut mucosa could either lead to development of IBD, particularly UC, or that these proteins are markers for disease severity.

IL-33/ST2 signaling has been shown to enhance epithelial proliferation and mucus production in the gut, suggesting that the increase in IL-33 in the colonic mucosa in active UC could be beneficial. However, in multiple mouse models of IBD, use of Il1rl1−/− mice led to amelioration of IBD compared to wild type (WT) controls. These results were verified using an IL-33 KO. Using bone marrow
chimeras, it was shown that ST2 signaling in non-hematopoietic cells was responsible for IBD. This was due to IL-33/ST2 signaling impairing epithelial barrier function and delayed wound healing. Lack of ST2 signaling in hematopoietic cells did not prevent UC development. A ST2 blocking antibody confirmed the findings from the KO experiments.\textsuperscript{170} Crohn’s disease (CD), however, shows opposite results from UC. In a trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis murine model, which mimics human CD, administration of recombinant IL-33 (rIL-33) ameliorated colonic tissue injury and clinical symptoms of colitis.\textsuperscript{80} Protection was shown to be through upregulation of type 2 cytokines, Foxp3\textsuperscript{+} T regulatory cells (Tregs), and CD103 dendritic cells, which promote Treg development. In patient colons with active IBD, Treg levels in the lamina propria are increased compared to healthy controls and function normally.\textsuperscript{171,172} It has recently been shown that colonic Tregs preferentially express ST2 and that signaling through IL-33/ST2 both promotes Treg accumulation and maintenance in the intestine and enhances their protective function.\textsuperscript{65} However, treatment with rIL-33 to promote Treg-mediated protection may be time-dependent, as rIL-33 treatment at onset of a DSS-induced colitis model exacerbated disease severity. rIL-33 treatment during recovery or chronic phases ameliorated DSS-induced colitis.\textsuperscript{173} Given this data, selective treatment of ST2\textsuperscript{+} Tregs with IL-33 could provide therapeutic benefits.
1.7.2: Acute graft-versus-host disease

aGVHD is a common occurrence in patients who undergo HCT as treatment for both malignant and non-malignant diseases of the blood and bone marrow. The pathogenesis of aGVHD has been well documented and is now thought to occur in three steps: 1) activation of antigen presenting cells (APCs) caused by tissue damage from the conditioning regimen leading to the release of proinflammatory cytokines and danger signals, 2) allo-activation of donor T cells leading to their proliferation and differentiation into type 1 and type 17 T cells, and 3) tissue destruction by alloreactive T cells through release of cytolytic molecules leading to donor cell apoptosis, mainly in the mucosal tissues. Discovering prognostic and diagnostic biomarkers for GVHD has been successful with sST2 being one of the most validated to date. Blocking sST2 with a blocking antibody during the peritransplant period decreased aGVHD morbidity and mortality in both minor histocompatibility and humanized murine models (Figure 5). Importantly, the ST2 blocking antibody, which inhibits the full length ST2 protein and not specifically sST2, maintained protective ST2-expressing T cells while also not impairing the graft-vs-leukemia activity, suggesting that addition of anti-ST2 ab or a small molecule inhibitor of ST2 could show efficacy in reducing GVHD-related morbidity and mortality in patients. Using IL-33 as a treatment seems to be time-dependent, as injection with IL-33 during the peak inflammatory response in a murine model led to increased morbidity and mortality in mice due to increased migration and increased proinflammatory cytokine production. IL-33 treatment pre-conditioning, however, increased the number of ST2+ Tregs which persisted
after irradiation in a murine model. This led to decreased aGVHD severity and mortality. Adoptive transfer of ST2\(^+\) vs ST2\(^-\) Tregs showed that aGVHD protection is increased by ST2\(^+\) and not ST2\(^-\) Tregs.\(^82\) Given that IL-33 is pleiotropic, IL-33 treatment for aGVHD seems to be dependent on both timing and the state of inflammation present.

1.7.3: Other gut diseases

IL-33/ST2 signaling has been implicated in protection from various infections which could impact the gut. Studies have shown that treatment of mice with recombinant IL-33 led to epithelial cell hyperplasia in the gut along with infiltration of eosinophils and mononuclear cells in the lamina propria.\(^66,175\) These effects are thought to be mediated by IL-13, which becomes overexpressed after IL-33 treatment.\(^30\) Treatment of mice with IL-33 after *Trichuris muris* infection increased parasite clearance through increased Th2 cytokine response.\(^175\) Other infections which can impact the gut, including *Toxoplasma gondii,\(^176\) Leptospira,\(^177\) and Pseudomonas aeruginosa,\(^178\) have shown that loss of ST2 or high sST2 levels led to higher morbidity and mortality with increased Th1 cytokine profiles. Recent studies have shown that gut epithelial barrier dysfunction and immune activation independently predict mortality during treated HIV infection.\(^179\) A later study showed that patients during the early stage of HIV infection, defined as being within 180 days of the date of infection, had higher levels of sST2 in their plasma and was highly correlated with CD8 T cell count and levels of gut mucosal damage, but not with viral load or CD4 T cell count.\(^96\)
sST2 increase has also been implicated during small bowel transplant rejection.\textsuperscript{50} Patients who had rejection of small bowel transplants had higher serum levels of sST2 during rejection compared to that during rejection-free time points, and that rejection increased allograft ST2 expression. Increase in sST2 in the allograft was predicted by Pathway and Network Analysis to be caused by TNF-\(\alpha\) and IL-1\(\beta\) signaling.\textsuperscript{50} However, this data does not implicate sST2 as a mediator of disease but rather a biomarker of occurring transplant rejection.
**Figure 5. Pathogenesis of aGVHD**

The gut and other issues are damaged during irradiation or chemotherapy, leading to the release of various DAMPs, PAMPs, and cytokines, including IL-33. These DAMPs, PAMPs, and cytokines activate both host and donor antigen presenting cells (APCs), which then activate the donor T cells. The APCs are also secreting various cytokines which promotes T cell differentiation toward a type 1 and type 17 response. These activated type 1 and type 17 T cells are able to secrete various proinflammatory cytokines, leading to apoptosis of healthy tissue, mainly in the gut, liver, and skin, which can be exacerbated by free IL-33. Furthermore, sST2 is produced by both type 1 and type 17 T cells, and while this may sequester free IL-33 from the type 1 and type 17 T cells, sST2 can also prevent the potential beneficial effects from IL-33/ST2 signaling in Th2 cells, Tregs, and ILC2s.
1.8: Research Goals

Acute graft-versus-host disease (aGVHD) hinders the efficacy of allogeneic hematopoietic cell transplantation (HCT). The transfer of donor T cells in the graft and their recognition of recipient antigen and recipient major histocompatibility complex are the main drivers of aGVHD. Until recently, determining risk of aGVHD for patients undergoing HCT has been difficult until it was found that plasma soluble serum stimulation-2 (sST2) predicted aGVHD-related mortality in HCT patients. Excess sST2 sequesters IL-33, shown to increase sST2 producing T cells (Th1/Th17) and decrease membrane ST2 (ST2) expressing cells (Th2/Tregs) at onset of aGVHD. Blockade of excess ST2 inverted these phenotypes.

Myeloid differentiation primary response 88 (MyD88) is an adapter protein vital for both IL-1 superfamily receptor signaling and most toll-like receptor (TLR) signaling. ST2, as a member of the IL-1 superfamily, signals through MyD88. Loss of MyD88 in CD4 conventional T cells (Tcons) has been shown to decrease ovalbumin or 2W peptide-stimulated Th1/Th17 cells via the IL-1 receptor (IL-1R). Regulatory T cells (Tregs) have been shown to keep their suppressive capabilities when MyD88 is lost. Intrinsic MyD88 signaling in T cells is also important for optimal T cell response to some viral infections.

MyD88 signaling during aGVHD has been studied in the context of both donor and host dendritic cells with mixed results. The role of MyD88 signaling in
the donor T cells is not understood. Given the importance of MyD88 for optimal T cell response, we used two mouse models of aGVHD to dissect the role of MyD88 in donor T cells after HCT. We hypothesized that absence of MyD88 signaling would protect against aGVHD through IL-1R, ST2, or both.
CHAPTER 2: MATERIALS AND METHODS

Mice

Boy/J (C57BL/6.Ptprca, H-2b, CD45.1) and C57BL/6 (H-2b, CD45.2) mice were purchased from the *In Vivo* Therapeutics Core at the Indiana University School of Medicine. BALB/c (H-2d, CD45.2), C3H.SW (H-2b, CD45.2), and B6.B10ScN-*Tlr4*los-del/*JthJ* (TLR4−/−) mice were purchased from Jackson Laboratory. MyD88−/− mice provided by Dr. Steve Kunkel (University of Michigan), ST2−/− mice provided by Dr. Andrew McKenzie (University of Cambridge), and IL-1R−/− mice provided by Dr. Travis Jerde (Indiana University Purdue University of Indianapolis) were backcrossed on C57BL/6 background for at least 10 generations. The Institutional Animal Care and Use Committee approved all animal protocols.

CD4 T cell differentiation

Naïve CD4+ CD62L+ T cells were purified from spleens of WT or MyD88−/− mice using the murine CD4+CD62L+ T Cell Isolation Kit (Miltenyi Biotec). Isolated naïve CD4 T cells cultured in RPMI 1640 supplemented with 10% (vol/vol) FBS (Hyclone), 1mM glutamine (ThermoFisher Scientific), 100 μg/mL Streptomycin (ThermoFisher Scientific), 100 U/mL Penicillin (ThermoFisher Scientific), 1 mM sodium pyruvate (ThermoFisher Scientific), 10 mM HEPES (ThermoFisher Scientific), and 50 μM 2-Mercaptoethanol (ThermoFisher Scientific) were stimulated with plate-bound αCD3 (2 μg/ml – 2C11) and soluble αCD28 (5 μg/ml
– 37.51) under Th0 (no additional cytokines), Th1 (20 ng/ml IL-12, 2 ng/ml IL-2), Th2 (20 ng/ml IL-4), or Th17 (4 ng/mL TGF-β, 20 ng/ml IL-6, 20 ng/ml IL-1β) conditions. On day 3 cells were expanded with media alone, except for cells under Th17 conditions, which received half the original cytokine cocktail along with media. On day 5 cells were harvested for analysis.

**aGVHD induction and assessment**

In the major MHC-mismatched model (B6 → BALB/c), BALB/c recipient mice received 900 cGy of total body irradiation (137Cs as source) at day −1. In the miHA-mismatched aGVHD model (B6 → C3H.SW), C3H.SW recipient mice received 1100 cGy of total body irradiation at day −1. Then, recipient mice were injected intravenously with WT B6 T cell–depleted (TCD) BM cells (5 × 10⁶) plus WT, MyD88⁺/-, IL-1R⁺/-, TLR4⁺/-, or ST2⁺/- splenic total T cells (1 × 10⁶ for BALB/c and 2 × 10⁶ for C3H.SW, unless indicated otherwise) from either syngeneic or allogeneic donors at day 0. T cells from donor mice were enriched using the murine Pan T Cell Isolation Kit (Miltenyi Biotec), and TCD BM was prepared with CD90.2 Microbeads (Miltenyi Biotec). For some experiments, donor T cells were first labeled with CFSE before injection. In adoptive transfer models, wild-type, MyD88⁺/- and ST2⁺/- B6 donor Tcons or Tregs were purified first using the murine Pan T Cell Isolation Kit followed by the murine CD4⁺ CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec). Purities of Tcons, defined as CD3⁺CD25⁻Foxp3⁻ cells, and Tregs, defined as CD3⁺CD4⁺CD25⁺Foxp3⁺ cells, were >98% and >92%, respectively. For the MyD88 small molecule inhibitor experiment, the
ST2825 compound (MedChem Express) was first reconstituted in 100% DMSO, followed by dilution to a working amount (5 mg/kg/200uL in 0.1% DMSO in PBS). ST2825 was administered intraperitoneally twice daily from day (-1) to day 9 post-HCT. The mice were housed in sterilized microisolator cages and maintained on acidified water (pH <3) for 3 weeks. Survival was monitored daily and clinical aGVHD scores were assessed weekly.

ELISA

We measured concentrations of murine plasma IFN-γ using DuoSet Kit and sST2 using Quantikine Kit (R&D Systems) according to manufacturer’s protocols.

Isolation and sorting of intestinal CD4 T cells

We prepared single-cell suspensions of mononuclear cells from intestines as previously described.75 Isolated intestines were flushed with cold PBS to remove mucus and feces. The intestines were cut into <0.5 cm fragments and digested in 10 mL of DMEM containing 4% bovine serum albumin (Sigma), 2 mg/mL collagenase type B (Roche), and 10 μg/mL DNase I (Roche) at 37°C with shaking (250rpm) for 90 minutes. The digested mixture was diluted with 30 mL DMEM, filtered through a 100 μm strainer, and centrifuged for 10 minutes at 850g. The cells were resuspended in 5 mL of 80% Percoll (GE Healthcare) and overlaid with 8 mL of 42% Percoll. The cells were spun at 4°C for 20 minutes at 800g without braking. The interface, which contains the live mononuclear cells was collected and washed twice with PBS. Live CD4+ T cells (Fixed Viability Dye-
CD90.2⁺CD4⁺; all from eBioscience) were stained with fluorescent antibodies and sorted on the BD FACSAria (BD Biosciences).

**Flow cytometry analysis**

All antibodies and reagents for flow cytometry were purchased from eBioscience, unless stated otherwise. Single cell suspensions were preincubated with purified anti-mouse CD16/CD32 mAb for 10 to 20 min at 4°C to prevent nonspecific binding of antibodies. The cells were subsequently incubated for 30 min at 4°C with antibodies for surface staining. Fixable viability dye (FVD) was used to distinguish live cells from dead cells. The Foxp3/Transcription Factor Staining Buffer Set and the Fixation and Permeabilization Kit were used for intracellular transcription factor and cytokine staining. For cytokine staining, cells were restimulated with phorbol myristate acetate (PMA, 50 ng/ml) and ionomycin (1 μg/ml; Sigma-Aldrich) for 4 to 6 hours, with the addition of brefeldin A during the last 2 hours of stimulation, before any staining. Staining antibodies against mouse antigens included: anti-CD45.1, anti-CD45.2, anti-CD90.2, anti-CD4, anti-CD8, anti-Foxp3, anti-IL-4, anti-IFNγ, anti-IL-17, anti-GM-CSF, annexin V, anti-CCR5, and anti-α4β7. Cells were analyzed using BD LSRFortessa (BD Biosciences) and results were analyzed using FlowJo (Tree Star).

**Nanostring analysis**

Sorted intestinal CD4 T cells were prepared and analyzed as previously described. Sorted intestinal CD4 T cells from either recipients of WT or MyD88⁻
allogeneic donor T cells were directly lysed in RLT buffer (Qiagen) on ice. Cell concentration for each sample was $2 \times 10^3$ cells/μL. Preparation of samples for analysis was then performed according to the Nanostring Technologies protocol for gene expression. Plates were run on the nCounter SPRINT ProfilerAnalysis System and the data analysis using nSolver 3.0. The nCounter Mouse Immunology Kit, which includes 561 immunology-related mouse genes, was used in the study.

**Quantitative PCR**

Total RNA from sorted intestinal T cells (Fixed Viability Dye-CD3+, all from eBioscience), were isolated using the RNeasy Plus Mini Kit (Qiagen). Complementary DNA (cDNA) was prepared with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Using an ABI Prism7500HT (Applied Biosystems), quantitative real-time PCR was performed with the SYBR Green PCR mix. Thermocycler conditions included 2-min incubation at 50°C, then at 95°C for 10 min; this was followed by a two-step PCR program: 95°C for 5 s and 60°C for 60 s for 40 cycles. β-Actin was used as an internal control to normalize for differences in the amount of total cDNA in each sample. The primer sequences were as follows: actin forward, 5′-CTCTGGCTCTAGCACCATGAAGA-3′; actin reverse, 5′-GTAAAACGCAGCTCAGTAGCTCCGTAACAGTCCG-3′; ST2 forward, 5′-AAGGCACACCATAAGGCTGA-3′; ST2 reverse, 5′-TCGTAGAGCTTGCCCATCGT-3′; sST2 forward, 5′-
TCGAAATGAAAGTTCCAGCA-3'; sST2 reverse, 5'-
TGTGTGAGGGACACTCCTTAC-3'.

**Western Blot**

CD4 and CD8 T cells were isolated from WT B6 spleens using CD4 microbeads and CD8 microbeads (both from Miltenyi Biotec), respectively, following manufacturer’s protocols. Purities of CD4 and CD8 T cells after selection were >95%. Sorted cells were lysed in RIPA buffer (Pierce Biotechnology) with Pierce Phosphatase Inhibitor MiniTablets (Pierce Biotechnology) and Protease Inhibitor Cocktail Tablets (Roche). Samples were boiled, electrophoretically separated, and transferred on Immobilon-FL polyvinylidene difluoride membranes (Millipore). The blots were blocked with Odyssey Blocking Buffer (LI-COR) for 1 hour at room temperature and incubated with specific primary antibodies: rabbit MyD88 mAb (D80F5, Cell Signaling Technology) and anti–β-actin mAb (LI-COR), both at 4°C overnight. IRDye 800CW goat anti-rabbit (LI-COR) and IRDye 680RD goat anti-mouse IgG polyclonal antibodies (LI-COR) were used as secondary detection antibodies for MyD88 and β-actin, respectively. Fluorescence from blots was then developed with the Odyssey CLx Imaging System (LI-COR) according to the manufacturer’s instructions.

**Statistical Analysis**

Log-rank test was used for survival analysis. Differences between two groups were compared using 2-tailed unpaired t tests. Bonferroni correction was used
when comparing multiple groups. All statistical analyses were performed using
GraphPad Prism, version 7.02. Data in graphs represent mean ± standard error
of the mean (SEM). P values less than 0.05 were considered significant.
CHAPTER 3: RESULTS

3.1 MyD88⁻/⁻ T cells reduce aGVHD morbidity and mortality in multiple murine models

First, we tested whether loss of MyD88 affected normal splenic T cells in naïve mice. We found no difference in splenic T cell numbers; CD4/CD8 frequency; or naïve, memory, and effector frequencies (Figure 6a). Ability to polarize toward Th1, Th2, or Th17 cells in vitro was also not affected by the absence of MyD88 as shown by IFN-γ, IL-4, or IL-17 production, respectively (Figure 6b). To explore the role of MyD88 signaling in the donor T cells in vivo following HCT, we used two clinically relevant murine HCT models: C57BL/6 → BALB/c and C57BL/6 → C3H.SW. In both models splenic T cells were isolated and bone marrow cells were depleted of T cells. In the MHC-major mismatch model C57BL/6 → BALB/c, mice receiving WT T cells quickly developed and succumbed to severe aGVHD (median survival time: 14 days). However, mice receiving MyD88⁻/⁻ T cells had decreased aGVHD scores and mortality (median survival time: >30 days) compared to mice receiving WT T cells (Table 1; Figure 7a,b). IFNγ production by donor T cells in the intestine at day 10 post-HCT was lower in the MyD88⁻/⁻ T cells than the WT T cells (Figure 7c). Systemically, plasma levels of IFNγ and sST2 were lower in recipients of MyD88⁻/⁻ T cells than those receiving WT T cells (Figure 7d). Using the miHA model, C57BL/6 → C3H.SW, we observed a similar decrease in aGVHD mortality (median survival time: WT - 43 days; MyD88⁻/⁻ - >60 days; Figure 8a,b). Similarly to that seen in the major mismatch model, both
IFNγ production by MyD88−/− donor T cells was lower compared to that from WT T cells and plasma levels of IFNγ and sST2 were lower in recipients of MyD88−/− T cells compared to those receiving WT T cells (Figure 8c,d). These results show that signaling through MyD88 in the donor T cells is critical in the pathogenesis of aGVHD.
Figure 6. Comparison of splenic T cells from naïve WT or MyD88⁻/⁻ mice

(A) Cell number and frequencies of total, CD4, or CD8 T cells (left); naïve, memory, and effector CD4 or CD8 populations (right) harvested from spleen of WT or MyD88⁻/⁻ mice (mean ± SEM; n=3). (B) Naïve CD4 T cells were stimulated with plate-bound αCD3 (2 μg/ml) and soluble αCD28 (5 μg/ml) under Th0 (no additional cytokines), Th1 (20 ng/ml IL-12, 2 ng/ml IL-2), Th2 (20 ng/ml IL-4), or Th17 (4 ng/mL TGF-β, 20 ng/ml IL-6, 20 ng/ml IL-1β) conditions for 5 days. Graphs show frequency of IFN-γ (left), IL-4 (middle), and IL-17 (right) expression (mean ± SEM; n=3).
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B6 WT or MyD88<sup>−/−</sup> Donor 1 x 10<sup>6</sup> T cells 5 x 10<sup>6</sup> TCD BM cells BALB/cRecipient

B

Days post-HCT

GVHD Score

0 2 4 6
0 10 20 30

Days post-HCT

Survival %

0 50 100
0 10 20 30

WT MyD88<sup>−/−</sup>

< Syn

C

WT MyD88<sup>−/−</sup>

IFN<sub>γ</sub>

CD4

0 12.6 32.3
0 87.4 67.7

% IFN<sub>γ</sub>+

WT MyD88<sup>−/−</sup>

D

Days post-HCT

IFN<sub>γ</sub> (pg/mL)

0 1000 2000 3000
5 10

Days post-HCT

sST2 (ng/mL)

0 200 400 600 800
5 10

WT MyD88<sup>−/−</sup>
Figure 7. aGVHD assessment using WT or MyD88<sup>−/−</sup> donor T cells in a major MHC mismatch model

(A) Schematic for allo-transplantation using WT or MyD88<sup>−/−</sup> donor T cells and WT BM in the B6 → BALB/c major MHC mismatch model. (B) Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10⁶ TCD-BM cells and 1 x 10⁶ donor T cells from WT or MyD88<sup>−/−</sup> B6 mice for allogeneic transplant or WT BALB/c TCD BM and donor T cells from WT BALB/c mice for syngeneic transplant (Syn). aGVHD score (left) and survival (right); BALB/c → BALB/c (n=5), B6 WT (n=15), or B6 MyD88<sup>−/−</sup> total T cells (n=15) groups. (C) Representative flow plots (left) and column scatter plot (right) showing frequency of IFN-γ positive total T cells in the intestine of recipient BALB/c mice transplanted with WT or MyD88<sup>−/−</sup> T cells at day 10 post-HCT. (D) IFN-γ (left) and sST2 (right) protein levels in the plasma of recipient mice transplanted with WT or MyD88<sup>−/−</sup> T cells collected at days 5 and 10 post-HCT. *p < 0.05, **p < 0.01, ***p < 0.001
A

B6 WT or MyD88<sup>−/−</sup> Donor 5 x 10<sup>6</sup> TCD BM cells 2 x 10<sup>5</sup> T cells  
Recipient C3H.SW

B

![Graphs showing GVHD Score, Survival, and IFN-γ](image)

C

WT MyD88<sup>−/−</sup>

![Flow cytometry data](image)

D

![Graphs showing IFN-γ and sST2 levels](image)
Figure 8. aGVHD assessment using WT or MyD88−/− donor T cells in a minor MHC mismatch model

(A) Schematic for allo-transplantation using WT or MyD88−/− donor T cells and WT BM in the B6 → C3H.SW minor MHC mismatch model. (B) Lethally irradiated C3H.SW mice (1100 cGy) were given 5 × 10^6 TCD-BM cells and 2 × 10^6 donor T cells from WT or MyD88−/− B6 mice for allogeneic transplant or WT C3H.SW TCD-BM and donor T cells from WT C3H.SW mice for syngeneic transplant. aGVHD score (left) and survival (right); C3H.SW → C3H.SW (n=5), B6 WT (n=6), or B6 MyD88−/− total T cells (n=6). (C) Representative flow plots (left) and column scatter plot (right) showing frequency of IFN-γ positive total T cells in the intestine of recipient C3H.SW mice transplanted with WT or MyD88−/− T cells at day 10 post-HCT. (D) IFN-γ (left) and sST2 (right) protein levels in the plasma of recipient mice transplanted with WT or MyD88−/− T cells collected at days 5 and 10 post-HCT. *p < 0.05, **p < 0.01
3.2 MyD88\textsuperscript{-/-} donor T cells do not have defects in their proliferation, apoptosis, migration, or Th2 and Treg frequencies following HCT

To determine if the donor MyD88\textsuperscript{-/-} T cells had a defect in proliferation, apoptosis, or migration following HCT, we stained the CD45.1 WT T cells and CD45.2 MyD88\textsuperscript{-/-} T cells with carboxyfluorescein succinimidyl ester (CFSE) right before transplantation and injected them into lethally irradiated recipients at a 1:1 ratio. At day 3 post-HCT, we did not observe a difference in proliferation between groups (Figure 9a). We then isolated cells from the spleen, liver, mesenteric lymph nodes, and intestine at day 10 post-HCT to test for differences in apoptosis, measured using annexin V. We found no differences between groups in apoptosis of T cells in the intestine at days 3, 5, and 10 post-HCT (Figure 9b). We also did not observe any differences in the expression of the chemokine receptor CCR5 or the integrin \(\alpha 4\beta 7\) in the intestine at day 10 post-HCT (Figure 9c), both of which have been implicated in the migration of T cells to the intestine.\textsuperscript{186,187} Th2\textsuperscript{188} and Treg\textsuperscript{189} cells have been shown to be protective against aGVHD; however, we found no difference in IL-4 production (Figure 10a) or Foxp3 expression (Figure 10b) in the donor T cells from the intestine at 10 days post-HCT. Together, these data show that following HCT the expansion, apoptosis, migration, or Th2 and Treg differentiation of MyD88\textsuperscript{-/-} donor T cells is different from WT donor T cells.
A

[Graph showing CFSE distribution with markers for Pre-transplant, WT, and MyD88-/-]

B

[Graph showing Annexin V+ (%) over days 3, 5, and 10 with markers for WT and MyD88-/-]

C

[Graphs showing CCR5+ (%) and CD4+7+ (%) with markers for WT and MyD88-/-, both labeled ns]
Figure 9. Proliferation, apoptosis, and migration of transplanted WT vs MyD88⁻/⁻ T cells

Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10⁶ TCD-BM cells and 1 x 10⁶ CFSE labeled donor T cells from WT CD45.1 or MyD88⁻/⁻ CD45.2 B6 mice. (A) Proliferation of CFSE labeled T cells from WT CD45.1 or MyD88⁻/⁻ CD45.2 donors harvested from the intestine at day 3 post-HCT. (B) Annexin V staining of T cells from WT CD45.1 or MyD88⁻/⁻ CD45.2 donors harvested from the intestine at days 3, 5, and 10 post-HCT (mean ± SEM, n=2). (C) CCR5 and α4β7 expression on T cells from WT CD45.1 or MyD88⁻/⁻ CD45.2 donors harvested from the intestine at day 5 post-HCT (mean ± SEM, n=2).
Figure 10. Th2 and Treg frequencies in the intestine of mice receiving WT or MyD88\(^{-/-}\) donor T cells

Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10\(^6\) TCD-BM cells and 1 x 10\(^6\) donor T cells from WT or MyD88\(^{-/-}\) B6 mice for allogeneic transplant. T cells were harvested from the intestine at day 10 post-HCT and stained for expression of (A) CD4 and Foxp3 or (B) CD4 and IL-4 (mean ± SEM, n=3).
3.3 Loss of IL-1R or TLR4, both upstream of MyD88, on T cells does not alleviate aGVHD

To elucidate the mechanism as to why MyD88\(^{-/-}\) donor T cells induce less severe aGVHD, we targeted upstream receptors of MyD88. The upstream receptors for MyD88 include the IL-1 receptor superfamily and the toll-like receptor (TLR) family, with the exception of TLR3.\(^{190,191}\) One group has shown that MyD88\(^{-/-}\) CD4 T cells produce less IFN-\(\gamma\) and proliferate less than WT CD4 T cells after immunization and this was due to defective IL-1R signaling.\(^{180}\) Another group found that, in an aGVHD model, recipients of IL-1R\(^{-/-}\) T cells survived longer than recipients of WT T cells.\(^{192}\) Thus, we next asked whether the phenotype observed using MyD88\(^{-/-}\) donor T cells is mediated through IL-1R. In our models, we found no difference between groups in clinical score or survival from mice receiving WT or IL-1R\(^{+/}\) donor T cells in either the MHC-major mismatch model (median survival time: WT - 14 days; IL-1R\(^{-/-}\) - 28 days; Figure 11a) or the miHA model (median survival time: WT - 43 days; IL-1R\(^{-/-}\) - 39 days; Figure 11b).

Another group showed that recipients of TLR4\(^{+/}\) BM and T cells together reduced aGVHD severity compared to WT recipients through defective donor APC response, but did not test whether TLR4\(^{+/}\) T cell response compared to WT was also affected.\(^{193}\) So, we asked whether loss of TLR4 on the donor T cells could affect aGVHD severity and mortality. Recipients of TLR4\(^{+/}\) donor T cells both models did not reduce aGVHD severity and mortality (Major model median survival time: WT - 10 days; TLR4\(^{+/}\) - 9 days; Figure 12a; Minor model median survival time: WT - 18 days; TLR4\(^{+/}\) - 33 days; Figure 12b). These data show that
IL-1R and TLR4 signaling in donor T cells do not play major roles for aGVHD induction.
A  Major HCT Model

Days post-HCT

GVHD Score

Days post-HCT

Survival %

B  Minor HCT Model

Days post-HCT

GVHD Score

Days post-HCT

Survival %
Figure 11. IL-1R⁻/⁻ donor T cells in aGVHD

(A) Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10⁶ TCD-BM cells and 1 x 10⁶ donor T cells from WT or IL-1R⁻/⁻ B6 mice for allogeneic transplant or WT BALB/c TCD-BM and donor T cells from WT BALB/c mice for syngeneic transplant. aGVHD score (left) and survival (right); BALB/c → BALB/c (n=5), B6 WT (n=15), or B6 IL-1R⁻/⁻ total T cells (n=8). (B) Lethally irradiated C3H.SW mice (1100 cGy) were given 5 x 10⁶ TCD-BM cells and 2 x 10⁶ donor T cells from WT or IL-1R⁻/⁻ B6 mice for allogeneic transplant or WT C3H.SW TCD-BM and donor T cells from WT C3H.SW mice for syngeneic transplant. aGVHD score (left) and survival (right); C3H.SW → C3H.SW (n=5), B6 WT (n=6), or B6 IL-1R⁻/⁻ total T cells (n=6).
A

Major HCT Model

B

Minor HCT Model
Figure 12. TLR4⁻/⁻ donor T cells in aGVHD

(A) Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10⁶ TCD-BM cells and 1 x 10⁶ donor T cells from WT or TLR4⁻/⁻ B6 mice for allogeneic transplant. aGVHD score (left) and survival (right); B6 WT (n=8) or B6 TLR4⁻/⁻ total T cells (n=8). (B) Lethally irradiated C3H.SW mice (1100 cGy) were given 5 x 10⁶ TCD-BM cells and 2 x 10⁶ donor T cells from WT or TLR4⁻/⁻ B6 mice for allogeneic transplant. aGVHD score (left) and survival (right); B6 WT (n=8) or B6 TLR4⁻/⁻ total T cells (n=8).
3.4 Transplantation of donor MyD88^{−/−} CD4 T cells, but not CD8 T cells, reduces aGVHD severity independent of intrinsic MyD88 signaling

MyD88 signaling in T cells has been characterized in both the CD4 and the CD8 compartments. We found higher expression of MyD88 in CD4 than CD8 T cells (Figure 13a). The importance of MyD88 signaling in donor CD4 and CD8 cells in the context of aGVHD has not been studied. To determine if MyD88 in CD4 T cells, CD8 T cells, or both is important for aGVHD development, we isolated WT CD4, WT CD8, MyD88^{−/−} CD4, and MyD88^{−/−} CD8 T cells from naïve mice. Transplanting MyD88^{−/−} CD4 T cells with WT CD8 T cells increased the survival of the recipient mice compared to transplanting WT CD4 T cells with MyD88^{−/−} CD8 T cells (median survival time: MyD88^{−/−} CD4 - >60 days; MyD88^{−/−} CD8 - 36 days; Figure 13b,c), the former showing a similar phenotype to total WT T cell recipients. These data show that MyD88 signaling in CD4 T cells, but not CD8 T cells, is needed for optimal aGVHD induction. GM-CSF expression by T cells has been implicated in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, through a STAT5 dependent mechanism. We found that production of GM-CSF in the intestine 10 days post-HCT is decreased when transplanting MyD88^{−/−} CD4 cells compared to WT CD4 cells (Figure 13d).

The CD4 T cell compartment consists of both pro-inflammatory Tcons and anti-inflammatory Tregs. MyD88^{+/+} Tregs prolong allograft survival in both organ transplantation and chronic GVHD (cGVHD) through a cell-intrinsic
mechanism.\textsuperscript{198} We next explored the cell-intrinsic role of MyD88\textsuperscript{-/-} Tregs in aGVHD. We did not observe a difference in survival using using WT Tcons with WT or MyD88\textsuperscript{-/-} Tregs (median survival time: WT Tregs - 26 days; MyD88\textsuperscript{-/-} Tregs - 24 days; Figure 14a). Intrinsic MyD88 signaling in CD4 T cells has also been implicated in mounting a proper antiviral response.\textsuperscript{182} After transplanting Treg-depleted WT or MyD88\textsuperscript{-/-} Tcons, we did not observe a difference in aGVHD severity or mortality (median survival time: WT Tcon - 10 days; MyD88\textsuperscript{-/-} Tcon - 10 days; Figure 14b). These data indicate that intrinsic MyD88 signaling in donor T cells does not impact aGVHD.
A

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B

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B6 WT, CD4 MyD88^−/^− or CD8 MyD88^−/^− Donor
5 x 10^6 TCD BM cells
2 x 10^6 T cells
C3H.SW
```

C

```
Days post-HCT

GVHD Score

Survival %

Days post-HCT

Syn MyD88^−/^− CD4 MyD88^−/^− CD8
```

D

```
WT Total T cells

MyD88^+/− CD4 + WT CD8

WT CD4 + MyD88^−/^− CD8

GM-CSF (%)

CD4

GMT CD4 MyD88^−/^− CD4

* 
Figure 13. CD4 vs CD8 MyD88-/- donor T cells in aGVHD

(A) Representative Western blot of MyD88 from freshly isolated CD4 or CD8 T cells from a WT B6 spleen. (B) Schematic for allo-transplantation using WT or MyD88-/- donor CD4 and CD8 cells and WT BM in the B6 → C3H.SW minor MHC mismatch model. (C) Lethally irradiated C3H.SW mice (1100 cGy) were given 5 x 10^6 TCD-BM cells and a mixture of 2 x 10^6 WT CD4 + MyD88-/- CD8 or MyD88-/- CD4 + WT CD8 donor T cells from B6 mice for allogeneic transplant or WT C3H.SW TCD-BM and donor T cells from WT C3H.SW mice for syngeneic transplant. aGVHD score (left) and survival (right); C3H.SW → C3H.SW (n=6), B6 WT CD4 + MyD88-/- CD8 T cells (n=6), or B6 MyD88-/- CD4 + WT CD8 T cells (n=6). (D) T cells were harvested from the intestine at day 10 post-HCT and stained for live GM-CSF producing CD4 T cells.
A

B
Figure 14. Intrinsic MyD88 signaling in Tcons and Tregs during aGVHD

(A) Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10^6 TCD-BM cells and 1 x 10^6 donor Tcons without Tregs from WT or MyD88^−/− B6 mice for allogeneic transplant or WT BALB/c TCD-BM and donor T cells from WT BALB/c mice for syngeneic transplant. aGVHD score (left) and survival (right); BALB/c → BALB/c (n=5), B6 WT Tcons (n=7), or B6 MyD88^−/− Tcons (n=7). (B) Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10^6 TCD-BM cells and a 5:1 mixture of WT Tcon + WT or MyD88^−/− Tregs totaling 1 x 10^6 donor T cells from B6 mice for allogeneic transplant or WT BALB/c TCD-BM and donor T cells from WT BALB/c mice for syngeneic transplant. aGVHD score (left) and survival (right); BALB/c → BALB/c (n=5), B6 WT Tcons and B6 WT Tregs (n=7), or B6 WT Tcons and B6 MyD88^−/− Tregs (n=7).
3.5 MyD88$^{-/-}$ Tcons require the presence of Tregs for full alleviation of aGVHD

Immunization of CD4 specific MyD88$^{+/+}$ mice has been shown to result in decreased IFN-γ production by CD4 T cells compared to WT CD4 T cells; however, IFN-γ levels after immunization were the same between WT and MyD88$^{-/-}$ CD4 T cells when Tregs were absent. As the survival of mice receiving WT of MyD88$^{-/-}$ Tcons without Tregs was not different, we tested whether the presence of Tregs is necessary for protection when using MyD88$^{-/-}$ donor Tcons. Indeed, use of MyD88$^{-/-}$ Tcons with WT or MyD88$^{-/-}$ Tregs, led to aGVHD amelioration (Figure 15a). These data show that loss of extrinsic MyD88 signaling in Tcons in the presence of Tregs reduces aGVHD severity and mortality. Transcriptome analysis from day 10 post-HCT comparing WT or MyD88$^{-/-}$ CD4 Tcons recovered from the intestines showed that MyD88$^{-/-}$ CD4 Tcons express lower levels of genes involved in the inflammatory response, including $Il1rl1$ (gene of ST2), $Ifng$, $Csf2$ (gene of GM-CSF), $Stat5$, and $Jak2$ (Figure 15b). MyD88$^{-/-}$ T cells recovered from the intestine at day 10 post-HCT expressed less sST2 and more ST2 compared to WT T cells (Figure 15c, left). Systemic levels of IFN-γ and sST2 in recipients of MyD88$^{-/-}$ T cells were also decreased at days 5 and 10 post-HCT compared to recipients of WT T cells (Figure 15c, right).
**Figure 15. MyD88⁻/⁻ Tcons versus MyD88⁻/⁻ Tregs during aGVHD**

(A) Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10⁶ TCD-BM cells and a 10:1 mixture of WT or MyD88⁻/⁻ Tcon + WT or MyD88⁻/⁻ Tregs totaling 1 x 10⁶ donor T cells from B6 mice for allogeneic transplant. aGVHD score (left) and survival (right); WT or MyD88⁻/⁻ Tcons + either WT or MyD88⁻/⁻ Tregs (all groups n=6). (B) Transcriptome analysis comparing intestinal WT and MyD88⁻/⁻ CD4 T cells harvested 10 days post-HCT (n=2 per group). (C,D) Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10⁶ TCD-BM cells and 1 x 10⁶ donor T cells from WT or MyD88⁻/⁻ B6 mice for allogeneic transplant. (C) Relative expression of sST2 and mST2 from WT or MyD88⁻/⁻ T cells harvested from the intestine 10 days post-HCT (mean ± SEM, n=4). (D) Kinetics of plasma levels of sST2 and IFN-γ in BALB/c mice collected at days 5 and 10 post-HCT (mean ± SEM, n=3). *p < 0.05, **p < 0.01, ***p < 0.001
3.6 ST2/MyD88 signaling in Tcons is necessary for aGVHD development

We hypothesized that this protective phenotype observed when transplanting MyD88−/− Tcons in the presence of Tregs was mediated by a lack of ST2 signaling on donor Tcons. Recipients of ST2−/− Tcons with either WT or ST2−/− Tregs had lower aGVHD mortality, mirroring the phenotype seen using MyD88−/− Tcons (Figure 16A). Clinical score and survival of recipients of total MyD88−/− and ST2−/− donor T cells phenocopy each other (Figure 16B). These data confirm that alloreactive T cells in the intestines produce sST2, as we previously suggested, and that targeting ST2/MyD88 signaling in Tcons could alleviate aGVHD while sparing Treg function.
Figure 16. ST2 vs MyD88 signaling during aGVHD

(A) Lethally irradiated C3H.SW mice (1100 cGy) were given $5 \times 10^6$ TCD-BM cells and a 10:1 mixture of WT or ST2$^{-/-}$ Tcon + WT or ST2$^{-/-}$ Tregs totaling $2 \times 10^6$ donor T cells from WT or ST2$^{-/-}$ B6 mice for allogeneic transplant. aGVHD score (left) and survival (right) (all groups n=6). (B) Lethally irradiated BALB/c mice (900 cGy) were given $5 \times 10^6$ TCD-BM cells and $1 \times 10^6$ donor T cells from WT, MyD88$^{-/-}$, or ST2$^{-/-}$ B6 mice for allogeneic transplant. aGVHD score (left) and survival (right) (all groups n=6). *p < 0.05, **p < 0.01
3.7 MyD88 signaling blockade using a small molecule inhibitor

We tested blocking MyD88 signaling \textit{in vivo} using the small molecule inhibitor ST2825, which prevents the homodimerization of MyD88 at their TIR domain, preventing downstream signaling.\textsuperscript{199} MyD88 signaling blockade using ST2825 injected intraperitoneally twice a day (5 mg/kg per injection) from day (-1) to day 9 post-HCT did not affect aGVHD severity or mortality in the B6 $\rightarrow$ BALB/c model (Figure 17).
Figure 17. Blockade of MyD88 signaling using ST2825 during aGVHD

Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10^6 TCD-BM cells from WT B6 mice and 1 x 10^6 donor total T cells from B6 WT or MyD88^-/- mice for allogeneic transplant. Recipient mice receiving WT T cells were injected with either vehicle control or ST2825 (5 mg/kg) intraperitoneally twice a day for 10 days beginning at day (-1). Recipient mice receiving MyD88^-/- T cells were injected with vehicle control intraperitoneally twice a day for 10 days beginning at day (-1). aGVHD score (left) and survival (right); WT (n=5), MyD88^-/- (n=5), or ST2825 (n=5). p > 0.05 between WT and ST2825.
4.1 Summary of Results

Genetic knockouts of ST2 on T cells and blocking of ST2 using a neutralizing antibody has been shown to ameliorate aGVHD.\textsuperscript{75} However, these experiments used knockout mice that lacked both membrane ST2 and sST2 and the neutralizing antibody also was not specific for either form of ST2. These experiments also did not show mechanistically how loss of ST2 reduces aGVHD. Here, we show that ST2/MyD88 signaling in donor T cells during HCT is important for aGVHD progression through prevention of Treg-mediated suppression of effector T cells. We used two murine models of aGVHD to show how loss of MyD88 in the donor T cells decreases aGVHD severity and mortality. Unlike in other models, which has suggested a role of IL-1 receptor/MyD88\textsuperscript{180,192} and TLR4/MyD88\textsuperscript{193} signaling in promoting an optimal T cell response \textit{in vivo}, we did not observe a difference in the development of aGVHD using donor T cells from IL-1 receptor or TLR4 knockout mice. Neither loss of MyD88 in Tcons adoptively transferred alone without Tregs nor loss of MyD88 in adoptively transferred Tregs with WT Tcons affected aGVHD progression. Amelioration of aGVHD was only observed when transplanting MyD88\textsuperscript{-/-} Tcons in the presence of Tregs, suggesting that loss of MyD88 in Tcons sensitizes them to Treg-mediated suppression. Strikingly, results using ST2\textsuperscript{-/-} donor Tcons mimic the MyD88\textsuperscript{-/-} donor Tcon phenotype, suggesting that loss of MyD88 in donor Tcons ameliorates murine aGVHD in an ST2-dependent manner.
4.2 MyD88 in T cells

Previous studies using MyD88^{-/-} T cells have shown that MyD88 is necessary for optimal CD4 and CD8 T cell responses in vivo.^{180-182,200} We found no differences in the ability of T cells to produce IFN-γ, IL-4, or IL-17 under Th1, Th2, or Th17 polarizing conditions, respectively, when using αCD3/αCD28 polyclonal stimulation. However, during antigen-specific responses, T cells require MyD88 for differentiation of Th1 and Th17 cells.^{180-182,194,200} In the context of aGVHD, we have shown that MyD88 in T cells is necessary for optimal allo-response. Loss of MyD88 in the donor T cells leads to decreased aGVHD severity and mortality in two different murine models: a major-MHC mismatch model and a minor-MHC mismatch model. However, our results are different from those previously published, in which a haploidentical aGVHD murine model, C57BL/6 (H-2^{b}) → B6D2F1 (H-2^{bid}) was used. Using this model, no difference in aGVHD severity was observed when transplanting MyD88^{-/-} T cells.^{201} We believe the discrepancy in results to be due to low number of T cells injected into the irradiated B6D2F1 recipients compared to what is normally used in that model.^{202,203} Using a lower number of T cells during HCT can lengthen the kinetics of aGVHD development and reduce the severity and mortality of aGVHD. The delayed onset and reduced severity and mortality may not have allowed any differences in aGVHD to be observed.

It has been shown that diminished Th1 and Th17 responses due to loss of MyD88 are a product of loss of IL-1R signaling on CD4 T cells.^{180} As well, WT T
cells upregulate IL-1R on their surface by day 3 post-HCT and transplanting IL-1R<sup>-/-</sup> donor T cells alleviated aGVHD in a major-mismatch model. However, we have found that IL-1R<sup>-/-</sup> donor T cells have no significant loss of effector function, as aGVHD was not attenuated in our two models. Our results are more in accordance with what has been shown examining MyD88 signaling in T cells in response to viral infection. After transfer of IL-1R<sup>-/-</sup> or MyD88<sup>-/-</sup> T cells into RAG<sup>-/-</sup> mice and infecting with vaccinia virus, mice with IL-1R<sup>-/-</sup> CD8 T cells were able to respond to the infection normally while mice with MyD88<sup>-/-</sup> CD8 T cells mounted a reduced response. Similar results were found during LCMV infection in CD8 T cells. Looking at WT, MyD88<sup>-/-</sup>, and IL-1R<sup>-/-</sup> CD4 T cells in absence of CD8 T cells in response to LCMV infection, WT and IL-1R<sup>-/-</sup> mice developed wasting disease and had lower virus levels while MyD88<sup>-/-</sup> mice did not develop wasting disease and had higher virus levels due to failure to induce LCMV-specific CD4 T cell response. This response was not due to impaired APC function. Our results also are in accordance with a clinical trial that observed no difference in aGVHD outcomes in HCT patients when using prophylactic treatment with IL-1 receptor antagonist, an IL-1R inhibitor. This discrepancy in our results along with the results in the literature looking at anti-viral response and a clinical trial with those shown others could be due to the difference in models. One used an immunization model, while our models and others use alloresponses and viral responses for stimulation, respectively. It is possible that the different use of antigens could impact which receptors become upregulated on T cells. IL-1R is upregulated on 2W:I-A<sup>b</sup> tetramer-positive T cells but not 2W:I-A<sup>b</sup> tetramer-
negative T cells after antigen stimulation. The immunization model also does not take into account the presence of a variety of other molecules that would be present in a diseased state. PAMPs, DAMPs, and alarmins released during viral infection or during conditioning for allo-transplant but not during immunization could impact T cell response to IL-1 signaling. A difference in the microbiota of the recipient mice could also explain this difference. Recent work has shown that the makeup of the intestinal microbiome can affect aGVHD severity.\textsuperscript{154,205-207} The mice from commercial vendors which are purchased for experiments can have significantly different microbiotas which can impact immune response.\textsuperscript{208} Aberrant IL-1/IL-1R signaling has been shown to alter the microbiota in mice.\textsuperscript{209} We purchased the BALB/c mice in our experiments from The Jackson Laboratory while the other group purchased their BALB/c mice from Charles River, Harlan, or from the local stock of the animal facility at Freiburg University Medical Center.\textsuperscript{192} It is possible that the difference in phenotype we saw compared what has been published is in part due to differences in intestinal microbiota of the recipients.

It is also possible that there is an unknown link between MyD88 and the TCR complex that is explaining our difference. Clearly, cross-linking CD3 with \(\alpha CD3\) stimulation in the presence of \(\alpha CD28\) leads to no noticeable difference between WT and MyD88\textsuperscript{-/-} T cell differentiation and cytokine production \textit{in vitro}, as mentioned earlier. Most of the work showing a difference has used APCs to stimulate TCR. No work has yet been done looking at T cell co-receptors and if
there is any change in their expression or signaling in MyD88\(^{-/-}\) T cells. An importance for MyD88 recruitment during B cell synapse formation has been identified, as loss of MyD88/DOCK8 signaling impairs ICAM-1 accumulation,\(^{210,211}\) a known integrin at immune synapses.\(^{212}\) Recently, it has been shown that DOCK8 is important for Treg immune synapse formation and loss of DOCK8 selectively in Tregs lead to autoimmune disease. DOCK8\(^{-/-}\) mice do not develop autoimmunity, however, suggesting that DOCK8 signaling may be important in optimal Tcon function as well.\(^{213}\) It is therefore possible that MyD88/DOCK8 signaling in T cells may play a similar or an unidentified role at the immune synapse.

During conditioning pre-HCT, intestinal mucosa is injured leading to the release of DAMPs, PAMPs, and alarmins. Tight junctions are damaged and LPS, among other bacterial products, is released into the body. LPS signals through TLR4 and MyD88. TLR4 is found on both human and murine CD4 T cells, but its function is not well understood. One study showed that only naïve murine T cells and not activated T cells express TLR4.\(^{214}\) However, TLR4 on human T cells was only detected in activated CD4 T cells.\(^{215}\) In a murine model of EAE, TLR4\(^{-/-}\) T cells transferred into RAG1\(^{-/-}\) followed by EAE induction did not produce disease.\(^{216}\) However, in a spontaneous model of colitis, IL-10\(^{-/-}\)/TLR4\(^{-/-}\) T cells transferred into RAG1\(^{-/-}\) mice accelerated disease progression.\(^{217}\) During aGVHD, we found that TLR4 signaling in donor T cells was not necessary. A lack of TLR4 expression on activated T cells could explain why we didn’t see any
difference when using TLR4<sup>−/−</sup> donor T cells, but we did not test for TLR4 surface expression post-HCT. Our data contrasts that found by others, who found that using TLR4<sup>−/−</sup> as donors does indeed protect against aGVHD.<sup>193</sup> We believe this difference is caused by the use of TLR4<sup>−/−</sup> BM and TLR4<sup>−/−</sup> T cells together compared to our use of WT BM and TLR4<sup>−/−</sup> T cells. As donor dendritic cells are present in the BM during transplantation and as it is well documented that TLR4 stimulation of dendritic cells triggers their maturation and cytokine expression, we believe that the protective phenotype observed is caused by TLR4<sup>−/−</sup> on these dendritic cells.

MyD88 signaling has been shown to be important in both CD4 and CD8 T cell responses to viruses, as MyD88<sup>−/−</sup> T cells show impaired anti-viral clearance;<sup>181,182,200</sup> however, its role in CD4 and CD8 T cells during aGVHD is not known. When transplanting WT CD4 T cells with MyD88<sup>−/−</sup> CD8 T cells, we observed no difference in aGVHD severity or mortality. When transplanting MyD88<sup>−/−</sup> CD4 T cells with WT CD8 T cells, we observed a decrease in aGVHD severity and an increase in survival. This is in accordance with the findings that MyD88<sup>−/−</sup> CD4 T cells have impaired function during coronavirus encephalomyelitis while MyD88<sup>−/−</sup> CD8 T cells appear normal.<sup>218</sup> Transcriptome analysis using Nanostring of CD4<sup>+</sup> T cells from the intestine 10 days post-HCT also showed that genes responsible for a potent type 1 response to be downregulated in mice receiving MyD88<sup>−/−</sup> T cells. Interestingly, GM-CSF production was lower in MyD88<sup>−/−</sup> CD4 T cells but not CD8 T cells. It has recently
been shown that loss of GM-CSF in the donor T cells attenuates aGVHD and that GM-CSF production in the donor T cells is mediated through basic leucine zipper transcription factor, ATF-like (BATF) signaling. Our Nanostring data suggests that loss of MyD88 impacted BATF expression, as BATF expression is much lower in MyD88−/− CD4 T cells than in WT CD4 T cells. Exploration of MyD88/BATF/GM-CSF regulation would help to understand how MyD88 affect GM-CSF production.

The CD4 T cell compartment consists of both Tcons and Tregs, with Tcons promoting aGVHD and Tregs alleviating aGVHD. In a colitis model in which naïve CD4+ CD45RB+ T cells are transplanted into RAG1−/− recipients, which lack mature T and B cells, MyD88−/− cells were unable to induce severe colitis. Our transplantation of WT or MyD88−/− Tcons without Tregs, however, demonstrated no difference in aGVHD severity or mortality. The discrepancy observed could be due to the difference in pro-inflammatory cytokines observed between models. This colitis model is dependent on IL-17 production from Th17 cells, and MyD88−/− CD4 T cells did indeed produce less IL-17. A difference in the Th1 cytokine IFN-γ was not observed. Our aGVHD model is dependent on IFN-γ and other type 1 cytokine production from Th1 cells, and IFN-γ was significantly lower in the plasma of MyD88−/− recipients. It has been shown that Treg-specific MyD88−/− cells have no impairment of suppressive capability compared to WT Treg. Meanwhile, it has also been shown that MyD88−/− Tregs during skin transplantation and cGVHD are deficient in their suppressive capabilities.
MyD88\(^{-/-}\) Tregs also protect less against colitis compared to WT Tregs;\(^{220}\) however, we found that transplantation with donor WT Tcons and either WT or MyD88\(^{-/-}\) Tregs did not alleviate aGVHD. The difference in our data could be due to the kinetics of disease. In the skin transplant model, Treg frequencies were similar early after transplant and only started to decrease after 21 days post-skin transplant.\(^{198}\) In the colitis model, a difference in disease severity using MyD88\(^{-/-}\) Tregs compared to WT Tregs was not observed until 9 weeks post-transplant into RAG1\(^{-/-}\) mice.\(^{220}\) In our aGVHD model, we start seeing severe aGVHD as early as 10 days post-HCT. In the immunization model, the authors waited only seven days before measuring Tcon proliferation and pro-inflammatory cytokine production.\(^{180}\) We cannot eliminate the possibility based off our data that, in a slower disease progression setting, MyD88\(^{-/-}\) Tregs do indeed develop a suppressive defect. Therefore, the importance of MyD88 signaling in Tregs may be highly disease and time dependent.

It has been shown that naïve CD4 T cells require MyD88 signaling through the IL-1R in order to overcome Treg-mediated suppression for induction of a Th1 response.\(^{180}\) While our data suggests that IL-1R signaling in T cells is not required for aGVHD induction, left open was the possibility that MyD88 signaling in Tcons is required for Treg-mediated suppression in aGVHD. Indeed, when transplanting MyD88\(^{-/-}\) Tcons with Tregs, we did observe a decrease in aGVHD severity and mortality. Interestingly, this phenotype did not depend on MyD88 in the Tregs, as transplanting WT or MyD88\(^{-/-}\) Tregs with MyD88\(^{-/-}\) Tcons showed no
difference in aGVHD severity or mortality. We believe that this may be caused by loss of signaling through soluble factors, such as IL-6 or TNFα, that act directly or indirectly through or on MyD88. Deficiency of IL-1β/MyD88 signaling has already been mentioned, but IL-6 has also been implicated in Tcon resistance to Treg-mediated suppression.\textsuperscript{221-223} It has been suggested that this is due to blocking of Treg-mediated inhibition of IL-2Rα on Tcons.\textsuperscript{223} We did not check for IL-2Rα expression on Tcons during our experiments. Loss of IL-6-producing T cells, but not bone marrow cells or non-hematopoietic cells, also prevents aGVHD mortality in a murine model, although the mechanism behind this remains unexplored.\textsuperscript{224} Interestingly, similar to our data with MyD88\textsuperscript{-/-} donor T cells, it was also found that the absence of IL-6 did not affect the expansion of T cells. As IL-6 is known to be upregulated by multiple TLR/MyD88 signaling pathways, it is possible that reduced IL-6 in MyD88\textsuperscript{-/-} donor T cells could explain our phenotype. However, we did not check for IL-6 production in T cells in our models. IL-6 signaling is known to activate STAT3.\textsuperscript{225} It is also possible that the absence of MyD88 signaling reduces phosphorylated STAT3 levels in the Tcons, which has been shown to be important in Tcons for their resistance to Treg suppression.\textsuperscript{226} Although STAT3 is not classically thought to be downstream of MyD88, it has recently been shown that activation of TLR4 through MyD88, TLR7, or TLR9 directly leads to phosphorylation of STAT3.\textsuperscript{211,227,228} Indeed, pSTAT3 Y705 is increased significantly in patient CD4 T cells before onset of aGVHD.\textsuperscript{229} IL-7 and IL-15 may also play a role in Tcons-resistance to Tregs during aGVHD.\textsuperscript{230-232} Adoptive transfer of T cells into lymphopenic hosts, as would be after irradiation
in our aGVHD models, leads to increased availability of IL-7 and IL-15 for the transferred T cells. Although a link between IL-7 and MyD88 has yet to be made, it has been shown that IL-15 promotes MyD88 expression in T cells. How IL-15 causes MyD88 upregulation and the effect of MyD88 upregulation by IL-15 has yet to be explored. Tcons could be using these pathways mentioned involving MyD88 to redundantly prevent their Treg-mediated suppression, which would explain why loss of IL-1R or TLR4 alone was insufficient.

A common convergence of all these pathways is the phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. This pathway helps control many cellular processes, such as proliferation, survival, migration, and metabolism. It has been suggested that hyper-activation of PI3K leads to Tcon resistance to Treg-mediated suppression. Indeed, in murine models that have genetic deficiencies in proteins that negatively regulate PI3K signaling, Tcons are more resistant to Treg-mediated suppression. Several cytokine receptors, TNF receptors, TLRs, and T cell costimulatory receptors have been shown to activate PI3K signaling. While MyD88 has not been implicated in all these pathways, we suspect that loss of MyD88 may affect enough to prevent hyper-activation of PI3K/ATK/mTOR signaling, thus rendering Tcons susceptible to Treg-mediated suppression (Figure 18). Direct targeting of mTOR using rapamycin (drug name: Sirolimus) has been extensively studied in GVHD and is given to patients routinely as a prophylaxis, with some studies suggesting efficacy as a treatment option of aGVHD. Recently, in a murine
model of aGVHD, direct pan-PI3K inhibition using a small molecule inhibitor prevented severe aGVHD development, in part through controlling T cell activation. However, how pan-PI3K inhibition works on Tcons and Tregs specifically was not studied nor the direct mechanism of how pan-PI3K inhibition of T cells prevented severe aGVHD development.
Figure 18. Proposed mechanism

Various receptor signaling pathways have been shown to use MyD88 as an adaptor protein, including all TLRs except TLR3, the IL-1 superfamily of receptors, and recently the IL-15 receptor. We propose a two-pronged approach as to how MyD88 Tcons are able to resist Treg-mediated suppression. First, signaling through MyD88 activates the PI3K/AKT pathway. Shortly after conditioning for transplant, the damage caused by the conditioning leads to release of various DAMPs, PAMPs, and alarmins that can activate TLR and IL-1R superfamily signaling. The loss of lymphocytes after conditioning also causes excessive IL-15 to be available. IL-6 is produced and released through IL-1R and TLR signaling and can bind to the IL-6R on other Tcons. Both IL-6 signaling and MyD88 signaling can activate the PI3K/AKT pathway. Through a yet to be defined mechanism, others have proposed that hyper-activation of PI3K/AKT signaling promotes resistance of Tcons to suppression by Tregs. Second, we show that sST2 production in Tcons is reduced in MyD88⁻/⁻ Tcons; however, how MyD88 regulates sST2 production is still unknown. sST2 released by the Tcon can bind free IL-33, preventing IL-33/ST2 signaling on Tregs. IL-33/ST2 signaling on Tregs has been shown by numerous groups to promote Treg function. Blocking sST2 with a neutralizing antibody has been shown to increase Treg frequency and ameliorate experimental aGVHD. We hypothesize that MyD88⁻/⁻ Tcons have less PI3K/AKT activation and secrete less sST2, allowing the Tcons to be better repressed by Treg cells. Solid lines: direct effect; Dashed lines: indirect effect; dotted lines: proposed effect.
4.3 ST2 on T cells

ST2 on T cells has been found primarily on the Th2 and Treg subsets. ST2 is a member of the IL-1R superfamily and signals through MyD88/IL-33/ST2 signaling enhances Th2 and Treg activity through increased IL-5 and IL-13 production in Th2 cells\textsuperscript{30,86,111,112} and increased $Foxp3$ expression in Tregs.\textsuperscript{65} A soluble form of ST2, sST2, sequesters free IL-33 and does not signal. Recently, we have shown that T cells, specifically type 1 and type 17 T cells, can produce sST2.\textsuperscript{75} We and others have shown that total ST2\textsuperscript{-/-} T cells ameliorate aGVHD.\textsuperscript{75,121} We asked whether the phenotype of MyD88\textsuperscript{-/-} Tcons in the presence of WT or MyD88\textsuperscript{-/-} Tregs observed before would be phenocopied when using ST2\textsuperscript{-/-} Tcons and WT or ST2\textsuperscript{-/-} Tregs. Indeed, transplanting ST2\textsuperscript{-/-} Tcons alleviated aGVHD in a similar manner regardless of using WT or ST2\textsuperscript{-/-} Tregs, suggesting that ST2/MyD88 signaling is required for Tcons to overcome Treg-mediated suppression. We did notice a small but non-statistically significant difference in aGVHD severity and mortality when using ST2\textsuperscript{-/-} Tregs with WT or ST2\textsuperscript{-/-} Tcons compared to using WT Tregs with WT or ST2\textsuperscript{-/-} Tcons, suggesting that loss of ST2 on Tregs may impact their suppressive capabilities. This would be in line with what has previously been shown.\textsuperscript{65,241} We also show that isolated MyD88\textsuperscript{-/-} CD4 T cells from the intestine at day 10 post-HCT express less sST2 and more ST2 compared to similarly collected WT CD4 T cells. However, whether MyD88 signaling is directly important in sST2 expression by CD4 T cells or whether the decrease in sST2 expression is due to a decrease in Th1 response is not clear. We hypothesize that sST2 produced by Tcons, in a yet-to-be-discovered MyD88-dependent
mechanism, is binding to free IL-33, preventing IL-33 binding to ST2 on Tregs. This may in part explain the protective phenotype we observed (Figure 18). There is evidence that STAT3 and ERK signaling, both of which can be activated through MyD88, influence ST2 proximal promoter activity.\textsuperscript{242} We have not tested for STAT3 or ERK activity in our models. As well, the increase in ST2 expression may be a compensatory mechanism by the CD4 T cells trying to overcome the loss of MyD88.

We and others have attempted to look for ST2 expression via flow cytometry on Th1 during aGVHD settings without success. However, recent reports have shown that ST2 can indeed be present on Th1 cells.\textsuperscript{121,147,243} ST2 signaling on Th1 cells helps clear LCMV infection through increased IFN-γ production and is dependent on T-bet and STAT4.\textsuperscript{243} The effect of IL-33 on Th1 differentiation was later confirmed using an OVA-immunization murine model as well as human \textit{in vitro} cell cultures.\textsuperscript{147} The expression of ST2 on the surface only occurred during times of inflammation.\textsuperscript{243} During aGVHD, IL-33 administration during peak inflammatory response (days 3-7 post-HCT) enhanced aGVHD severity and mortality,\textsuperscript{121} while IL-33 administration during the peri-transplant period ameliorated aGVHD through enhanced ST2\textsuperscript{+} Treg response.\textsuperscript{82} This suggests that ST2 may be only transiently expressed on Th1 cells, while it is more stably expressed on Th2 cells and Tregs. Although an inflammatory response is clearly occurring during aGVHD, perhaps this transient expression is the reason that we were not able to detect ST2 in our aGVHD model. As we’ve only looked for ST2
expression via flow cytometry after day 10 post-HTC and an inflammatory response in the host begins as early as day (-)1 pre-transplant after irradiation, it is possible that we missed the timepoint in which ST2 is expressed on Th1 cells. Further work needs to be done to assess a potential role of ST2/MyD88 signaling in promoting a Th1 response early during aGVHD.

A weakness of our mouse model is that the ST2^/- mouse we use has a loss of both the membrane and soluble forms of ST2. It is therefore difficult to determine whether sST2 production by Tcons or if indeed ST2 is present on Th1 cells and loss of ST2 on these cells is more important in the phenotype we observed. Development of distinct sST2^/- and membrane ST2^/- mice could really help answer these questions.
4.4 Therapeutic avenues

Small molecule inhibitors of MyD88 exist and have been tested in vitro and in murine models, with the peptido-mimetic compound ST2825 being the most validated.\textsuperscript{199,244-247} This compound works by preventing MyD88 TIR domain homodimerization.\textsuperscript{199} Treatment of human B cells with ST2825 prevented their proliferation when stimulated with CpG in vitro.\textsuperscript{199} As well, addition of ST2825 to astragalus polysaccharide-stimulated RAW 264.7 macrophage cells prevented their secretion of proinflammatory cytokines.\textsuperscript{247} In vivo treatment with this inhibitor has been tested in an experimental acute myocardial infarction model,\textsuperscript{244} experimental traumatic brain injury model,\textsuperscript{245} and an experimental seizure model;\textsuperscript{246} however, they have never been tested in an experimental aGVHD model nor in a phase 1/2 clinical trial. We used ST2825 in the major mismatch model of aGVHD and found no difference in aGVHD severity or mortality. However, there could be multiple reasons as to why this experiment did not show any differences. First, the length of the treatment period may have been insufficient. Beginning earlier or lengthening the treatment period could lead to better results. Second, the dosing may not have been correct. We used the recommended dose 5 mg/kg twice a day,\textsuperscript{244} but it may not be a high enough dose to see an effect as the pharmacokinetics between naïve and GVHD could be quite different (up to 5 times fold difference, i.e. clinical trial on HDAC).\textsuperscript{248} Studying the pharmacokinetics and adapting the dose could lead to protection against aGVHD.\textsuperscript{249}
Undoubtedly, treatment of patients with MyD88 inhibitors could be problematic because MyD88 has been shown to be essential for proper myeloid-derived suppressor cell (MDSC) protective function during aGVHD. MyD88 is also well-known for its role in anti-infection immunity against intracellular pathogens. Loss of MyD88 signaling post-HCT could greatly increase incidences and mortalities due to infection, which can be difficult to treat when the immune system is yet to fully recover. We argue that using a ST2 neutralizing antibody, as we have previously shown, would be better as it would help avoid the limitations of using a MyD88 inhibitor.
CHAPTER 5: FUTURE DIRECTIONS

Our data suggests that MyD88−/− Tcons are more susceptible to Treg-mediated suppression through loss of sST2 production by the donor Tcons. However, the exact mechanism for this is still not understood. Our data shows that MyD88−/− CD4 T cells isolated from the intestine at day 10 post-HCT express less sST2 than WT cells collected similarly. The link between MyD88 and sST2 production has yet to be elucidated. It’s possible that a transcription factor downstream of MyD88 can bind to the promoter region of exon 1B of the ST2 gene, leading to expression of sST2. To determine the protein(s) responsible for this link, we could start with a thorough search of potential transcription factors that bind to the ST2 exon 1B using ENCODE. From this data we would need to find transcription factors that are known to be downstream of MyD88 and confirm using chromatin immunoprecipitation from CD4 T cells isolated from the intestine after aGVHD induction. As well, development of a double knockout of ST2 and MyD88 and comparing transcriptome analyses using RNA-seq between the double knockout, MyD88−/− T cells, and WT T cells isolated from the intestine post-HCT could also help to shed some light on the link between MyD88 and sST2 production.

New data has suggested that ST2 is present on Th1 cells transiently during an inflammatory response and that ST2 signaling on Th1 cells promotes IFN-γ production through a Tbet and STAT4 dependent mechanism. Although we
have been unable to detect ST2 expression via flow cytometry on Th1 cells during aGVHD, this does not mean that ST2 expression did not occur previously in these cells. It is possible that we just missed the time point in which ST2 is expressed on these cells. To test this we could transplant WT donor BM and T cells into lethally irradiated recipients and check in the spleen, MLN, and intestine at various early timepoints post-HCT. If we do find expression of ST2 on Th1 cells, we could perform a flow cytometry analysis, gating first on T-bet+ CD4+ T cells, followed by analyzing ST2+ or ST2- cells, and finally look at the IFN-γ production from these cells. This would help to show whether ST2+ Th1 cells in our aGVHD model express higher IFN-γ, as shown in an LCMV model. This finding would also help explain in part why loss of MyD88 in the donor Tcons ameliorates aGVHD in our mouse models. This also brings up the question as to whether ST2+ Th1 cells are resistant to Treg-mediated suppression, as we found that ST2-/- Tcons were also susceptible to Treg-mediated suppression. To answer this, we could first try to in vitro culture Th1 cells with IL-12 alone or IL-12 and IL-33, followed by stainings for ST2 and IFN-γ. If we cannot find ST2 staining, we should at least see increased IFN-γ, as previously suggested. After culturing for 5 days under these conditions, we can remove the media containing the cytokines, label these Th1 polarized cells with CFSE, and perform a suppressive assay with varying amounts of isolated Tregs. If ST2+ Th1 cells, or at least IL-33 responsive Th1 cells, are resistant to Treg-mediated suppression, we would expect more dilute CFSE than Th1 cells cultured without IL-33.
Although our experiments show that MyD88 is indeed important in Tcons for an optimal aGVHD response, the work so far has been confined to murine models. Previous work in the lab has shown that blockade of ST2 using a neutralizing antibody in a humanized murine model of aGVHD in which the donor T cells were of human origin protects against aGVHD, in part through decreased sST2 production. As our work suggests that MyD88 is necessary for optimal sST2 production by donor T cells during aGVHD, confirming these results in a humanized model of aGVHD would be beneficial to help make this work more translational. To perform this experiment, we would have to knockdown MyD88 in isolated human total T cells and confirm with western blot. After confirming sufficient knockdown, we would irradiate NOD.Cg-Prkdc^{scid} Il2rg^{-/-}/SzJ (NSG) mice, which are deficient for mature lymphocytes and have extremely low NK cell activity, and transplant the MyD88 knockdown human T cells and control T cells into these mice and record their weight loss and survival.


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CURRICULUM VITAE

Brad Griesenauer

Education

Ph.D.: Immunology
Indiana University - Indianapolis, Indiana 2018

B.S.: Biotechnology
Indiana University - Bloomington, Indiana 2012

Training

Graduate Research Fellow, Department of Microbiology and Immunology
Dr. Sophie Paczesny, Indiana University - Indianapolis, Indiana 2013-2018

Undergraduate Research Fellow, Biology Department
Dr. Gregory Velicer, Indiana University, Bloomington, Indiana 2011-2012

Lab Assistant, Integrated Freshman Learning Experience
Dr. James Drummond, Indiana University, Bloomington, Indiana 2008

Research Interests

1. Mucosal inflammation and development of targeted therapies toward inflammatory diseases - Understanding how inflammatory bowel diseases and allergies, some of the most prevalent chronic conditions in the world, impact mucosal surfaces and how we can alleviate these conditions either therapeutically or preventatively.
2. Development of immunotherapies for malignancies - Learning and eventual experimentation of human immunotherapies targeted toward blood malignancies, such as leukemias and lymphomas.

3. Innovative therapies for GVHD treatment without hampering the beneficial GVL effect - Discovering novel mechanisms of action of proteins in the context of GVHD and GVL based on patient proteomic studies for therapeutic development.

Peer-Reviewed Publications


3. Griesenauer B and Paczesny S. The role of the ST2/IL-33 axis in immune cells during inflammatory diseases. *Frontiers in Immunology* 2017 Apr 24; 8(475)


7. Griesenauer B, Zhang J, Ramadan AM, Egbousuba JC, Campa KA, Paczesny S. ST2/MyD88 signaling is a therapeutic target alleviating murine acute graft-versus-host disease sparing T regulatory cell function. *(Submitted)*


**Oral Presentations**

“MyD88 KO in donor T cells alleviates graft-vs-host disease”

Autumn Immunology Conference, Chicago, IL Nov 2014

“Deficiency in MyD88 Signaling in Donor T Cells Attenuates GVHD”
Hematologic Malignancies & Stem Cell Biology Group Meeting, Indianapolis, IN Oct 2015
“MyD88 KO in donor T cells alleviates graft-vs-host disease”

Autumn Immunology Conference, Chicago, IL Nov 2015
“Deficiency of MyD88 Signaling in CD4 Tconvs Increases Tregs Suppression through Loss of ST2 Signaling” Autumn Immunology Conference, Chicago, IL Nov 2016

“Deficiency of MyD88 Signaling in Conventional CD4 T Cells Increases Tregs Suppression through Loss of ST2 Signaling” BMT Tandem Meetings, Orlando, FL Feb 2017

“Deficiency of MyD88 Signaling in Conventional CD4 T Cells Increases Tregs Suppression through Loss of ST2 Signaling” Immunology 2017, Washington, D.C. May 2017

**Authored Posters**

“MyD88 KO in donor T cells alleviates graft-vs-host disease”

Autumn Immunology Conference, Chicago, IL

*Authors: Brad Griesenauer, Abdulraouf Ramadan, Jilu Zhang, Sophie Paczesny* Nov 2014

“MyD88 KO in donor T cells alleviates graft-vs-host disease”

Innovation to Enterprise Showcase & Forum, Indianapolis, IN

*Authors: Brad Griesenauer, Abdulraouf Ramadan, Jilu Zhang, Sophie Paczesny* Nov 2014
“ST2 blockade reduces soluble ST2 producing cells while maintaining the protective ST2L expressing T cells during graft-versus-host disease”

BMT Tandem Meetings, San Diego, CA

Authors: Jilu Zhang, Abdulraouf Ramadan, Brad Griesenauer, Wei Li, Chen Liu, Reuben Kapur, Helmut Hanenberg, Bruce Blazar, Isao Tawara, Sophie Paczesny

Feb 2015

“IL-33/ST2 activation of IL-9-secreting T cells alters the balance of fatal immunity and tumor immunity”

Immunology 2015, New Orleans, LA

Authors: Abdulraouf Ramadan, Jilu Zhang, Brad Griesenauer, Reuben Kapur, Helmut Hanenberg, Jie Sun, Mark Kaplan, Sophie Paczesny

May 2015

“MyD88 KO in donor T cells alleviates graft-vs-host disease”

12th Regional Midwest Blood Club Symposium, French Lick, IN

Authors: Brad Griesenauer, Abdulraouf Ramadan, Jilu Zhang, Sophie Paczesny

Oct 2015

“MyD88 KO in donor T cells alleviates graft-vs-host disease”

Autumn Immunology Conference, Chicago, IL

Authors: Brad Griesenauer, Abdulraouf Ramadan, Jilu Zhang, Sophie Paczesny

Nov 2015

“MyD88 KO in donor T cells alleviates graft-vs-host disease”

Cancer Research Day, IUPUI, Indianapolis, IN
Authors: **Brad Griesenauer, Abdulraouf Ramadan, Jilu Zhang, Sophie Paczesny**

May 2016

“Deficiency of MyD88 Signaling in CD4 Tconvs Increases Tregs Suppression through Loss of ST2 Signaling”

Autumn Immunology Conference, Chicago, IL

Authors: **Brad Griesenauer, Abdulraouf M Ramadan, Jilu Zhang, Jane Egbosiuba, Sophie Paczesny**

Nov 2016

“Deficiency of MyD88 Signaling in Conventional CD4 T Cells Increases Tregs Suppression through Loss of ST2 Signaling”

Immunology 2017, Washington, D.C.

Authors: **Brad Griesenauer, Jilu Zhang, Abdulraouf Ramadan, Jane Egbosiuba, Sophie Paczesny**

May 2017

**Teaching**

Co-led immunology seminars teaching first and second year medical students how to read primary scientific literature 2014

Lectured 37 students in “J210: Microbiology and Immunology” lab section 2015

Advised 1st year medical student in the CUPID (Cancer in Under-Privileged, Indigent, or Disadvantaged) Summer Translational Oncology Program for 7 weeks on a project entitled: Donor MyD88+ Tregs Alleviate GVHD 2016
Advised 1st year medical student in the CUPID (Cancer in Under-Privileged, Indigent, or Disadvantaged) Summer Translational Oncology Program for 7 weeks on a project entitled: Role of TLR4 in the Development of Acute Graft-Versus-Host Disease 2017

Advised 3rd year medical student on writing for grants 2017

HONORS AND AWARDS

Indiana School of Medicine Biomedical Gateway Program Travel Fellowship 2012

Accepted onto Dr. Hal Broxmeyer’s NIH training grant T32 DK007519 entitled: "Regulation of Hematopoietic Cell Production" 2016

AAI Trainee Abstract Award 2017

Best Presenter as voted by Immunology faculty at IU School of Medicine for Immunology Journal Club 2017