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

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Brad Griesenauer

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

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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LIST OF ABBREVIATIONS

aGVHD	Acute Graft-versus-Host Disease
AP-1	Activator protein 1
APC	Antigen presenting cell
BATF	Basic leucine zipper transcription factor, ATF-like
BCL6	B cell lymphoma 6
Bcl-xL	B-cell lymphoma-X large
BCR	B cell receptor
BM	Bone marrow
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CD	Crohn's Disease
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
cGVHD	chronic Graft-versus-Host Disease
ChIP	Chromatin immunoprecipitation
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAMP	Danger associated molecular pattern
DC	Dendritic cell

DNA	Deoxyribonucleic acid
DOCK8	Dedicator of cytokinesis 8
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
Fc	Fragment crystallizable
Foxp3	Forkhead box P3
FVD	Fixable viability dye
GATA	GATA binding protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GRK2	G protein-coupled receptor kinase
HBV	Hepatitis B virus
HCT	Hematopoietic cell transplant
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICAM1	Intracellular adhesion molecule 1
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1R	Interleukin 1 receptor
IL-1RAP	Interleukin 1 receptor accessory protein

IL-15R	Interleukin 15 receptor
IL-6R	Interleukin 6 receptor
ILC	Innate lymphoid cell
iNKT	Invariant natural killer T cell
IRF	Interferon regulatory factor
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MCMV	Mouse cytomegalovirus
MD2	Lymphocyte antigen 96
MHC	Major histocompatibility complex
miHA	Minor histocompatibility antigen
MLR	Mixed leukocyte reaction
MOG	Myelin oligodendrocyte glycoprotein
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
ns	Not significant
nTreg	Natural T regulatory cell
OX40L	Tumor necrosis factor superfamily member 4

PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
PI3K	Phosphatidylinositol-3 kinase
PRR	Pathogen recognition receptor
pTreg	Peripheral T regulator cell
PU.1	Purine-rich box 1
RAG	Recombination-activating genes
RNA	Ribonucleic acid
RORγt	Retinoic acid receptor-related orphan receptor gamma t
SEM	Standard error of the mean
SIGIRR	Single Ig IL-1-related receptor
sST2	Soluble serum stimulation-2
ST2	Serum stimulation-2
ST2LV	Serum stimulation-2 long variant
ST2V	Serum stimulation-2 variant
STAT	Signal transducer and activator of transcription
Syn	Syngeneic
T-bet	T-box transcription factor TBX 21
Tc	T cytotoxic
TCD	T cell depleted
Tcon	T conventional cell

TCR	T cell receptor
Tfh	T follicular helper
TGF-β	Transforming growth factor beta
Th	T helper
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor associated factors
Treg	T regulatory cells
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
WT	Wild type

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.[1] 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

cells.[2] 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.[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.[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).[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_2O_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.[6] Eosinophils are granulocytes which release various chemical mediators such as peroxidase, neurotoxin, ribonuclease, and major basic protein; growth factors; and cytokines.[7] Basophils are granulocytes which release histamine, proteoglycans, and elastase and secrete various cytokines and lipid mediators.[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.[3] 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.[9] 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.[13] Th1 cells produce both interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) 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.[14] TSLP is not necessary for Th2 cell differentiation or function in already mature Th2 cells,[15] but is important for their generation/maintenance *in vivo*.[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.[17] These cells require transforming growth factor beta (TGF-β) 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.[18] Like Th2 cells, Th9 cells contribute to allergic diseases if left unchecked.[19]

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.[20]

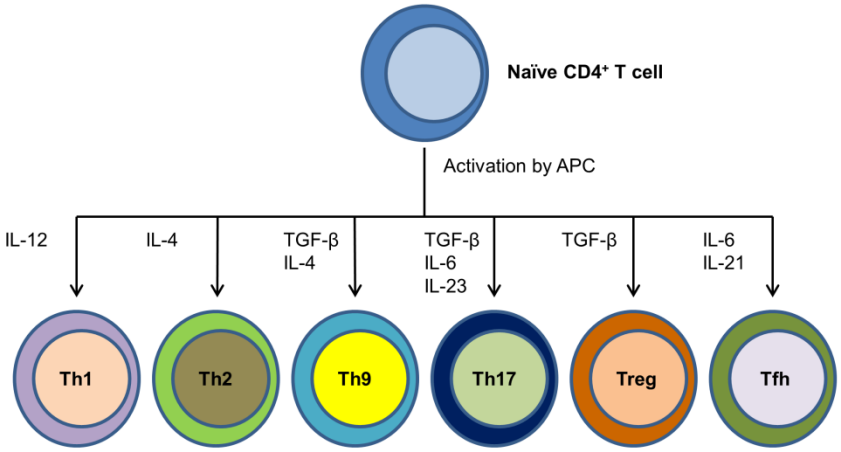
Tregs are critical for suppressing the immune response after clearance of pathogens and for maintaining tolerance to self-antigens. Tregs can be classified into two groups, depending on where they originate: natural or thymic Tregs (nTregs or tTregs) and peripheral or induced Tregs (pTregs or iTregs). nTregs develop in the thymus while pTregs develop extrathymically at peripheral sites.[21] 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.[22] 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.[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.[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.[1] 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]



Transcription Factors	Tbet, STAT4	GATA3, STAT5, STAT6	PU.1, STAT6, IRF4	RORγt, STAT3	Foxp3, STAT5	Bcl6, STAT3
Cytokines	TNF-α, IFN-γ	IL-4, IL-5, IL-13	IL-9, IL-10	IL-17, IL-22, IL-21	IL-10, TGF-β	IL-4, IL-21
Chemokine Receptors	CCR5, CXCR3	CCR3, CCR4, CCR8	CCR3, CCR6, CXCR3	CCR6	CCR4, CCR6	CXCR5
Protective Function	Intracellular Pathogens	Extracellular Parasites	Helminths	Fungus	Tolerance, Suppression	B Cell Support
Pathogenic Function	Inflammation	Allergy	Allergy	Inflammation	Uncontrolled Growth of Pathogen or Tumor	Autoreactive Antibodies

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-κB 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.[32]

Dysregulation of IL-33/ST2 signaling and sST2 production have been implicated in a variety of inflammatory diseases such as cardiac disease,[34-37] intestinal bowel disease (IBD),[38-41] graft-versus-host disease (GVHD),[42-49] small bowel transplant rejection,[50] and type-2 diabetes.[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.[54-56] Little is known about ST2V other than it is expressed highly in gastrointestinal organs.[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.[58] Other information on ST2LV is currently lacking.

By cloning the *Il1rl1* gene in rat and sequencing sST2 and ST2 cDNAs, it was found that sST2 and ST2 have different exon 1 sequences.[54] Mapping the promoter regions for *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).[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 *Il1rl1* genes.[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.[60] PU.1 and GATA2 cooperatively transactivate the distal ST2 promoter inducing expression of ST2, but not sST2.[60] Loss of PU.1 significantly decreased ST2 expression.[60] Conversely, a PMA-responsive element has been found near the proximal promoter region of ST2 in the mouse fibroblast line NIH 3T3.[61] Similarly, activating the human fibroblast line TM12, which only uses the proximal promoter for *Il1rl1* transcription, led to sST2 expression.[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.[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[63] and a large variety of immune cells, including T conventional cells, particularly type 2,[64] T regulatory cells (Tregs),[65] innate helper 2 cells (ILC2),[66] M2 polarized macrophages,[67] mast cells,[68] eosinophils,[69] basophils,[70] neutrophils,[70] NK,[71] and iNKT cells.[71] Signaling through ST2 in immune cells induces type-2 and Treg immune responses, IgE production, and eosinophilia.[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.[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,[73] but can also be produced after activation with IL-33 in mast cells[74] or anti-CD3/anti-CD28 in both CD4 and CD8 conventional T cells.[75] In a murine aGVHD model, it has recently been shown that intestinal Th17 and Tc17 cells produced large amounts of sST2 following alloreactivity.[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.[76]

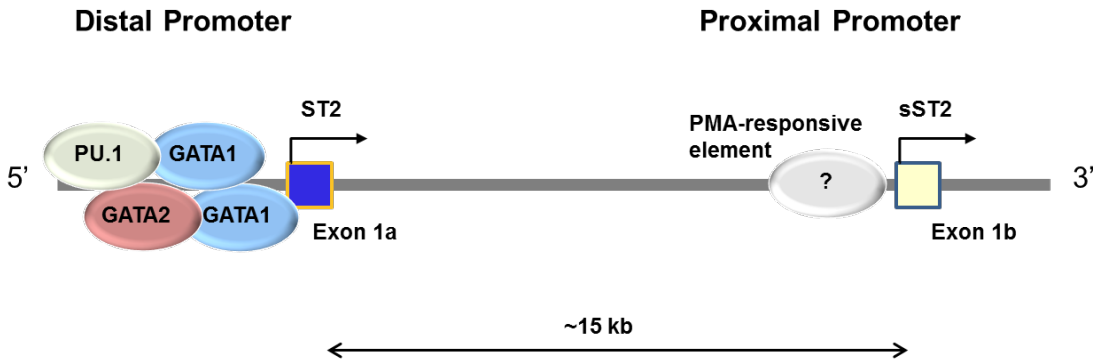

Distal Promoter
Proximal Promoter
ST2
sST2
PMA-responsive element
PU.1
GATA1
GATA2
GATA1
?
5'
3'
Exon 1a
Exon 1b
~15 kb

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,[83] IRF1 signaling can inhibit Tregs by binding to the *Foxp3* promoter and preventing *Foxp3* transcription in murine T cells;[84] 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.[83] 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*.[31,85] IL-33 binding to ST2 has also been shown to negatively regulate ST2 through protein polyubiquitination, internalization, and degradation.[86]

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.[87] Another group used IL-33-treated cardiomyocytes and observed blocked pro-hypertrophic effects of angiotensin II or phenylephrines in the presence of sST2.[88] 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.[73] In a

human endotoxin model, healthy donors injected with LPS (2 ng/kg) had increased sST2 in their plasma within 24 hours of injection.[73] Fibroblast growth factor 2 enhanced sST2 production in the human breast adenocarcinoma cell line MCF-7 through MEK/ERK signaling.[89] Lysophosphatidic acid has also been shown to increase sST2 production by human bronchial epithelial cells in an NF-κB or JNK-dependent manner.[90] Enhanced sST2 plasma circulation has been correlated with pulmonary fibrosis,[91] acute myocardial infarction,[63] subclinical brain injury and stroke,[92] celiac disease,[93] gastric cancer,[94] HBV-related acute-on-chronic liver failure,[95] HIV progression,[96] and GVHD.[42-49]

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;[33,97,98] however, some hematopoietic cells like dendritic cells have also been shown to express IL-33 when activated.[30] 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.[33] 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,[33] leading to activation of the immune system through IL-33/ST2 signaling.[33,99]

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.[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.[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.[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.[101] Loss of the nuclear localization domain of IL-33 led to non-resolving lethal inflammation.[102] However, IL-33$^{-/-}$ 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.[103,104] Surprisingly, caspase-1, caspase-3, or caspase-7 processing actually leads to IL-33 inactivation.[105,106] 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.[104,107,108]

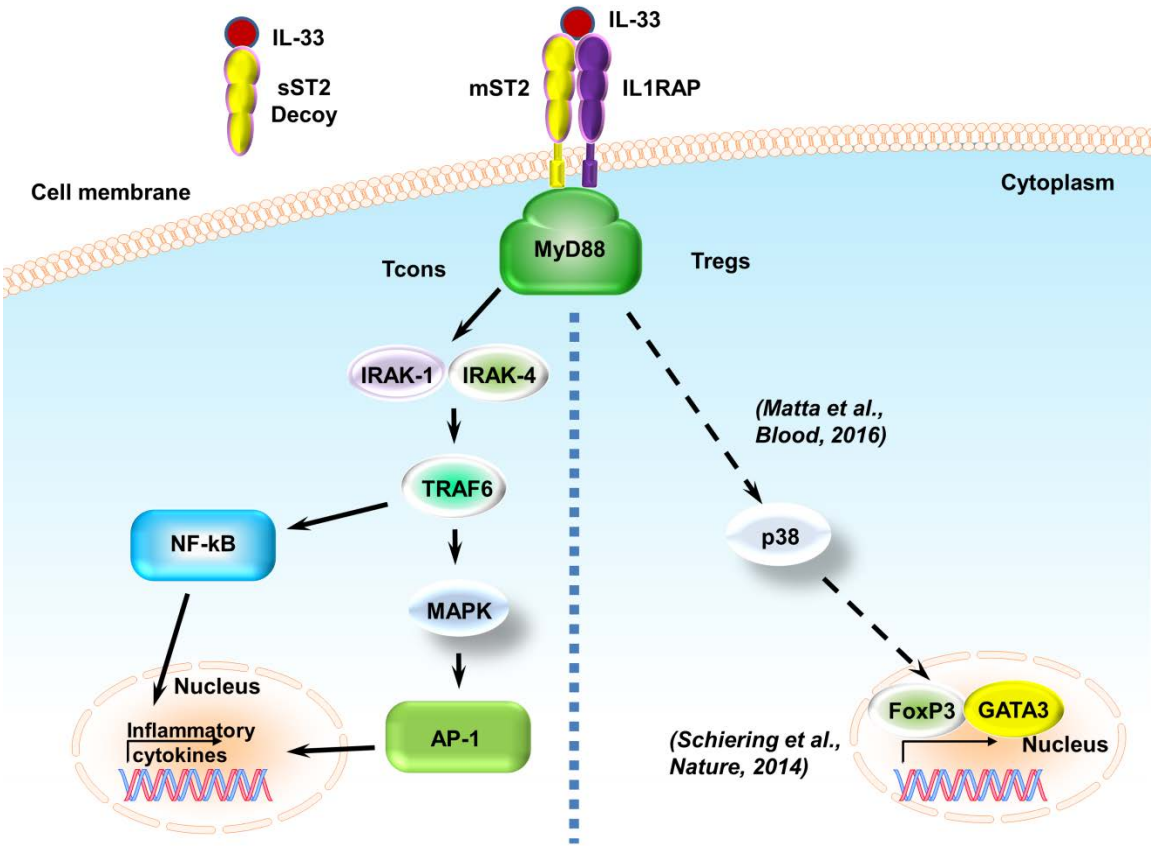

IL-33
sST2
Decoy
IL-33
mST2
IL1RAP
Cell membrane
Cytoplasm
MyD88
Tcons
Tregs
IRAK-1
IRAK-4
(Matta et al.,
Blood, 2016)
TRAF6
NF-kB
p38
MAPK
Nucleus
Inflammatory
cytokines
AP-1
(Schiering et al.,
Nature, 2014)
FoxP3
GATA3
Nucleus

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.[64,109] 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.[64] ST2 expression on Th2 cells is dependent on GATA3 signaling[110] and is enhanced by IL-6, IL-1, TNF-α, and IL-5.[29] 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.[29] IL-33 stimulation of Th2 cells *in vitro* increased the amount of IL-5 and IL-13 produced.[30] 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.[111] 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.[86,112] Adoptive transfer of these cells into naïve $IL\text{-}4^{-/-}$ mice still triggered airway inflammation.[112] *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.[30] 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.[113] 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.[114] 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.[65] 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.[24,125] $ST2^+$ 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.[126] 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.[82] Treatment of mice receiving a heart transplant with IL-33 prolonged graft survival through increased Treg and myeloid derived-suppressor cell numbers.[127,128] 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.[129] 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,[75] 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[130] and negatively regulates *Foxp3* transcription,[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.[66,131] These cells have been shown to play a protective role against helminth infection and regulate metabolic homeostasis.[132] In humans $ST2^+$ ILC2s were later found in the lung and gut[133] and these ILC2s produced IL-5 and IL-13. During ILC2 activation ST2 is upregulated in a GATA3 and Gfi1-dependent manner.[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.[66] IL-33/ST2 signaling enhancement was shown to expand ILC2s *in vivo*.[66,136] This group also found that ILC2s are major producers of type 2 cytokines after *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.[75,80,145] 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.[145] IL-33/ST2 signaling has also been shown to enhance CD8 T cell antitumor activity.[146] During aGVHD, however, IL-33 treatment during peak inflammation significantly increased aGVHD severity and mortality in part through increased expansion of Tc1 cells.[121] Given that IL-33 can increase type 1 responses when IL-12 levels are high,[147] 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.[148] 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.[149] Adoptive transfer of these IL-33-treated, IL-10 producing B cells prevented spontaneous colitis in IL-$10^{-/-}$ mice without affecting Treg frequency.[149]

1.4.7: iNKT cells and NK cells

IL-33/ST2 signaling in murine iNKT cells causes their expansion and activation.[150] Mice treated with IL-33 had twice as many iNKT cells in the spleen

and liver compared to untreated mice.[150] Unexpectedly, ST2 signaling in iNKT cells induced IFN-γ instead of IL-4 upon TCR engagement, which synergized in the presence of IL-12.[71,150].This effect was also seen in Vα24^{+} human iNKT cells.[71] NK cells constitutively express ST2 and IL-33/ST2 signaling increases IFN-γ levels synergistically with IL-12.[71,150] Loss of ST2 in Ly49H^{+} NK cells did not affect their development but did impair their ability to expand and protect against MCMV.[151] These data have not yet been translated to human disease.

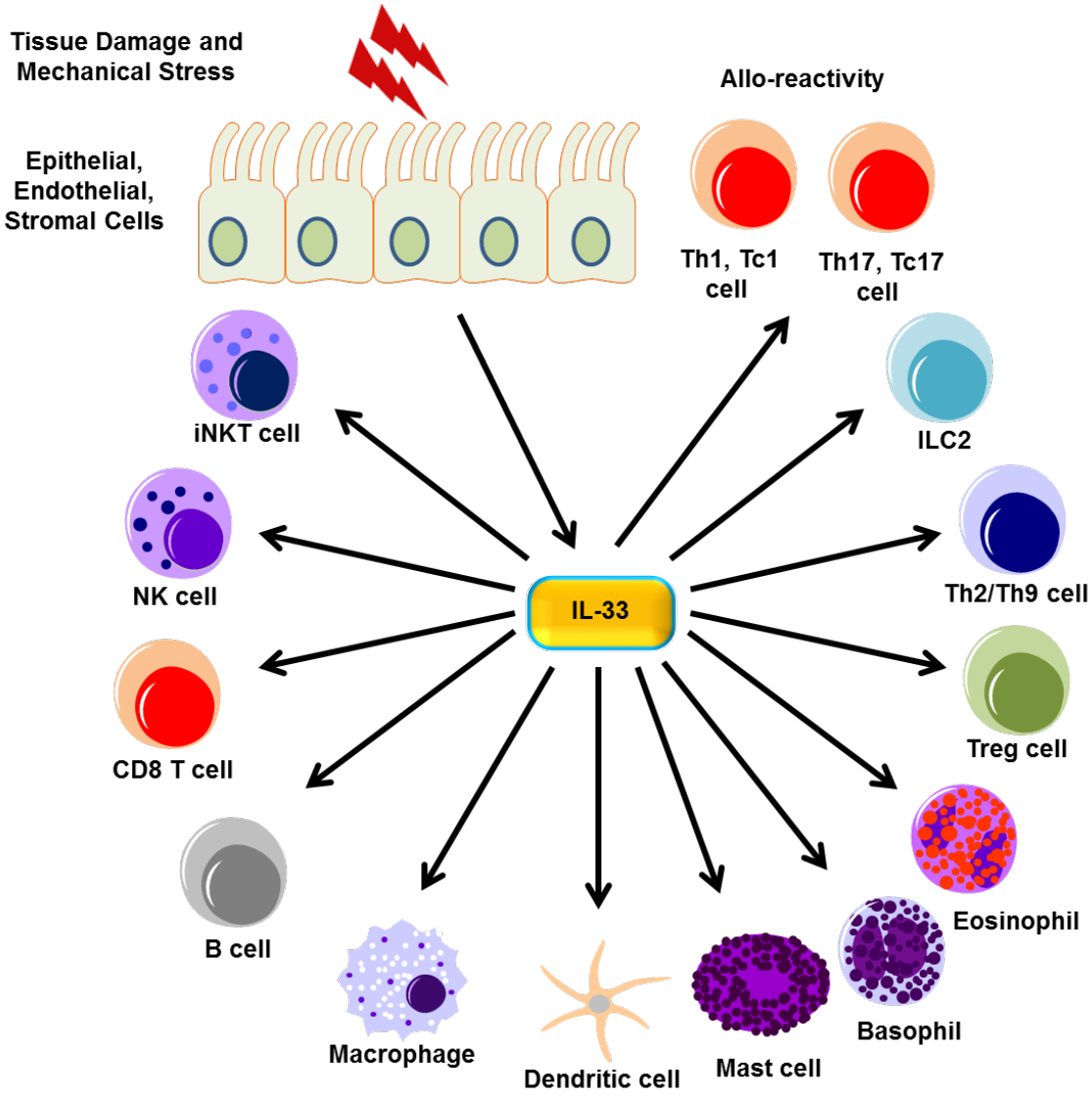

Tissue Damage and
Mechanical Stress
Epithelial,
Endothelial,
Stromal Cells
Allo-reactivity
Th1, Tc1
cell
Th17, Tc17
cell
iNKT cell
ILC2
NK cell
Th2/Th9 cell
IL-33
CD8 T cell
Treg cell
B cell
Eosinophil
Basophil
Macrophage
Dendritic cell
Mast cell

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 *in vitro* and *in vivo* after HCT.[75] Blocking ST2 with a blocking antibody *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.[75] 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.[75]

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 *in vitro* and after HCT due to alloreactivity.[75] 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 *in vivo* after HCT.[75] 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.[96] 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.[67-70,154] IL-33 amplifies the expression of M2 markers on murine macrophages.[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.[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.[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).[157] IL-33/ST2 signaling also promotes mast cell activation and maturation, as IL-33 treatment of $CD34^+$ mast cell precursors accelerated their maturation *in vitro* and induced GM-CSF, IL-5, IL-13, CXCL8, CCL17, CCL22, and CCL2 secretion.[158,159] These cytokine and chemokine secretions may be NFAT and AP-1 signaling-dependent.[160] It is well documented that mast cells can produce a variety of type 2 cytokines after ST2 signaling;[24,161,162] however, IL-33/ST2 signaling on mast cells during airway inflammation has also been shown to promote a Th17 response.[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.[163] Basophils can also release sST2 after activation via IL-3 and C5a or anti-FcεRIα antibody, while IL-33 prevents sST2 release.[163] IL-33 induces the degranulation of eosinophils and production of superoxide,[69] controls their responsiveness to Siglec 8,[24] and increases IL-13, TGF-β, CCL3, CCL17, and CCL24 in the lungs during airway inflammation.[164] Treatment with anti-ST2 antibodies prevented the upregulation of CD11b and also decreased the survival of eosinophils.[70]

1.6.4: Dendritic cells

Dendritic cells (DCs) express low basal levels of ST2 on their cell surface;[154] however, activation of DCs with rapamycin strongly upregulates ST2 through autocrine IL-1β signaling.[165] Treatment of DCs with IL-33 has been shown to increase surface levels of MHC-II, CD40, CD80, CD86, OX40L, and CCR7.[35,154,166] IL-33/ST2 signaling in DCs also increases their production of IL-4, IL-5, IL-13, CCL17, TNF-α, and IL-1β.[35] 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.[154,166] 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.[167] 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,[126] 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.[75]

1.6.5: Neutrophils

While ST2 has been shown to be present on neutrophils,[70,168] 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.[168] IL-33 injected into the ears of mice induced neutrophil recruitment to the skin;[169] 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.[38-41] However, in IBD patients, ST2 expression in the colonic mucosa remained similar to that of healthy patients.[38] In the lamina propria of active ulcerative colitis (UC) patients, ST2 predominately came from $CD11b^+$ and $CD4^+$ cells.[39] 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,[30] 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.[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.[80] Protection was shown to be through upregulation of type 2 cytokines, $Foxp3^+$ 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.[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.[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.[173] Given this data, selective treatment of $ST2^+$ 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.[174] Discovering prognostic and diagnostic biomarkers for GVHD has been successful with sST2 being one of the most validated to date.[42-49] 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,[75] 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.[121] 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.[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-α and IL-1β signaling.[50] However, this data does not implicate sST2 as a mediator of disease but rather a biomarker of occurring transplant rejection.

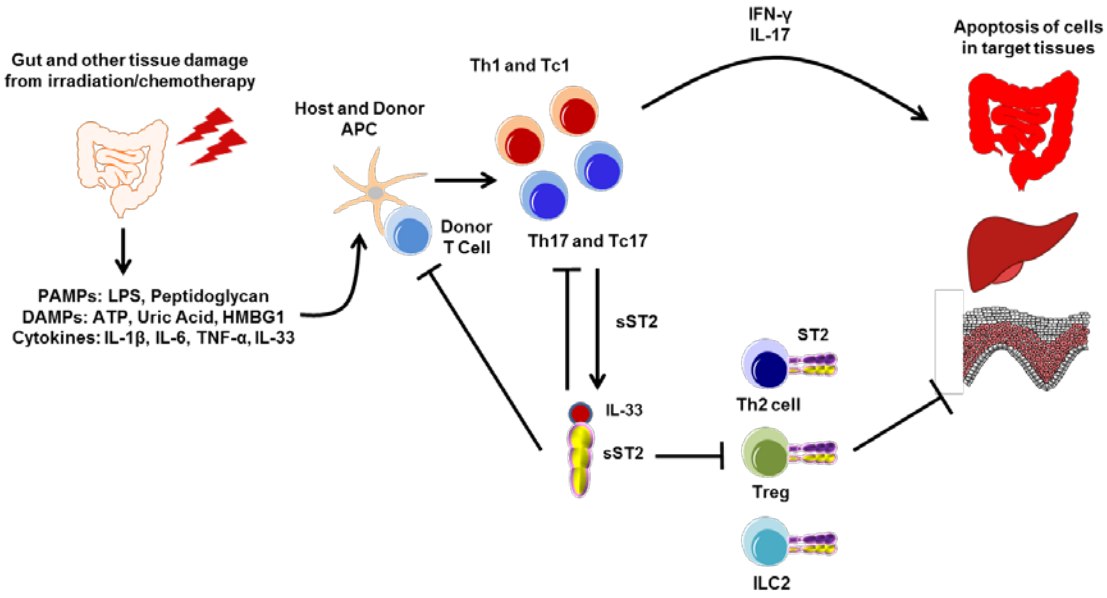

Gut and other tissue damage
from irradiation/chemotherapy
PAMPs: LPS, Peptidoglycan
DAMPs: ATP, Uric Acid, HMBG1
Cytokines: IL-1β, IL-6, TNF-α, IL-33
Host and Donor
APC
Donor
T Cell
Th1 and Tc1
Th17 and Tc17
IFN-γ
IL-17
Apoptosis of cells
in target tissues
sST2
IL-33
sST2
ST2
Th2 cell
Treg
ILC2

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.[42] 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.[75]

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.[180] Intrinsic MyD88 signaling in T cells is also important for optimal T cell response to some viral infections.[110,181,182]

MyD88 signaling during aGVHD has been studied in the context of both donor and host dendritic cells with mixed results.[100,154] 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-2^b, CD45.1) and C57BL/6 (H-2^b, CD45.2) mice were purchased from the *In Vivo* Therapeutics Core at the Indiana University School of Medicine. BALB/c (H-2^d, CD45.2), C3H.SW (H-2^b, CD45.2), and B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ (TLR4$^{-/-}$) mice were purchased from Jackson Laboratory. MyD88$^{-/-}$ mice provided by Dr. Steve Kunkel (University of Michigan),[183] ST2$^{-/-}$ mice provided by Dr. Andrew McKenzie (University of Cambridge),[184] and IL-1R$^{-/-}$ mice provided by Dr. Travis Jerde (Indiana University Purdue University of Indianapolis)[185] 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 (^{137}Cs 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 (5×10^6) plus WT, MyD88$^{-/-}$, IL-1R$^{-/-}$, TLR4$^{-/-}$, or ST2$^{-/-}$ splenic total T cells (1×10^6 for BALB/c and 2×10^6 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 850*g*. 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 800*g* 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.[75] 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 2×10^3 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′-CTCTGGCTCCTAGCACCATGAAGA-3′; actin reverse, 5′- GTAAAACGCAGCTCAGTAACAGTCCG-3′; ST2 forward, 5′-AAGGCACACCATAAGGCTGA-3′; ST2 reverse, 5′-TCGTAGAGCTTGCCATCGTT-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

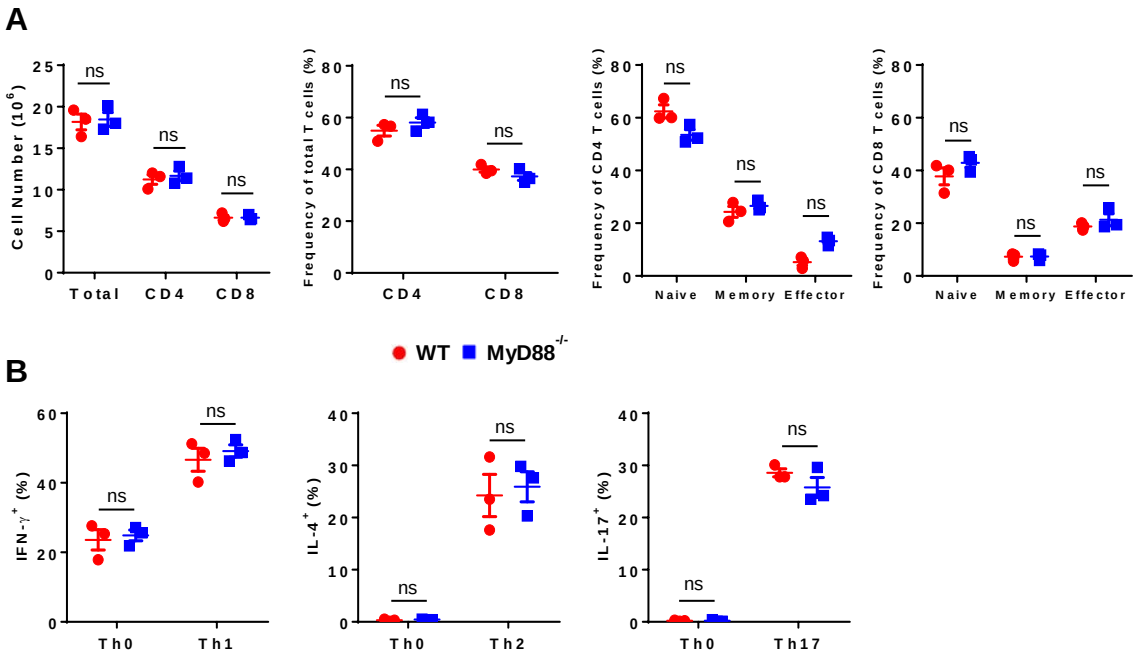
when comparing multiple groups. All statistical analyses were performed using GraphPad Prism, version 7.02. Data in graphs represent mean ± standard error of the mean (SEM). P values less than 0.05 were considered significant.

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.


A
Cell Number (10^6)
25
20
15
10
5
0
ns
ns
ns
Total
CD4
CD8
Frequency of total T cells (%)
80
60
40
20
0
ns
ns
CD4
CD8
Frequency of CD4 T cells (%)
80
60
40
20
0
ns
ns
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Naive
Memory
Effector
Frequency of CD8 T cells (%)
80
60
40
20
0
ns
ns
ns
Naive
Memory
Effector
WT
MyD88-/-
B
IFN-γ+ (%)
60
40
20
0
ns
ns
Th0
Th1
IL-4+ (%)
40
30
20
10
0
ns
ns
Th0
Th2
IL-17+ (%)
40
30
20
10
0
ns
ns
Th0
Th17

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).

Table 1. aGVHD Clinical Score Assessment in Transplanted Mice

Criteria	Grade 0	Grade 0.5	Grade 1.0	Grade 1.5	Grade 2.0
Weight loss	<10%	N/A	10-24.9%	N/A	≥25%
Posture	No hunch	Slight hunch, straightens when walks	Animal stays hunched when walks	Animal does not straighten out	Animal tends to stand on rear toes.
Mobility	Very mobile, hard to catch	Slower than naïve, easier to catch	Not moving, but will move when poked	Not moving, moves slightly when poked	Not moving, won't move if poked
Skin	No redness, abrasions, lesions or scaling present	Redness in one area only	Abrasions in 1 area, or mild abrasions in 2 areas	Bad abrasions in 2 areas	Extremely bad abrasion, cracking skin, dried blood etc.
Fur	No fur pathology	Ridging on the side of belly or nape of neck	Ridging across or the side of belly + neck	Unkempt matted and ruffled fur	Badly matted fur on belly, and on top

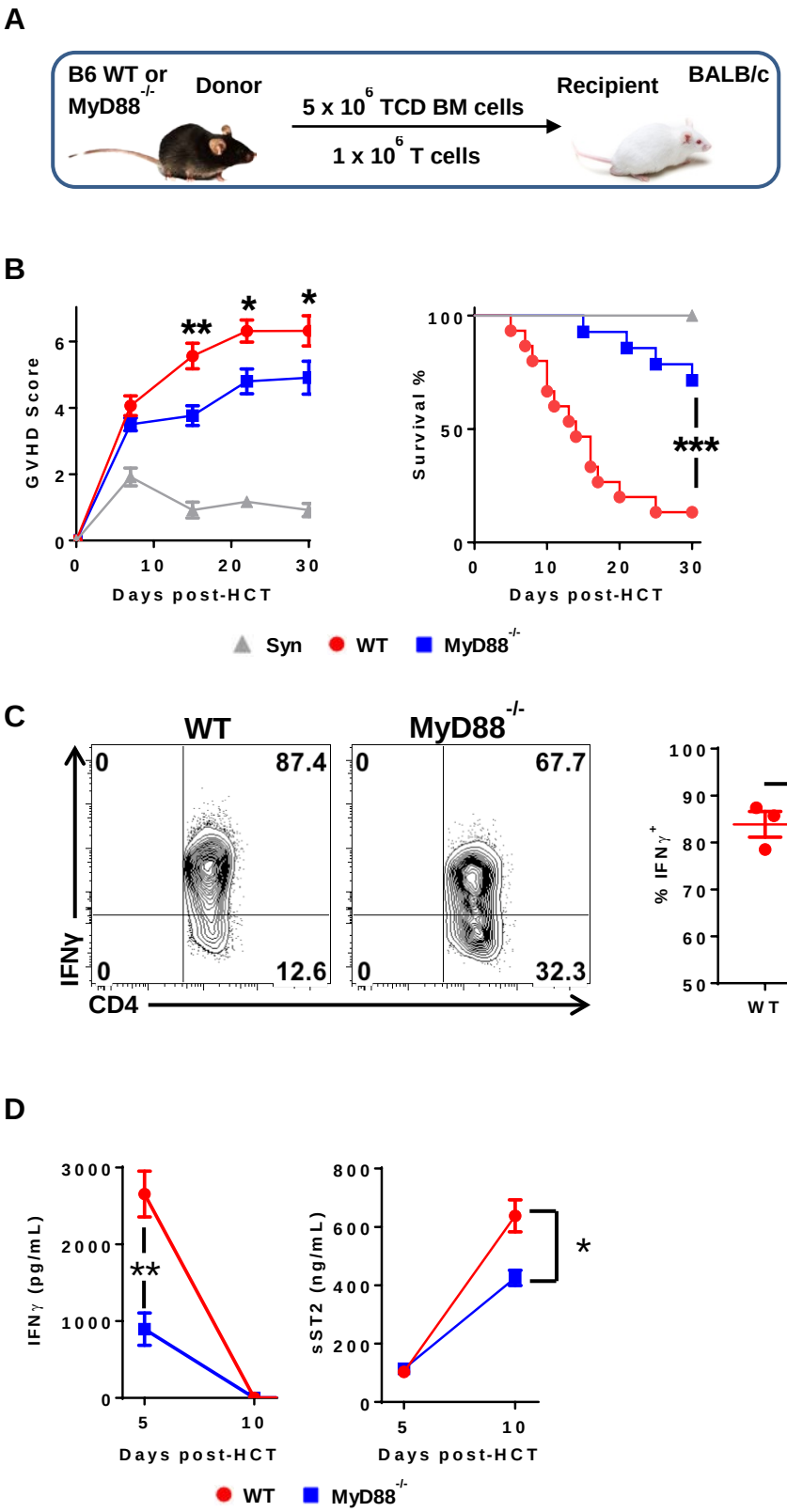

A
B6 WT or MyD88-/-
Donor
5 x 10^6 TCD BM cells
1 x 10^6 T cells
Recipient
BALB/c
B
GVHD Score
Days post-HCT
Survival %
Days post-HCT
Syn
WT
MyD88-/-
C
WT
MyD88-/-
87.4
12.6
67.7
32.3
IFNγ
CD4
% IFNγ+
WT
MyD88-/-
D
IFNγ (pg/mL)
Days post-HCT
sST2 (ng/mL)
Days post-HCT
WT
MyD88-/-

Figure 7. aGVHD assessment using WT or MyD88$^{-/-}$ donor T cells in a major MHC mismatch model

(A) Schematic for allo-transplantation using WT or MyD88$^{-/-}$ 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 10^6 TCD-BM cells and 1 x 10^6 donor T cells 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 (Syn). aGVHD score (left) and survival (right); BALB/c → BALB/c (n=5), B6 WT (n=15), or B6 MyD88$^{-/-}$ 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$^{-/-}$ 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. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

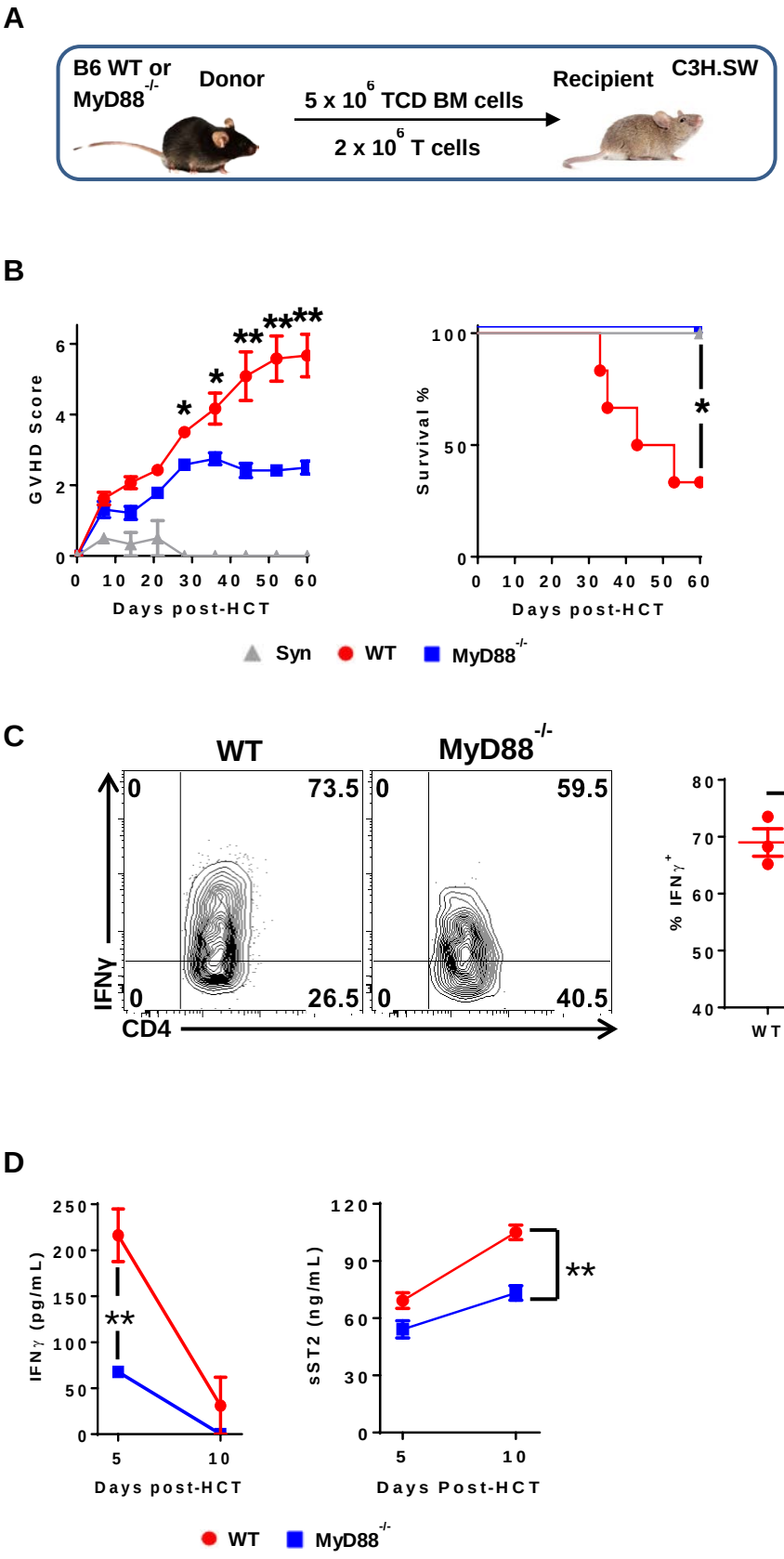

A
B6 WT or MyD88-/-
Donor
5 x 10^6 TCD BM cells
2 x 10^6 T cells
Recipient
C3H.SW
B
GVHD Score
Days post-HCT
Survival %
Days post-HCT
Syn
WT
MyD88-/-
C
WT
MyD88-/-
IFNγ
CD4
0
73.5
26.5
59.5
40.5
% IFNγ+
WT
MyD88-/-
D
IFNγ (pg/mL)
Days post-HCT
sST2 (ng/mL)
Days Post-HCT
WT
MyD88-/-

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 x 10^6 TCD-BM cells and 2 x 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$^{-/-}$ 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$^{-/-}$ T cells had a defect in proliferation, apoptosis, or migration following HCT, we stained the CD45.1 WT T cells and CD45.2 MyD88$^{-/-}$ 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 α4β7 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.[186,187] Th2[188] and Treg[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$^{-/-}$ donor T cells is different from WT donor T cells.

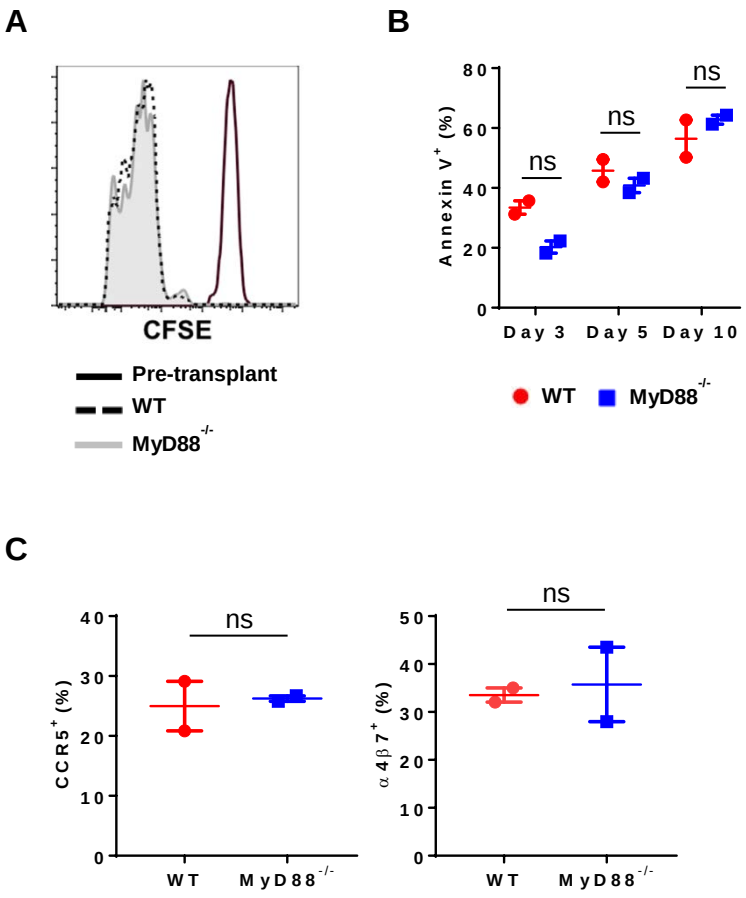

A
B
CFSE
Pre-transplant
WT
MyD88-/-
Annexin V+ (%)
ns
ns
ns
Day 3
Day 5
Day 10
WT
MyD88-/-
C
CCR5+ (%)
ns
WT
MyD88-/-
α4β7+ (%)
ns
WT
MyD88-/-

Figure 9. Proliferation, apoptosis, and migration of transplanted WT vs MyD88$^{-/-}$ T cells

Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10^6 TCD-BM cells and 1 x 10^6 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).

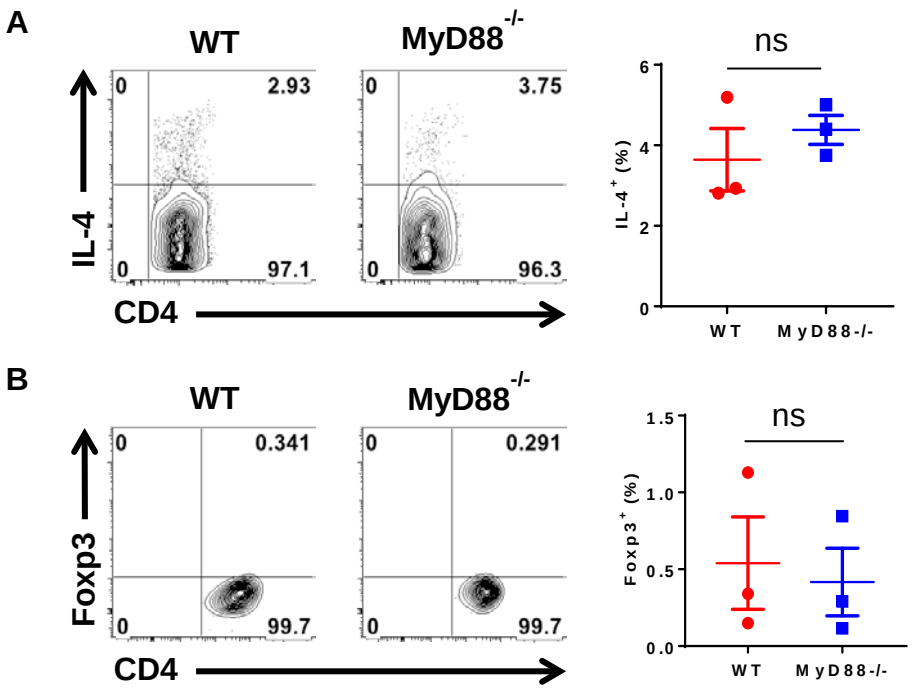

A
WT
MyD88-/-
0
2.93
97.1
3.75
96.3
IL-4
CD4
ns
IL-4+ (%)
6
4
2
0
WT
MyD88-/-
B
WT
MyD88-/-
0.341
99.7
0.291
99.7
Foxp3
CD4
ns
Foxp3+ (%)
1.5
1.0
0.5
0.0
WT
MyD88-/-

Figure 10. Th2 and Treg frequencies in the intestine of mice receiving WT or MyD88$^{-/-}$ donor T cells

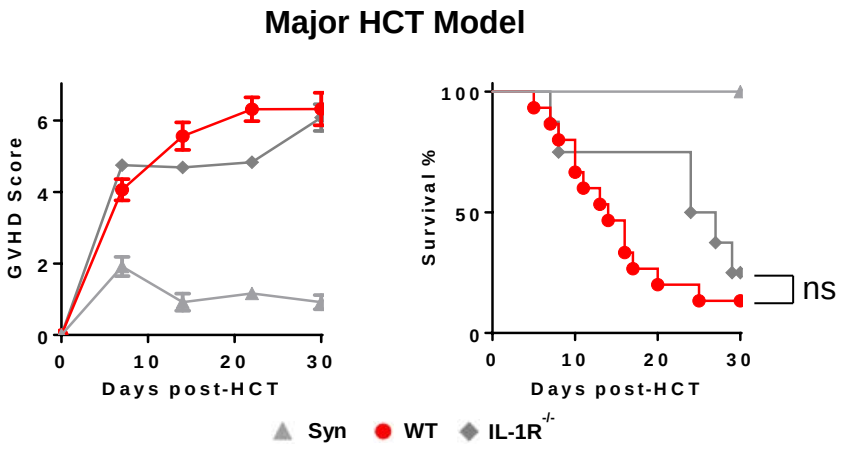
Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10^6 TCD-BM cells and 1 x 10^6 donor T cells from WT or MyD88$^{-/-}$ B6 mice for allogeneic transplant. T cells were harvested from the intestine at day 10 post-HCT and stained for expression of (A) CD4 and Foxp3 or (B) CD4 and IL-4 (mean ± SEM, 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-γ 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
GVHD Score
Days post-HCT
Survival %
ns
Syn
WT
IL-1R-/-

B

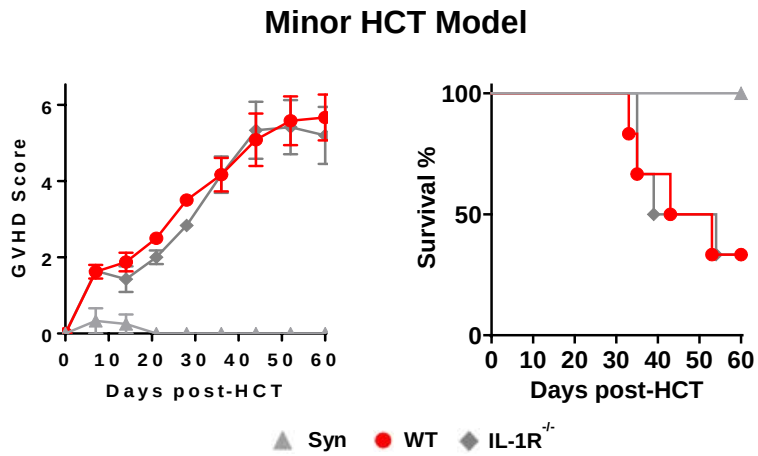
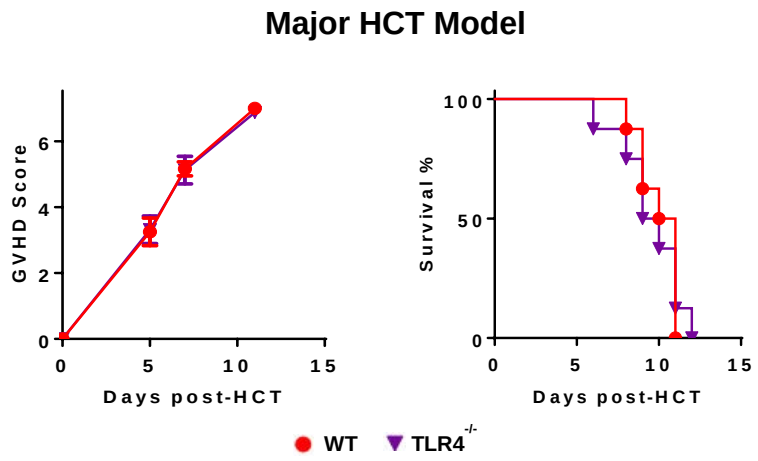

Minor HCT Model
GVHD Score
Days post-HCT
Survival %
Syn
WT
IL-1R-/-

Figure 11. IL-1R$^{-/-}$ donor T cells in aGVHD

(A) 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 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 10^6 TCD-BM cells and 2 x 10^6 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
GVHD Score
0
2
4
6
0
5
10
15
Days post-HCT
Survival %
0
50
100
0
5
10
15
Days post-HCT
WT
TLR4-/-

B

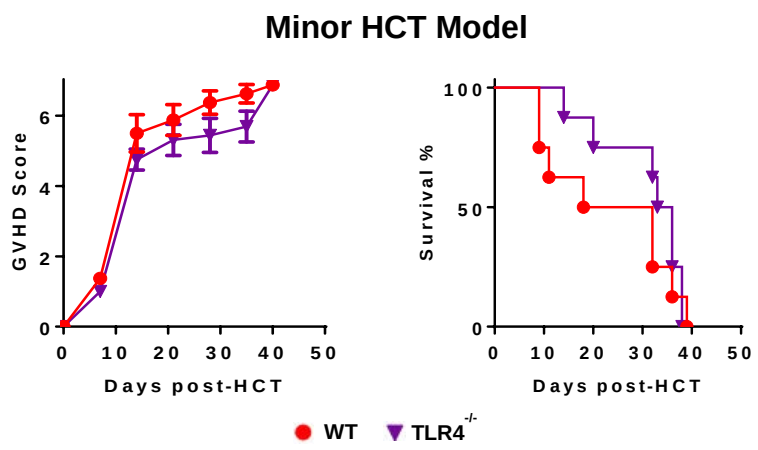

Minor HCT Model
GVHD Score
0
2
4
6
0
10
20
30
40
50
Days post-HCT
Survival %
0
50
100
0
10
20
30
40
50
Days post-HCT
WT
TLR4-/-

Figure 12. TLR4$^{-/-}$ donor T cells in aGVHD

(A) 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 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 10^6 TCD-BM cells and 2 x 10^6 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.[180,181,194,195] 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.[196,197] 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.[198] We next explored the cell-intrinsic role of MyD88$^{-/-}$ Tregs in aGVHD. We did not observe a difference in survival using using WT Tcons with WT or MyD88$^{-/-}$ Tregs (median survival time: WT Tregs - 26 days; MyD88$^{-/-}$ Tregs - 24 days; Figure 14a). Intrinsic MyD88 signaling in CD4 T cells has also been implicated in mounting a proper antiviral response.[182] After transplanting Treg-depleted WT or MyD88$^{-/-}$ Tcons, we did not observe a difference in aGVHD severity or mortality (median survival time: WT Tcon - 10 days; MyD88$^{-/-}$ Tcon - 10 days; Figure 14b). These data indicate that intrinsic MyD88 signaling in donor T cells does not impact aGVHD.

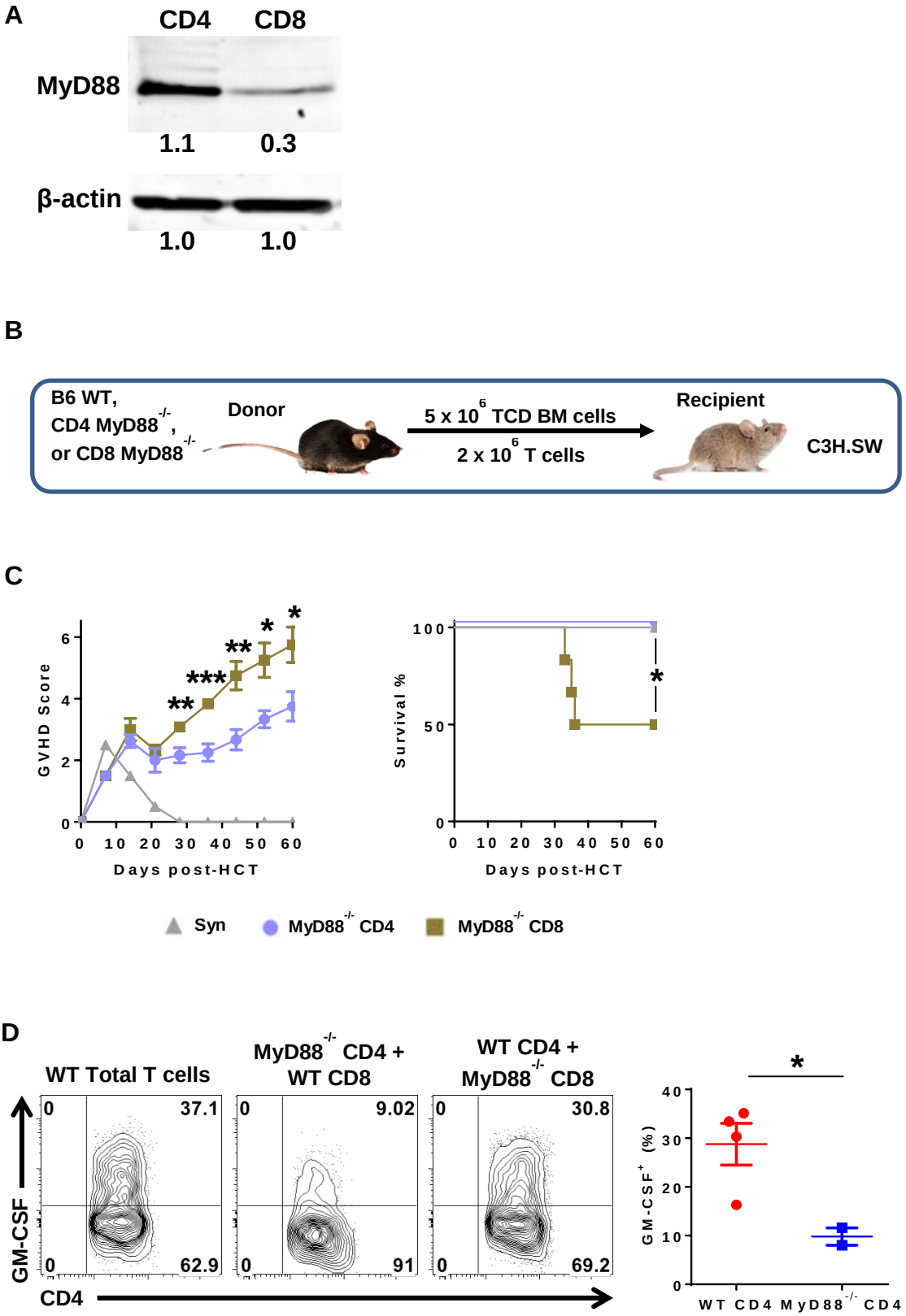
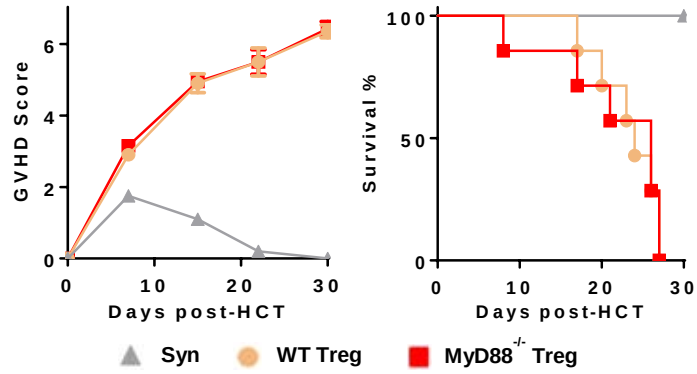

A
CD4
CD8
MyD88
1.1
0.3
β-actin
1.0
1.0
B
B6 WT,
CD4 MyD88-/-,
or CD8 MyD88-/-
Donor
5 x 10^6 TCD BM cells
2 x 10^6 T cells
Recipient
C3H.SW
C
GVHD Score
Days post-HCT
Survival %
Days post-HCT
Syn
MyD88-/- CD4
MyD88-/- CD8
D
WT Total T cells
MyD88-/- CD4 + WT CD8
WT CD4 + MyD88-/- CD8
GM-CSF
CD4
37.1
62.9
9.02
91
30.8
69.2
GM-CSF+ (%)
WT 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


GVHD Score
0
2
4
6
0
10
20
30
Days post-HCT
Survival %
100
50
0
0
10
20
30
Days post-HCT
Syn
WT Treg
MyD88-/- Treg

B

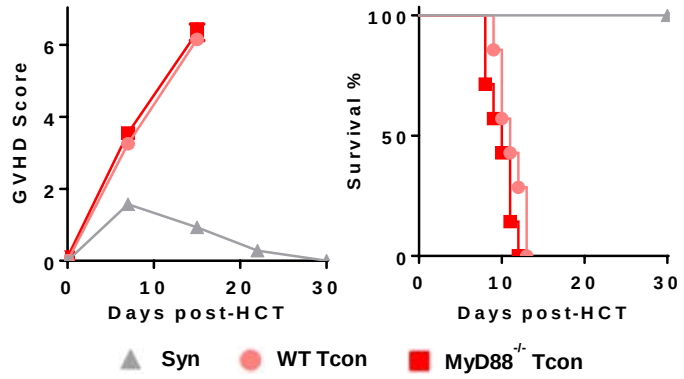
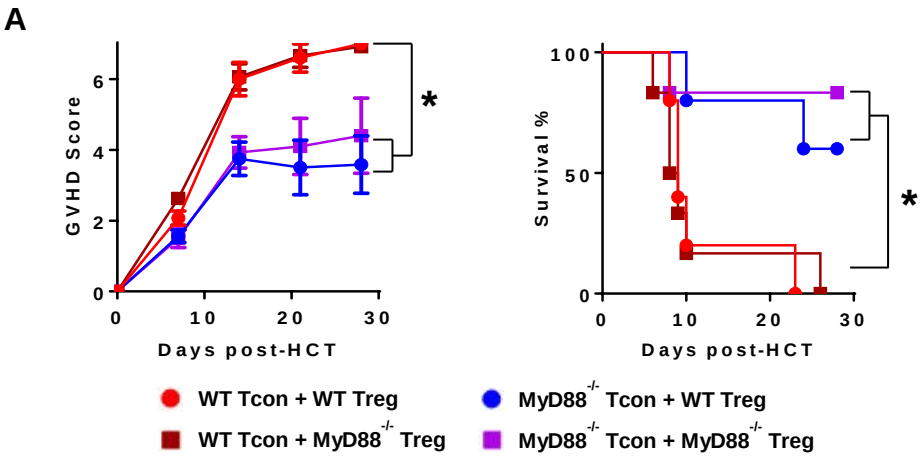

GVHD Score
0
2
4
6
0
10
20
30
Days post-HCT
Survival %
100
50
0
0
10
20
30
Days post-HCT
Syn
WT Tcon
MyD88-/- Tcon

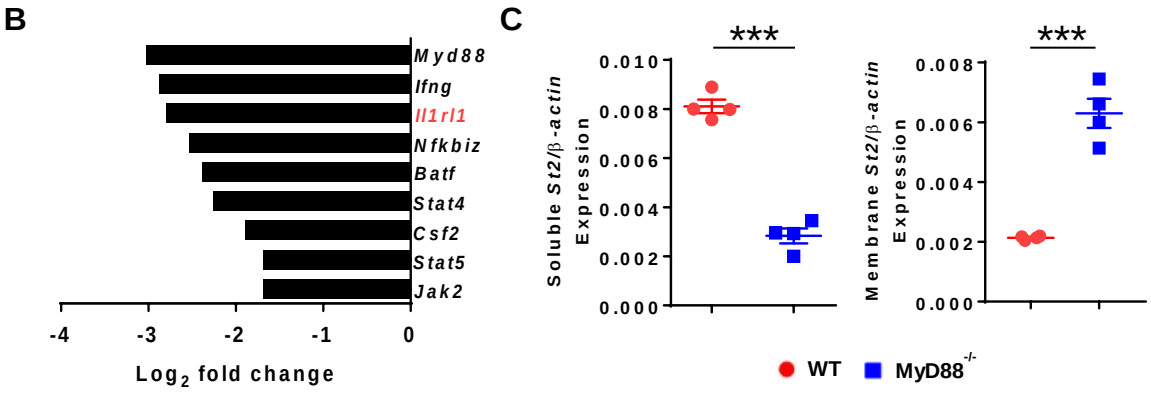
Figure 14. Intrinsic MyD88 signaling in Tcons and Tregs during aGVHD

(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.[180] 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).


A
GVHD Score
Days post-HCT
Survival %
Days post-HCT
WT Tcon + WT Treg
MyD88-/- Tcon + WT Treg
WT Tcon + MyD88-/- Treg
MyD88-/- Tcon + MyD88-/- Treg


B
Myd88
Ifng
Il1rl1
Nfkbiz
Batf
Stat4
Csf2
Stat5
Jak2
Log2 fold change
C
Soluble St2/β-actin Expression
Membrane St2/β-actin Expression
WT
MyD88-/-

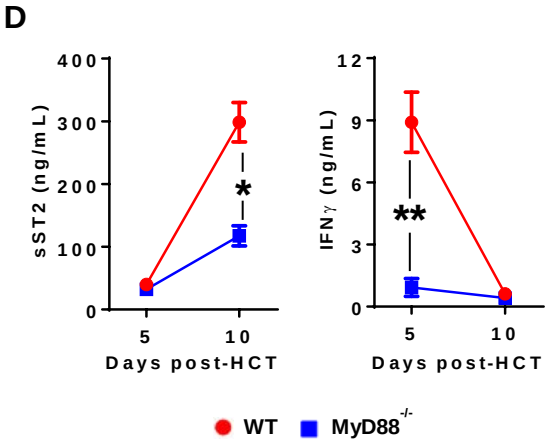

D
sST2 (ng/mL)
Days post-HCT
IFNγ (ng/mL)
Days post-HCT
WT
MyD88-/-

Figure 15. MyD88$^{-/-}$ Tcons versus MyD88$^{-/-}$ Tregs during aGVHD

(A) Lethally irradiated BALB/c mice (900 cGy) were given 5 x 10^6 TCD-BM cells and a 10:1 mixture of WT or MyD88$^{-/-}$ Tcon + WT or MyD88$^{-/-}$ Tregs totaling 1 x 10^6 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 10^6 TCD-BM cells and 1 x 10^6 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,[75] and that targeting ST2/MyD88 signaling in Tcons could alleviate aGVHD while sparing Treg function.

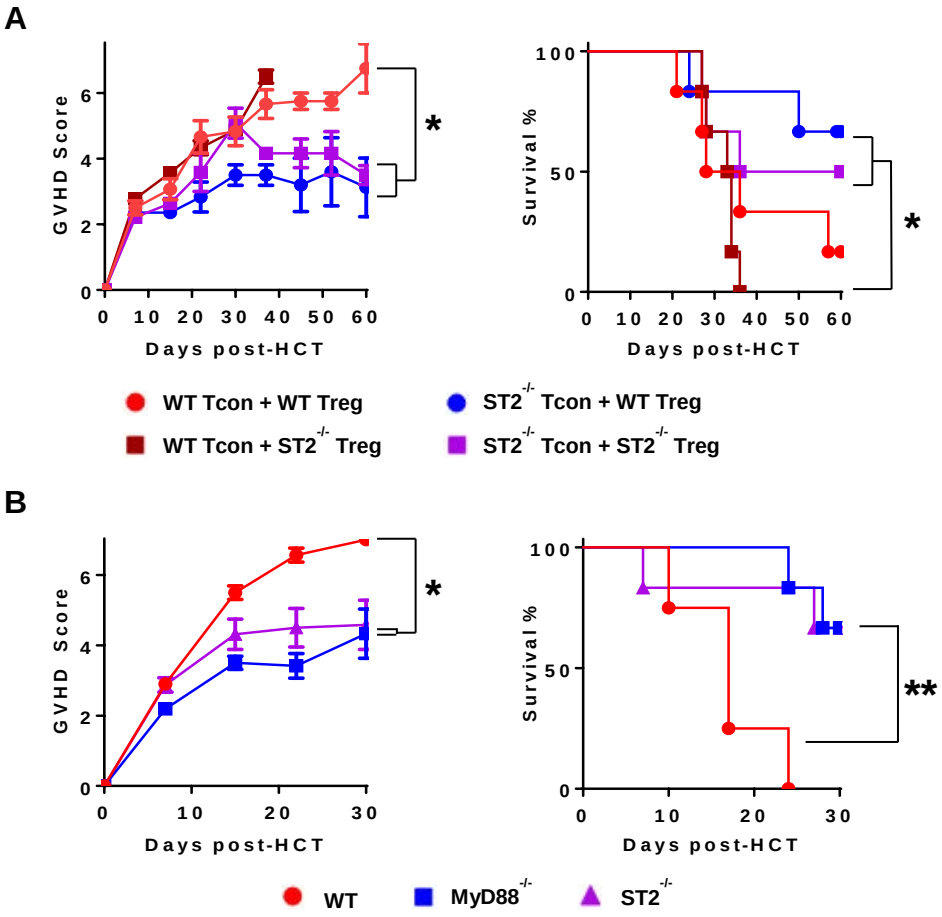

A
GVHD Score
Days post-HCT
Survival %
Days post-HCT
*
*
WT Tcon + WT Treg
ST2-/- Tcon + WT Treg
WT Tcon + ST2-/- Treg
ST2-/- Tcon + ST2-/- Treg
B
GVHD Score
Days post-HCT
Survival %
Days post-HCT
*
**
WT
MyD88-/-
ST2-/-

Figure 16. ST2 vs MyD88 signaling during aGVHD

(A) Lethally irradiated C3H.SW mice (1100 cGy) were given 5 x 10^6 TCD-BM cells and a 10:1 mixture of WT or $ST2^{-/-}$ Tcon + WT or $ST2^{-/-}$ Tregs totaling 2 x 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 x 10^6 TCD-BM cells and 1 x 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 *in vivo* using the small molecule inhibitor ST2825, which prevents the homodimerization of MyD88 at their TIR domain, preventing downstream signaling.[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 → BALB/c model (Figure 17).

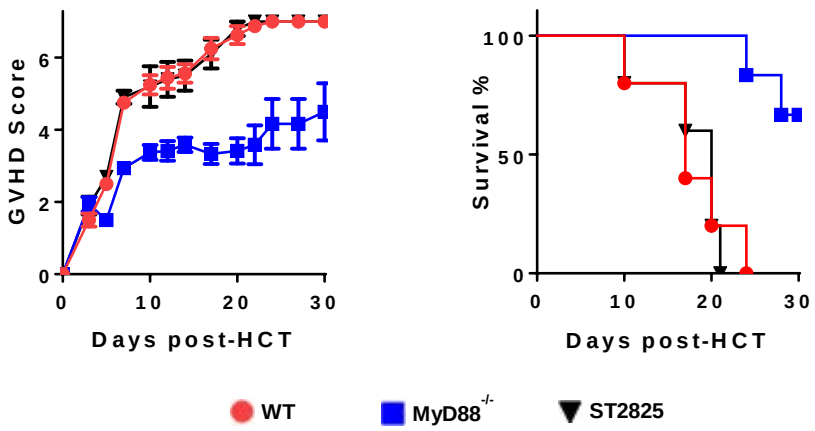

GVHD Score
0
2
4
6
0
10
20
30
Days post-HCT
Survival %
0
50
100
0
10
20
30
Days post-HCT
WT
MyD88-/-
ST2825

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

CHAPTER 4: DISCUSSION

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.[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[180,192] and TLR4/MyD88[193] signaling in promoting an optimal T cell response *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$^{-/-}$ Tcons in the presence of Tregs, suggesting that loss of MyD88 in Tcons sensitizes them to Treg-mediated suppression. Strikingly, results using ST2$^{-/-}$ donor Tcons mimic the MyD88$^{-/-}$ 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$^{b/d}$) 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$^{-/-}$ donor T cells alleviated aGVHD in a major-mismatch model.[192] However, we have found that IL-1R$^{-/-}$ 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$^{-/-}$ or MyD88$^{-/-}$ T cells into RAG$^{-/-}$ mice and infecting with vaccinia virus, mice with IL-1R$^{-/-}$ CD8 T cells were able to respond to the infection normally while mice with MyD88$^{-/-}$ CD8 T cells mounted a reduced response.[200] Similar results were found during LCMV infection in CD8 T cells.[181] Looking at WT, MyD88$^{-/-}$, and IL-1R$^{-/-}$ CD4 T cells in absence of CD8 T cells in response to LCMV infection, WT and IL-1R$^{-/-}$ mice developed wasting disease and had lower virus levels while MyD88$^{-/-}$ 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.[182] 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.[204] 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^{b} tetramer-positive T cells but not 2W:I-A^{b} 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.[154,205-207] The mice from commercial vendors which are purchased for experiments can have significantly different microbiotas which can impact immune response.[208] Aberrant IL-1/IL-1R signaling has been shown to alter the microbiota in mice.[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.[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 αCD3 stimulation in the presence of αCD28 leads to no noticeable difference between WT and MyD88$^{-/-}$ T cell differentiation and cytokine production *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$^{-/-}$ 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$^{-/-}$ as donors does indeed protect against aGVHD.[193] We believe this difference is caused by the use of TLR4$^{-/-}$ BM and TLR4$^{-/-}$ T cells together compared to our use of WT BM and TLR4$^{-/-}$ 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$^{-/-}$ on these dendritic cells.

MyD88 signaling has been shown to be important in both CD4 and CD8 T cell responses to viruses, as MyD88$^{-/-}$ T cells show impaired anti-viral clearance;[181,182,200] however, its role in CD4 and CD8 T cells during aGVHD is not known. When transplanting WT CD4 T cells with MyD88$^{-/-}$ CD8 T cells, we observed no difference in aGVHD severity or mortality. When transplanting MyD88$^{-/-}$ 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$^{-/-}$ CD4 T cells have impaired function during coronavirus encephalomyelitis while MyD88$^{-/-}$ CD8 T cells appear normal.[218] Transcriptome analysis using Nanostring of CD4^{+} 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$^{-/-}$ T cells. Interestingly, GM-CSF production was lower in MyD88$^{-/-}$ 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.[219] 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.[220] 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.[220] 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.[180] Meanwhile, it has also been shown that MyD88$^{-/-}$ Tregs during skin transplantation and cGVHD are deficient in their suppressive capabilities.[198]

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.[221-223] It has been suggested that this is due to blocking of Treg-mediated inhibition of IL-2Rα on Tcons.[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.[224] Interestingly, similar to our data with MyD88$^{-/-}$ 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$^{-/-}$ 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.[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.[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.[211,227,228] Indeed, pSTAT3 Y705 is increased significantly in patient CD4 T cells before onset of aGVHD.[229] IL-7 and IL-15 may also play a role in Tcons-resistance to Tregs during aGVHD.[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.[233] 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.[234] It has been suggested that hyper-activation of PI3K leads to Tcon resistance to Treg-mediated suppression.[235] Indeed, in murine models that have genetic deficiencies in proteins that negatively regulate PI3K signaling, Tcons are more resistant to Treg-mediated suppression.[236,237] Several cytokine receptors, TNF receptors, TLRs, and T cell costimulatory receptors have been shown to activate PI3K signaling.[238] 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.[239] 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.[240] 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.

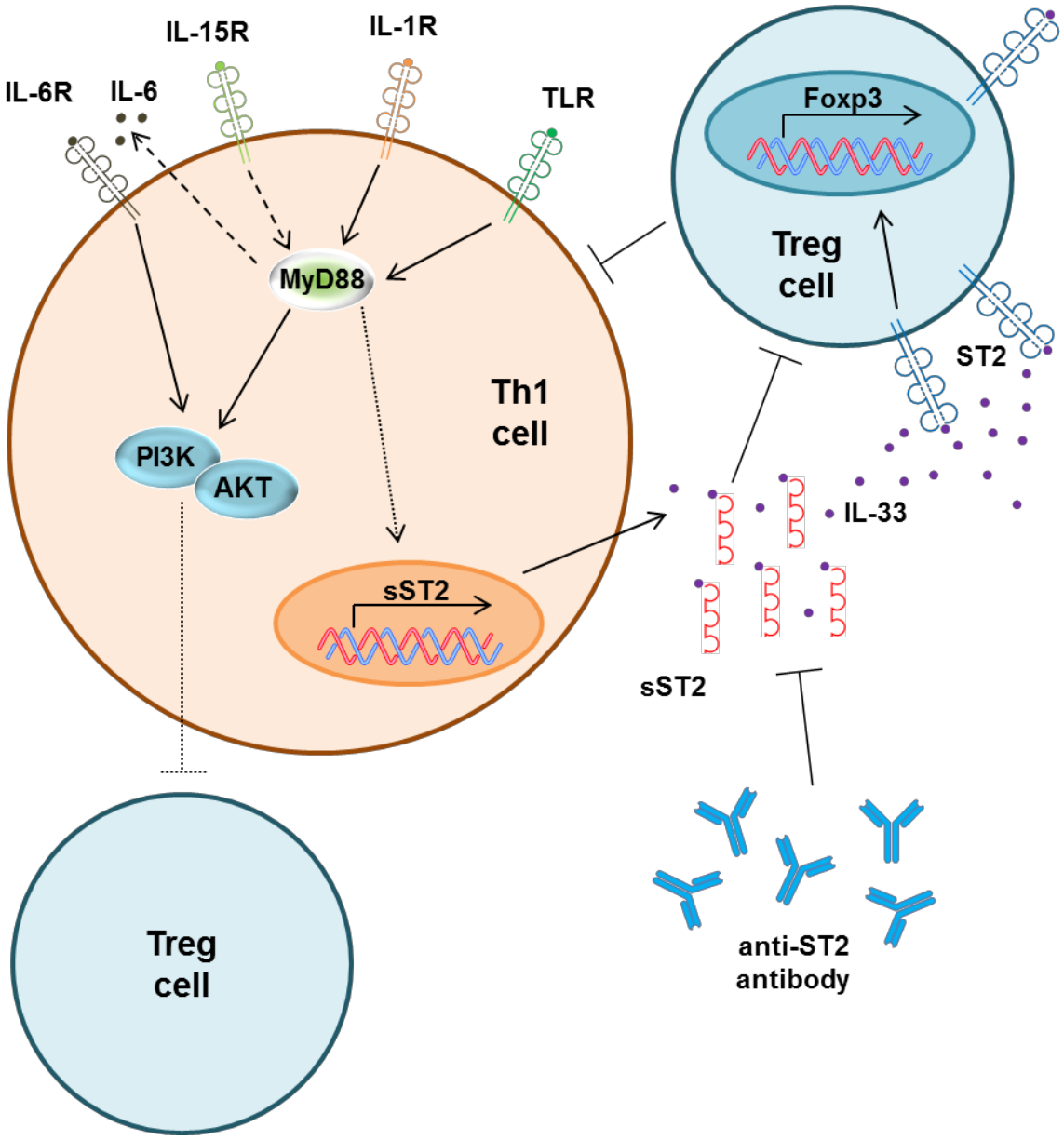

IL-15R
IL-1R
IL-6R
IL-6
TLR
MyD88
PI3K
AKT
Th1
cell
sST2
Foxp3
Treg
cell
ST2
IL-33
sST2
anti-ST2
antibody
Treg
cell

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[30,86,111,112] and increased *Foxp3* expression in Tregs.[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.[75] We and others have shown that total ST2$^{-/-}$ T cells ameliorate aGVHD.[75,121] We asked whether the phenotype of MyD88$^{-/-}$ Tcons in the presence of WT or MyD88$^{-/-}$ Tregs observed before would be phenocopied when using ST2$^{-/-}$ Tcons and WT or ST2$^{-/-}$ Tregs. Indeed, transplanting ST2$^{-/-}$ Tcons alleviated aGVHD in a similar manner regardless of using WT or ST2$^{-/-}$ 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$^{-/-}$ Tregs with WT or ST2$^{-/-}$ Tcons compared to using WT Tregs with WT or ST2$^{-/-}$ Tcons, suggesting that loss of ST2 on Tregs may impact their suppressive capabilities. This would be in line with what has previously been shown.[65,241] We also show that isolated MyD88$^{-/-}$ 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.[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. [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.[243] The effect of IL-33 on Th1 differentiation was later confirmed using an OVA-immunization murine model as well as human *in vitro* cell cultures.[147] The expression of ST2 on the surface only occurred during times of inflammation.[243] During aGVHD, IL-33 administration during peak inflammatory response (days 3-7 post-HCT) enhanced aGVHD severity and mortality,[121] while IL-33 administration during the peri-transplant period ameliorated aGVHD through enhanced $ST2^+$ Treg response.[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.[199,244-247] This compound works by preventing MyD88 TIR domain homodimerization.[199] Treatment of human B cells with ST2825 prevented their proliferation when stimulated with CpG *in vitro*.[199] As well, addition of ST2825 to astragalus polysaccharide-stimulated RAW 264.7 macrophage cells prevented their secretion of proinflammatory cytokines.[247] *In vivo* treatment with this inhibitor has been tested in an experimental acute myocardial infarction model,[244] experimental traumatic brain injury model,[245] and an experimental seizure model;[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,[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).[248] Studying the pharmacokinetics and adapting the dose could lead to protection against aGVHD.[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.[250] MyD88 is also a well-known for its role in anti-infection immunity against intracellular pathogens.[251] 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,[75] 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.[243] 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.[243] 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.[147] 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.[75] 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^{tm1Wjl}/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.

REFERENCES

1 Murphy, K., Janeway, C. A. & Mowat, A. *Janeway's immunobiology*. (Garland Science, 2012).

2 Medzhitov, R. & Janeway, C., Jr. Innate immune recognition: mechanisms and pathways. *Immunological reviews* **173**, 89-97. (2000).

3 Janeway, C. A., Jr. & Medzhitov, R. Innate immune recognition. *Annual review of immunology* **20**, 197-216, doi:10.1146/annurev.immunol.20.083001.084359 (2002).

4 Nguyen, L. T., Haney, E. F. & Vogel, H. J. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends in biotechnology* **29**, 464-472, doi:10.1016/j.tibtech.2011.05.001 (2011).

5 Owen, J. A., Punt, J., Stranford, S. A., Jones, P. P. & Kuby, J. *Kuby immunology*. (W.H. Freeman, 2013).

6 Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nature reviews. Immunology* **13**, 159-175, doi:10.1038/nri3399 (2013).

7 Rothenberg, M. E. & Hogan, S. P. The eosinophil. *Annual review of immunology* **24**, 147-174, doi:10.1146/annurev.immunol.24.021605.090720 (2006).

8 Siracusa, M. C., Kim, B. S., Spergel, J. M. & Artis, D. Basophils and allergic inflammation. *The Journal of allergy and clinical immunology* **132**, 789-801; quiz 788, doi:10.1016/j.jaci.2013.07.046 (2013).

9 Starr, T. K., Jameson, S. C. & Hogquist, K. A. Positive and negative selection of T cells. *Annual review of immunology* **21**, 139-176, doi:10.1146/annurev.immunol.21.120601.141107 (2003).

10 Melchers, F. *et al.* Positive and negative selection events during B lymphopoiesis. *Current opinion in immunology* **7**, 214-227 (1995).

11 Luckheeram, R. V., Zhou, R., Verma, A. D. & Xia, B. CD4(+)T cells: differentiation and functions. *Clinical & developmental immunology* **2012**, 925135, doi:10.1155/2012/925135 (2012).

12 Dustin, M. L. & Long, E. O. Cytotoxic immunological synapses. *Immunological reviews* **235**, 24-34, doi:10.1111/j.0105-2896.2010.00904.x (2010).

13 Thieu, V. T. *et al.* Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. *Immunity* **29**, 679-690, doi:10.1016/j.immuni.2008.08.017 (2008).

14 Omori, M. & Ziegler, S. Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. *Journal of immunology* **178**, 1396-1404 (2007).

15 Jang, S., Morris, S. & Lukacs, N. W. TSLP promotes induction of Th2 differentiation but is not necessary during established allergen-induced pulmonary disease. *PloS one* **8**, e56433, doi:10.1371/journal.pone.0056433 (2013).

16 Wang, Q., Du, J., Zhu, J., Yang, X. & Zhou, B. TSLP Signaling in CD4(+) T cells is Required for Th2 Memory. *The Journal of allergy and clinical immunology* **135**, 781-791 e783 (2015).

17 Purwar, R. *et al.* Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. *Nature medicine* **18**, 1248-1253, doi:10.1038/nm.2856 (2012).

18 Kaplan, M. H. Th9 cells: differentiation and disease. *Immunological reviews* **252**, 104-115, doi:10.1111/imr.12028 (2013).

19 Soroosh, P. & Doherty, T. A. Th9 and allergic disease. *Immunology* **127**, 450-458, doi:10.1111/j.1365-2567.2009.03114.x (2009).

20 Zambrano-Zaragoza, J. F., Romo-Martinez, E. J., Duran-Avelar Mde, J., Garcia-Magallanes, N. & Vibanco-Perez, N. Th17 cells in autoimmune and infectious diseases. *International journal of inflammation* **2014**, 651503, doi:10.1155/2014/651503 (2014).

21 Shevach, E. M. & Thornton, A. M. tTregs, pTregs, and iTregs: similarities and differences. *Immunological reviews* **259**, 88-102, doi:10.1111/imr.12160 (2014).

22 Burchill, M. A., Yang, J., Vogtenhuber, C., Blazar, B. R. & Farrar, M. A. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *Journal of immunology* **178**, 280-290 (2007).

23 Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T cells and immune tolerance. *Cell* **133**, 775-787, doi:10.1016/j.cell.2008.05.009 (2008).

24 Amin, K. The role of mast cells in allergic inflammation. *Respiratory medicine* **106**, 9-14, doi:10.1016/j.rmed.2011.09.007 (2012).

25 De Silva, N. S. & Klein, U. Dynamics of B cells in germinal centres. *Nature reviews. Immunology* **15**, 137-148, doi:10.1038/nri3804 (2015).

26 Werenskiold, A. K., Hoffmann, S. & Klemenz, R. Induction of a mitogen-responsive gene after expression of the Ha-ras oncogene in NIH 3T3 fibroblasts. *Molecular and cellular biology* **9**, 5207-5214 (1989).

27 Tominaga, S. A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS letters* **258**, 301-304 (1989).

28 Tominaga, S.-i., Ohta, S. & Tago, K. Soluble form of the ST2 gene product exhibits growth promoting activity in NIH-3T3 cells. *Biochemistry and Biophysics Reports* **5**, 8-15, doi:http://dx.doi.org/10.1016/j.bbrep.2015.11.020 (2016).

29 Meisel, C. *et al.* Regulation and function of T1/ST2 expression on CD4+ T cells: induction of type 2 cytokine production by T1/ST2 cross-linking. *Journal of immunology* **166**, 3143-3150 (2001).

30 Schmitz, J. *et al.* IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23**, 479-490, doi:S1074-7613(05)00311-0 [pii]

10.1016/j.immuni.2005.09.015 (2005).

31 Liew, F. Y., Girard, J. P. & Turnquist, H. R. Interleukin-33 in health and disease. *Nature reviews. Immunology* **16**, 676-689, doi:10.1038/nri.2016.95 (2016).

32 Carriere, V. *et al.* IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 282-287, doi:0606854104 [pii] 10.1073/pnas.0606854104 (2007).

33 Moussion, C., Ortega, N. & Girard, J. P. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PloS one* **3**, e3331, doi:10.1371/journal.pone.0003331 (2008).

34 Caselli, C. Inflammation in cardiac disease: focus on Interleukin-33/ST2 pathway. *Inflammation and Cell Signaling* **1**, e149, doi:http://dx.doi.org/10.14800/ics.149 (2014).

35 Ciccone, M. M. *et al.* A novel cardiac bio-marker: ST2: a review. *Molecules (Basel, Switzerland)* **18**, 15314-15328, doi:10.3390/molecules181215314 (2013).

36 Shimpo, M. *et al.* Serum levels of the interleukin-1 receptor family member ST2 predict mortality and clinical outcome in acute myocardial infarction. *Circulation* **109**, 2186-2190, doi:10.1161/01.cir.0000127958.21003.5a (2004).

37 Weinberg, E. O. *et al.* Identification of serum soluble ST2 receptor as a novel heart failure biomarker. *Circulation* **107**, 721-726 (2003).

38 Beltran, C. J. *et al.* Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflammatory bowel diseases* **16**, 1097-1107, doi:10.1002/ibd.21175 (2010).

39 Pastorelli, L. *et al.* Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 8017-8022, doi:0912678107 [pii] 10.1073/pnas.0912678107 (2010).

40 Diaz-Jimenez, D. *et al.* Soluble ST2: a new and promising activity marker in ulcerative colitis. *World J Gastroenterol* **17**, 2181-2190, doi:10.3748/wjg.v17.i17.2181 (2011).

41 Diaz-Jimenez, D. *et al.* Soluble ST2 is a sensitive clinical marker of ulcerative colitis evolution. *BMC gastroenterology* **16**, 103, doi:10.1186/s12876-016-0520-6 (2016).

42 Vander Lugt, M. T. *et al.* ST2 as a Marker for Risk of Therapy-Resistant Graft-versus-Host Disease and Death. *New England Journal of Medicine* **369**, 529-539, doi:10.1056/NEJMoa1213299 (2013).

43 Ponce, D. M. *et al.* High day 28 ST2 levels predict for acute graft-versus-host disease and transplant-related mortality after cord blood transplantation. *Blood* **125**, 199-205, doi:10.1182/blood-2014-06-584789 (2015).

44 McDonald, G. B. *et al.* Plasma biomarkers of acute GVHD and nonrelapse mortality: predictive value of measurements before GVHD onset and treatment. *Blood* **126**, 113-120, doi:10.1182/blood-2015-03-636753 (2015).

45 Levine, J. E. *et al.* A Prognostic Score for Acute Graft-Versus-Host Disease Based on Biomarkers: A Multicenter Study. *Lancet Haematol* **2**, e21-e29, doi:10.1016/S2352-3026(14)00035-0 (2015).

46 Yu, J. *et al.* Biomarker Panel for Chronic Graft-Versus-Host Disease. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **34**, 2583-2590, doi:10.1200/JCO.2015.65.9615 (2016).

47 Abu Zaid, M. *et al.* Plasma biomarkers of risk for death in a multicenter phase 3 trial with uniform transplant characteristics post-allogeneic HCT. *Blood* **129**, 162-170, doi:10.1182/blood-2016-08-735324 (2017).

48 Hartwell, M. J. *et al.* An early-biomarker algorithm predicts lethal graft-versus-host disease and survival. *JCI insight* **2**, e89798, doi:10.1172/jci.insight.89798 (2017).

49 Kanakry, C. G. *et al.* Plasma-derived proteomic biomarkers in HLA-haploidentical or HLA-matched bone marrow transplantation using post-transplantation cyclophosphamide. *Haematologica*, doi:10.3324/haematol.2016.152322 (2017).

50 Mathews, L. R. *et al.* Elevated ST2 Distinguishes Incidences of Pediatric Heart and Small Bowel Transplant Rejection. *American journal of transplantation : official journal of the American Society of Transplantation*

and the American Society of Transplant Surgeons **16**, 938-950, doi:10.1111/ajt.13542 (2016).

51 Pei, C. *et al.* Emerging role of interleukin-33 in autoimmune diseases. *Immunology* **141**, 9-17, doi:10.1111/imm.12174 (2014).

52 Johnpulle, R. A. *et al.* Metabolic Complications Precede Alloreactivity and Are Characterized by Changes in Suppression of Tumorigenicity 2 Signaling. *Biol Blood Marrow Transplant* **23**, 529-532, doi:10.1016/j.bbmt.2016.12.627 (2017).

53 Miller, A. M. *et al.* Soluble ST2 associates with diabetes but not established cardiovascular risk factors: a new inflammatory pathway of relevance to diabetes? *PloS one* **7**, e47830, doi:10.1371/journal.pone.0047830 (2012).

54 Bergers, G., Reikerstorfer, A., Braselmann, S., Graninger, P. & Busslinger, M. Alternative promoter usage of the Fos-responsive gene Fit-1 generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor. *The EMBO journal* **13**, 1176-1188 (1994).

55 Thomassen, E. *et al.* Role of cell type-specific promoters in the developmental regulation of T1, an interleukin 1 receptor homologue. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **6**, 179-184 (1995).

56 Iwahana, H. *et al.* Different promoter usage and multiple transcription initiation sites of the interleukin-1 receptor-related human ST2 gene in UT-7 and TM12 cells. *Eur J Biochem* **264**, 397-406 (1999).

57 Tago, K. *et al.* Tissue distribution and subcellular localization of a variant form of the human ST2 gene product, ST2V. *Biochemical and biophysical research communications* **285**, 1377-1383, doi:10.1006/bbrc.2001.5306 (2001).

58 Iwahana, H. *et al.* Molecular cloning of the chicken ST2 gene and a novel variant form of the ST2 gene product, ST2LV. *Biochimica et biophysica acta* **1681**, 1-14, doi:10.1016/j.bbaexp.2004.08.013 (2004).

59 Gachter, T., Werenskiold, A. K. & Klemenz, R. Transcription of the interleukin-1 receptor-related T1 gene is initiated at different promoters in mast cells and fibroblasts. *The Journal of biological chemistry* **271**, 124-129 (1996).

60 Baba, Y. *et al.* Involvement of PU.1 in mast cell/basophil-specific function of the human IL1RL1/ST2 promoter. *Allergology international : official journal of the Japanese Society of Allergology* **61**, 461-467, doi:10.2332/allergolint.12-OA-0424 (2012).

61 Trub, T., Kalousek, M. B., Frohli, E. & Klemenz, R. Growth factor-mediated induction of the delayed early gene T1 depends on a 12-O-tetradecanoylphorbol 13-acetate-responsive element located 3.6 kb upstream of the transcription initiation site. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 3896-3900 (1994).

62 Yanagisawa, K., Takagi, T., Tsukamoto, T., Tetsuka, T. & Tominaga, S. Presence of a novel primary response gene ST2L, encoding a product

highly similar to the interleukin 1 receptor type 1. *FEBS letters* **318**, 83-87 (1993).

63 Weinberg, E. O. *et al.* Expression and regulation of ST2, an interleukin-1 receptor family member, in cardiomyocytes and myocardial infarction. *Circulation* **106**, 2961-2966 (2002).

64 Lohning, M. *et al.* T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6930-6935 (1998).

65 Schiering, C. *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564-568, doi:10.1038/nature13577 (2014).

66 Neill, D. R. *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367-1370, doi:nature08900 [pii] 10.1038/nature08900 (2010).

67 Kurowska-Stolarska, M. *et al.* IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *Journal of immunology* **183**, 6469-6477, doi:10.4049/jimmunol.0901575 (2009).

68 Moritz, D. R., Rodewald, H. R., Gheyselinck, J. & Klemenz, R. The IL-1 receptor-related T1 antigen is expressed on immature and mature mast cells and on fetal blood mast cell progenitors. *Journal of immunology* **161**, 4866-4874 (1998).

69 Cherry, W. B., Yoon, J., Bartemes, K. R., Iijima, K. & Kita, H. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *The Journal of allergy and clinical immunology* **121**, 1484-1490, doi:10.1016/j.jaci.2008.04.005 (2008).

70 Suzukawa, M. *et al.* An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *Journal of immunology* **181**, 5981-5989 (2008).

71 Smithgall, M. D. *et al.* IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *International immunology* **20**, 1019-1030, doi:dxn060 [pii] 10.1093/intimm/dxn060 (2008).

72 Komai-Koma, M. *et al.* Interleukin-33 amplifies IgE synthesis and triggers mast cell degranulation via interleukin-4 in naive mice. *Allergy* **67**, 1118-1126, doi:10.1111/j.1398-9995.2012.02859.x (2012).

73 Mildner, M. *et al.* Primary sources and immunological prerequisites for sST2 secretion in humans. *Cardiovascular research* **87**, 769-777, doi:10.1093/cvr/cvq104 (2010).

74 Bandara, G., Beaven, M. A., Olivera, A., Gilfillan, A. M. & Metcalfe, D. D. Activated mast cells synthesize and release soluble ST2-a decoy receptor for IL-33. *European journal of immunology* **45**, 3034-3044, doi:10.1002/eji.201545501 (2015).

75 Zhang, J. *et al.* ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host

disease. *Sci Transl Med* **7**, 308ra160, doi:10.1126/scitranslmed.aab0166 (2015).

76 Oshikawa, K., Yanagisawa, K., Tominaga, S. & Sugiyama, Y. Expression and function of the ST2 gene in a murine model of allergic airway inflammation. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **32**, 1520-1526, doi:1494 [pii] (2002).

77 Chackerian, A. A. *et al.* IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *Journal of immunology* **179**, 2551-2555 (2007).

78 Ali, S. *et al.* IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 18660-18665, doi:10.1073/pnas.0705939104 (2007).

79 Funakoshi-Tago, M. *et al.* TRAF6 is a critical signal transducer in IL-33 signaling pathway. *Cellular signalling* **20**, 1679-1686, doi:10.1016/j.cellsig.2008.05.013 (2008).

80 Duan, L. *et al.* Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3(+) regulatory T-cell responses in mice. *Mol Med* **18**, 753-761, doi:10.2119/molmed.2011.00428 (2012).

81 Peine, M., Marek, R. M. & Lohning, M. IL-33 in T Cell Differentiation, Function, and Immune Homeostasis. *Trends in immunology* **37**, 321-333, doi:10.1016/j.it.2016.03.007 (2016).

82 Matta, B. M. *et al.* Peri-alloHCT IL-33 administration expands recipient T-regulatory cells that protect mice against acute GVHD. *Blood* **128**, 427-439, doi:10.1182/blood-2015-12-684142 (2016).

83 Negishi, H. *et al.* Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 15136-15141, doi:10.1073/pnas.0607181103 (2006).

84 Fragale, A. *et al.* IFN regulatory factor-1 negatively regulates CD4+ CD25+ regulatory T cell differentiation by repressing Foxp3 expression. *Journal of immunology* **181**, 1673-1682 (2008).

85 Bulek, K. *et al.* The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response. *Journal of immunology* **182**, 2601-2609, doi:10.4049/jimmunol.0802729 (2009).

86 Yang, Z. *et al.* IL-33-induced alterations in murine intestinal function and cytokine responses are MyD88, STAT6, and IL-13 dependent. *American journal of physiology. Gastrointestinal and liver physiology* **304**, G381-389, doi:10.1152/ajpgi.00357.2012 (2013).

87 Hayakawa, H., Hayakawa, M., Kume, A. & Tominaga, S. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *The Journal of biological chemistry* **282**, 26369-26380, doi:M704916200 [pii] 10.1074/jbc.M704916200 (2007).

88 Sanada, S. *et al.* IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. *The Journal of clinical investigation* **117**, 1538-1549, doi:10.1172/JCI30634 (2007).

89 Kunze, M. M. *et al.* sST2 translation is regulated by FGF2 via an hnRNP A1-mediated IRES-dependent mechanism. *Biochimica et biophysica acta* **1859**, 848-859, doi:10.1016/j.bbagrm.2016.05.005 (2016).

90 Zhao, J. *et al.* Lysophosphatidic acid increases soluble ST2 expression in mouse lung and human bronchial epithelial cells. *Cellular signalling* **24**, 77-85, doi:10.1016/j.cellsig.2011.08.004 (2012).

91 Tajima, S., Oshikawa, K., Tominaga, S. & Sugiyama, Y. The increase in serum soluble ST2 protein upon acute exacerbation of idiopathic pulmonary fibrosis. *Chest* **124**, 1206-1214 (2003).

92 Andersson, C. *et al.* Associations of Circulating Growth Differentiation Factor-15 and ST2 Concentrations With Subclinical Vascular Brain Injury and Incident Stroke. *Stroke; a journal of cerebral circulation* **46**, 2568-2575, doi:10.1161/strokeaha.115.009026 (2015).

93 Lopez-Casado, M. A., Lorite, P., Palomeque, T. & Torres, M. I. Potential role of the IL-33/ST2 axis in celiac disease. *Cellular & molecular immunology*, doi:10.1038/cmi.2015.85 (2015).

94 Bergis, D., Kassis, V. & Radeke, H. H. High plasma sST2 levels in gastric cancer and their association with metastatic disease. *Cancer biomarkers : section A of Disease markers* **16**, 117-125, doi:10.3233/cbm-150547 (2016).

95 Lei, Z. *et al.* Soluble ST2 plasma concentrations predict mortality in HBV-related acute-on-chronic liver failure. *Mediators of inflammation* **2015**, 535938, doi:10.1155/2015/535938 (2015).

96 Mehraj, V. *et al.* The plasma levels of soluble ST2 as a marker of gut mucosal damage in early HIV infection. *AIDS (London, England)* **30**, 1617-1627, doi:10.1097/qad.0000000000001105 (2016).

97 Wood, I. S., Wang, B. & Trayhurn, P. IL-33, a recently identified interleukin-1 gene family member, is expressed in human adipocytes. *Biochemical and biophysical research communications* **384**, 105-109, doi:10.1016/j.bbrc.2009.04.081 (2009).

98 Pichery, M. *et al.* Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: in situ analysis using a novel Il-33-LacZ gene trap reporter strain. *Journal of immunology* **188**, 3488-3495, doi:10.4049/jimmunol.1101977 (2012).

99 Liew, F. Y., Pitman, N. I. & McInnes, I. B. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nature reviews. Immunology* **10**, 103-110, doi:10.1038/nri2692 (2010).

100 Ali, S. *et al.* The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *Journal of immunology* **187**, 1609-1616, doi:10.4049/jimmunol.1003080 (2011).

101 Shao, D. *et al.* Nuclear IL-33 regulates soluble ST2 receptor and IL-6 expression in primary human arterial endothelial cells and is decreased in idiopathic pulmonary arterial hypertension. *Biochemical and biophysical research communications* **451**, 8-14, doi:10.1016/j.bbrc.2014.06.111 (2014).

102 Bessa, J. *et al.* Altered subcellular localization of IL-33 leads to non-resolving lethal inflammation. *Journal of autoimmunity* **55**, 33-41, doi:10.1016/j.jaut.2014.02.012 (2014).

103 Talabot-Ayer, D., Lamacchia, C., Gabay, C. & Palmer, G. Interleukin-33 is biologically active independently of caspase-1 cleavage. *The Journal of biological chemistry* **284**, 19420-19426, doi:10.1074/jbc.M901744200 (2009).

104 Lefrancais, E. *et al.* IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 1673-1678, doi:10.1073/pnas.1115884109 (2012).

105 Cayrol, C. & Girard, J. P. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 9021-9026, doi:10.1073/pnas.0812690106 (2009).

106 Luthi, A. U. *et al.* Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* **31**, 84-98, doi:10.1016/j.immuni.2009.05.007 (2009).

107 Lefrancais, E. *et al.* Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 15502-15507, doi:10.1073/pnas.1410700111 (2014).

108 Waern, I., Lundequist, A., Pejler, G. & Wernersson, S. Mast cell chymase modulates IL-33 levels and controls allergic sensitization in dust-mite induced airway inflammation. *Mucosal immunology* **6**, 911-920, doi:10.1038/mi.2012.129 (2013).

109 Xu, D. *et al.* Selective Expression of a Stable Cell Surface Molecule on Type 2 but Not Type 1 Helper T Cells. *The Journal of experimental medicine* **187**, 787-794 (1998).

110 Guo, L. *et al.* IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 13463-13468, doi:10.1073/pnas.0906988106 (2009).

111 Piehler, D. *et al.* T1/ST2 promotes T helper 2 cell activation and polyfunctionality in bronchopulmonary mycosis. *Mucosal immunology* **6**, 405-414, doi:10.1038/mi.2012.84 (2013).

112 Kurowska-Stolarska, M. *et al.* IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *Journal of immunology* **181**, 4780-4790, doi:181/7/4780 [pii] (2008).

113 Komai-Koma, M. *et al.* IL-33 is a chemoattractant for human Th2 cells. *European journal of immunology* **37**, 2779-2786, doi:10.1002/eji.200737547 (2007).

114 Hoshino, K. *et al.* The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. *The Journal of experimental medicine* **190**, 1541-1548 (1999).

115 Veldhoen, M. *et al.* Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature immunology* **9**, 1341-1346, doi:10.1038/ni.1659 (2008).

116 Dardalhon, V. *et al.* IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nature immunology* **9**, 1347-1355, doi:10.1038/ni.1677 (2008).

117 Chang, H. C. *et al.* The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nature immunology* **11**, 527-534, doi:10.1038/ni.1867 (2010).

118 Staudt, V. *et al.* Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* **33**, 192-202, doi:10.1016/j.immuni.2010.07.014 (2010).

119 Brustle, A. *et al.* The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nature immunology* **8**, 958-966, doi:10.1038/ni1500 (2007).

120 Rengarajan, J. *et al.* Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *The Journal of experimental medicine* **195**, 1003-1012 (2002).

121 Reichenbach, D. K. *et al.* The IL-33/ST2 axis augments effector T-cell responses during acute GVHD. *Blood* **125**, 3183-3192, doi:10.1182/blood-2014-10-606830 (2015).

122 Wu, B. *et al.* IL-9 is associated with an impaired Th1 immune response in patients with tuberculosis. *Clinical immunology (Orlando, Fla.)* **126**, 202-210, doi:10.1016/j.clim.2007.09.009 (2008).

123 Blom, L., Poulsen, B. C., Jensen, B. M., Hansen, A. & Poulsen, L. K. IL-33 induces IL-9 production in human CD4+ T cells and basophils. *PloS one* **6**, e21695, doi:10.1371/journal.pone.0021695 (2011).

124 Gerlach, K. *et al.* TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nature immunology* **15**, 676-686, doi:10.1038/ni.2920 (2014).

125 Jiang, H. R. *et al.* IL-33 attenuates EAE by suppressing IL-17 and IFN-gamma production and inducing alternatively activated macrophages. *European journal of immunology* **42**, 1804-1814, doi:10.1002/eji.201141947 (2012).

126 Matta, B. M. *et al.* IL-33 is an unconventional Alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells. *Journal of immunology* **193**, 4010-4020, doi:10.4049/jimmunol.1400481 (2014).

127 Brunner, S. M. *et al.* Interleukin-33 prolongs allograft survival during chronic cardiac rejection. *Transplant international : official journal of the European Society for Organ Transplantation* **24**, 1027-1039, doi:10.1111/j.1432-2277.2011.01306.x (2011).

128 Turnquist, H. R. *et al.* IL-33 expands suppressive CD11b+ Gr-1(int) and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival. *Journal of immunology* **187**, 4598-4610, doi:10.4049/jimmunol.1100519 (2011).

129 Gajardo, T., Morales, R. A., Campos-Mora, M., Campos-Acuna, J. & Pino-Lagos, K. Exogenous interleukin-33 targets myeloid-derived suppressor cells and generates periphery-induced Foxp3(+) regulatory T cells in skin-transplanted mice. *Immunology* **146**, 81-88, doi:10.1111/imm.12483 (2015).

130 Lal, G. *et al.* Distinct inflammatory signals have physiologically divergent effects on epigenetic regulation of Foxp3 expression and Treg function. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **11**, 203-214, doi:10.1111/j.1600-6143.2010.03389.x (2011).

131 Moro, K. *et al.* Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* **463**, 540-544, doi:nature08636 [pii] 10.1038/nature08636 (2010).

132 Stehle, C., Saikali, P. & Romagnani, C. Putting the brakes on ILC2 cells. *Nature immunology* **17**, 43-44, doi:10.1038/ni.3353 (2016).

133 Mjosberg, J. M. *et al.* Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nature immunology* **12**, 1055-1062, doi:10.1038/ni.2104 (2011).

134 Yagi, R. *et al.* The transcription factor GATA3 is critical for the development of all IL-7Ralpha-expressing innate lymphoid cells. *Immunity* **40**, 378-388, doi:10.1016/j.immuni.2014.01.012 (2014).

135 Spooner, C. J. *et al.* Specification of type 2 innate lymphocytes by the transcriptional determinant Gfi1. *Nature immunology* **14**, 1229-1236, doi:10.1038/ni.2743 (2013).

136 Price, A. E. *et al.* Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 11489-11494, doi:10.1073/pnas.1003988107 (2010).

137 Wilhelm, C. *et al.* An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nature immunology* **12**, 1071-1077, doi:10.1038/ni.2133 (2011).

138 Salimi, M. *et al.* A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *The Journal of experimental medicine* **210**, 2939-2950, doi:10.1084/jem.20130351 (2013).

139 Matsuki, A. *et al.* T-bet inhibits innate lymphoid cell-mediated eosinophilic airway inflammation by suppressing IL-9 production. *The Journal of allergy and clinical immunology*, doi:10.1016/j.jaci.2016.08.022 (2016).

140 Monticelli, L. A. *et al.* Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature immunology* **12**, 1045-1054, doi:10.1031/ni.2131 (2011).

141 Imai, Y. *et al.* Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 13921-13926, doi:10.1073/pnas.1307321110 (2013).

142 Rak, G. D. *et al.* IL-33-Dependent Group 2 Innate Lymphoid Cells Promote Cutaneous Wound Healing. *The Journal of investigative dermatology* **136**, 487-496, doi:10.1038/jid.2015.406 (2016).

143 McHedlidze, T. *et al.* Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity* **39**, 357-371, doi:10.1016/j.immuni.2013.07.018 (2013).

144 Jovanovic, I. P. *et al.* Interleukin-33/ST2 axis promotes breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells. *International journal of cancer* **134**, 1669-1682, doi:10.1002/ijc.28481 (2014).

145 Bonilla, W. V. *et al.* The alarmin interleukin-33 drives protective antiviral CD8(+) T cell responses. *Science* **335**, 984-989, doi:10.1126/science.1215418 (2012).

146 Gao, Q., Li, Y. & Li, M. The potential role of IL-33/ST2 signaling in fibrotic diseases. *Journal of leukocyte biology* **98**, 15-22, doi:10.1189/jlb.3RU0115-012R (2015).

147 Komai-Koma, M. *et al.* Interleukin-33 promoting Th1 lymphocyte differentiation dependents on IL-12. *Immunobiology* **221**, 412-417, doi:10.1016/j.imbio.2015.11.013 (2016).

148 Komai-Koma, M. *et al.* IL-33 activates B1 cells and exacerbates contact sensitivity. *Journal of immunology* **186**, 2584-2591, doi:10.4049/jimmunol.1002103 (2011).

149 Sattler, S. *et al.* IL-10-producing regulatory B cells induced by IL-33 (Breg(IL-33)) effectively attenuate mucosal inflammatory responses in the gut. *Journal of autoimmunity* **50**, 107-122, doi:10.1016/j.jaut.2014.01.032 (2014).

150 Bourgeois, E. *et al.* The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *European journal of immunology* **39**, 1046-1055, doi:10.1002/eji.200838575 (2009).

151 Nabekura, T., Girard, J. P. & Lanier, L. L. IL-33 receptor ST2 amplifies the expansion of NK cells and enhances host defense during mouse cytomegalovirus infection. *Journal of immunology* **194**, 5948-5952, doi:10.4049/jimmunol.1500424 (2015).

152 Palmer, G. *et al.* Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis. *Arthritis and rheumatism* **60**, 738-749, doi:10.1002/art.24305 (2009).

153 Leung, B. P., Xu, D., Culshaw, S., McInnes, I. B. & Liew, F. Y. A novel therapy of murine collagen-induced arthritis with soluble T1/ST2. *Journal of immunology* **173**, 145-150 (2004).

154 Heimesaat, M. M. *et al.* MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. *Gut* **59**, 1079-1087, doi:59/8/1079 [pii] 10.1136/gut.2009.197434 (2010).

155 Espinassous, Q. *et al.* IL-33 enhances lipopolysaccharide-induced inflammatory cytokine production from mouse macrophages by regulating lipopolysaccharide receptor complex. *Journal of immunology* **183**, 1446-1455, doi:10.4049/jimmunol.0803067 (2009).

156 Joshi, A. D. *et al.* Interleukin-33 contributes to both M1 and M2 chemokine marker expression in human macrophages. *BMC Immunol* **11**, 52, doi:1471-2172-11-52 [pii] 10.1186/1471-2172-11-52 (2010).

157 Gao, X. *et al.* Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells. *Journal of immunology* **194**, 438-445, doi:10.4049/jimmunol.1401344 (2015).

158 Allakhverdi, Z., Smith, D. E., Comeau, M. R. & Delespesse, G. Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *Journal of immunology* **179**, 2051-2054 (2007).

159 Sabatino, G. *et al.* Impact of IL -9 and IL-33 in mast cells. *Journal of biological regulators and homeostatic agents* **26**, 577-586 (2012).

160 Andrade, M. V. *et al.* Amplification of cytokine production through synergistic activation of NFAT and AP-1 following stimulation of mast cells

with antigen and IL-33. *European journal of immunology* **41**, 760-772, doi:10.1002/eji.201040718 (2011).

161 Sawaguchi, M. *et al.* Role of mast cells and basophils in IgE responses and in allergic airway hyperresponsiveness. *Journal of immunology* **188**, 1809-1818, doi:10.4049/jimmunol.1101746 (2012).

162 Saluja, R., Khan, M., Church, M. K. & Maurer, M. The role of IL-33 and mast cells in allergy and inflammation. *Clinical and translational allergy* **5**, 33, doi:10.1186/s13601-015-0076-5 (2015).

163 Pecaric-Petkovic, T., Didichenko, S. A., Kaempfer, S., Spiegl, N. & Dahinden, C. A. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* **113**, 1526-1534, doi:10.1182/blood-2008-05-157818 (2009).

164 Stolarski, B., Kurowska-Stolarska, M., Kewin, P., Xu, D. & Liew, F. Y. IL-33 exacerbates eosinophil-mediated airway inflammation. *Journal of immunology* **185**, 3472-3480, doi:10.4049/jimmunol.1000730 (2010).

165 Turnquist, H. R. *et al.* IL-1beta-driven ST2L expression promotes maturation resistance in rapamycin-conditioned dendritic cells. *Journal of immunology* **181**, 62-72 (2008).

166 Besnard, A. G. *et al.* IL-33-activated dendritic cells are critical for allergic airway inflammation. *European journal of immunology* **41**, 1675-1686, doi:10.1002/eji.201041033 (2011).

167 Nagata, A. *et al.* Soluble ST2 protein inhibits LPS stimulation on monocyte-derived dendritic cells. *Cellular & molecular immunology* **9**, 399-409, doi:10.1038/cmi.2012.29 (2012).

168 Alves-Filho, J. C. *et al.* Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nature medicine* **16**, 708-712, doi:10.1038/nm.2156 (2010).

169 Hueber, A. J. *et al.* IL-33 induces skin inflammation with mast cell and neutrophil activation. *European journal of immunology* **41**, 2229-2237, doi:10.1002/eji.201041360 (2011).

170 Sedhom, M. A. *et al.* Neutralisation of the interleukin-33/ST2 pathway ameliorates experimental colitis through enhancement of mucosal healing in mice. *Gut* **62**, 1714-1723, doi:10.1136/gutjnl-2011-301785 (2013).

171 Makita, S. *et al.* CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells. *Journal of immunology* **173**, 3119-3130 (2004).

172 Holmen, N. *et al.* Functional CD4+CD25high regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. *Inflammatory bowel diseases* **12**, 447-456 (2006).

173 Grobeta, P., Doser, K., Falk, W., Obermeier, F. & Hofmann, C. IL-33 attenuates development and perpetuation of chronic intestinal inflammation. *Inflammatory bowel diseases* **18**, 1900-1909, doi:10.1002/ibd.22900 (2012).

174 Ferrara, J. L., Cooke, K. R. & Teshima, T. The pathophysiology of acute graft-versus-host disease. *Int J Hematol* **78**, 181-187 (2003).

175 Humphreys, N. E., Xu, D., Hepworth, M. R., Liew, F. Y. & Grencis, R. K. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *Journal of immunology* **180**, 2443-2449 (2008).

176 Jones, L. A. *et al.* IL-33 receptor (T1/ST2) signalling is necessary to prevent the development of encephalitis in mice infected with Toxoplasma gondii. *European journal of immunology* **40**, 426-436, doi:10.1002/eji.200939705 (2010).

177 Wagenaar, J. F. *et al.* Soluble ST2 levels are associated with bleeding in patients with severe Leptospirosis. *PLoS neglected tropical diseases* **3**, e453, doi:10.1371/journal.pntd.0000453 (2009).

178 Huang, X., Du, W., Barrett, R. P. & Hazlett, L. D. ST2 is essential for Th2 responsiveness and resistance to pseudomonas aeruginosa keratitis. *Investigative ophthalmology & visual science* **48**, 4626-4633, doi:10.1167/iovs.07-0316 (2007).

179 Hunt, P. W. *et al.* Gut epithelial barrier dysfunction and innate immune activation predict mortality in treated HIV infection. *The Journal of infectious diseases* **210**, 1228-1238, doi:10.1093/infdis/jiu238 (2014).

180 Schenten, D. *et al.* Signaling through the adaptor molecule MyD88 in CD4+ T cells is required to overcome suppression by regulatory T cells. *Immunity* **40**, 78-90, doi:10.1016/j.immuni.2013.10.023 (2014).

181 Rahman, A. H. *et al.* MyD88 plays a critical T cell-intrinsic role in supporting CD8 T cell expansion during acute lymphocytic choriomeningitis virus infection. *Journal of immunology* **181**, 3804-3810 (2008).

182 Zhou, S. *et al.* MyD88 intrinsically regulates CD4 T-cell responses. *Journal of virology* **83**, 1625-1634, doi:10.1128/jvi.01770-08 (2009).

183 Adachi, O. *et al.* Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**, 143-150, doi:S1074-7613(00)80596-8 [pii] (1998).

184 Townsend, M. J., Fallon, P. G., Matthews, D. J., Jolin, H. E. & McKenzie, A. N. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *The Journal of experimental medicine* **191**, 1069-1076 (2000).

185 Jerde, T. J. & Bushman, W. IL-1 induces IGF-dependent epithelial proliferation in prostate development and reactive hyperplasia. *Science signaling* **2**, ra49, doi:10.1126/scisignal.2000338 (2009).

186 Li, W. *et al.* Proteomics analysis reveals a Th17-prone cell population in presymptomatic graft-versus-host disease. *JCI insight* **1**, doi:10.1172/jci.insight.86660 (2016).

187 Petrovic, A. *et al.* LPAM (alpha 4 beta 7 integrin) is an important homing integrin on alloreactive T cells in the development of intestinal graft-versus-host disease. *Blood* **103**, 1542-1547, doi:10.1182/blood-2003-03-0957 (2004).

188 Foley, J. E., Mariotti, J., Ryan, K., Eckhaus, M. & Fowler, D. H. Th2 cell therapy of established acute graft-versus-host disease requires IL-4 and IL-10 and is abrogated by IL-2 or host-type antigen-presenting cells. *Biol Blood Marrow Transplant* **14**, 959-972, doi:S1083-8791(08)00250-4 [pii] 10.1016/j.bbmt.2008.06.007 (2008).

189 Di Ianni, M. *et al.* Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* **117**, 3921-3928, doi:10.1182/blood-2010-10-311894 (2011).

190 Akira, S. & Takeda, K. Toll-like receptor signalling. *Nature reviews. Immunology* **4**, 499-511 (2004).

191 Garlanda, C., Dinarello, C. A. & Mantovani, A. The interleukin-1 family: back to the future. *Immunity* **39**, 1003-1018, doi:10.1016/j.immuni.2013.11.010 (2013).

192 Jankovic, D. *et al.* The Nlrp3 inflammasome regulates acute graft-versus-host disease. *The Journal of experimental medicine* **210**, 1899-1910, doi:10.1084/jem.20130084 (2013).

193 Zhao, Y. *et al.* TLR4 inactivation protects from graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Cellular & molecular immunology* **10**, 165-175, doi:10.1038/cmi.2012.58 (2013).

194 Schnare, M. *et al.* Toll-like receptors control activation of adaptive immune responses. *Nature immunology* **2**, 947-950 (2001).

195 Zhou, S. *et al.* MyD88 is critical for the development of innate and adaptive immunity during acute lymphocytic choriomeningitis virus

infection. *European journal of immunology* **35**, 822-830, doi:10.1002/eji.200425730 (2005).

196 Sheng, W. *et al.* STAT5 programs a distinct subset of GM-CSF-producing T helper cells that is essential for autoimmune neuroinflammation. *Cell research* **24**, 1387-1402, doi:10.1038/cr.2014.154 (2014).

197 Ponomarev, E. D. *et al.* GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. *Journal of immunology* **178**, 39-48 (2007).

198 Borges, C. M. *et al.* Regulatory T cell expressed MyD88 is critical for prolongation of allograft survival. *Transplant international : official journal of the European Society for Organ Transplantation* **29**, 930-940, doi:10.1111/tri.12788 (2016).

199 Loiarro, M. *et al.* Pivotal Advance: Inhibition of MyD88 dimerization and recruitment of IRAK1 and IRAK4 by a novel peptidomimetic compound. *Journal of leukocyte biology* **82**, 801-810, doi:10.1189/jlb.1206746 (2007).

200 Zhao, Y. *et al.* The adaptor molecule MyD88 directly promotes CD8 T cell responses to vaccinia virus. *Journal of immunology* **182**, 6278-6286, doi:10.4049/jimmunol.0803682 (2009).

201 Lim, J. Y. *et al.* Differential Effect of MyD88 Signal in Donor T Cells on Graft-versus-Leukemia Effect and Graft-versus-Host Disease after Experimental Allogeneic Stem Cell Transplantation. *Molecules and cells* **38**, 966-974, doi:10.14348/molcells.2015.0158 (2015).

202 Blaser, B. W. *et al.* Donor-derived IL-15 is critical for acute allogeneic graft-versus-host disease. *Blood* **105**, 894-901 (2005).

203 Gartlan, K. H. *et al.* Tc17 cells are a proinflammatory, plastic lineage of pathogenic CD8+ T cells that induce GVHD without antileukemic effects. *Blood* **126**, 1609-1620, doi:10.1182/blood-2015-01-622662 (2015).

204 Antin, J. H. *et al.* Interleukin-1 blockade does not prevent acute graft-versus-host disease: results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation. *Blood* **100**, 3479-3482 (2002).

205 Jenq, R. R. *et al.* Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. *The Journal of experimental medicine* **209**, 903-911, doi:10.1084/jem.20112408 (2012).

206 Holler, E. *et al.* Metagenomic analysis of the stool microbiome in patients receiving allogeneic stem cell transplantation: loss of diversity is associated with use of systemic antibiotics and more pronounced in gastrointestinal graft-versus-host disease. *Biol Blood Marrow Transplant* **20**, 640-645, doi:10.1016/j.bbmt.2014.01.030 (2014).

207 Taur, Y. *et al.* The effects of intestinal tract bacterial diversity on mortality following allogeneic hematopoietic stem cell transplantation. *Blood* **124**, 1174-1182, doi:10.1182/blood-2014-02-554725 (2014).

208 Ivanov, II *et al.* Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* **139**, 485-498, doi:S0092-8674(09)01248-3 [pii] 10.1016/j.cell.2009.09.033 (2009).

209 Rogier, R. *et al.* Aberrant intestinal microbiota due to IL-1 receptor antagonist deficiency promotes IL-17- and TLR4-dependent arthritis. *Microbiome* **5**, 63, doi:10.1186/s40168-017-0278-2 (2017).

210 Randall, K. L. *et al.* Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. *Nature immunology* **10**, 1283-1291, doi:10.1038/ni.1820 (2009).

211 Jabara, H. H. *et al.* DOCK8 functions as an adaptor that links TLR-MyD88 signaling to B cell activation. *Nature immunology* **13**, 612-620, doi:10.1038/ni.2305 (2012).

212 Grakoui, A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221-227 (1999).

213 Janssen, E. *et al.* DOCK8 enforces immunological tolerance by promoting IL-2 signaling and immune synapse formation in Tregs. *JCI insight* **2**, doi:10.1172/jci.insight.94298 (2017).

214 Gelman, A. E., Zhang, J., Choi, Y. & Turka, L. A. Toll-like receptor ligands directly promote activated CD4+ T cell survival. *Journal of immunology* **172**, 6065-6073 (2004).

215 Komai-Koma, M., Jones, L., Ogg, G. S., Xu, D. & Liew, F. Y. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3029-3034, doi:10.1073/pnas.0400171101 (2004).

216 Reynolds, J. M., Martinez, G. J., Chung, Y. & Dong, C. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Proceedings of*

the National Academy of Sciences of the United States of America **109**, 13064-13069, doi:10.1073/pnas.1120585109 (2012).

217 Gonzalez-Navajas JM, F. S., Law J, Datta SK, Nguyen KP, Yu M, Corr M, Katakura K, Eckman L, Lee J, Raz E. TLR4 signaling in effector CD4+ T cells regulates TCR activation and experimental colitis in mice. *The Journal of clinical investigation* **120**, 12, doi:10.1172/JCI40055 (2010).

218 Butchi, N. *et al.* Myd88 Initiates Early Innate Immune Responses and Promotes CD4 T Cells during Coronavirus Encephalomyelitis. *Journal of virology* **89**, 9299-9312, doi:10.1128/jvi.01199-15 (2015).

219 Ullrich, E. *et al.* BATF-dependent IL-7RhiGM-CSF+ T cells control intestinal graft-versus-host disease. *The Journal of clinical investigation*, doi:10.1172/jci89242 (2018).

220 Fukata, M. *et al.* The myeloid differentiation factor 88 (MyD88) is required for CD4+ T cell effector function in a murine model of inflammatory bowel disease. *Journal of immunology* **180**, 1886-1894 (2008).

221 Trinschek, B. *et al.* Kinetics of IL-6 production defines T effector cell responsiveness to regulatory T cells in multiple sclerosis. *PloS one* **8**, e77634, doi:10.1371/journal.pone.0077634 (2013).

222 Schneider, A. *et al.* In active relapsing-remitting multiple sclerosis, effector T cell resistance to adaptive T(regs) involves IL-6-mediated signaling. *Science Translational Medicine* **5**, 170ra115, doi:10.1126/scitranslmed.3004970 (2013).

223 Nish, S. A. *et al.* T cell-intrinsic role of IL-6 signaling in primary and memory responses. *eLife* **3**, e01949, doi:10.7554/eLife.01949 (2014).

224 Tawara, I. *et al.* Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation. *Clin Cancer Res* **17**, 77-88, doi:1078-0432.CCR-10-1198 [pii] 10.1158/1078-0432.CCR-10-1198 (2011).

225 Lutticken, C. *et al.* Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science* **263**, 89-92 (1994).

226 Goodman, W. A., Young, A. B., McCormick, T. S., Cooper, K. D. & Levine, A. D. Stat3 phosphorylation mediates resistance of primary human T cells to regulatory T cell suppression. *Journal of immunology* **186**, 3336-3345, doi:10.4049/jimmunol.1001455 (2011).

227 Yamawaki, Y., Kimura, H., Hosoi, T. & Ozawa, K. MyD88 plays a key role in LPS-induced Stat3 activation in the hypothalamus. *American journal of physiology. Regulatory, integrative and comparative physiology* **298**, R403-410, doi:10.1152/ajpregu.00395.2009 (2010).

228 Liu, B. S., Stoop, J. N., Huizinga, T. W. & Toes, R. E. IL-21 enhances the activity of the TLR-MyD88-STAT3 pathway but not the classical TLR-MyD88-NF-kappaB pathway in human B cells to boost antibody production. *Journal of immunology* **191**, 4086-4094, doi:10.4049/jimmunol.1300765 (2013).

229 Betts, B. C. *et al.* CD4+ T cell STAT3 phosphorylation precedes acute GVHD, and subsequent Th17 tissue invasion correlates with GVHD severity and therapeutic response. *Journal of leukocyte biology* **97**, 807-819, doi:10.1189/jlb.5A1114-532RR (2015).

230 van Amelsfort, J. M. *et al.* Proinflammatory mediator-induced reversal of CD4+,CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. *Arthritis and rheumatism* **56**, 732-742, doi:10.1002/art.22414 (2007).

231 Ben Ahmed, M. *et al.* IL-15 renders conventional lymphocytes resistant to suppressive functions of regulatory T cells through activation of the phosphatidylinositol 3-kinase pathway. *Journal of immunology* **182**, 6763-6770, doi:10.4049/jimmunol.0801792 (2009).

232 Ruprecht, C. R. *et al.* Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. *The Journal of experimental medicine* **201**, 1793-1803, doi:10.1084/jem.20050085 (2005).

233 Strengell, M., Sareneva, T., Foster, D., Julkunen, I. & Matikainen, S. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. *Journal of immunology* **169**, 3600-3605 (2002).

234 Wullschleger, S., Loewith, R. & Hall, M. N. TOR Signaling in Growth and Metabolism. *Cell* **124**, 471-484 (2006).

235 Wohlfert, E. A. & Clark, R. B. 'Vive la Resistance!'--the PI3K-Akt pathway can determine target sensitivity to regulatory T cell suppression. *Trends in immunology* **28**, 154-160, doi:10.1016/j.it.2007.02.003 (2007).

236 Wohlfert, E. A., Callahan, M. K. & Clark, R. B. Resistance to CD4+CD25+ regulatory T cells and TGF-beta in Cbl-b-/- mice. *Journal of immunology* **173**, 1059-1065 (2004).

237 King, C. G. *et al.* TRAF6 is a T cell-intrinsic negative regulator required for the maintenance of immune homeostasis. *Nature medicine* **12**, 1088-1092, doi:10.1038/nm1449 (2006).

238 Mercadante, E. R. & Lorenz, U. M. Breaking Free of Control: How Conventional T Cells Overcome Regulatory T Cell Suppression. *Front Immunol* **7** (2016).

239 Abouelnasr, A., Roy, J., Cohen, S., Kiss, T. & Lachance, S. Defining the role of sirolimus in the management of graft-versus-host disease: from prophylaxis to treatment. *Biol Blood Marrow Transplant* **19**, 12-21, doi:10.1016/j.bbmt.2012.06.020 (2013).

240 Herrero-Sánchez Mª, C. *et al.* Targeting of PI3K/AKT/mTOR pathway to inhibit T cell activation and prevent graft-versus-host disease development. *Journal of Hematology & Oncology* **9** (2016).

241 Siede, J. *et al.* IL-33 Receptor-Expressing Regulatory T Cells Are Highly Activated, Th2 Biased and Suppress CD4 T Cell Proliferation through IL-10 and TGFbeta Release. *PloS one* **11**, e0161507, doi:10.1371/journal.pone.0161507 (2016).

242 Tago, K. *et al.* STAT3 and ERK pathways are involved in cell growth stimulation of the ST2/IL1RL1 promoter. *FEBS Open Bio* **7**, 293-302 (2017).

243 Baumann, C. *et al.* T-bet- and STAT4-dependent IL-33 receptor expression directly promotes antiviral Th1 cell responses. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 4056-4061, doi:10.1073/pnas.1418549112 (2015).

244 Van Tassell, B. W. *et al.* Pharmacologic inhibition of myeloid differentiation factor 88 (MyD88) prevents left ventricular dilation and hypertrophy after experimental acute myocardial infarction in the mouse. *Journal of cardiovascular pharmacology* **55**, 385-390, doi:10.1097/FJC.0b013e3181d3da24 (2010).

245 Zhang, H. S. *et al.* Inhibition of myeloid differentiation factor 88(MyD88) by ST2825 provides neuroprotection after experimental traumatic brain injury in mice. *Brain Res* **1643**, 130-139, doi:10.1016/j.brainres.2016.05.003 (2016).

246 Wang, N. *et al.* Myeloid differentiation factor 88 is up-regulated in epileptic brain and contributes to experimental seizures in rats. *Experimental neurology* **295**, 23-35, doi:10.1016/j.expneurol.2017.05.008 (2017).

247 Zhou, L. *et al.* Astragalus polysaccharides exerts immunomodulatory effects via TLR4-mediated MyD88-dependent signaling pathway in vitro and in vivo. *Sci Rep* **7**, 44822, doi:10.1038/srep44822 (2017).

248 Choi, S. W. *et al.* Vorinostat plus tacrolimus and mycophenolate to prevent graft-versus-host disease after related-donor reduced-intensity conditioning allogeneic haemopoietic stem-cell transplantation: a phase

1/2 trial. *Lancet Oncol* **15**, 87-95, doi:10.1016/S1470-2045(13)70512-6 (2014).

249 Betts, B. C. *et al.* Targeting JAK2 reduces GVHD and xenograft rejection through regulation of T cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 1582-1587, doi:10.1073/pnas.1712452115 (2018).

250 Lim, J. Y. *et al.* MyD88 in donor bone marrow cells is critical for protection from acute intestinal graft-vs.-host disease. *Mucosal immunology* **9**, 730-743, doi:10.1038/mi.2015.96 (2016).

251 Picard, C., Casanova, J. L. & Puel, A. Infectious diseases in patients with IRAK-4, MyD88, NEMO, or IkappaBalpha deficiency. *Clinical microbiology reviews* **24**, 490-497, doi:10.1128/cmr.00001-11 (2011).

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

1. Ramadan AM, **Griesenauer B**, Adom D, Kapur R, Hanenberg H, Liu C, Kaplan M, Paczesny S. Specifically differentiated T cell subset promotes tumor immunity over fatal immunity. *J Exp Med*. 2017 Dec 4;214(12):3577-3596
2. Forcade E, Paz K, Flynn R, **Griesenauer B**, Amet T, Li W, Liu L, Jiang D, Chu HW, Lobera M, Yang J, Wilkes DS, Du J, Gartlan K, Hill GR, MacDonald KP, Espada EL, Blanco P, Cutler CS, Antin JH, Soiffer RJ, Ritz J, Paczesny S, Blazar BR. An activated Th17-prone T-cell subset involved in chronic graft-versus-host disease sensitive to pharmacological inhibition. *JCI Insight*. 2017 Jun 15;2(12).
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)

4. Zhang J, Ramadan AM, **Griesenauer B**, Li W, Turner MJ, Liu C, Kapur R, Hanenberg H, Blazar BR, Tawara I, Paczesny S. ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease. *Science Translational Medicine* 2015 Oct 7; 7(308):308ra160
5. Chen IC**, Griesenauer B**, Yu YT, Velicer GJ. A recent evolutionary origin of a bacterial small RNA that controls multicellular fruiting body development. *Mol Phylogenet Evol.* 2014 Apr;73:1-9
6. Ramadan A, Daguindau E, Chinnaswamy K, Zhang J, Lura GL, **Griesenauer B**, Bolten Z, Stuckey J, Yang CY, Paczesny S. From proteomics to discovery of first-in-class ST2 inhibitors exhibiting in vivo activity. *(Submitted)*
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)*
8. Ramadan AM, Reichenbach DK, Yang J, Zhang J, **Griesenauer B**, Liu H, Blazar BR, Paczesny P. Reciprocal roles of ST2 and RORgt expression in intestinal CD4+ Foxp3+ regulatory T cells during inflammation. *(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