THE ROLE OF SUPPRESSOR OF CYTOKINE SIGNALING-1 IN MAINTAINING REGULATORY T CELL HOMEOSTASIS

By

ERIN LOUISE COLLINS

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To my family, Ted, Emily, Christy, Rachel, and Dodger
Thank you for your endless encouragement and support
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>13</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>15</td>
</tr>
<tr>
<td><strong>Immune Regulation</strong></td>
<td>15</td>
</tr>
<tr>
<td><strong>Suppressor of Cytokine Signaling</strong></td>
<td>17</td>
</tr>
<tr>
<td>SOCS1</td>
<td>18</td>
</tr>
<tr>
<td>Role of SOCS1 in Autoimmunity</td>
<td>19</td>
</tr>
<tr>
<td>Effects of SOCS1 Deficiency</td>
<td>20</td>
</tr>
<tr>
<td><strong>Regulatory T Cells</strong></td>
<td>20</td>
</tr>
<tr>
<td>Treg Transcription Factor Foxp3</td>
<td>21</td>
</tr>
<tr>
<td>Role of Tregs in Immune Regulation</td>
<td>23</td>
</tr>
<tr>
<td>Treg Associated Molecules</td>
<td>24</td>
</tr>
<tr>
<td>Effects of Treg Deficiency</td>
<td>26</td>
</tr>
<tr>
<td>Project Rationale and Design</td>
<td>27</td>
</tr>
<tr>
<td>2 ASSESS THE PRESENCE OF TREGS IN SOCS1/-/- MICE</td>
<td>33</td>
</tr>
<tr>
<td><strong>Background</strong></td>
<td>33</td>
</tr>
<tr>
<td>Results</td>
<td>35</td>
</tr>
<tr>
<td>SOCS1/-/- Mice are Deficient in Peripheral Foxp3+ Tregs.</td>
<td>35</td>
</tr>
<tr>
<td>Thymic Development of SOCS1/-/- Tregs Does Not Contribute to Peripheral Deficiency</td>
<td>36</td>
</tr>
<tr>
<td>Treg Deficiency is Correlated to Dysregulated Cytokine Production.</td>
<td>36</td>
</tr>
<tr>
<td>Summary</td>
<td>37</td>
</tr>
<tr>
<td>3 DEVELOPMENT OF TREATMENT TO DECREASE DISEASE SEVERITY IN SOCS1/-/- MICE</td>
<td>45</td>
</tr>
<tr>
<td><strong>Background</strong></td>
<td>45</td>
</tr>
<tr>
<td>Results</td>
<td>46</td>
</tr>
<tr>
<td>Combined SOCS1/+/+ CD4+ T Cell Adoptive Transfer and SOCS1-KIR Mimetic Treatment Delays Lethal Disease</td>
<td>46</td>
</tr>
</tbody>
</table>
CD4+/SOCS1-KIR Treatment Increased Weight Gain, Delayed Leukocyte Infiltration into Heart and Liver, and Reduced Serum IFNγ Levels in SOCS1-/- Mice

Summary

4 EXAMINATION OF TREG POPULATION IN TREATED SOCS1-/- MICE

Background
Results
Treatment of SOCS1-/- Mice with CD4+/SOCS1-KIR Treatment Restores Foxp3+ Treg Peripheral Frequency and Decreases Peripheral Effector CD4+ T Cells
CD4+/SOCS1-KIR Treatment Confers Enhanced Foxp3+ Treg Peripheral Homeostasis and Reverses Lymphopenia in SOCS1-/- Mice
Enhanced Survival of Other SOCS1-/- Mouse Models Correlates with Maintained Treg Homeostasis
SOCS1 Deficient Tregs Experience Lineage Specific Transcription Factor Plasticity
Summary

5 DISCUSSION

6 MATERIALS AND METHODS

Mice
Genotyping
Magnetic Cell Separation
Flow Cytometry
Peptide Synthesis
In vivo Mouse Treatments
RNA Isolation and RT-qPCR
Cytokine Secretion Analysis
Histology
Bone Marrow Chimeras
Transcription Factor Analysis
Statistical Analysis

LIST OF REFERENCES

BIOGRAPHICAL SKETCH
<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-1</td>
<td>83</td>
</tr>
<tr>
<td>Primers used and/or discussed in this study.</td>
<td></td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Suppressor of cytokine signaling conserved structure</td>
<td>30</td>
</tr>
<tr>
<td>1-2</td>
<td>Inhibition of cytokine signaling by suppressor of cytokine signaling</td>
<td>31</td>
</tr>
<tr>
<td>1-3</td>
<td>Defects in regulatory T cells cause increased susceptibility to autoimmunity</td>
<td>32</td>
</tr>
<tr>
<td>2-1</td>
<td>Tregs constitutively express more SOCS1 mRNA than conventional T cells</td>
<td>40</td>
</tr>
<tr>
<td>2-2</td>
<td>SOCS1-/- mice are deficient in peripheral Foxp3+ regulatory T cells</td>
<td>41</td>
</tr>
<tr>
<td>2-3</td>
<td>Periphera Foxp3+ Treg deficiency in SOCS1-/- mice is not due to inadequate thymic development</td>
<td>43</td>
</tr>
<tr>
<td>2-4</td>
<td>Dysregulated cytokine production by SOCS1-/- lymphocytes is correlated to a reduction in peripheral Tregs</td>
<td>44</td>
</tr>
<tr>
<td>3-1</td>
<td>SOCS1-KIR restores partial function of SOCS1 in the absence of the endogenous protein</td>
<td>50</td>
</tr>
<tr>
<td>3-2</td>
<td>Schematic diagram of mouse treatment strategies performed on SOCS1-/- mice</td>
<td>51</td>
</tr>
<tr>
<td>3-3</td>
<td>Survival curve of SOCS1-/- mice receiving treatment</td>
<td>52</td>
</tr>
<tr>
<td>3-4</td>
<td>CD4+/SOCS1-KIR treatment increases the overall health of 2 week old SOCS1-/- mice</td>
<td>53</td>
</tr>
<tr>
<td>3-5</td>
<td>CD4+/SOCS1-KIR treatment prevents leukocyte infiltration into the heart and liver of 2 week old SOCS1-/- mice</td>
<td>54</td>
</tr>
<tr>
<td>4-1</td>
<td>Treatment of SOCS1-/- mice with CD4+/SOCS1-KIR treatment increases peripheral Tregs and decreases activated CD4+CD25+Foxp3- cells</td>
<td>66</td>
</tr>
<tr>
<td>4-2</td>
<td>CD4+/SOCS1-KIR treatment increases total, CD4+, and CD4+Foxp3+ peripheral lymphocyte numbers in SOCS1-/- mice</td>
<td>67</td>
</tr>
<tr>
<td>4-3</td>
<td>SOCS1-/-IFNγ-/-mice have a sustained peripheral Treg population</td>
<td>69</td>
</tr>
<tr>
<td>4-4</td>
<td>SOCS1 KO Bone marrow chimerashave a sustained peripheral Treg population</td>
<td>70</td>
</tr>
<tr>
<td>4-5</td>
<td>No leukocytic infiltration present in SOCS1 KO bone marrow chimeras</td>
<td>71</td>
</tr>
<tr>
<td>4-6</td>
<td>SOCS1 deficient Tregs experience lineage specific transcription factor instability upon IFNγ stimulation</td>
<td>72</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CIS</td>
<td>Cytokine-inducible Scr homology 2 containing protein</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T Lymphocyte Antigen 4</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl terminus</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>DTR</td>
<td>Diphtheria toxin receptor</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
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<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ESS</td>
<td>Extended SH2 sequence</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box p3</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
</tbody>
</table>
IPEX = immunodysregulation-polyendocrinopathy-enteropathy X-linked syndrome
iTr35 = IL35 producing suppressor cell
iTreg = Inducible regulatory T cell
JAK = Janus Kinase
KIR = Kinase inhibitory region
KO = Knock out
LN = Lymph node
MACS = Magnetic-activated cell sorting
MHC = Major Histocompatibility Complex
MS = Multiple sclerosis
NOD = Non-obese diabetic
nTreg = Natural regulatory T cell
N-terminus = Amino terminus
PBS = Phosphate buffered saline
qPCR = Quantitative PCR
RA = Rheumatoid arthritis
RORγt = RAR-related orphan receptor gamma t
SCID = Severe Combined Immuno-Deficiency
SH2 = Src homology 2
SLE = Systemic lupus erythematosus
SOCS = Suppressor of cytokine signaling
STAT = Signal Transducer and Activator of Transcription
T1D = Type 1 diabetes
Tbet = T-box transcription factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factorβ</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2</td>
</tr>
<tr>
<td>Th3</td>
<td>T helper 3</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17</td>
</tr>
<tr>
<td>Tr1</td>
<td>Type 1 regulatory T cell</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
Suppressor of cytokine signaling-1 deficient mice (SOCS1-/-) die of a T cell mediated inflammatory, autoimmune disease by 3 weeks of age. In SOCS1-/- mice inflammation mediates excessive interferon (IFN)γ signaling and leukocyte infiltration, resulting in the destruction of many vital organs. Significantly, numerous mouse models of inflammatory autoimmune disease have been associated with a deficiency in Foxp3+ regulatory T cells (Tregs). Indeed, SOCS1-/- mice possessed a reduction in peripheral Tregs, despite enhanced thymic development. Notably, SOCS1-/- lymphocytes have dysregulated cytokine production, including reduced capacity to make interleukin (IL) 2, which is required for the survival of Tregs. The adoptive transfer of SOCS1+/+ Tregs or CD4+ T lymphocytes mediated an increased, yet limited survival in the SOCS1-/- mice. However, the adoptive transfer of SOCS1+/+ CD4+ T lymphocytes in conjunction with SOCS1-KIR, a mimetic peptide sufficient to partially restore SOCS1 function, resulted in 30% of the mice living beyond 5 weeks of age. Moreover, the combined treatment mediated a decrease in leukocytic infiltration into vital organs, IFNγ, and effector T cell frequency. Additionally, the decrease in inflammation was associated with an increase in the peripheral Foxp3+ regulatory T cell population. Collectively, these results suggest
that the CD4+ T cell/SOCS1-KIR treatment promoted long-term survival of the SOCS1-/- mice partly by decreasing inflammation and restoring peripheral Tregs. In fact, our results show that SOCS1 deficiency allows for the instability of lineage specific transcription factors in Tregs. Furthermore, these data propose a relationship between SOCS1 and the peripheral stability of Tregs under inflammatory conditions.
CHAPTER 1
INTRODUCTION

Immune Regulation

The immune system is an intricate web of immune cells, cytokines, and signaling cascades that our body uses to defend against potential pathogens. These invaders include bacteria, viruses, and large parasites, all of which can cause infection and subsequent inflammation in our tissues. Inflammation is a mechanism of innate and adaptive immunity that is achieved by the increased movement of various immune cells to the site of infection or tissue damage. A proper inflammatory response is designed to begin with quick and accurate identification of the foreign antigen. Upon proper identification, the antigen should be eliminated from the host by specific immune cells. Finally, cells that have been specifically activated by antigen should quickly clear immune waste from the site of inflammation. If cells cannot follow this procedure it can lead to excessive inflammation, damaging host tissues. In addition to defending against invading pathogens, our immune system has developed the ability to identify and eliminate host cells that have gone rogue and cause complications in our bodies. These host cells may be auto-reactive immune cells that have been poorly educated during their development and have specificity to self-antigens. The inability of our body to prevent the development and activation of auto-reactive cells contributes to autoimmunity.

Although inflammation is a necessary property of an immune response, excessive inflammation, due to faulty immune cell function, can trigger autoimmunity. Autoimmunity is the failure of our immune system to tolerate self (Khoruts and Fraser, 2005). Therefore, autoimmune cells will attack host tissues and cells by recognizing
self-antigens as foreign. In order to prevent autoimmunity, our immune system has developed a set of checkpoints to eliminate cells that can potentially be precursors to autoimmunity. More specifically, these are checkpoints are put in place to eliminate, or stop, auto-reactive T cells which are one of the agents contributing to autoimmunity. Although our immune system has a series of mechanisms to prevent autoimmunity, there are times when these regulatory mechanisms fail. This leads to the development of autoimmune diseases like type 1 diabetes (T1D), inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS) (Buckner, 2010).

Central and peripheral tolerance mechanisms are measures by which our immune system prevents development of autoimmunity by T cells. Central tolerance occurs during the thymic development of T cells. During thymic development, T cells that play an important role in cell-mediated immunity, undergo a stringent education and selection process. Throughout this selection process, they are exposed to various antigens, both foreign and self, and develop an exclusive specificity to one particular antigen. T cells that are reactive to self-antigens are eliminated during negative selection via programmed cell death, before export into the periphery. After the developmental selection process has occurred, and cells have moved into the periphery, peripheral tolerance mechanisms ensure reliability of immune cells. In the periphery, the suppressors of cytokine signaling (SOCS) proteins regulate cells responsiveness to cytokines (Kubo et al., 2003). In addition to cytokine regulation, cells with the ability to suppress immune responses, regulatory T cells (Tregs) can regulate cells at the site of inflammation (Miyara and Sakaguchi, 2007).
Suppressor of Cytokine Signaling

Suppressor of cytokine signaling (SOCS) and cytokine-inducible SH2 protein (CIS) are a family of intracellular proteins that are involved in modulating immune responses by reducing cytokine responsiveness by the cell (Yoshimura et al., 2007). There are 8 members of the CIS/SOCS protein family; CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, and SOCS7 (Krebs and Hilton, 2001). The members of this family have similar structure, all containing a carboxy-terminal domain, Src homology 2 (SH2) domain, and amino-terminal domain (Figure 1-1).

The carboxy-terminal domain of SOCS proteins is known as the SOCS box. The SOCS box is composed of 40 amino acids. The SOCS box mediates degradation of proteins that are associated through the amino-terminal regions. The SOCS box has three alpha helices bound to an E3 ubiquitin ligase complex. When combined with an E1 ubiquitin-activating enzyme, and E2 ubiquitin conjugating enzyme, this complex polyubiquitinates the SOCS binding partner and targets the receptor complex for proteasomal degradation (Zhang et al., 2001).

The SH2 domain of the SOCS proteins binds to phosphotyrosine residues on the target protein leading to inhibition of signal transduction. There is also a unique amino-extended SH2 sequence (N-ESS) domain that is next to the SH2 domain. This domain is a 15 residue alpha-helix that directly contacts the phosphotyrosine binding loop (Krebs and Hilton, 2001; Yoshimura et al., 2007; Croker and Kiu, 2008).

Lastly, there is the amino-terminal domain. This region contains the kinase inhibitory region (KIR) in SOCS1 and SOCS3. The KIR region is constituted of 12 amino acids. KIR is required for the inhibition of the Janus kinases (JAK) phosphorylation activity. JAK enzymatic activity is blocked when KIR is lodged into the catalytic cleft.
preventing subsequent phosphorylation and stopping the signaling cascade (Croker and Kiu, 2008).

SOCS proteins mediate their function through the three protein domains mentioned above: the SOCS-BOX, SH2, and KIR regions of the protein. These three domains allow the SOCS proteins to modulate cytokine responsiveness by the cell. Each member of the SOCS protein family is induced by and inhibits numerous cytokines. Although the various members of the SOCS family inhibit different cytokines, they possess similar means of suppression.

**SOCS1**

SOCS1 is one of the more heavily studied SOCS proteins. It is a cytokine-inducible negative regulator of the Janus kinas/signal transducer and activator of transcription (JAK/STAT) cytokine signaling pathway. Interleukin 2 (IL2), IL3, erythropoietin (EPO), granulocyte/monocyte colony-stimulating factor (GM-CSF), and IFNγ are a subset of cytokines that can induce and in turn, be inhibited by SOCS1, in a negative feedback fashion (Alexander et al., 1999). As shown in Figure 1-2, SOCS1 can stop signaling in the JAK/STAT pathways in two ways. As mentioned before SOCS1 can inhibit cytokine signaling by targeting the complex for degradation or by impeding phosphorylation of the JAK and STATs (Croker and Kiu, 2008). Both of these signaling prevention methods utilized by SOCS1 obstructs the transcription of cytokine responsive genes that can lead to further inflammation, proliferation, and cytokine production by the cell.

In addition to the inhibition of cytokine signaling, SOCS1 is also required during the development and differentiation of T cells. In fact, SOCS1 expression is vital for normal development in the thymus. SOCS1 is highly expressed in the thymus and is
necessary for proper T cell selection (Zhan et al., 2009). Increased SOCS1 expression in the thymus likely because it can prevent the effects of inflammatory cytokines produced by thymocytes that can perturb the selection process.

The role of SOCS1 has recently been studied in terms of its role in the differentiation of CD4+ T cell subsets (Palmer and Restifo, 2009). Since polarization into a T helper lineage is driven by a specific cytokine milieu, it is logical that a protein such as SOCS1 would have an effect on the differentiation process of these cells. Moreover, studies have proven that SOCS1 is required for the differentiation of CD4+ T cells into the Th17 phenotype, due to the ability of SOCS1 to inhibit the contradictory effects of IFNγ (Tanaka et al., 2008). Thus, SOCS1 plays a critical role in modulation of cytokine signals that can influence the development and differentiation of T cells.

**Role of SOCS1 in Autoimmunity**

SOCS1 is an essential regulator of immune responses because it is involved in inhibiting the signaling of a wide variety of immune cytokines. Since SOCS1 has an obvious role in maintaining immune homeostasis, it has been of particular interest to see if SOCS1 abnormalities are associated with autoimmune diseases. In fact, it was found that SOCS1 expression is reduced in NZBxNZW F1 mice, the murine lupus model (Sharabi et al., 2009). Additionally, expression of SOCS1 is also altered in human patients with rheumatoid arthritis or systemic lupus (Tsao et al., 2008). It is also suggested that decreased levels of induction of SOCS1 may play a role in determining the course of EAE in mice (Stark and Cross, 2006). Supporting studies across a broad range of autoimmune diseases suggest that SOCS1 plays a role in the onset or progression of autoimmunity.
**Effects of SOCS1 Deficiency**

The requirement of SOCS1 in maintaining immune homeostasis is supported in mice with a deficiency in SOCS1, as they experience severe T cell mediated autoimmunity and die by 3 weeks of age (Marine et al., 1999). Manifestations of SOCS1 knock out (KO) disease include autoimmune arthritis, joint inflammation, and severe growth retardation. It is known that the neonatal defects in SOCS1-/- mice are caused by excessive IFNγ signaling. This was determined by creating SOCS1-/-IFNγ-/- mice and finding that these mice experience delayed autoimmunity (Alexander et al., 1999). In addition, treatment of SOCS1-/- mice with anti-IFNγ antibodies ameliorated disease and allowed for extended survival of these mice, confirming the causative role of IFNγ in this disease.

T cell specific SOCS1-/- mice and SOCS1-/-IFNγ-/- mice also experience perturbed thymic selection of CD4+CD8- and CD4-CD8+ thymocytes. Moreover, it has been shown that increases in Treg selection can occur in thymocytes deficient in SOCS1 (Zhan et al., 2009). Although Treg numbers are higher in the periphery of these mice, they still succumb to autoimmunity, suggesting the requirement of SOCS1 in Treg function or stability (Zhan et al., 2009). In fact, studies looking at T cells deficient in SOCS1 have shown Tregs require SOCS1 to maintain a stable phenotype and suppressor functions (Takahashi et al., 2011). Phenotype, or lineage, stability is a method of SOCS1 regulation that goes beyond the traditional manipulation of cytokine signaling. Thus it is important to examine the role of SOCS1 in Tregs further.

**Regulatory T Cells**

One of the key players in controlling our immune system is regulatory T cells. The initial discovery of Tregs occurred when a subset CD4+ T cells was found that
constitutively expressed the IL2 receptor alpha-chain, cluster of differentiation 25 (CD25), and cytotoxic T lymphocyte antigen 4 (CTLA4) (Rudensky, 2011). These cells were originally thought to be subset of constantly activated T cells that served as a sink, depriving other immune cells of resources to promote inflammation. The suppressive function of these Tregs was elucidated when they were adoptively transferred into day 3 thymectomized mice. These thymectomized mice suffer from systemic inflammation. However, the addition of this suppressive subset of cells protected mice from inflammation (Asano et al., 1996). In vitro studies of these suppressor cells suggested that they were anergic, being unable to proliferate or produce IL2 upon T cell receptor (TCR) stimulation (Papiernik et al., 1998). Yet, when put into lymphopenic mice, they expanded vigorously (Cozzo et al., 2003). Tregs were finally accepted as a distinct suppressor T cell population after CD4+ cells were activated to induce CD25 but they were unable to rescue day 3 thymectomized mice from inflammation (Suri-Payer et al., 1998). These findings proposed that CD4+CD25+ Tregs were a thymically derived and had a true suppressive function.

**Treg Transcription Factor Foxp3**

As various subsets of T helper cells have transcription factors that are specific to their lineage, Tregs also possess this trait. Forkhead box p3 (Foxp3) is the transcription factor that identifies Tregs (Hori et al., 2003; Khattri et al., 2003). Foxp3 was found to be associated to Tregs after a loss of function mutation, in this X chromosome encoded transcription factor, lead to multi-organ autoimmunity in humans. This extreme inflammatory disorder in humans is called immunodysregulationpolyendocrinopathyenteropathy X-linked syndrome (IPEX). Like humans suffering from IPEX, mice with the Foxp3 mutation also suffer from a
comparable disease that has been named scurfy (Blair et al., 1994; Chatila et al., 2000; Wildin et al., 2001; McGinness et al., 2006; Torgerson and Ochs, 2007). These data establish an important role for the Foxp3 transcription factor in the maintenance of immune homeostasis.

Initial studies of the scurfy mouse revealed that the inflammatory disease caused by a Foxp3 mutation was T cell mediated. T cell mediated autoimmunity lead to the notion that a Foxp3 mutation could be impairing Treg function or development. This was confirmed when bone marrow from Foxp3 knockout and Foxp3 sufficient mice was mixed 1:1 and transferred into a depleted mouse. In the chimeric mouse, Foxp3 sufficient donor cells were the only cells that developed into CD4+CD25+ cells in the thymus (Fontenot et al., 2003; Hori et al., 2003). This showed the importance of Foxp3 in the development of thymically derived nTregs.

The above studies support the role of Foxp3 in Treg development, but the requirement of Foxp3 in Treg function was still unclear. This was addressed by studying mice, which still promoted Foxp3 expression but did not have a functional Foxp3 protein. These mice harbored a green fluorescent protein (GFP) coding DNA sequence knocked-in the Foxp3 locus (Gavin et al., 2007). Studies within these mice exposed Foxp3 as unnecessary for the survival/development of Treg precursors, but it was required for the suppressor function of Tregs. Additionally, high expression of Foxp3 was required to maintain the molecular markers of Tregs that are essential to their function and longevity. Studies supported this by showing lower levels of Foxp3 were correlated with reduced expression of CD25 and CTLA4 (Wan and Flavell, 2007). Likewise, Foxp3 reverses cell features that would be detrimental to Treg function. This
is shown when Foxp3 binds to RAR-related orphan receptor gamma t (RORγt), a transcription factor specific to T helper 17 (Th17) cells, and inhibits the expression of IL17 and a Th17 phenotype (Zhou et al., 2008). These studies confirm the importance of Foxp3 expression in not only development, but also in the suppressive functions and phenotype stability of Tregs.

**Role of Tregs in Immune Regulation**

Although thymic development of T cells works to eliminate auto-reactive T cells, some auto-reactive cells slip past the stringent selection process and reside in the periphery (Miller, 2002; Sprent and Kishimoto, 2002). This is where Tregs play an important role in preventing autoimmunity. Tregs control immune responses and maintain immune homeostasis by inhibiting lymphocyte activation and subsequent autoimmunity. The early peripheral role of Tregs is clear in the scurfy (Brunkow et al., 2001) and in the Day 3 thymectomized mouse (Min et al., 2003), as these mice both lack early Treg populations. The early deficiency in Tregs causes severe autoimmunity and even death in these mice. The presence of a Treg population is required to regulate immune homeostasis, during the development of the peripheral T cell repertoire.

In addition to the role of Tregs in the development of the peripheral T cell repertoire, they also play a role in maintaining immune homeostasis throughout the lifetime of an individual (Lahl et al., 2007). This was established by the elimination of Tregs which possess the human diphtheria toxin receptor (DTR) expressed under control of the Foxp3 locus (Foxp3DTR mice) (Kim et al., 2007). Treg elimination was attained in Foxp3DTR mice by the administration of diphtheria toxin (DT). Mice receiving DT from birth suffered from a disease similar to scurfy mice, where adult mice experienced autoimmunity characterized by lymphoproliferative disease within 3 weeks.
Moreover, antibody mediated T cell depletion of CD4+ T cells in conjunction with Treg deficiency prevented autoimmune disease onset (Kim et al., 2007). These results suggested that in the absence of Tregs, activation of autoreactive CD4+ T cells is what drove proliferation of adaptive cells of the immune system. Furthermore, the requirement of Tregs throughout the life of an individual was essential for immune regulation.

**Treg Associated Molecules**

Various cytokines and cell surface molecules play a fundamental role in the survival and suppressive function of Tregs. Tregs are able to suppress using soluble anti-inflammatory cytokines; for example IL10, IL35, and Transforming Growth Factorβ (TGFβ) are the three most heavily studied Treg cytokines (Collison et al., 2007; Vignali et al., 2008; Collison et al., 2009). The three anti-inflammatory cytokines used by Tregs to suppress seem to have much overlap in function. Tregs also suppress immune responses via cell-to-cell contact using cytotoxic T lymphocyte antigen 4 (CTLA4) (Sojka et al., 2009). In addition to these Treg associated inhibitory cytokines/molecules, Tregs also require IL2 for peripheral maintenance/survival (Malek et al., 2008).

IL10 is a homodimeric cytokine produced by a wide variety of cells and has a wide range of inhibitory actions. The regulatory activity of IL10 mainly affects APCs, however IL10 also functions as an inhibitor of T helper 1 (Th1) responses (Moore et al., 2001; Mosser and Zhang, 2008). IL10 deficient mice do not develop autoimmunity but they do develop colitis in response to some intestinal bacteria (Kühn et al., 1993). In experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, IL10 deficient mice experience intensified disease. IL10 made by Tregs is associated with the remission phase of EAE, and adoptive transfer of Tregs can prevent EAE via IL10.
signaling. IL10 is also involved in the generation of the Tr1 induced Treg population, which also produces IL10 (Allan et al., 2008).

TGFβ can inhibit Th1 and T helper 2 (Th2) responses, T cell proliferation, aid in nTreg maintenance (Marie et al., 2006; Li et al., 2007). TGFβ has been connected to Tregs suppressive function in type 1 diabetes (T1D) and inflammatory bowel disease (IBD) (Fahlén et al., 2005; Tonkin and Haskins, 2009). Furthermore, depletion of TGFβ with anti-TGFβ antibodies results in diminished Treg function in both humans and mice (Joetham et al., 2007; Strauss et al., 2007). TGFβ deficient mice succumb to spontaneous autoimmunity by 5 weeks of age, characterized by a dysregulated inflammatory response (Letterio et al., 1996; Kobayashi et al., 1999). In addition to its suppressive function, TGFβ can also induce the regulatory T cell subset, Th3.

IL35 is the most recently discovered suppressive cytokine of Tregs and can inhibit Th1, Th2, and Th17 responses. IL35 is a heterodimeric cytokine composed of the IL12α and IL27β chains (Collison et al., 2007). This cytokine is required for optimal regulatory function in vivo, as Tregs deficient in either chain of the cytokine are unable to effectively suppress T cell proliferation and inhibit inflammatory bowel disease (IBD) (Collison et al., 2007). Whether or not this cytokine has the ability to inhibit all T cell, B cell, and APC subsets has yet to be determined. Like IL10 and TGFβ, IL35 can induce a regulatory T cell population called iTr35 cells(Collison et al., 2010).

CTLA4 (cytotoxic T lymphocyte antigen 4) plays an important immunoregulatory role the immune system, particularly in Tregs, as it mediates contact dependent suppression(Sojka et al., 2009). CTLA4 is present on T cells and is similar to the CD28 costimulatory protein, as it binds to CD80 and CD86 on APCs. In contrast to CD28,
CTLA4 transmits an inhibitory signal to the cell (Walker and Sansom, 2011). CTLA4 is found intracellularly in Tregs and is an important aspect of their contact dependent suppressive function. In fact, CTLA4 deficient Tregs are unable to control expansion of CD4+ T cells in a lymphopenic environment and cannot prevent colitis. These CTLA4 deficient Tregs also failed to suppress cytokine production associated with T cell expansion (Sojka et al., 2009). These results show the significance of CTLA4 in the regulatory capabilities of Tregs.

Although IL2 is not an anti-inflammatory cytokine or suppressive molecule used by Tregs, it is necessary for their expansion and survival. IL2 is also necessary for the development of T cell memory as well as being required for the development and maintenance of Tregs in the thymus and periphery (Malek et al., 2008). Production of IL2 is achieved when antigen binds to the TCR of conventional T cells. Although Tregs cannot produce their own IL2, they require it to expand and maintain suppressive function. IL2 helps to maintain Treg homeostasis by the downstream phosphorylation of STAT5, which leads to the upregulation of Foxp3, heightening suppressive function (Malek et al., 2008). In the absence of IL2, Tregs have reduced suppressive capabilities and decreased survival.

**Effects of Treg Deficiency**

An underlying cause of autoimmunity is thought to be due to a deficiency in Tregs, either in numbers, function, or the presence of effector cells resistance to Tregs (Figure 1-3). This reduction in Tregs may not be systemic and may be restricted to the site of inflammation. There are many plausible causes for a Treg deficiency. A disturbed population of Tregs in the periphery could be due to a defect in the selection of Tregs, leading to fewer cells in the periphery. A pro-inflammatory environment can
also hinder Tregs from eliciting their suppressive function. Regardless of how a Treg deficiency develops, the inability of Tregs to suppress leads to the development of excessive inflammation and eventual autoimmunity.

Although some studies examining the correlation of Tregs and autoimmunity have not shown significant results regarding Treg populations, these results are debatable due to the limitations in tissue availability for examination. Despite the varied results from these studies, low levels of Tregs are correlated with various autoimmune diseases such as type 1 diabetes (Tonkin and Haskins, 2009). Additionally, the adoptive transfer of Tregs has proven successful in preventing autoimmunity in mouse models of SLE, MS, IBD, and T1D (Brusko et al., 2008). Due to Tregs’ importance in controlling autoimmunity, there has been much research on finding novel ways to develop therapeutic strategies using Treg cells.

**Project Rationale and Design**

The maintenance of immune homeostasis is critical for a healthy immune system. One should be able to mount a robust immune response and shut it down upon clearance of the pathogen. The appropriate regulation of an immune response inhibits excessive inflammation, which could potentially lead to autoimmunity. Immune cells can be regulated in the periphery by both cytokine and peripheral regulation mechanisms.

SOCS1 plays a critical role in the modulation of cytokine signaling intracellularly. A deficiency in SOCS1 causes excessive inflammation resulting in a severe autoimmune disease leading to death by 21 days in mice (Marine et al., 1999). In addition to immune regulation by SOCS1, Tregs also play an essential role in maintaining immune homeostasis. A deficiency in Tregs allows for disrupted regulation
of immune cells further leading to autoimmunity (Wing and Sakaguchi, 2010).

Furthermore, mice with a genetic mutation rendering their Tregs nonfunctional suffer from autoimmunity resulting in death (Brunkow et al., 2001).

The importance of both the SOCS1 and Treg immune regulatory mechanisms cannot be ignored. However, the relationship between these two pathways has not been fully examined. We hypothesize that SOCS1 plays an essential role in the maintenance of Tregs. The purpose of this study was to analyze the effects of SOCS1 deficiency on Treg homeostasis.

The first goal of this project was to assess the presence of Tregs in SOCS1-/- mice. Since SOCS1 deficient mice suffer from a severe autoimmune disease due to excessive inflammation, we wanted to examine whether disease was caused by a deficiency in Tregs. The second goal of this project was to develop a treatment to decrease inflammation in SOCS1-/- mice. Due to the early onset of severe autoimmunity and death by 3 weeks of SOCS1-/- mice, we wanted to develop a treatment strategy to reduce inflammation and prolong life of our mouse model. By doing this, we will have an increased window of time for examination of cell populations. The third goal of this project was to examine the Treg population in treated SOCS1-/- mice. We wanted to examine Tregs post treatment because the excessive inflammation of the untreated mouse may be detrimental to the SOCS1 deficient Treg population.

This work is designed to determine the role that SOCS1 plays in the maintenance of a peripheral Treg population. By understanding the interplay between the SOCS1 and Treg immune regulatory pathways, one can further examine the
mechanism by with SOCS1 regulates the regulators. SOCS1 can potentially be used as a target for the development of Treg specific therapeutics.
Figure 1-1. Suppressor of cytokine signaling conserved structure. The SOCS family of proteins have a relatively conserved structure. SOCS proteins possess a SH2 domain that binds to phosphotyrosines on the cytokine receptor blocking transcription factor binding. They also have a SOCS-BOX region, which can target receptor complexes for proteasomal degradation. SOCS1, SOCS3, and possibly SOCS5 have a kinase inhibitory region (KIR) that can bind to the activation loop of janus kinases (JAKs) to inhibit JAKs ability to phosphorylate. All of these regions of the SOCS protein work to inhibit cytokine signals.
Figure 1-2. Inhibition of cytokine signaling by suppressor of cytokine signaling-1. Upon a cytokine’s interaction with its corresponding receptor, JAKs can phosphorylate themselves, one another, and the receptor. STATs bind to the phosphorylated receptor and then homo- or hetero-dimerize and move into the nucleus to initiate the transcription of cytokine specific genes. One of these genes is for the SOCS1 protein. Upon upregulation, SOCS1 can inhibit cytokine signaling by inhibiting phosphorylation of the JAKs or by targeting the receptor complex for degradation.
Figure 1-3. Defects in regulatory T cells cause increased susceptibility to autoimmunity. Defects in Tregs include deficiency in number/percentage, lack of suppressor function, and resistance of effector cells to being suppressed. Any one, or a combination, of these defects promote the development of autoimmunity.
CHAPTER 2
ASSESS THE PRESENCE OF TREGSINSOCS1/- MICE

Background

Thymic development, as mentioned previously, is an important aspect of the immune system that allows for proper development of T cells to ensure elimination of auto reactive cells that could potential induce autoimmunity (Miller, 2002; Aspinall et al., 2010). Upon export from the thymus, T cells should have specificity to foreign antigen without being auto-reactive. Regulatory T cells can develop in two accepted ways. Tregs can develop in the thymus with conventional T cells and are termed natural Tregs (nTreg) or they can be induced in the periphery from naïve CD4+ T cells and are termed induced Tregs (iTreg). Regulatory T cells develop in the thymus in a manner slightly different than conventional T cells. While conventional T cells are targeted for deletion when their TCR has self-antigen specificity, Tregs are not targeted for deletion. The nTreg TCR is specific for host-antigens (Caton et al., 2004; Cabarrocas et al., 2006). T cells that have a host antigen specific TCR and also express the Treg transcription factor, forkhead box p3 (Foxp3), will not be deleted during negative selection in the thymus (Bettini and Vignali, 2010). Instead, these Foxp3+ T cells will be released into the periphery with suppressive function, being denoted as nTregs. Although they are released from the thymus slightly later than conventional T cells, the thymic development of Tregs is critical in early immune repertoire development. This fact is confirmed by day 3 thymectomized mice that die of severe inflammation due to a lack of Tregs. This is because the thymus is removed before Tregs can be exported (Suri-Payer et al., 1998). The presence of Tregs early on in the periphery is required to prevent autoimmunity/inflammation.
In addition to Tregs that develop naturally in the thymus, Tregs can also be induced from naïve T cells in the periphery. The distinction between nTreg and iTreg is site their of development and antigen specificity. Unlike nTregs, iTregs do not require costimulation to become activated. This is due to the cytokine environment present when this Treg subset develops. iTregs are also different from nTregs in that they do not have TCR specificity to self-antigens. Indeed, iTregs have specificity for non-self antigens similar to that of effector T cells, and they become suppressor cells at the site of inflammation (Belkaid et al., 2002; Suvas et al., 2004; Suffia, 2006; Belkaid, 2008).

Both nTregs and iTregs play an important role in maintaining immune homeostasis (Vignali et al., 2008; Betts et al., 2012). nTregs control autoreactive T cells responding to self-antigens. Conversely, iTregs are usually found at the site of inflammation and suppress inflammation in response to non-self antigens.

There are three types of iTregs currently recognized: type 1 regulatory cells (Tr1) (Tang and Bluestone, 2008), T helper-3 T cells (Th3) (Vignali et al., 2008), and IL35 producing suppressor cells (iTr35) (Collison et al., 2010). Each of these subsets requires a unique cytokine environment to facilitate induction. Tr1 cells do not express Foxp3, are induced by IL10, and suppress via IL10 and TGFβ (Mandapathil and Whiteside, 2011). Th3 cells are induced by TGFβ and express both Foxp3 and TGFβ after transformation (Xu et al., 2003). Lastly, iTr35 cells express Foxp3 are induced by and produce IL35 (Collison et al., 2010; Gravano and Vignali, 2011). Although these iTreg subtypes are developed differently, they all possess the ability to control the development of excessive inflammation/autoimmunity.
Regardless of the means of Treg development, their presence is necessary for the maintenance of immune homeostasis. A deficiency in Tregs allows for the development of autoimmunity. In this chapter, we identify a deficiency in Tregs in SOCS1-/- mice, which succumb to lethal autoimmunity by 21 days. The experiments provide evidence suggesting Treg deficiency in SOCS1-/- mice is likely not due to poor thymic development, but rather dysregulation of the peripheral cytokine environment.

**Results**

**SOCS1-/- Mice are Deficient in Peripheral Foxp3+ Tregs.**

Tregs are critical inhibitors of inflammation. Without this ability to alleviate inflammation, autoimmunity can develop. Since SOCS1-/- mice die of an inflammation mediated autoimmune disease, we wanted to examine the presence of Tregs in the periphery of SOCS1 deficient mice at 2 weeks of age. Tregs are CD4+CD25+Foxp3+ cells. We initially examined SOCS1 expression in WT CD4+CD25- conventional T cells and WT CD4+CD25+ Tregs. As shown in Figure 2-1, WT CD4+CD25+ Tregs express more Foxp3 mRNA than conventional T cells. These Tregs also express more SOCS1 mRNA than the CD4+CD25- T cells. We observed that the frequency of Tregs is reduced from 11% to 8% in the lymph nodes and 13% to 5% in the spleen from WT and SOCS1 deficient mice, respectively (Figure 2-2). Furthermore, despite the comparable absolute numbers in the lymph nodes of total and CD4+ T cells in 2 week old WT and SOCS1-/- mice, there is a significant decrease of the absolute number of Tregs in SOCS1-/- mice when compared to WT littermate controls (6.6x10^4 versus 1.5x10^5; p=.02; Figure 2-2A). Consistent with previous studies, SOCS1-/- mice are lymphopenic, with total and CD4+ lymphocytes in spleens of 2 week old SOCS1-/- mice being significantly reduced in contrast to WT controls (Figure 2-2B). Notably,
there was a reduction in Treg numbers in the spleen of SOCS1−/− mice to WT (2x10^4 versus 1.2x10^5; p=.0003; Figure 2-2B). Together these results show the importance of SOCS1 in Tregs and that SOCS1−/− mice have both a frequency and numeric deficiency in secondary lymphoid organs.

**Thymic Development of SOCS1−/− Tregs Does Not Contribute to Peripheral Deficiency.**

The thymus plays a vital role in the development of the peripheral T cell repertoire, and more importantly, in the early establishment of peripheral Tregs. We next determined whether the peripheral reduction of Tregs was due to defective development in the thymus. Consistent with previous studies, analysis of the thymus by flow cytometry shows higher frequencies of mature CD4+CD8− and CD8+CD4− lymphocytes in SOCS1−/− mice in comparison to WT mice at 2 weeks of age (Figure 2-3A) (Zhan et al., 2009; 2009). The increase in mature lymphocytes could be attributed to a decrease in immature CD4+CD8+ thymocytes (85% WT to 32% KO) and a decrease in total thymocytes (4.8x10^7 WT versus 1.5x10^7 KO; p=.0001; Figure 3-3C). Regardless of the reduction of total thymocytes in SOCS1−/− mice, the numbers of CD4+CD8− and CD4+CD8−Foxp3+ Tregs were comparable between SOCS1−/− and WT mice (Figure 2-3C). In fact, Foxp3+ Treg percentage was also comparable in WT and SOCS1−/− (Figure 2-3B) and these Tregs are CD4+CD8− (Figure 2-3A).

Collectively, this data suggests that improper thymic development of Tregs is not likely a contributing factor in the peripheral deficiency of Tregs in the SOCS1−/− mice.

**Treg Deficiency is Correlated to Dysregulated Cytokine Production.**

Since SOCS1 is an inhibitor of cytokine signaling, we next examined the cytokine production of lymphocytes in the absence of SOCS1. *Ex vivo* analysis of IL2 (which is
essential for Treg survival) and pro-inflammatory cytokines IL17 and IFNγ show no significant difference between SOCS1-/- and WT littermates at 2 weeks (Figure 2-4). We next measured the ability of lymphocytes collected from 2 week old SOCS1-/- and WT mice to produce IFNγ, IL17 and IL2 by ELISA. As seen in Figure 2-4, there is no statistical difference between unstimulated lymphocytes. However, stimulated SOCS1-/- lymphocytes have a dysregulated cytokine production characterized by decreased production of IL17 and increased production in IFNγ in comparison to WT controls (Figure 2-4). Strikingly, SOCS1-/- lymphocytes produce significantly less IL2 than WT lymphocytes upon TCR stimulation. These results collectively show that lymphocytes deficient in SOCS1 have an enhanced capacity to produce IFNγ and a reduced ability to produce IL17 and IL2. Thus suggesting that the lack of cytokine regulation is likely contributing to the peripheral reduction of Tregs in SOCS1-/- mice.

**Summary**

SOCS1-/- mice succumb to severe T cell mediated autoimmunity resulting in death by 21 days (Marine et al., 1999). This disease is characterized by leukocytic infiltration into vital organs, excessive IFNγ production, and extreme lymphocyte activation. In addition to the previously addressed indicators of SOCS1-/- disease, this study has shown that SOCS1 deficient mice also suffer from lymphopenia, particularly in peripheral Foxp3+ Tregs (Figure 2-2). The deficiency in peripheral Tregs is in contrast to previous studies utilizing T cell specific deficiency of SOCS1 (Zhan et al., 2009). Since we saw this deficiency in peripheral Tregs, we further examined the thymus of SOCS1-/- mice, as the development of thymically derived Tregs is critical for healthy peripheral homeostasis early on. Strikingly, we found increased percentages and
numbers of Tregs in the thymus of SOCS1-/- mice in comparison to their WT littermate controls (Figure 2-3). These results were consistent with previous studies examining the thymic development of Tregs in T cell specific SOCS1 deficient mice (Zhan et al., 2009). Our findings suggest that the Treg deficiency seen in the SOCS1-/- mouse was not due to thymic development but rather from a peripheral influence.

The peripheral cytokine environment influences cells to elicit their function. In T cells, for example, various cytokines present upon T cell activation can guide the cell to differentiate into various T cell subsets such as, Th1, Th2, Th17 or iTreg. Once differentiated, cells may require cytokines that are critical for their survival. Tregs, for example, require the presence of IL2 in order to survive (Vignali et al., 2008). Moreover, the presence of cytokines which are associated with inflammation have recently been shown to cause plasticity of T cell subtypes (Zhou et al., 2009a). Due to the importance of the cytokine environment for the maintenance of Tregs, we then decided to examine the cytokine profiles of lymphocytes deficient in SOCS1.

Our studies revealed an increased capacity of SOCS1-/- cells to produce IFNγ upon TCR stimulation in comparison to SOCS1 sufficient lymphocytes. These results were consistent with studies showing a predominant Th1 bias in SOCS1-/- mice that was determined by excessive IFNγ production (Alexander et al., 1999) (Figure 2-4). In addition to the excessive ability of SOCS1-/- lymphocytes to produce IFNγ, we found a decreased ability to produce IL17 (Figure 2-4). This is expected due to the reciprocal relationship of IL17 and IFNγ. One of the most striking findings was the inability of SOCS1-/- mice to produce IL2, a cytokine required for Treg survival, to the same extent as SOCS1+/+ mice (Figure 2-4). Together these results conclusively show that
SOCS1/- mice suffer from dysregulation of several cytokines, one of which (IL2) is required for survival and peripheral expansion of Tregs.
Figure 2-1. Tregs constitutively express more SOCS1 mRNA than conventional T cells. Comparison of Foxp3 and SOCS1 relative expression between WT CD4+CD25+ and CD4+CD25- lymphocytes. Axillary, brachial, inguinal, superficial cervical, and mesenteric lymph nodes were isolated from WT mice, followed by magnetic separation of CD4+CD25+ and CD4+CD25- T lymphocytes. Graph showing relative expression of Foxp3 and SOCS1 in CD4+CD25- and CD4+CD25+ lymphocytes relative to β actin. Data shown is representative of 4 independent experiments. Statistical comparisons between CD4+CD25- and CD4+CD25+ cell fractions were performed using unpaired, two-tailed t test with statistical significance denoted by asterisks. *, p ≤ 0.05; ** , p ≤0.005.
Figure 2-2. SOCS1-/- mice are deficient in peripheral Foxp3+ regulatory T cells. (A) Axillary, brachial, inguinal, superficial cervical, and mesenteric lymph nodes were isolated from SOCS1-/- (n=12) or WT littermate controls (n=12) at 2 weeks. Top: Histogram showing percentages of CD4+Foxp3+ lymphocytes in SOCS1-/- and littermate controls. Bottom: Graphs showing absolute numbers of total, CD4+, and CD4+Foxp3+ lymphocytes. Each dot is representative of an individual mouse with averages denoted by lines. (B) Spleens were isolated from mice indicated in A. Top: Histogram comparing percentages of CD4+Foxp3+ lymphocytes in SOCS1-/- and littermate controls. Bottom: Graphs showing absolute numbers of total, CD4+, and CD4+Foxp3+ lymphocytes. Each dot is representative of an individual mouse with averages denoted by lines. Statistical comparisons between WT and SOCS1-/- mice were performed using unpaired, two-tailed t test with statistical significance denoted by asterisks. *, p ≤ 0.05; ***, p ≤ 0.0005.
Figure 2-3. Periperal Foxp3+ Treg deficiency in SOCS1-/- mice is not due to inadequate thymic development. WT (n=14) and SOCS1-/- (n=11) mice were sacrificed at 2 weeks afterbirth. Thymii were removed and analyzed. (A) Dot plots showing CD4 versus CD8 expression in total (top) and Foxp3+ (bottom) thymocytes. (B) Histograms showing Foxp3 expression in CD4+CD8- thymocytes. (C) Graphs showing absolute cell numbers of total, CD4+CD8-, and CD4+CD8-Foxp3+ thymocytes. Each dot represents an individual mouse with lines denoting averages. Statistical comparisons between WT and SOCS1-/- mice were performed using unpaired, two-tailed t test with statistical significance denoted by asterisks. ***,$p<0.0005$. 
Dysregulated cytokine production by SOCS1-/- lymphocytes is correlated to a reduction in peripheral Tregs. Dysregulated cytokine production by SOCS1-/- lymphocytes. Single cell lymph node suspensions were isolated from SOCS1-/- (n=4) or WT littermate controls (n=7) at 2 weeks. Analysis of the production of IFNγ, IL17, or IL2 cytokine message and/or protein by cells ex vivo or after culture, in the presence or absence of TCR stimulation. Left: Graphs showing mRNA expression of IFNγ, IL2 and IL17 relative to β-actin. Graphs showing production of IFNγ, IL2, and IL17 by SOCS1-/- and WT lymphocytes in the absence (middle) or presence (right) of TCR stimulation. Statistical comparisons between WT and SOCS1-/- mice were performed using unpaired, two-tailed t test with statistical significance denoted by asterisks. *, p ≤ 0.05; **, p ≤ 0.005; ***, p ≤ 0.0005.
Background

SOCS1 is able to control hyperactive cytokine signaling, which has been associated with the onset and pathogenesis of autoimmune disease. However, in situations where this regulation goes awry, development of autoimmunity can occur in the form of MS, RA or IBD. In SOCS1-/- mice the development of autoimmunity is so severe it leads to death by 3 weeks of age. Because regulation of cytokine signals is so critical in maintaining immune homeostasis, SOCS1 and their mimetics could serve as a potential treatment option to delay autoimmunity in the SOCS1 deficient mouse model.

Mimetics of the SOCS1 kinase inhibitory region, SOCS1-KIR, have been developed by the lab of Dr. H.M. Johnson. SOCS1-KIR is the 12 residue sequence that makes up the endogenous SOCS1 kinase inhibitory region (Waiboci et al., 2007). SOCS1-KIR (sequence: DTHFRTFRSHSDYRRI) has a lipophilic palmitoyl-lysine and arginine group that allows uptake into the cell to act intracellularly.

SOCS1-KIR acts by inhibiting intracellular tyrosine kinases just as the endogenous SOCS1 kinase inhibitory region does (Figure 3-1). This mimetic peptide was shown to inhibit the IFNγ-induced STAT1 phosphorylation by binding to the autophosphorylation site of JAK2 (Waiboci et al., 2007). SOCS1-KIR has also been shown to be an effective treatment of Experimental Autoimmune Encephalomyelitis (EAE), which is the mouse model for MS (Jager et al., 2011).

In this chapter, we develop a treatment using the SOCS1-KIR mimetic peptide in combination with SOCS1+/+ CD4+ T cell adoptive transfer. The CD4+/SOCS1-KIR combined treatment worked to both prolong life and significantly reduce inflammation in
the SOCS-/- mouse. The effects of the combined treatment allowed us to further examine SOCS1 deficiency on target Treg populations in the absence of severe inflammation early on.

Results

Combined SOCS1+/+ CD4+ T Cell Adoptive Transfer and SOCS1-KIR Mimetic Treatment Delays Lethal Disease.

Regulatory T cells play a critical role in maintaining immune homeostasis in mice. Due to a deficiency in peripheral Tregs in SOCS1-/- mice we then examined whether adoptive transfer of CD4+ T cells (10% Tregs) into SOCS1-/- mice could delay the onset of the disease. We adoptively transferred magnetically sorted SOCS1 WT CD4+CD25+ Tregs or CD4+ Tcells into SOCS1-/- mice 48 hours after birth (Figure 3-2). As seen in Figure 3-3A, the adoptive transfer of either SOCS1 sufficient CD4+CD25+ Tregs or total CD4+ T cells offered a significant, but limited, increase in survival of SOCS1-/- mice ($p=0.002$).

The kinase inhibitory region of SOCS1 is involved in inhibition of cytokine signals. Due to the dysregulation seen in SOCS1 deficient cells, we suspected that by restoring SOCS1 kinase inhibitory function we could delay morbidity in SOCS1-/- mice. We used a 16 amino acid peptide mimicking the KIR region of SOCS1, SOCS1-KIR, which contains a lipophilic group that allows the peptide to enter the cell and work intracellularly. We then treated SOCS1-/- mice with 10 $\mu$g/g of mouse weight daily to examine its ability to prevent disease lethality. Treatment of SOCS1-/- mice with SOCS1-KIR resulted in an increase in the lifespan that was similar to the adoptive transfer of CD4+ T cells or Tregs (Figure 3-3A). The control peptide, SOCS1-KIR2A, containing a two-alanine substitution in critical regions of KIR function, has no effect on
SOCS1/- mouse survival (data not shown). Jointly, these data show that SOCS1-KIR elicits a peptide specific effect on increasing the lifespan of SOCS1/- mice.

Because treatment with CD4+ T cell/CD4+CD25+ Treg or SOCS1-KIR peptide facilitated a similar increased survival of SOCS1/- mice, we next examined whether a combination of these treatments could further enhance the survival of SOCS1/- mice. Figure 3-3B shows that the combined treatment of CD4+ T cells and SOCS1-KIR (CD4+/SOCS1-KIR treatment) indeed mediated an enhanced survival. In fact, SOCS1/- mice receiving the treatment had a maximum life span of 77 days rather than the 17 days seen in the untreated mice. Collectively, this shows that CD4+/SOCS1-KIR treatment could extend the lifespan of 20% of the treated SOCS1/- mice beyond 30 days, which is 3 fold more than CD4+ T cells or SOCS1-KIR treated mice with lifespan extended to 24 days.

**CD4+/SOCS1-KIR Treatment Increased Weight Gain, Delayed Leukocyte Infiltration into Heart and Liver, and Reduced Serum IFNγ Levels in SOCS1/- Mice.**

Since SOCS1/- disease is characterized by stunted growth, leukocytic infiltration into vital organs, and aberrant IFNγ signaling, we determined the effect of CD4+/SOCS1-KIR treatment in these specific areas. CD4+ T cell, CD4+CD25+ Treg, and SOCS1-KIR treated SOCS1/- mice all experienced a statistically significant increase in weight and improved overall appearance in comparison to untreated SOCS1/- mice (Figure 3-4). We next examined the effect of the CD4+/SOCS1-KIR treatment on leukocytic infiltration in the heart and liver by performing hemotoxylin and eosin staining of the heart and liver of WT, SOCS1/-, and CD4+/SOCS1-KIR treated SOCS1/- mice. Figure 3-5A shows that SOCS1/- mice have infiltration and damage in 100% of tissues examined. In contrast, SOCS1/- mice receiving the CD4+/SOCS1-KIR
treatment had reduced leukocytic infiltration in 40 percent of heart tissue and 20 percent of liver tissue that was examined. We then examined the sera collected from mice at 2 weeks of age to assess the presence of inflammatory cytokines by ELISA. Despite IL17 levels being unaffected by the combined treatment, IFNγ was significantly lower in SOCS1-/- mice receiving the combined treatment in comparison to untreated SOCS1-/- mice ($p=0.0292$; Figure 3-5B). Thus, there is a correlation between long-term survival and healthy tissues in CD4+/SOCS1-KIR treated SOCS1-/- mice. Together, these data propose that CD4+/SOCS1-KIR treatment not only significantly prolongs the lives of SOCS1-/- mice but it also reduces leukocytic infiltration and IFNγ production.

**Summary**

In this chapter, we develop a therapy to enhance the survival of SOCS1-/- mice that die by 3 weeks of age. Although Tregs are able to suppress autoimmunity when adoptively transferred into mouse models of inflammation, Tregs or CD4+ T cells alone were only able to mediate a limited, but significant increase in survival (Figure 3-3A). Likewise, Treatment with the SOCS1-KIR peptide could only enhance survival similar to the T cell/Treg adoptive transfer treatment strategy (Figure 3-3A). We then performed a combined treatment of SOCS1-KIR and adoptive transfer of SOCS1+/+ CD4+ T cells. Strikingly, our CD4+/SOCS1-KIR treatment enhanced the survival of SOCS1-/- mice from 100% death by 18 days to a maximum life span of 77 days (Figure 3-3B).

In addition to an increased lifespan, SOCS1-/- mice receiving combined treatment experienced enhanced overall health, which is noted by increased weight and healthier appearance when compared to untreated SOCS1-/- controls (Figure 3-4). The long-term survival that was observed in SOCS1-/- mice receiving the combined treatment was correlated to reduced leukocytic infiltration into the heart and liver as well as a
significant reduction in serum IFNγ (Figure 3-5). These results suggest that the CD4+/SOCS1-KIR treatments mediated improved survival of SOCS1-/- mice by reducing IFNγ signaling thus deferring destruction of vital organs by infiltrating leukocytes.
Figure 3-1. SOCS1-KIR restores partial function of SOCS1 in the absence of the endogenous protein. SOCS1-KIR, a mimetic peptide of the kinase inhibitory region of SOCS1, successfully inhibits cytokine signaling by inhibiting phosphorylation of JAKs.
Figure 3-2. Schematic diagram of mouse treatment strategies performed on SOCS1-/- mice. A single cell suspension of splenocytes obtained from 6-8 week old WT mice was enriched for CD4+ or CD4+CD25+ T lymphocytes by magnetic separation (MiltenyiBiotec). 5 x 10^6 CD4+ or CD4+CD25+ T lymphocytes cells were injected into SOCS1-/- pups bi-weekly starting on day 2 after birth in the presence or absence of daily ip injections of SOCS1-KIR (10µg/g).
Figure 3-3. Survival curve of SOCS1−/− mice receiving treatment. (A) Kaplan Meier curve showing survival of SOCS1−/− (black square; n=9), mice receiving injection of $5 \times 10^5$ purified CD4+CD25+ Tregs (ip) twice a week (green diamond; n=5), mice receiving injection of $5 \times 10^5$ purified CD4+ T cells (ip) twice a week (blue square; n=12), or daily SOCS1-KIR peptide (10 µg/g) treatment (red triangle, n=7). (B) Kaplan Meier curve showing survival of SOCS1−/− (black square; n=7) or CD4+/SOCS1-KIR treatment (purple triangles, n=12). Numbers of mice in each group are indicated in parentheses. Mice were euthanized when moribund. SOCS1−/− survival curve is being compared to various treatment survival curves by Mantel-Cox comparison. Statistical significance denoted by asterisks. *, p ≤0.05; **, p ≤0.005.
Figure 3-4. CD4+/SOCS1-KIR treatment increases the overall health of 2 week old SOCS1-/- mice. (A) Graph showing average daily weights of SOCS1-/- mice untreated (n=16), receiving 5 x 10^5 WT Tregs (n=6), 5 x 10^5 WT CD4+ T cells (n=12), SOCS1-KIR peptide treatment (n=7), CD4+/SOCS1-KIR treatment(n=12), or WT littermate controls (n=9) over a two week period. (B) Photographs of 2 week old SOCS1-/- mice, SOCS1-/- mice receiving dual treatment, and WT littermate controls. Shown are two photos, each depicting the range of visual appearance under each condition. Statistical significance denoted by asterisks. ***, p ≤ 0.0005.
Figure 3-5. CD4+/SOCS1-KIR treatment prevents leukocyte infiltration into the heart and liver of 2 week old SOCS1-/- mice. (A) Photographs of H&E stains at 20x magnification depicting heart and liver tissues of 2 week old SOCS1-/-, SOCS1-/- mice receiving dual treatments, or littermate control mice. Photographs shown of H&E stained liver and heart of untreated SOCS1-/- (n=8) and WT (n=7) mice are representative of 100% of samples analyzed. Photographs shown of H&E stained liver and heart of SOCS1-/- mice receiving combined treatments are representative of 2 out of 5 mice in regard to heart samples and 1 out of 5 mice in regards to liver tissue. Please note that these percentages are consistent with SOCS1-/- mice with extended survival. (B) Graphs showing IFNγ and IL17 levels within the sera of 2 week old WT (n=7) or SOCS1-/- mice with (n=8) and without (n=10) CD4+/SOCS1-KIR treatment. IFNγ and IL17 cytokine levels were analyzed by ELISA. Statistical comparisons between WT and SOCS1-/- mice were performed using unpaired, two-tailed t test with statistical significance denoted by asterisks. *, p ≤0.05.
CHAPTER 4
EXAMINATION OF TREG POPULATION IN TREATED SOCS1-/- MICE

Background

Foxp3 expression is accepted as Treg specific cell marker. As long as Foxp3 is expressed, Tregs can develop and possess suppressive function with the capability of reversing severe inflammation or autoimmunity. However, recent studies have clarified what happens when once conventional Tregs fail to maintain Foxp3 expression. These studies involve Cre recombinase (Cre) mediated deletion of the Foxp3 allele in fully differentiated Tregs. These “ex-Tregs”, no longer expressing Foxp3, undergo cell divisions and lose characteristic Treg markers (Yang et al., 2008; Sharma et al., 2010). Moreover, ex-Tregs acquire the ability to begin producing effector T cell cytokines such as IL17, IFNγ, and IL4. In fact, when these ex-Tregs are transferred into lymphopenic hosts without functional Tregs, they cause tissue lesions and wasting disease, similar to the adoptive transfer of conventional T cells.

These findings ignited an important question on whether or not Tregs, like other T cell subtypes, have phenotype plasticity. Another concern was whether inflammatory conditions could influence loss of Foxp3 expression in Tregs, converting them to potentially pathogenic effector cells. Recent studies have proposed wavering answers to the question on Treg stability. Treg phenotype instability first came from an in vitro study showing that Tregs exposed to IL1 and IL6 down regulate Foxp3 and begin producing IL17, a Th17 signature cytokine (Yang et al., 2008). In another study, Tregs expressing GFP-Foxp3, expressed GFP, but not Foxp3 following exposure to an inflammatory environment (Hori, 2011). This would suggest that upon inflammatory
conditions that Foxp3 expression might diminish allowing for an unstable Treg phenotype.

To further investigate induction and loss of Foxp3 expression, one study used GFP and yellow fluorescent protein (YFP) reporters for Foxp3. In these studies, cells that are currently Foxp3+ Tregs are expressing both GFP and YFP, but cells that are no longer expressing Foxp3 are only YFP+ cells. The population of YFP+GFP- cells that stopped expressing Foxp3, are referred to as “ex-Tregs”. Ex-Tregs displayed effector T cell functions, such as production of IFNγ and IL17, and had the ability to stimulate autoimmune inflammation in diabetes-prone NOD mice (Zhou et al., 2009).

Another group used a similar model with inducible Treg cells in knock-in mice that had GFP and YFP reporters at the Foxp3 locus. In contrast to the previous study, analysis of these mice revealed an extremely stable expression of Foxp3 in Treg cells under various immune conditions. Included were basal conditions, lymphopenia, and Listeria infection. In addition to the conservation of Foxp3 expression in these Tregs, they did not produce IL17 or IFNγ when transferred into autoimmune prediabetic, lymphoreplete NOD mice (Rubtsov et al., 2010).

T cell plasticity is the swapping of a T cell lineage when presented with a certain cytokine milieu. For example, Th17 cells expressing RORγt, a Th17 specific transcription factor, can “switch” to Th1 cells expressing Tbet, the Th1 specific transcription factor. Moreover, it has been implicated that Foxp3+ Tregs, when in the appropriate cytokine microenvironment, may experience a loss of Foxp3 expression (Zhou et al., 2009). Furthermore, loss of Foxp3 expression may allow for the expression of pro-inflammatory T cell transcription factors that can promote the
production of IFNγ or IL17. Phenotype stability is particularly important nTregs, which are specific for self-antigen. nTregs must maintain a stable phenotype to not transition into auto-reactive T cells. Therefore, in order to maintain immune homeostasis and prevent autoimmunity, it is critical that Tregs remain functional, and not develop this plastic phenotype at sites of inflammation.

Because it is evident that inflammation can have a detrimental effect on Tregs, it was essential for us to further examine the effects of the combined treatment on the peripheral Treg population of SOCS1-/- mice. In this chapter, we observe the restoration of the peripheral Foxp3+ Treg population as well as a decrease in the percentage of CD4+CD25+Foxp3- effector T cells. Additionally, we found that the enhanced survival and reduced inflammation of SOCS1-/-IFNγ-/- and SOCS1 KO bone marrow chimeras was correlated to a maintained peripheral Treg population. Lastly, we examined the levels of mRNA for lineage specific transcription factors in Foxp3+ Tregs. Results confirm, upon culturing in vitro with IFNγ, that SOCS1 deficient Foxp3+ Tregs experience transcription factor plasticity.

Results

Treatment of SOCS1-/- Mice with CD4+/SOCS1-KIR Treatment Restores Foxp3+ Treg Peripheral Frequency and Decreases Peripheral Effector CD4+ T Cells.

Since SOCS1-/- mice are lymphopenic and have a significant reduction in peripheral Foxp3+ Tregs (refer to Figure 2-2), we next determined whether the delayed organ infiltration and decrease in IFNγ with CD4+/SOCS1-KIR treatment was due to restoration of Treg homeostasis. Indeed, a statistically significant increase in the frequency of Tregs was seen in the lymph nodes of SOCS1-/- mice with combined treatment in comparison to untreated SOCS1-/- mice (p=0.05; Figure 4-1A/C).
Furthermore, a statistically significant decrease in the frequency of CD4+CD25+Foxp3-effector cells was observed in the spleens of SOCS1-/- mice with treatment (p=0.05; Figure 4-1B/C). These results collectively confirm that CD4+/SOCS1-KIR treatment increased Foxp3+ Treg frequency and decreased activated CD4+ effector T cells in SOCS1-/- mice.

**CD4+/SOCS1-KIR Treatment Confers Enhanced Foxp3+ Treg Peripheral Homeostasis and Reverses Lymphopenia in SOCS1-/- Mice.**

Since autoimmunity has been attributed to a defect in Tregs and lymphopenia, we next determined whether the increased survival we saw in SOCS1-/- mice receiving treatment was due to homeostatic restoration of lymphocytes. Figure 4-2 illustrates that CD4+/SOCS1-KIR treatment on SOCS1+/+ mice creates no differences in total cell numbers, CD4+ lymphocytes, or CD4+Foxp3+ Tregs in secondary lymphoid organs. Despite the lack of differences in SOCS1+/+ mice with combined treatment, SOCS1-/- mice receiving treatment had a significant increase in the total, and CD4+ lymphocytes of peripheral lymphoid organs (Figure 4-2 top and middle). Likewise, SOCS1-/- mice experienced enhanced CD4+Foxp3+ Tregs in the lymph nodes upon combined treatment (Figure 4-2 bottom). Interestingly, CD4+ T cell and CD4+CD25+ Treg transfer or SOCS1-KIR alone was unable to mediate this homeostatic restoration (data not shown). This data together suggests that CD4+/SOCS1-KIR treatment was successful in facilitating the reversal of lymphopenia in SOCS1-/- mice. Additionally, SOCS1-/- mice receiving combined treatment had significantly increased Foxp3+ Tregs in lymph nodes compared to untreated SOCS1-/- controls.
**Enhanced Survival of Other SOCS1−/− Mouse Models Correlates with Maintained Treg Homeostasis.**

SOCS1−/− mice receiving the CD4+/SOCS1-KIR treatment experience enhanced survival due to decreased inflammation and increased peripheral Treg homeostasis. Other SOCS1 deficient mouse models, such as the SOCS1−/−IFNγ−/− mice, do not experience peri-lethal death that is associated with the traditional global SOCS1 deficient mouse. Due to this enhanced survival, we examined the peripheral Treg population in SOCS1−/−IFNγ−/− and SOCS1−/− bone marrow chimeric mice. Figure 4-3 shows a sustained CD4+Foxp3+ Treg population in the lymph node (A) and spleen (B) of SOCS1−/−IFNγ−/− mice (10% and 25%) when compared to littermate controls (8% and 19% at 6-8 weeks of age). Thus, it is concluded that SOCS1−/−IFNγ−/− mice, which do not experience autoimmunity early on, have a sustained peripheral regulatory T cell population.

Importantly, SOCS1−/−IFNγ−/− mice are unable to produce IFNγ, a key component in the early onset autoimmunity seen in the conventional global SOCS1−/− mouse. We developed SOCS1 deficient bone marrow chimeras in order to account for this genetic difference. Bone marrow chimeras were generated by lethally irradiating C57BL/6 mice and reconstituting them with 5x10^5 enriched stem cells from 2 week old SOCS1−/− (KO) mice and littermate controls (WT). 8 weeks post transfer, mice were sacrificed for analysis. Interestingly, we saw a comparable percentage of CD4+Foxp3+ regulatory T cells in the lymph node and spleen of either SOCS1 WT or SOCS1 KO bone marrow chimeras (Figure 4-4A). Treg percentages were supported with similar absolute numbers of CD4+Foxp3+ regulatory T cells in the lymph nodes and spleen of SOCS1 KO and SOCS1 WT bone marrow chimeras (Figure 4-4B). Since the SOCS1 KO bone
marrow chimeric mice experienced a sustained Treg population, we further assessed the state of inflammation in these mice by looking at organ infiltration. Figure 4-5 shows that mice receiving SOCS1 KO bone marrow cells did not experience leukocytic infiltration into the heart and liver at 8 weeks post transfer. Lack of infiltration indicates that severe inflammation is not yet occurring in the bone marrow chimeras at this time point. This data shows that SOCS1 deficient Treg numbers and percentages were similar in comparison to control mice in both the SOCS1-/-IFNγ-/- and SOCS1 KO bone marrow chimera models. Moreover, both of these models of SOCS1 deficiency have delayed onset of autoimmune inflammation and increased survival when compared to the traditional SOCS1-/-mouse. This suggests that the enhanced survival of these other mouse models of SOCS1 deficiency is correlated to a sustained peripheral regulatory T cell population.

**SOCS1 Deficient Tregs Experience Lineage Specific Transcription Factor Plasticity.**

Foxp3 expression is required for the suppressive function of regulatory T cells (Zheng and Rudensky, 2007). Additionally, the expression of T cell lineage specific transcription factors is influenced by the cytokine environment (Zhou et al., 2009; Murphy and Stockinger, 2010). Due to this emerging trend of T cell lineage plasticity we examined the expression of transcription factors specific to Th1, Th17, and Treg cells in CD4+CD25+ regulatory T cells isolated from SOCS1-/-IFNγ-/- mice. After magnetic separation, isolated Tregs were examined *ex vivo* and after being cultured with TCR stimulation in the presence and absence of 100 U/ml IFNγ, the cytokine responsible for peri-lethal death of SOCS1-/- mice. After 48 hours, cells were collected and RNA isolation and RT-qPCR were performed to measure the levels of mRNA expression of
Foxp3 (Treg), Tbet (Th1), and RORγt (Th17) transcription factors specific for various T cell lineages. Initial isolation of CD4+CD25+ cells from either SOCS1−/−IFNγ−/− mice or littermate controls showed that the CD25+ T cell fraction expressed higher levels of Foxp3 than the CD25− T cell fraction, indicating that the CD25+ population was indeed predominantly regulatory T cells before being cultured (Figure 4-6A). Figure 4-6B shows that although ex vivo SOCS1−/− CD25+ cells express significantly less Foxp3 than WT controls, the addition of IFNγ significantly increases Foxp3 expression in SOCS1−/− CD25+ T cells whereas the Foxp3 expression in the WT CD25+ cells remains the same. Since Foxp3 expression seemed less stable in SOCS1 deficient Tregs upon the addition of the pro-inflammatory cytokine IFNγ, we then examined Th1 specific transcription factor Tbet under the same conditions. Both ex vivo and IFNγ stimulating conditions showed a significantly higher Tbet expression in SOCS1−/− Tregs in comparison to WT Tregs (Figure 4-6C left). Heightened Tbet expression suggested that the SOCS1−/− cells have a Th1 bias, even in the regulatory T cell population. We next examined Th17 specific transcription factor RORγt to determine if differences were also present in the expression of this lineage specific marker. As expected, ex vivo SOCS1−/− Tregs cells expressed less RORγt in comparison to WT (Figure 4-6C right), this is consistent with previously studies showing the reciprocal relationship of Tbet and RORγt (Zhou et al., 2009). However, upon IFNγ stimulation there was no significant difference in the expression of RORγt between SOCS1 WT and deficient Tregs, although RORγt expression did decrease in WT Tregs in the presence of IFNγ (Th1 specific cytokine). Together, these data show that Tregs deficient in SOCS1 have a dysregulation in lineage specific transcription factors upon stimulation with Th1 pro-
inflammatory cytokines. This dysregulation in the presence of IFN\(\gamma\) suggests plasticity in SOCS1 deficient regulatory T cells could contribute to the absence of Treg homeostasis during SOCS1 KO disease.

**Summary**

Due to the severe inflammatory environment of SOCS1-/- mice, we suspected that the reduced Treg maintainance in the periphery was caused by excessive inflammation. As stated previously, upon treatment of SOCS1-/- mice with the CD4+/SOCS1-KIR treatment, we saw reduced inflammation. Since combined treatment of SOCS1-/- mice successfully decreased disease severity/inflammation, we then examined the peripheral T cell populations present in these mice. SOCS1 sufficient mice, with or without combined treatment, had no significant differences in effector T cell or Treg populations in secondary lymphoid organs (Figure 4-1). This consistency is likely because WT mice do not suffer from lymphopenia, thus expansion and effects of adoptive transfer are not seen. SOCS1-/- mice, which are lymphopenic, did in fact experience a modified peripheral T cell repertoire following CD4+/SOCS1-KIR treatment. This modified peripheral T cell repertoire of treated SOCS1-/- mice included increased percentage of Foxp3+ Tregs in the lymph nodes and decreased percentage of effector T cells in the spleen (Figure 4-1). In addition to a percentage increase of Foxp3+ Tregs, we also saw a significant numeric increase in Tregs in the lymph nodes in relation to untreated SOCS1-/- controls (Figure 4-2A). Interestingly, we also revealed a reversal of the lymphopenia that was associated with disease in the SOCS1-/- mouse. Total and CD4+ T cell numbers were significantly amplified in the lymph node and spleen of SOCS1-/- mice getting the combined treatment (Figure 4-2).
In addition to the traditional global SOCS1-/- mouse, we examined SOCS1-/-IFNγ-/- mice as well as SOCS1 KO bone marrow chimeras for the presence of a peripheral Treg population. Because SOCS1-/-IFNγ-/- mice experience delayed autoimmunity and reduced inflammation early in life, we examined whether reduced inflammation in this model of SOCS1 deficiency also provided maintenance of the SOCS1-/- Treg population. In fact, we saw that Tregs were present in the lymph nodes and spleen of these mice. Furthermore, no significant difference was seen in the Treg percentage of SOCS1 deficient and WT mice (Figure 4-3). SOCS1-/-IFNγ-/- mice cannot produce IFNγ, which is responsible for the aggressive disease seen early on in the global SOCS1-/- mouse. To further examine the effects of SOCS1 deficiency on the peripheral Treg population, we developed bone marrow chimeras, which still possess the ability of producing a Th1 inflammatory response. We show that at 8 weeks post bone marrow transfer, not only are Tregs present in both percentage and absolute number in the lymph node and spleen of both SOCS1 KO and WT bone marrow chimeras, but that this presence was correlated to the absence of inflammation (Figure 4-4/5). These studies strongly suggest that an enhancement in survival, and decrease in inflammation during SOCS1 deficiency is correlated to a sustained Treg population.

As previously mentioned, the peripheral cytokine environment plays a vital role in the differentiation and maintenance of T cells (Zhou et al., 2009). Inflammation has been shown to affect the stability of not only effector T cell, but also the regulatory T cell lineage (Josefowicz and Rudensky, 2009; Pillai et al., 2011). Since SOCS1 plays a critical role in regulating the effects of various cytokines, including IFNγ, we explored the possibility that the deficiency in Tregs was due to lineage plasticity caused by
dysregulated transcription factor expression. Our data indicates, upon culturing with Th1 pro-inflammatory cytokine IFNγ, that magnetically isolated Tregs experience a dysregulation of lineage specific transcription factors in the absence of SOCS1 (Figure 4-6). Upregulation of Tbet, a Th1 cell specific transcription factor, upon IFNγ stimulation suggests that regulatory T cells (like effector T cells) have a Th1 bias when cells are SOCS1 deficient. These results propose a likely plasticity of SOCS1-/- Tregs, and that they may be shifting from a traditional regulatory phenotype into a pro-inflammatory type of cell.
Figure 4-1. Treatment of SOCS1-/- mice with CD4+/SOCS1-KIR treatment increases peripheral Tregs and decreases activated CD4+CD25+Foxp3- cells. Flow cytometry analysis was performed on lymph node and spleen isolated from 2 week old WT or SOCS1-/- mice with and without CD4+/SOCS1-KIR treatment. Foxp3 vs. CD25 dot plot of CD4+ cells present in (A) lymph nodes or (B) spleen. (C) Bar graphs showing percentage of CD4+CD25+Foxp3+ Tregs and CD4+CD25+Foxp3- effector cells in SOCS1-/- mice with or without treatment. Statistical comparisons between SOCS1-/- mice with or without treatment were performed using unpaired, two-tailed t test with statistical significance denoted by asterisks. *, p ≤0.05. Data is representative of at least 5 mice in each group.
Figure 4-2. CD4+/SOCS1-KIR treatment increases total, CD4+, and CD4+Foxp3+ peripheral lymphocyte numbers in SOCS1-/- mice. (A) Graphs showing absolute numbers of total, CD4+, and CD4+Foxp3+ lymphocytes present in the lymph nodes of two week old SOCS1-/- and WT littermate control mice with or without CD4+/SOCS1-KIR treatment and WT 6-8 week old adult mice. (B) Graphs showing total, CD4+, and CD4+Foxp3+ splenocytes present in mice denoted in (A). Each dot is representative of an individual mouse with averages denoted by lines. Statistics were performed using unpaired, two-tailed t test comparing treated to untreated SOCS1-/- mice at 2wks with statistical significance denoted by asterisks. *, p ≤ 0.05; **, p ≤ 0.005; ***, p ≤ 0.0005.
Figure 4-3. SOCS1-/IFNγ-/-mice have a sustained peripheral Treg population. (A) Axillary, brachial, inguinal, superficial cervical, and mesenteric lymph nodes were isolated from SOCS1-/IFNγ-/- (n=3) or SOCS1+/+IFNγ-/- littermate controls (n=2) at 6-8 weeks. Histogram showing percentages of CD4+Foxp3+ lymphocytes in SOCS1-/IFNγ-/- and littermate controls. (B) Spleens were isolated from mice indicated in (A). Histogram comparing percentages of CD4+Foxp3+splenocytes in SOCS1-/IFNγ-/- and littermate controls.
Figure 4-4. SOCS1 KO Bone marrow chimeras have a sustained peripheral Treg population. Axillary, brachial, inguinal, superficial cervical, and mesenteric lymph nodes or spleen were isolated from C57BL/6 mice receiving SOCS1 KO (n=6) or SOCS1 WT (n=5) bone marrow 8 weeks post transfer. Histogram showing percentages of CD4+Foxp3+ Tregs in lymph nodes (left) and spleen (right) in SOCS1 KO and SOCS1 WT bone marrow chimeras. (B) Graphs showing absolute numbers of CD4+Foxp3+ regulatory T cells in lymph node and spleens of mice denoted in (A).
Figure 4-5. No leukocytic infiltration present in SOCS1 KO bone marrow chimeras six weeks post transfer. (A) Photographs of H&E stains depicting heart and liver tissues at 20x magnification of SOCS1 WT or SOCS1 KO mice 8 weeks post transfer. Photographs shown of H&E stained liver and heart of irradiated C57B/6 mice receiving SOCS1 KO (N=6) and SOCS1 WT (N=5) bone marrow are representative of 100% of samples analyzed.
Figure 4-6. SOCS1 deficient Tregs experience lineage specific transcription factor instability upon IFNγ stimulation. Lymph nodes and spleen were isolated from SOCS1−/−IFNγ−/− (n=3) or SOCS1+/+IFNγ−/− littermate controls (n=2) at 6-8 weeks and CD4+CD25+ Tregs were magnetically purified. Cells were analyzed ex vivo or after culture in the presence of TCR stimulation (3μg/ml anti-CD3 and 1μg/ml anti-CD28) and 100U/ml IFNγ, for the production of Foxp3, Tbet, and RORγt message. (A) Graphs showing mRNA expression of Foxp3 in the CD25+ or CD25− cell fractions. (B) Graphs showing mRNA expression of Foxp3 in the CD25+ cell fraction ex vivo and after TCR stimulation. (C) Graphs showing mRNA expression of Tbet and RORγt in the CD25+ cell fraction ex vivo and after TCR stimulation. Expression is relative to β-actin. Statistical comparisons between mice were performed using unpaired, two-tailed t test with statistical significance denoted by asterisks. *, p ≤0.05; **, p ≤0.005.
CHAPTER 5
DISCUSSION

It is accepted that SOCS1 and Tregs play a critical role in maintaining immune homeostasis. Mice with a deficiency in either of these two regulatory mechanisms suffer from severe autoimmunity and eventual death by a T cell mediated disease similar to one another (Marine et al., 1999; Brunkow et al., 2001). In this project, we begin to address the interrelationship of these two regulatory pathways. We examine the peripheral population of Tregs in mice with a global deficiency in SOCS1. Our data shows that SOCS1-/- mice, suffering from severe autoimmunity, do not have a maintained peripheral population of Tregs. Furthermore, the deficiency of Tregs is not due to faulty thymic development. Upon further examination, we expose that SOCS1-/- lymphocytes not only have a Th1 bias, producing pro-inflammatory cytokine IFNγ, but that they also have a reduced capacity to produce IL2. IL2 is an essential survival cytokine for Tregs. The reduced ability of SOCS1-/- lymphocytes to make IL2, and increased production of IFNγ, propose that the peripheral cytokine environment of SOCS-/- mice is a likely cause of Treg deficiency.

SOCS1-/- mice die of lethal autoimmune disease by 3 weeks of age (Marine et al., 1999). Because of this early and excessive inflammation, it is hard to examine the SOCS1 deficient Tregs in this mouse model. In order to combat this difficulty, we developed a treatment strategy that allowed for the enhanced survival and reduction of inflammation in SOCS1-/- mice. This treatment involved the adoptive transfer of SOCS1+/+ CD4+ T cells in combination with the SOCS1-KIR mimetic peptide. The combined treatment allowed for a significant increase in survival of the SOCS1-/- mice, prolonging the life of 30% of the mice past 24 days, whereas 100% of untreated mice
were dead by 18 days. Strikingly, the survival we observed with our combined treatment is comparable to the survival of SOCS-box deficient mice, suggesting that we are successful in the partial restoration of SOCS1 function (Zhang et al., 2001). In addition to enhanced survival of treated SOCS1-/- mice, combined treatment significantly reduced the leukocytic infiltrates into the heart and liver as well as decreased serum IFNγ levels. Together these data suggest that our CD4+/SOCS1-KIR combined treatment was able to ameliorate disease in SOCS1-/- mice, providing us with a less severe model of inflammation to examine SOCS1 deficient Tregs.

Since combined treatment was able to successfully reduce disease severity in SOCS1-/- mice, we now examined the effects of reduced inflammation on the SOCS1 deficient Treg population. Our results demonstrate an increase in the percentages and absolute numbers of Treg in the lymph nodes of SOCS1-/- mice receiving our combined treatment when compared to the untreated controls. In addition to the increase in the peripheral Treg population, treated SOCS1-/- mice experienced a reduced percentage of effector T cells. These results further propose that inflammation was reduced in SOCS1-/- mice receiving the combined treatment. The inability of SOCS1 Tregs to persist in the inflammatory environment of the SOCS1-/- mouse suggests that SOCS1 plays a role in the maintenance of Tregs during inflammation.

To confirm the role of SOCS1 in the maintenance of a Treg population during inflammation, we examined two other SOCS1 deficient mouse models. The first mouse model examined was the SOCS1-/-IFNγ-/- mouse. This mouse does not suffer from early onset autoimmunity (Alexander et al., 1999). Our data supports the notion that in absence of IFNγ, and excessive autoimmunity, Tregs are present in the peripheral
lymphoid organs. We also examined the presence of Tregs in SOCS1 KO and WT bone marrow chimeras. Previous studies have shown that SOCS1 KO bone marrow chimeras do not experience the early onset autoimmunity seen in the global SOCS1-/- mouse (Metcalf et al., 2003). As suspected, in the absence of excessive inflammation, Foxp3+ Tregs are present in the peripheral lymphoid organs of SOCS1 KO bone marrow chimeras. Moreover, the absence of autoimmunity was confirmed in the bone marrow chimeric mice by examining infiltration into vital organs by histology. Histology shows no infiltration in the heart and liver of mice receiving SOCS1 KO or WT bone marrow cells.

Lastly, due to the essential role that SOCS1 plays in the modulation of cytokine signaling and T cell differentiation, we examined the effects of Th1 inflammation on SOCS1-/- Tregs (Tanaka et al., 2008; Palmer and Restifo, 2009). Our results confirmed that SOCS1 deficiency results in the instability of lineage specific transcription factors, Foxp3 and Tbet, in Tregs upon stimulation with IFNγ. These results are novel in that it suggests a role for SOCS1 in the preservation of the Treg phenotype. The suppressive anti-inflammatory phenotype of Tregs is important in safeguarding immune homeostasis. During times of inflammation, Tregs must remain stable in order to regulate an immune response when clearance is necessary. The inability of Tregs to modulate this immune clearance can lead to excessive inflammation resulting in autoimmunity. The instability of the Treg phenotype during inflammation may also result in Tregs transitioning into auto-reactive effector T cells, enhancing the immune response at the site of inflammation.
The results of this project collectively reveal a role for SOCS1 in the maintenance of Treg homeostasis, especially during times of heightened inflammation. The importance of Tregs and SOCS1 in conserving immune homeostasis has been proven time and again. However, the discovery of their joint importance is recent. This data supports the hypothesis that in the absence of SOCS1, Tregs are less fit to survive during inflammation. Moreover, SOCS1 deficient Tregs are likely less fit because of the inability of the cell to inhibit the influences of pro-inflammatory cytokines. The influences of these cytokines can lead to the instability of lineage specific transcription factors, perhaps resulting in phenotype plasticity of SOCS1 deficient Tregs. These studies give insight into the relationship of the SOCS1 and Treg immune regulatory pathways. Although additional studies examining this interrelationship would be required, the modification of immune responsiveness by either manipulation of Tregs or SOCS1 could prove to be an innovative way to develop therapeutics for autoimmunity in the future.
CHAPTER 6
MATERIALS AND METHODS

Mice

SOCS1+/- mice on a C57BL/6 genetic background were purchased from the St. Jude Animal facility (Memphis, TN) and mated in the University of Florida Cancer and Genetics Animal Facility, generating SOCS1-/-, SOCS1+/-, and SOCS1+/+(WT) mice. SOCS1+/-IFNγ-/- mice used for generation of SOCS1-/-IFNγ-/- and SOCS1+/+IFNγ-/- mice were purchased from Jackson labs (Bar Harbor, ME). C57BL/6 mice used in adoptive transfers and bone marrow chimeras were purchased from Jackson Labs (Bar Harbor, ME). Mice were maintained in sterile micro-isolators under specific pathogen free conditions at the University of Florida Cancer and Genetics Animal Facility. Mice undergoing various treatments were weighed daily and general health assessed. Mice becoming morbid or exhibiting a 20% weight loss were euthanized. Procedures described in manuscript were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and experiments were performed in strict accordance to the approved protocols.

Genotyping

Quantitative PCR was used to determine the presence of the SOCS1 gene in mice. Tail clips (1mm) isolated from 1 week old SOCS1+/-, SOCS1+/-, or SOCS1-/- mice were degraded using the DNAeasy Blood and Tissue Kit (Qiagen, Valencia, CA). ABSolute QPCR SYBR Green Mix (ABgene Epsom, Surrey, UK) and primers specific for SOCS1 (F – 5’-GACACTCACTTCCGCACCTT-3’, SOCS1 R – 5’-GAAGCAGTTCCGTTGGCGACT-3’) or housekeeping gene β-Actin (F – 5’- CCACAGCACTGTAGGGTTTA-3’, β-Actin R – 5’- ATTGTCTTTCTTCTGCCGTTCTC-
(200nM) were used to amplify relative amounts of DNA on a PTC-200 Peltier Thermal Cycler with a CHROMO 4 Continuous Fluorescence Detector (BIORAD, Hercules, CA, USA). The amplification was performed by one 15 minute cycle at 95°C which was required for enzyme activation; followed by forty-seven cycles of denaturation (95°C, 15s), annealing (57°C; 30s), and extension (72°C, 30s). Phenotype of mice was determined by relative expression of SOCS1. Melting curve analysis was performed to confirm amplicon specificity. The fold-change in expression was calculated using the double ΔCT method (i.e. using the equation 2- ΔΔCT) using BIORAD software.

**Magnetic Cell Separation**

CD4+ and CD4+CD25+ T lymphocytes were enriched using either a CD4+ or CD4+CD25+ T cell isolation kit (MiltenylBiotec, BergischGladbach, Germany) respectively, according to manufacturer’s instructions. Briefly, a single cell suspension of pooled lymph node (axillary, inguinal, brachial, mesenteric, and superficial cervical) and spleen was obtained from mice followed by magnetic activated cell sorting (MACS) column enrichment performed under aseptic conditions. The enriched CD4+ T cell population was obtained via MACS column negative selection using CD4 T cell isolation kit (MiltenyiBiotec). In the case of CD4+CD25+ T cell enrichment, an enriched CD4+ lymphocyte population was first obtained through MACS column negative selection, followed by MACS column positive selection of CD4+CD25+ cells according to manufactures instructions. The purities for the enriched CD4+ and CD4+CD25+ populations were typically ≥ 80% pure.
Flow Cytometry

Single cell suspensions of pooled lymph nodes (LN) (axillary, inguinal, brachial, mesenteric, and superficial cervical), spleen, and thymus cells were stained with the following mAbs for flow-cytometric analysis: anti-CD4-Pacific Blue (RM4-5; BD PharMingen, San Diego, CA), anti-CD8a-Alexa Flour 700 (53-6.7; BD PharMingen), anti-CD25-APC (PC61; BD PharMingen), and anti-CD45R(B220)-FITC (RA3-6B2; eBioscience, San Diego, CA) Ab. Foxp3 intracellular staining was performed as previously described (Larkin et al., 2008). Briefly, cells were fixed and permeabilized using the reagents provided with the anti-Foxp3-PE (FJK-16s; eBioscience) or anti-Foxp3-FITC (FJK-16s; eBioscience) Ab. 50,000-100,000 live events were collected on a LSRII (BD PharMingen) and analyzed using FlowJo® software (Tree Star, San Carlos, CA). The absolute numbers of cells recovered from various organs was determined by multiplying the total number of cells isolated from various tissues by the percentage of total cells bearing a lineage specific marker denoted by flow cytometry.

Peptide Synthesis

Peptides SOCS1-KIR (53DTHFRTFRSHSDYRRI) and SOCS1-KIR2A (53DTHARTARSHSDYRRI) were synthesized using conventional fluorenlymethy carbonyl chemistry as previously described (Szente et al., 1994) using an Applied Biosystems 431A automated peptide synthesizer (Applied Biosystems, Carlsbad, CA). Using a semi-automated protocol (Thiam et al., 1999), a lipophilic group (palmitoyl-lysine) for cell penetration was added to the N-terminus as a final step. Peptides were characterized using mass spectrometry and purified by high-performance liquid chromatography (HPLC). Peptides were dissolved in DMSO or PBS (Sigma-Aldrich, St. Louis, MO) prior to use.
In vivo Mouse Treatments

Intraperitoneal injections of SOCS1/- or WT (SOCS1+/+) littermate mice with SOCS1-KIR peptide (10μg/g) and/or MACS purified CD4+ or CD4+CD25+ lymphocytes (5 x 10^5) began 24 hours after birth. IP injections of SOCS1-KIR were performed daily, while lymphocyte adoptive transfers were performed twice weekly. In some experiments SOCS1-KIR2A (10μg/g) was administered as a specificity control.

RNA Isolation and RT-qPCR

Total RNA was extracted from the lymph nodes of SOCS1/- mice, WTagematched littermates, or magnetically separated CD4+CD25- and CD4+CD25+ T lymphocytes using the SV Total RNA Isolation System (Promega, Corp., Madison, WI, USA). The concentrations and purity of the total RNA were determined using a SmartSpecPlus Spectrophotometer (BioRad, Hercules, CA, USA). Quality and integrity of the total RNA was assessed by a 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) and a RNA integrity number of 7 or greater was routinely obtained. First-strand cDNA synthesis was performed using ImProm-II Reverse Transcription System (Promega, Corp., Madison, WI, USA). iQ SYBR Green Supermix(Bio-Rad, Hercules, CA, USA) and gene specific primers (Table 6-1) at 200nM were used to amplify relative amounts of cDNA on a PTC-200 Peltier Thermal Cycler with a CHROMO 4 Continuous Flourescence Detector (BIORAD, Hercules, CA, USA). The amplification was performed by one 5 minute cycle at 95°C which was required for enzyme activation; followed by fifty-one cycles of denaturation (95°C, 15s), annealing (55°C of 57°C; 30s), and extension (72°C, 30s). Melting curve analysis was performed to confirm amplicon specificity. The fold-change in expression was calculated using the double ΔCT method (i.e. using the equation 2^\(-\Delta\Delta CT\) using BIORAD software.
Cytokine Secretion Analysis

Lymph nodes were isolated from SOCS1-/- mice and WT littermates, followed by the generation of single cell suspensions. 2x10^5 cells were plated with or without 3µg/ml anti-CD3 and 1µg/mL anti-CD28 (BD Biosciences, San Diego, CA). After 72 hours supernatants were collected from wells. Cytokine ELISAs were subsequently performed, as previously described (Lau et al., 2011) on harvested supernatants. IL-2 (555148) ELISA kits, capture (555068) and detection (555067) mAb for IL-17A, and cytokine standard for IFNγ (554587) were obtained from BD Biosciences. IL17 cytokine standard (14-8171-80), and IFNγ capture (167313-85) and detection (13-7311-85) mAbs were purchased from eBioscience.

Histology

Histology and immuno-histochemistry was performed under the advisement of the University of Florida histology core lab. Heart and liver were isolated from treated and untreated two-week old SOCS1+/+ and SOCS1-/- mice or SOCS1 KO and SOCS1 WT bonemarrow chimeras 6 weeks post transfer and stored for 24 hours in PBS containing 2% paraformaldehyde. Organs were subsequently transferred into 70% ethanol for long-term storage. Organs were paraffin embedded, sectioned at a thickness of 3 µm, and stained for H&E’s. Photos were taken at 20x magnification using the Leica DM 2500 Microscope equipped with an Optronics color camera and MagnaFire software (Optronics, Goleta, CA).

Bone Marrow Chimeras

Bone marrow was removed from the femur and tibia bones of SOCS1 WT and SOCS1 KO mice at 2 weeks of age and washed. Progenitor cells were purified using
BD IMag mouse hematopoietic progenitor (stem) cell purification kit (BD Biosciences, San Diego, CA). 24 hours post lethal irradiation (850 RAD) C57BL/6 mice were reconstituted with 5x10^5 enriched hematopoietic stem cells from SOCS1 WT and SOCS1 KO donor mice. Mice were maintained in sterile micro-isolators under specific pathogen free conditions at the University of Florida Cancer and Genetics Animal Facility. Mice undergoing irradiation were given .2 mg/ml Neomycin Sulfate in drinking water, weighed daily, and general health assessed. Mice becoming morbid or exhibiting a 20% weight loss were euthanized. Chimeric mice were sacrificed 8 weeks post transfer for analysis.

**Transcription Factor Analysis**

Lymph nodes and spleen were isolated from SOCS1-/-IFNγ-/- and SOCS1+/+IFNγ-/- mice, followed by the generation of single cell suspensions. CD4+CD25+ T lymphocytes were isolated using the CD4+CD25+ T cell isolation kit (MiltenylBiotec) as previously described. 1x10^5 CD4+CD25+Tregs were plated with 3µg/ml anti-CD3 and 1µg/mLanti-CD28 (BD Biosciences, San Diego, CA) with or without 100 U/ml IFNγ (eBioscience). After 72 hours cells were collected from wells and RNA isolation and RT-qPCR looking at relative expression of Foxp3, Tbet, and RORγt were performed as described above.

**Statistical Analysis**

Graph Pad Prism® v.5 was used to calculate the statistically significance differences between different groups using unpaired two-tailed Student’s t test. For Kaplan Meier survival curve type experiments, Mantel-Cox or two-way ANOVA analyses was applied. A 95% confidence limit, defined by p values ≤.05, was considered significant and is indicated within figures.
Table 6-1. Primers used and/or discussed in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Temperature (°C)</th>
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<tbody>
<tr>
<td>Actin</td>
<td>F: 5’-CCT TCC TTC TTG GGT ATG CA-3’</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GGA GGA GCA ATG ATC TTG AT-3’</td>
<td>55</td>
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<tr>
<td>Foxp3</td>
<td>F:5’-TCTGTGGCCTCAATGGACAA-3’</td>
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<tr>
<td></td>
<td>R:5’-GAAGAACTCTGGGAAGGAACATA-3’</td>
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<tr>
<td>IFNγ</td>
<td>F:5’-AACTATTTTAACTCAAGTGGAAT-3’</td>
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<td>R:5’-AGGTGTGATTCAATGACG-3’</td>
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<tr>
<td>IL2</td>
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<td></td>
<td>R:5’-GTGTGGTCAGAGCCCTTTAG-3’</td>
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<td>IL17A</td>
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<td></td>
<td>R:5’-CTCTTCAGGACCAGGAT-3’</td>
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<tr>
<td>RORγt</td>
<td>F: 5’-ACAGCCACTGCATTCACAGTT-3’</td>
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<td></td>
<td>R: 5’-TCTCGGAAGGACTTGCAATGAT-3’</td>
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<tr>
<td>SOCS1</td>
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<td></td>
<td>R:5’-GAAGCAGTTCCGTTGGCGACT-3’</td>
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<td>Tbet</td>
<td>F: 5’-GGGAGAAGTTTTGAGTCCA-3’</td>
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<tr>
<td></td>
<td>R: 3’-GAAGGTGCGGGGTAGAAA-5’</td>
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BIOGRAPHICAL SKETCH

Erin Louise Collins was born in Port Huron, Michigan in 1985. In 2003 Erin graduated from Port Huron High School. Following high school, she attended Bowling Green State University for her undergraduate studies, completing a Bachelor of Science degree in microbiology in the spring of 2007. Inspired by research topics revolving around benchside to bedside translational studies, she received her Ph.D. from the University of Florida in the spring of 2012, focusing on suppressors of cytokine signaling-1 and regulatory T cells. She plans to pursue a career in biomedical research.