



---

Publicly Accessible Penn Dissertations

---


1-1-2016

# Factors Influencing Regulatory T Cell Maintenance for the Control of Autoimmunity

Theresa Marie Leichner

University of Pennsylvania, leichner@mail.med.upenn.edu

Follow this and additional works at: <http://repository.upenn.edu/edissertations>

 Part of the [Allergy and Immunology Commons](#), [Immunology and Infectious Disease Commons](#), and the [Medical Immunology Commons](#)

---

## Recommended Citation

Leichner, Theresa Marie, "Factors Influencing Regulatory T Cell Maintenance for the Control of Autoimmunity" (2016). *Publicly Accessible Penn Dissertations*. 1840.

<http://repository.upenn.edu/edissertations/1840>

This paper is posted at Scholarly Commons. <http://repository.upenn.edu/edissertations/1840>

For more information, please contact [libraryrepository@pobox.upenn.edu](mailto:libraryrepository@pobox.upenn.edu).

---

# Factors Influencing Regulatory T Cell Maintenance for the Control of Autoimmunity

## **Abstract**

Regulatory T cells (Tregs) are a subset of CD4+ T cells with suppressive function and are critical in limiting autoimmunity. Increasing Treg numbers can be beneficial in the treatment of several inflammatory disorders. Here, we investigate the roles played by various factors on the control of Treg homeostasis. We provide evidence that the skin can exert strong systemic effects on Treg numbers by producing the cytokine thymic stromal lymphopoietin (TSLP) in response to topical administration of the vitamin D3 analog MC903. Widespread increases in Tregs were observed in mice treated topically but not systemically with MC903. TSLP receptor (TSLP-R) but not hematopoietic vitamin D receptor signaling was important for this increase in Treg numbers and MC903 treatment did not lead to changes in Treg development, but drove increased Treg proliferation. However, TSLP-R expression by Tregs themselves was not required for the expansion induced by MC903 treatment. Rather, TSLP promotes Treg proliferation by affecting dendritic cell (DC)/Treg interactions, as mice lacking DCs did not have an increase in Tregs after MC903 treatment, and TSLP enhanced proliferation of Tregs co-cultured with DCs. To test whether MC903 could influence progression of autoimmunity, non-obese diabetic (NOD) mice were treated topically with MC903 and it was found that this treatment significantly lowered the incidence of diabetes. Other than TSLP-driven expansion, Treg numbers are known to rely on the cytokine interleukin-2 (IL-2) and T cell receptor (TCR) signals. We found that Foxp3- conventional T cells (Tconvs) produce IL-2 in response to self-peptides and that Tconvs possessing TCRs with greater self-reactivity express more IL-2 at baseline. Furthermore, selective disruption of TCR signaling in Tconvs led to a trend towards decreased expression of IL-2 and diminished the ability of Tconvs to maintain Treg numbers. These data suggest that the role of TCR in Treg maintenance includes the ability of Tconvs to signal in response to self-peptides. Together, this work investigates multiple factors that have important effects on Treg maintenance. These findings have potential implications on development of therapies that seek to modulate immune activation in autoimmune settings.

## **Degree Type**

Dissertation

## **Degree Name**

Doctor of Philosophy (PhD)

## **Graduate Group**

Immunology

## **First Advisor**

Taku Kambayashi

## **Keywords**

Interleukin 2, Regulatory T cells, Type 1 Diabetes

---

**Subject Categories**

Allergy and Immunology | Immunology and Infectious Disease | Medical Immunology

FACTORS INFLUENCING REGULATORY T CELL MAINTENANCE FOR THE CONTROL OF  
AUTOIMMUNITY

Theresa M. Leichner

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2016

Supervisor of Dissertation

---

Taku Kambayashi, MD, PhD

Assistant Professor, Pathology and Laboratory Medicine

Graduate Group Chairperson

---

David Allman, PhD

Associate Professor, Pathology & Laboratory Medicine

Dissertation Committee

Edward M. Behrens, MD, Joseph Hollander Associate Professor in Pediatric Rheumatology

Terri M. Laufer (Chair), MD, Associate Professor of Medicine

Jonathan S. Maltzman, MD, PhD, Associate Professor of Medicine

Paula M. Oliver, PhD, Associate Professor of Pathology and Laboratory Medicine

FACTORS INFLUENCING REGULATORY T CELL MAINTENANCE FOR THE CONTROL OF  
AUTOIMMUNITY

COPYRIGHT

2016

Theresa M Leichner

This work is licensed under the  
Creative Commons Attribution-  
NonCommercial-ShareAlike 3.0  
License

To view a copy of this license, visit

<http://creativecommons.org/licenses/by-nc-sa/3.0/>

## ACKNOWLEDGEMENTS

I would like to thank everyone that has had a part in making the work that went into this thesis possible. I am incredibly thankful for all the members of the Kambayashi lab for making the daily work in the laboratory easier and more enjoyable. I would like to especially thank Atsushi Satake for all his invaluable help in training me during my initial time in the lab as well as for his integral contributions that he made in the early stages of my thesis work. Additionally, I must extend a huge amount of appreciation to Mariko Okumura. Without her, I would not have been able to complete anywhere near the amount of work I did. I would like to thank Gary Koretzky all the members of the Behrens, Jordan, Oliver, and Bassiri groups for helpful discussions in joint lab meeting. I am incredibly grateful for the insight provided by all the members of my thesis committee while I was completing my work; Terri Laufer, Paula Oliver, Jonathan Maltzman, Avinash Bhandoola, and Ed Behrens. I am thankful to all of the labs in John Morgan Building for help in countless ways. A huge thank you to all of my friends and especially my family for being so supportive and understanding throughout this work. I want to thank EnJun Yang for being my lab buddy; it has been a privilege to go through this process with you. Finally and most importantly, I would like to extend my greatest appreciation to my mentor, Taku Kambayashi, for allowing me to study and learn from him in his lab. He has been an amazing teacher and supporter in all aspects and I am glad to have been able to complete this thesis work under his guidance.

## **ABSTRACT**

### **FACTORS INFLUENCING REGULATORY T CELL MAINTENANCE FOR THE CONTROL OF AUTOIMMUNITY**

Theresa Lechner

Taku Kambayashi

Regulatory T cells (Tregs) are a subset of CD4<sup>+</sup> T cells with suppressive function and are critical in limiting autoimmunity. Increasing Treg numbers can be beneficial in the treatment of several inflammatory disorders. Here, we investigate the roles played by various factors on the control of Treg homeostasis. We provide evidence that the skin can exert strong systemic effects on Treg numbers by producing the cytokine thymic stromal lymphopoietin (TSLP) in response to topical administration of the vitamin D<sub>3</sub> analog MC903. Widespread increases in Tregs were observed in mice treated topically but not systemically with MC903. TSLP receptor (TSLP-R) but not hematopoietic vitamin D receptor signaling was important for this increase in Treg numbers and MC903 treatment did not lead to changes in Treg development, but drove increased Treg proliferation. However, TSLP-R expression by Tregs themselves was not required for the expansion induced by MC903 treatment. Rather, TSLP promotes Treg proliferation by affecting dendritic cell (DC)/Treg interactions, as mice lacking DCs did not have an increase in Tregs after MC903 treatment, and TSLP enhanced proliferation of Tregs co-cultured with DCs. To test whether MC903 could influence progression of autoimmunity, non-obese diabetic (NOD) mice

were treated topically with MC903 and it was found that this treatment significantly lowered the incidence of diabetes. Other than TSLP-driven expansion, Treg numbers are known to rely on the cytokine interleukin-2 (IL-2) and T cell receptor (TCR) signals. We found that Foxp3<sup>+</sup> conventional T cells (Tconvs) produce IL-2 in response to self-peptides and that Tconvs possessing TCRs with greater self-reactivity express more IL-2 at baseline. Furthermore, selective disruption of TCR signaling in Tconvs led to a trend towards decreased expression of IL-2 and diminished the ability of Tconvs to maintain Treg numbers. These data suggest that the role of TCR in Treg maintenance includes the ability of Tconvs to signal in response to self-peptides. Together, this work investigates multiple factors that have important effects on Treg maintenance. These findings have potential implications on development of therapies that seek to modulate immune activation in autoimmune settings.



# TABLE OF CONTENTS

<b>ACKNOWLEDGEMENTS.....</b>	<b>iii</b>
<b>ABSTRACT.....</b>	<b>iv</b>
<b>LIST OF FIGURES.....</b>	<b>viii</b>
<b>CHAPTER I: Introduction</b>	
<b>T cells in the adaptive immune system.....</b>	<b>1</b>
<b>T cell development and central tolerance.....</b>	<b>2</b>
<b>Discovery of regulatory T cells and their role in peripheral         tolerance.....</b>	<b>4</b>
<b>Generation of regulatory T cells.....</b>	<b>7</b>
<b>Peripheral homeostasis of regulatory T cells.....</b>	<b>10</b>
<b>Regulatory T cells for therapeutic use.....</b>	<b>15</b>
<b>Skin and the immune system.....</b>	<b>17</b>
<b>Vitamin D and the immune system.....</b>	<b>19</b>
<b>Structure of the thesis.....</b>	<b>21</b>
<b>CHAPTER II: Skin mediated control of systemic regulatory T cell numbers and protection against type 1 diabetes</b>	
<b>Summary.....</b>	<b>22</b>
<b>Introduction.....</b>	<b>24</b>
<b>Results.....</b>	<b>28</b>
<b>Discussion.....</b>	<b>40</b>
<b>Figures.....</b>	<b>45</b>

**CHAPTER III: TCR signaling by conventional CD4+ T cells is required for optimal maintenance of peripheral regulatory T cell numbers**

Summary.....	55
Introduction.....	56
Results.....	58
Discussion.....	62
Figures.....	66

**CHAPTER IV: Discussion**

Overview.....	69
Skin mediated control of systemic regulatory T cell numbers.....	71
Role of TSLP in expansion of regulatory T cells.....	73
Dendritic cells are intermediates to TSLP driven regulatory T cell proliferation.....	77
Skin mediated protection from type 1 diabetes.....	80
TCR requirements for regulatory T cell maintenance.....	81
Implications for regulatory T cell therapeutics.....	84
Conclusions.....	85

**APPENDIX**

I: List of abbreviations.....	87
-------------------------------	----

**II: Materials and Methods**

Chapter II.....	91
-----------------	----

Chapter III.....	96
------------------	----

<b>BIBLIOGRAPHY.....</b>	<b>100</b>
--------------------------	------------

# LIST OF FIGURES

## Chapter II

- Figure 2.1 *Topical MC903 treatment increases Treg numbers independent of VDR expression on hematopoietic cells*
- Figure 2.2 *Treg increases are dependent on TSLPR signaling*
- Figure 2.3 *Treg increases do not occur due to tTreg or pTreg generation*
- Figure 2.4 *Tregs are proliferative and gain an effector phenotype in MC903 treated mice*
- Figure 2.5 *Treg increases occur independently of the Th2 response to topical MC903*
- Figure 2.6 *Tregs are increased with topical MC903 treatment indirectly through TSLP and dendritic cells*
- Figure 2.7 *TSLP stimulates Treg proliferation in vitro through dendritic cells*
- Figure 2.8 *TSLP stimulates upregulation of DC costimulatory receptors*
- Figure 2.9 *Long-term topical MC903 treatment increases Tregs in NOD mice and decreases the incidence of diabetes*
- Figure 2.10 *Model of topical MC903 mediated Treg increases.*

## Chapter III

Figure 3.1 *IL-2 is induced by stimulation of CD4<sup>+</sup> Tconvs by self peptide-MHC-II complexes*

Figure 3.2 *Lack of IL-2 Production in MHCII deficient environment leads to decreased Treg percentages*

Figure 3.3 *Selective ablation of TCR signaling in CD4<sup>+</sup> Tconvs leads to decreased Treg numbers*

## **CHAPTER I:**

### **Introduction**

#### **T cells in the adaptive immune system**

The survival of an organism is critically dependent on its ability to protect from and defend against exposure to a large variety of pathogens. In order to effectively achieve this protection, there are two branches of the immune system that work in concert. When exposed to a pathogen, the innate immune system acts in a rapid, non-specific manner using germ-line encoded receptors to clear the threat. In contrast, cells of the adaptive immune system, called lymphocytes, express receptors of diverse specificities that are able to detect a wide array of potential pathogens. Recognition of foreign pathogens by these antigen specific receptors on lymphocytes triggers the cells to become activated and divide. In this way, the proliferation of the antigen specific lymphocytes help to clear the pathogen.

One type of lymphocyte important in the adaptive immune system is the CD4<sup>+</sup> T lymphocyte, or CD4<sup>+</sup> T cell. CD4<sup>+</sup> T cells express the T cell receptor (TCR) on their surface and develop in a specialized immune organ, the thymus. In the course of an immune response, the ability of CD4<sup>+</sup> T cells to recognize antigen depends on interaction of the TCR with another receptor known as major histocompatibility complex class II (MHC-II) present on specialized antigen

presenting cells (APCs). APCs such as dendritic cells (DCs) first recognize pathogen, process it and present fragments of the foreign peptides on its surface bound to the MHC-II molecules. It is only within the context of MHC-II on APCs that pathogens can be detected by CD4<sup>+</sup> T cells, resulting in activation of this arm of the immune response.

### **T cell development and central tolerance**

The development of CD4<sup>+</sup> T cells is centered on the formation of the antigen specific TCR. Every T cell that develops has to go through a process of recombining germ-line gene segments found within the TCR gene locus. In a process termed VDJ recombination, for the gene segments used in the process (Variable, Diversity, and Joining), specialized proteins select one each of V, D, and J segments to form a complete TCR. The combinatorial diversity that arises from joining these segments in a largely random manner leads to the ability of the immune system to develop a large number of unique TCRs with 25 million different TCR specificities (Arstila et al., 1999). The large diversity of TCRs affords protection against the vast majority of foreign pathogens that an organism may face, while also putting the host at risk of developing T cells with TCRs that react against host-derived antigens.

The risk associated with the random development of the TCR is in part corrected for through selection of T cells during the development in the thymus. The process of T cell selection ensures both the functionality of the TCR as well

as the removal of T cells containing TCRs that are reactive against host-derived antigens. T cells that do not bind with a high enough affinity to the MHC molecules present in the thymus do not survive and perish in a process called “death by neglect”. T cells that bind to MHC in the thymic cortex and induce weak TCR signals are positively selected and proceed to the thymic medulla (Hogquist et al., 1994; Hedrick, 2012). It is here that negative selection of T cells occurs, in which T cells that bind too strongly to self-antigen/MHC complexes are removed from the pool of mature T cells (Starr et al., 2003; Palmer, 2003). Through these stages of selection, T cells with self-reactive TCRs are removed from existence, and the rest of the T cells are allowed to migrate out of the thymus and into the periphery.

Despite this complex process, there is still evidence of self-reactive T cells being found peripherally (Semana et al., 1999; Lohmann et al., 1996; Zehn and Bevan, 2006; Bouneaud et al., 2000). While mechanisms have developed in order express and present self-antigens within the thymus during the process of selection, the production of self-reactive T cells during their development remains dangerous due to the inability of certain tissue or temporally specific antigens to be expressed as part of the selection process. To correct for these gaps in central tolerance resulting from T cell selection, there exist peripheral tolerance mechanisms to prevent activation of potentially self-reactive T cells existing in the periphery (Xing and Hogquist, 2012).

One way in which peripheral tolerance is maintained is through peripheral deletion of self-reactive T cell clones. This is mediated through an induced death of T cells that receive prolonged TCR stimulation, as the majority of self-reactive cells would, through Fas-mediated signals (Strasser and Pellegrini, 2004; Kawabe and Ochi, 1991). A second method to peripherally induce tolerance is to inactivate self-reactive cells through a process known as anergy (Schwartz, 2003). Anergy occurs when a T cell receives a TCR mediated signal, but lacks the second signals necessary to induce full activation of the cell. The result of this is alteration of the signaling capabilities of the T cell such that it survives in the periphery, but remains in a hyporesponsive state (Lechler et al., 2001). Anergy can also be achieved not through loss of the second signal, but by receiving this signal through a different receptor, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (Perez et al., 1997). Both deletion and anergy serve to remove the threat that peripheral self-reactive T cells pose and although these preventative methods are important, an additional method of dominant tolerance is still required in order to truly prevent activation of the immune system by self-antigens (Rudensky, 2005; Walker and Abbas, 2002).

### **Discovery of regulatory T cells and their role in peripheral tolerance**

In 1969 and 1970, two important studies were performed to begin to understand the role of T cells in establishing immune tolerance (Sakaguchi et al., 2007). It was discovered that there existed a cell produced in the thymus that had



the ability to suppress antibody mediated immune responses (Gershon and Kondo, 1970). Additionally, work by Nishizuka and Sakakura illuminated the need for thymus derived cells throughout life, as symptoms suggesting a loss of tolerance to the ovaries arose in mice thymectomized at day 3 after birth (Nishizuka and Sakakura, 1969). Further studies illuminated the presence of a thymus-derived factor important in preventing autoimmunity in many different organs that was not present in the mice thymectomized at day 3 after birth (Kojima and Prehn, 1981). Evidence of thymectomy causing a loss of tolerance was also present in rodent models of type 1 diabetes (T1D) (Penhale et al., 1973; 1990). Interestingly, protection against the autoimmune symptoms was conferred with a transfer of normal syngeneic CD4<sup>+</sup> T cells (Penhale et al., 1976; Sakaguchi et al., 1982). Together, these experiments suggested that the thymus produced both cells that mediated the effector immune response as well as a subset of CD4<sup>+</sup> T cells that were important in enforcing tolerance, likely through dominant suppression of the response of other immune cells (Sakaguchi et al., 1985).

The suppressive CD4<sup>+</sup> T cells important in maintaining tolerance were initially recognized by the expression of a set of surface receptors. Included in this phenotype were high expression of CD5 and low expression of CD45RB (Sugihara et al., 1988; Powrie and Mason, 1990; McKeever et al., 1990; Powrie et al., 1993; Morrissey et al., 1993). The lack of a functional marker for the suppressive cells led to difficulties in studying this cell type for many years, but the discovery of high expression of CD25 as a reliable marker for suppressive

CD4<sup>+</sup> T cells became a turning point in the study of these cells (Sakaguchi et al., 1995). It was recognized that depletion of CD25<sup>+</sup>CD4<sup>+</sup> T cells mimicked the neonatal thymectomy autoimmune model and that transfer of these cells specifically protected from autoimmunity in both the neonatal thymectomy model as well as in antigen specific autoimmune models (Itoh et al., 1999; Asano et al., 1996; Suri-Payer et al., 1998). With an easily measurable surface marker to label the suppressive CD4<sup>+</sup> T cells, they were given the name of regulatory T cells, or Tregs.

Discovery of the forkhead box P3 (Foxp3) gene as the mutated gene that lead to spontaneous autoimmunity in Scurfy mice lead to further breakthroughs in the study of Tregs (Brunkow et al., 2001). In conjunction with this discovery in mice, mutations in the Foxp3 gene were recognized as also being important in the development of an analogous human disease known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Chatila et al., 2000; Bennett et al., 2001; Wildin et al., 2001). With this knowledge, a number of groups in rapid succession identified the essential role that Foxp3 played in the development of Tregs in the mouse (Hori et al., 2003; Fontenot et al., 2003; Khattri et al., 2003).

The unearthing of Foxp3 as the most reliable marker for Tregs caused it to become a tool to better understand the biology of these cells. It was shown that the Foxp3 gene regulates hundreds of additional genes important in Treg development and function through direct and indirect methods (Zheng et al.,

2007; Wu et al., 2006; Ono et al., 2007; Marson et al., 2007). In fact, forcing expression of Foxp3 in Foxp3<sup>-</sup> conventional T cells (Tconvs) conferred suppressive ability, leading to the recognition of Foxp3 as a driver of the Treg lineage (Hori et al., 2003; Fontenot et al., 2003).

## **Generation of regulatory T cells**

### *Thymic Treg generation*

Tregs that develop within the thymus are termed thymically-derived Tregs (tTregs) and the factors required for this development have been extensively studied. Early experiments provided strong suggestions for TCR specificity in the development of Tregs. These experiments revealed that presence of a specific organ, such as a sex-related organ, was required to find Tregs that were able to suppress autoimmunity to that tissue (Garza et al., 2000; Taguchi et al., 1994). This requirement for TCR stimulation in tTreg development was further directly supported by studies in which double transgenic mice that expressed TCRs specific for the flu antigen hemagglutinin (HA) only developed Tregs when the HA antigen was also expressed within the thymus (Jordan et al., 2001). Resulting from these studies is a model where the majority of T cells arise with low TCR stimulation during thymic development, while Tregs develop within the range of TCR signal strength between negative and positive selection (Maloy and Powrie, 2001). In the wild type (WT) thymus, the protein called the autoimmune regulator (Aire) plays a critical role in expressing peripheral self-antigens for T cell negative

selection (Anderson et al., 2002). While it is likely that the expression of these self-antigens play some role in Treg development, it has not been conclusively shown that generation of tTregs requires Aire driven expression of tissue-specific antigens (Aschenbrenner et al., 2007; Anderson et al., 2005).

Aside from TCR signals, tTreg generation has also been shown to require cytokines and costimulation during development. As had been appreciated, Tregs express CD25, the high affinity subunit of the interleukin 2 (IL-2) receptor. The expression of this receptor is critical in the development of tTregs as they require IL-2 induced signal transducer and activator of transcription 5 (STAT5) signals to upregulate Foxp3 (Lio and Hsieh, 2008). Furthermore, the cytokine thymic stromal lymphopoietin (TSLP) has been implicated in the development of tTregs in humans. Through production by Hassal's corpuscles, TSLP is believed to instruct DCs to drive Foxp3 expression and tTreg development (Watanabe et al., 2005). The role that TSLP plays in Treg development is less clear in the mouse. While TSLP may drive the upregulation of Foxp3 in CD4+ single positive T cells *in vitro*, global removal of TSLPR does not change the ability of Tregs to develop *in vivo* (Mazzucchelli et al., 2008; Lee et al., 2007; Jiang et al., 2006). In addition to these cytokine requirements, tTreg generation has been shown to require CD28 costimulation (Tai et al., 2005). In mice lacking CD28 or the binding partners CD80 and CD86, the majority of tTreg generation is lost (Lio et al., 2010).

### *Peripheral Treg generation*

Tregs can be generated within the thymus as demonstrated, but they can also arise through induction of Foxp3 expression in naïve Tconvs in the periphery. Tregs that arise in this manner are called peripherally-derived Tregs, (pTregs). The proportion of total Tregs in a mouse that derive from tTregs or pTregs is not completely clear (Lee et al., 2011a). There are a number of studies looking in adoptive transfers as well as comparing TCR specificities between Foxp3<sup>+</sup> Tregs in the thymus and periphery that have concluded that pTregs make up somewhere between 4-7% of the total Treg population (Lathrop et al., 2008; Pacholczyk et al., 2006; Wong et al., 2007). A second method of measuring the contribution of pTregs to the total Treg population surfaced with the identification of Helios as a marker of tTregs. While the fidelity of this marker is controversial, its expression on Tregs suggested that pTregs may actually be about 30% of the peripheral Tregs (Thornton et al., 2010). In reality, the percentage of pTregs likely varies by the location studied, as in the gut it has been shown with Helios expression patterns that pTregs may comprise up to 80% of the total Tregs (Lee et al., 2011a; Richards et al., 2015).

It was appreciated initially that long-term treatment with low doses of antigen could cause antigen-specific naïve Tconvs to become CD25<sup>+</sup> suppressive cells (Apostolou and Boehmer, 2004). A number of other studies supported the notion that foreign antigen could induce Foxp3 expression in naïve Tconv cells under the right conditions (Mucida et al., 2005; Verginis et al., 2008; Kretschmer et al., 2005). Like the shared requirements of TCR stimulation, the

other conditions essential for pTreg generation are not that far different from those required for tTreg generation (Lee et al., 2011a). Cytokines play an important role in the production of these cells, in particular IL-2 and transforming growth factor beta (TGF $\beta$ ) have been shown to be required for optimal pTreg development (Chen et al., 2003; Knoechel et al., 2005; Zheng et al., 2010). Interestingly, the role of CD28 costimulation on Tconvs for pTreg generation is controversial with studies supporting both a positive as well as an inhibitory role for CD28 signals in inducing Foxp3 expression in *in vitro* induced Tregs (iTregs) (Guo et al., 2008; Liang et al., 2005; Kim and Rudensky, 2006). Despite the partially analogous requirements for tTreg and pTreg generation, stability of Foxp3 expression is much lower in pTregs due to complex epigenetic differences between these populations that are now beginning to be illuminated (Josefowicz et al., 2009; Floess et al., 2007; Zheng et al., 2010).

### **Peripheral homeostasis of regulatory T cells**

While development plays a large part in the overall number of Tregs, there are a several factors that play an important role in the maintenance of Tregs in the periphery, some of which overlap with those required for development (Smigiel et al., 2014b). The requirements for peripheral Tconv homeostasis have been broadly studied and despite the potential overlap with the mechanisms of Treg maintenance, details of the factors important in the homeostasis of Tregs are still not fully understood.

### *Role of IL-2*

The first and potentially most apparent factor important in Treg maintenance is the cytokine IL-2, as Tregs were initially distinguished by their expression of the high affinity subunit of the IL-2 receptor, CD25. While IL-2 has long been associated with the expansion of activated T cells, Tregs are unique in their ability to constitutively express CD25 through regulation by Foxp3 (Williams and Rudensky, 2007; Chen et al., 2006). Furthermore, STAT5 signaling through the IL-2 receptor leads to further stabilization of the expression of CD25 in Tregs (Kim et al., 2001; Boyman and Sprent, 2012). Despite this dependence on IL-2, Tregs are unable to produce this key cytokine themselves, and instead depend on the production of IL-2 by Tconvs (Cheng et al., 2011; Almeida et al., 2006; Setoguchi et al., 2005; Almeida et al., 2002). This was elucidated in mixed bone marrow chimeras in which it was observed that WT T cells could rescue the inability of IL-2 knock out (KO) cells to maintain Tregs (Almeida et al., 2002). Furthermore, neutralization of IL-2 using anti-IL-2 antibodies leads to an acute depletion of Tregs in the periphery, even in the setting of thymectomy to correct for the known role of IL-2 in Treg generation (Setoguchi et al., 2005). At a time when IL-2 was thought to be primarily immunostimulatory, it was intriguingly found that treatment with IL-2 was protective against autoimmune diabetes in the non-obese diabetic (NOD) mouse model (Serreze et al., 1989). With the discovery of the tolerogenic role that IL-2 plays in Treg biology, these autoimmune protective observations became clear. Additionally, it has been found that treatment of mice with IL-2 cytokine/anti-IL-2 antibody complexes (IL-2

IC) to target the high affinity IL-2 receptor lead to widespread proliferation and expansion of Tregs resulting in protection from autoimmunity in both the NOD diabetes model as well as experimental autoimmune encephalomyelitis (EAE) (Webster et al., 2009; Boyman et al., 2006; Tang et al., 2008).

Despite this knowledge, the exact role that IL-2 plays in Treg maintenance is only beginning to be clarified. While there are stark reductions in Tregs with mutations within the IL-2 signaling pathway, none are as complete as are seen with Foxp3 mutations, suggesting that while IL-2 is needed to maintain tolerance, there are likely IL-2 independent Tregs (Smigielski et al., 2014b). In fact, studies were done to demonstrate that Tregs are found in one of two subsets, termed central and effector Tregs. Maintenance of central Tregs is dependent on IL-2, while effector Tregs rely more heavily on binding of the surface receptor, inducible T cell costimulator (ICOS) (Smigielski et al., 2014a). Tregs overall are a highly proliferative subset of T cells, and with the ability of exogenous IL-2 to drive enhanced proliferation of Tregs, it was believed for a time that the role of IL-2 in Treg homeostasis was to drive this constant proliferation at the steady state. The studies on the subsets of Tregs demonstrated that IL-2 was required instead for the survival of the non-proliferating quiescent central Tregs (Smigielski et al., 2014a).



### *Role of DCs and Costimulation*

Tregs constitutively express several T cell specific costimulatory surface receptors. A number of these play a role in the homeostasis of both Tconv and Tregs. In particular, CD28 was recognized as having an important role in the maintenance of tolerance through Tregs with the initially paradoxical observations that CD28 KO mice develop widespread autoimmunity (Zhang et al., 2013; Tang et al., 2003; Salomon et al., 2000). Furthermore, the expression of ICOS that was identified as being important for the maintenance of effector Tregs has been appreciated in a number of other models as well. Both ICOS KO as well as blockade of ICOS lead to decreases in peripheral Treg numbers, while thymic Treg generation is preserved (Burmeister et al., 2008; Herman et al., 2004).

While the mechanisms by which costimulation maintains Tregs peripherally are largely unknown, there is evidence to suggest that many of the receptor engagement interactions occur between Tregs and DCs. A number of studies have demonstrated that DCs are a critical cell type in Treg maintenance with loss of DCs leading to decreases in Tregs as well as expansion of DCs leading to increases (Swee et al., 2009; Darrasse-Jèze et al., 2009). These studies varied in the conclusions about the specific role that DCs played in Treg maintenance, with one suggesting it was MHC-II/TCR mediated and the other suggesting that it was TCR independent. The true requirements may actually be a combination of TCR dependent and independent interactions between DCs and Tregs. Illustrating this, mixed chimera experiments showed that DCs are the

source of CD80 and CD86 molecules for CD28 driven Treg homeostasis *in vivo* (Bar-On et al., 2011). In addition, *in vitro* experiments have supported this notion, showing the DC-induced Treg expansion is CD80/86 and CD28 dependent (Smigiel et al., 2014b; Zou et al., 2010).

### *Role of TCR*

On the other hand, MHC-II expression is also required, as experiments in lymphopenic hosts have exhibited that MHC-II signals are required *in vivo* for both Treg survival and proliferation (Gavin et al., 2001; Bhandoola et al., 2002). In fact, Tregs receive constitutive TCR signals in the periphery as has been shown using Nur77 as a marker. Nur77 is an early, immediately upregulated gene with TCR stimulation (Osborne et al., 1994). Creation of a green fluorescent protein (GFP)-tagged Nur77 molecule demonstrated that Tregs receive constant TCR signals that are higher than that of Tconvs (Moran et al., 2011). Furthermore, studies have associated the need for TCR signaling molecules Ick and linker of activated T cells (LAT) in the homeostasis of Tregs independent of their thymic generation (Kim et al., 2009; Shen et al., 2010). Studies using antigen specific Tregs *in vivo* and *in vitro* illuminated that like the costimulatory requirement, the TCR stimulation was also provided by DCs (Fehervari and Sakaguchi, 2004; Yamazaki et al., 2003). Even within a polyclonal Treg TCR repertoire, it has been demonstrated that DC-derived TCR stimulation may play an important role in the proliferation critical for Treg homeostasis (Zou et al., 2010).

Many of the manipulations used to look at peripheral homeostasis of Tregs target important molecules in Tconv biology as well. This has led to confounding results in many studies. As had been demonstrated by multiple groups, the IL-2 required for Treg maintenance is produced by the Tconvs that share many of these target molecules. Despite this, the requirement for IL-2, costimulation, TCR, and DC-Treg interactions are clearly important in the peripheral homeostasis of Tregs. Discovery of additional factors important in this process will open new doors to understanding Treg biology and hold the potential to be able to better control Treg numbers.

### **Regulatory T cells for therapeutic use**

With the knowledge of cytokines and interactions that can alter the number of Tregs, this cell type quickly became an important target for therapeutic use. The ability to suppress the immune response with increases in Tregs as well as potentially to enhance responses through inhibiting Treg maintenance is appealing for its clinical implications. Despite the findings in mouse models linking Treg numbers to various forms of autoimmunity, few autoimmune patients exhibit decreases in Tregs (Smigiel et al., 2014b). While systemic lupus erythematosus (SLE) patients do in fact display decreases in Treg numbers, type 1 diabetic (T1D), rheumatoid arthritis (RA), psoriasis, Crohn's disease, and multiple sclerosis (MS) patient samples have exhibited conflicting results suggesting that human Treg therapy may not be as straight forward as it is in the

mouse (Brusko et al., 2007; Putheti et al., 2004; Lee et al., 2006; Miyara et al., 2005; Hahn et al., 2015). Of note, measurements of Tregs in these patients are within the circulating blood and not at the sites of immune activation. Furthermore, recognition of Tregs in humans is less clear than the mouse using current markers, as human activated effector T cells upregulate Foxp3 as well (Ziegler, 2007; R Walker et al., 2003; Morgan et al., 2005). It is only when looking at demethylation of the Foxp3 locus that it is clear if the Foxp3<sup>+</sup> T cell is a true Treg or an activated effector cell (Baron et al., 2007; Wieczorek et al., 2009).

While these data are suggestive of little causative effect in the development of autoimmunity, it does not rule out the ability of Treg increases to affect negative regulation of the immune response in human patients. There are still many barriers to being able to effectively use Treg transfers in human therapies. Creation of human iTregs *in vitro* for therapeutic use has not been successful due to stability of Foxp3 expression after transfer (Rossetti et al., 2014). A more promising approach that has shown to be effective in graft versus host disease (GVHD) and decreasing effector responses in T1D patients is the expansion of pre-existing Tregs either through *in vitro* or *in vivo* means (Rossetti et al., 2014; Koreth et al., 2011; Matsuoka et al., 2013; Rosenzweig et al., 2015). The studies performed with *in vivo* targets utilize treatment with low dose IL-2 to drive Treg expansion, similar to a number of mouse studies. Importantly, it has been recognized that in order for *ex vivo* Treg expansion and cell transfer approaches to be effective, there must exist the proper environment and minimal competition from pre-existing Tregs in the host (Cabello-Kindelan et al., 2014).

Overall, the potential use of Tregs as a therapy requires greater understanding of the factors and environment necessary to maintain and manipulate this population of complex immune cells.

### **Skin and the immune system**

While a large amount of the immune system exists within the primary and secondary immune organs, such as the bone marrow, thymus, spleen and lymph nodes, immune cells also reside in non-immune tissues. The roles these tissue resident immune cells play are varied and important for the development of appropriate immune responses. Within the skin, there are a number of interactions between the immune system and non-immune cells to control immune responses. This is an important communication due to the large size of the skin as an organ and the constant exposure to the external environment. Dysregulated immune responses in the skin can lead to a number of harmful inflammatory disorders including atopic dermatitis, contact hypersensitivity, and psoriasis (Gratz and Campbell, 2014). The need for the skin to be involved in the immune response requires both driving the activation of the immune system against pathogens as well as a protective regulatory response of tolerance to benign commensals (Matzinger and Kamala, 2011). This is an important balance, as a number of locations in the skin in fact harbor a more diverse set of commensals than those found in the gut and lungs (Costello et al., 2009; Grice et al., 2009).

Locally within the skin, signals in response to these commensals have been shown to be important in the steady state to control the frequency of Tregs (Naik et al., 2012). Furthermore, commensals are able to control the ability of the skin to mount protective Th1 immune responses during dermal infections (Belkaid and Naik, 2013). Skin resident DCs are found to be involved in many of these processes, as Langerhans cells are able to drive both tolerance through Tregs as well as immunity through memory T cells (Seneschal et al., 2012). Responses within the skin have also been observed to be able to control the immune system in more than just the local tissue, having additional effects on systemic immunity. This is not a totally foreign concept, as commensals in the gut have been shown to be able to affect the development of autoimmunity in distal sites (Wu et al., 2010; Lee et al., 2011b). Treatment of the skin with the chemical compound MC903, that signals through the Vitamin D Receptor (VDR), leads to the production of TSLP by keratinocytes (Li et al., 2006; 2009). While this results in the local development of Th2 inflammation resembling atopic dermatitis, it can also lead to systemic effects in the immune system through the driving of basophil hematopoiesis (Zhang et al., 2009; Siracusa et al., 2011). In all these ways, the skin and skin-derived commensals are able to interact with and control immune responses in both local as well as newly appreciated systemic manners.

In the reverse direction, immune cells are also known to play a role in the maintenance of skin cells through the production of keratinocyte growth factor (KGF) by  $\gamma\delta$  T cells for the growth of keratinocytes (Boismenu and Havran,

1994). This KGF pathway is similar to the recently appreciated role of growth factor production by innate lymphoid cells that is important in the healing of damaged epithelial cells in the gut in a model of colitis (Monticelli et al., 2015). Altogether, it is appreciated that within the skin there is a diverse interplay between immune cells, non-immune cells, and commensals that control the homeostasis of this large organ at the steady state.

### **Vitamin D and the immune system**

Vitamins have been shown to have effects on the immune system in specific (receptor mediated) and non-specific (antioxidant) manners in both the innate and the adaptive immune system (Mora et al., 2008). One of the early-appreciated vitamins to play a role in the immune system was vitamin D<sub>3</sub> (Lemire et al., 1984; Rigby et al., 1984). This effect was initially believed to be a direct interaction due to the observation that vitamin D receptor (VDR) is expressed within a number of immune cells including macrophages, DCs, T cells and B cells (Bikle, 2011). Furthermore, some activated immune cells have the ability to convert vitamin D<sub>3</sub> into the active form that is able to bind to the VDR (Mora et al., 2008). Due to these observations, the effect of vitamin D<sub>3</sub> on the immune system has primarily been appreciated and studied as a direct effect of this compound on immune cells.

Overall, the role of vitamin D<sub>3</sub> in the adaptive immune system is one of inhibition resulting from decreases in the proliferation of a number of cells types

and general switching of the immune response away from an inflammatory Th1 response (Rigby et al., 1987; Reichel et al., 1987). Furthermore, vitamin D<sub>3</sub> has been implicated in the promotion of suppressive immune responses through Tregs, some of which may have additional roles in autoimmunity and atherosclerosis. (Takeda et al., 2010; Meehan et al., 1992; Gorman et al., 2010). In fact, low serum levels of active vitamin D<sub>3</sub> have been associated with the development of autoimmune disorders including type 1 diabetes and SLE (Littorin et al., 2006; Muller et al., 1995). The possibility to treat autoimmunity in these patients by augmenting vitamin D<sub>3</sub> through oral administration has been plagued by the toxic effects of high levels of active vitamin D<sub>3</sub>, leading to the need for alternate analogues that have immunomodulatory effects without the negative outcome of hypercalcemia (van Etten and Mathieu, 2005). Alternatively, the effects of vitamin D<sub>3</sub> on the immune system have begun to be understood to not solely act through a direct effect on immune cells, but also through VDR signals in non-immune cells that are then able to alter the immune response (Zhang et al., 2009; Siracusa et al., 2011).

Better recognition of how exactly vitamin D<sub>3</sub> and vitamin D<sub>3</sub> analogues are able to exert inhibitory effects on the immune system will allow for safer and more effective future use of this known immunomodulatory compound in clinical settings of over-activation of the immune system.



## **Structure of the thesis**

Despite broad study of the maintenance of Tregs, there is still much to learn about the cell types and factors important in controlling the homeostasis of these cells. In this thesis, I investigate the role of factors deriving from non-Treg cells in the maintenance and expansion of Tregs. This examination will help to clarify the environment that is needed to best support Tregs in both the steady state as well as in therapeutic applications.

In chapter II, I examine the ability of local tissue responses to produce factors that can drive systemic expansion of Tregs. This expansion is achieved through a series of events involving a number of cell types located within the skin and elsewhere as well as cytokine mediated signals between them to drive Treg proliferation. In chapter III, I further investigate the previously accepted role that Tconvs play in Treg maintenance. Specifically, I look at the role of TCR signaling within Tconvs for the production of the essential cytokine IL-2 in the maintenance of Tregs.

Within chapter IV, I will go over the findings of the previous two chapters and discuss the implications of the results in the context of what is known about Treg homeostasis. Additionally, I will discuss the important future directions of this work and what it may mean for the ability to manipulate Treg maintenance for therapeutic uses.

## CHAPTER II:

### RESULTS

#### **Skin mediated control of systemic regulatory T cell numbers and protection against type 1 diabetes**

##### **Summary**

Regulatory T cells (Tregs) are a subset of CD4<sup>+</sup> T cells with suppressive function. Tregs are critical in limiting autoimmunity and increasing Treg numbers can be beneficial in the treatment of various inflammatory disorders. Here, we provide evidence that the skin can exert strong systemic effects on Treg numbers by producing the cytokine thymic stromal lymphopoietin (TSLP) in response to topical administration of the Vitamin D<sub>3</sub> analog MC903. A 2 fold increase in the proportion (out of all CD4<sup>+</sup> T cells) and absolute number of Tregs was observed in the blood, lymph nodes, and spleen of mice treated topically but not systemically with MC903. The increase in Treg numbers was dependent on TSLP-R signaling but not on Vitamin D receptor signaling in hematopoietic cells. However, TSLP-R expression by Tregs themselves was not required for the proliferation of Tregs induced by topical MC903 treatment. Rather, TSLP promotes Treg proliferation by affecting the DC/Treg interaction, as TSLP alone or in combination with IL-2 induced the proliferation of Tregs co-cultured with DCs. To test whether these effects of MC903 could influence progression of an autoimmune disorder, nonobese diabetic (NOD) mice were treated topically with

MC903. Treatment with MC903 compared to vehicle significantly lowered the incidence of diabetes from 100% to 40%. Together, these data demonstrate that the skin has the remarkable potential to control systemic immune responses and that topical MC903 treatment could serve as a novel strategy to induce systemic immunomodulation in autoimmune diseases.

## Introduction

The interaction between the immune system and local tissue has been investigated by a number of studies looking at the ability of skin and intestinal resident cells to alter the local immune response (Saenz et al., 2008). Tissues such as these that interface with the outside environment have a powerful ability to signal to and instruct the progress of a local, compartmentalized immune response (Belkaid and Naik, 2013). What has been recently appreciated is the ability of local responses to then alter the progression of systemic immune effects. In particular, the presence of segmented filamentous bacteria (SFB) in the intestine has been shown to play a role in the development of autoimmune diseases in the mouse at sites far from the SFB exposure (Wu et al., 2010; Lee et al., 2011b). In a comparable event, chemical treatment of the skin with a vitamin D<sub>3</sub> analogue has shown to have effects of the hematopoiesis of basophils (Siracusa et al., 2011).

This effect was mediated by the systemic increase in thymic stromal lymphopeptin (TSLP) resulting from the production of this cytokine by keratinocytes in the skin (Zhang et al., 2009). TSLP has traditionally been recognized as a Th2 driving cytokine, but the role that it may play in regulatory T cell (Treg) development has begun to be highlighted by a number of studies (Watanabe et al., 2005; Besin et al., 2008). While the factors effecting homeostasis of Tregs have primarily been investigated within immune sites,

these results introduce the possibility for additional factors dependent on cells situated within non-immune tissues to play a role in immune suppression.

Preservation of immunologic tolerance to self is a complex process that involves development of both central and peripheral mechanisms (Josefowicz et al., 2012; Sakaguchi et al., 2008; Bilate and Lafaille, 2012). Development and maintenance of Tregs is pivotal in this process (Smigiel et al., 2014b; Webster et al., 2009; Smigiel et al., 2014a; Kim et al., 2001; Gavin et al., 2001; Boyman and Sprent, 2012; Setoguchi et al., 2005; Fisson et al., 2003). Loss of Tregs leads to development of a widespread autoimmune syndrome in both mice and humans (Sakaguchi et al., 1995; Fontenot et al., 2003; Lahl et al., 2007; Brunkow et al., 2001). Furthermore, impaired homeostasis and function within the Treg subset leads to a number of other common autoimmune diseases such as type 1 diabetes (T1D), multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (Lindley et al., 2005; Viglietta, 2004; Ehrenstein, 2004; Horwitz, 2008). To this effect, increases in Treg number have been shown to be beneficial in limiting inflammation in mouse models of autoimmunity (Lepault and Gagnerault, 2000; Szanya et al., 2002; Besin et al., 2008; Webster et al., 2009).

Tregs depend on a number of other cell types in order to develop in the thymus as well as survive in the periphery. Two essential cell types are conventional T cells (Tconvs) and dendritic cells (DCs) that provide fundamental cytokines as well as cellular contacts for Tregs (Almeida et al., 2006; Swee et al., 2009; Zou et al., 2010). Despite this knowledge, there is likely a large, complex

network of interactions between immune cells and other potentially unidentified cell types important in the maintenance of Tregs in both steady state and inflammatory environments.

The capability to manipulate an easily accessible organ such as the skin provides a clear advantage in the development of clinical therapies for autoimmune diseases. In particular, the skin has been an important location in which vitamin D<sub>3</sub> therapy has been efficacious. Vitamin D<sub>3</sub> has been implicated as an immunosuppressive compound due to a number of *in vitro* studies (Mora et al., 2008). The ability to utilize vitamin D<sub>3</sub> as a therapy has been hindered by toxicity issues associated with oral administration of efficacious doses. In contrast, the vitamin D<sub>3</sub> analogue MC903 has been quite effective as an immune therapy when used as a topical treatment in psoriasis, an autoimmune disease of the skin. As mentioned, topical MC903 has been shown to have specific effects on hematopoiesis within the immune system in murine models, suggesting an ability for this compound locally to have widespread immune effects (Siracusa et al., 2011).

In this study, we assess the ability of local responses in the skin to alter systemic immunity. We discovered that topical application of MC903 led to production of the cytokine TSLP, which was found systemically within the serum of mice. This cytokine skewed DCs to a non-inflammatory phenotype that was able to stimulate the proliferation of Tregs both *in vivo* and *in vitro*. In the topical MC903 treatment model, the effect was solely through DC-driven Treg

proliferation, as Tregs did not need to sense the TSLP directly and the treatment did not stimulate production of Tregs either centrally or peripherally. The DC driven expansion of Tregs was partially dependent on CD80/CD86 interactions with Tregs. This topical treatment alone was able to decrease the incidence of diabetes in the non-obese diabetic (NOD) mouse model of T1D. Together, this work demonstrates a remarkable ability of the skin to produce immunomodulatory effects that can have therapeutic results in the setting of autoimmune diseases.

## Results

### Topical MC903 treatment increases Treg numbers through TSLP signaling

To test the hypothesis that systemic TSLP would promote increases in Treg numbers, we utilized the MC903 topical treatment model. MC903 is an analogue of vitamin D<sub>3</sub> with added modifications to optimize it as a topical therapy. MC903 is retained in the skin to a greater extent than vitamin D<sub>3</sub> and what little compound does make it into the circulation, has a significantly decreased half-life (Knutson et al., 1997; Kissmeyer and Binderup, 1991). Together, these effects provide MC903 less than 1% of the negative calcium metabolism related activity of vitamin D<sub>3</sub>, leading to greatly decreased toxicity in comparison. We tested the ability of this topical treatment in wild type (WT) mice to affect the levels of Tregs. WT mice were treated with 2 nmol MC903 or equivalent volume of EtOH vehicle on both ears daily for 7 days. On day 8, Treg percentages were measured as Foxp3<sup>+</sup>% of total CD4<sup>+</sup> T cells by flow cytometry in the spleen, blood, skin, and skin-draining cervical lymph nodes (dLN) (Fig. 2.1A). In all locations tested, we observed significant increases in the percentage of Foxp3<sup>+</sup>CD4<sup>+</sup> Tregs (Fig. 2.1B-C). Additionally, increases in absolute Treg numbers were seen in lymphoid organs with MC903 treatment in comparison to EtOH (Fig. 2.1D).

Vitamin D<sub>3</sub> is known to have effects on the immune system through direct interactions (Mora et al., 2008). To test the possibility that MC903 was driving



Treg increases due to it becoming systemic, we compared topical to systemic MC903 administration. The observed Treg increases were only obtained with topical treatment, as intraperitoneal (i.p.) injection of 4nmol of MC903 did not lead to any change in Treg proportions (Fig. 2.1E). The inability of MC903 to cause Treg increases with i.p. administration also suggested that the effects of treatment with the vitamin D<sub>3</sub> analogue were on a skin resident cell rather than on circulating immune cells. To further test this and to determine the requirement of vitamin D signals in this model, we created vitamin D receptor knock out (VDR KO) bone marrow (BM) chimeras. 4x10<sup>6</sup> bone marrow cells from either VDR KO or WT C57BL/6 mice were transferred into lethally irradiated C57BL/6.SJL mice. At 9-10 weeks post-transfer, mice were treated with either 2nmol MC903 or EtOH vehicle. Treg percentages were increased in VDR KO BM chimeras with MC903 topical treatment to a similar extent as WT BM chimeras (Fig. 2.1F). This demonstrates that Treg increases occur independently of VDR signaling in the hematopoietic system.

Instead this suggests that VDR signaling is required within a skin resident population. It has been shown previously that topical MC903 treatment induces TSLP found in the serum and that this is produced solely by keratinocytes (Li et al., 2006; 2009). We confirmed that TSLP was produced and found systemically within our model as well by looking in the serum at day 3 of topical MC903 or EtOH treatment. Consistent with previous reports, TSLP was undetectable in the serum by ELISA in EtOH treated animals, but found at high levels in MC903 treated mice (Fig. 2.2A). To determine if TSLP could be playing a role in the Treg

increases we observed, we treated WT and TSLP receptor knock out (TSLPR KO) mice with EtOH or MC903 topically for 7 days. On day 8, we observed increases in both Treg percentages as well as total numbers in WT mice, but not in the TSLPR KO mice (Fig. 2.2B-C). This demonstrated that the ability of Treg percentages to increase with topical MC903 treatment was dependent on the production of TSLP and the ability to signal through the TSLPR.

Together, these data suggest that the topical treatment with MC903 leads to TSLP production resulting from VDR signals within the skin. This leads to increases in Treg percentages systemically that are dependent on signals through the TSLPR.

### **Treg increases with topical MC903 treatment occur through proliferation of existing Tregs**

TSLP has been suggested to play a potential role in the thymic production of Tregs through studies in the human thymus (Watanabe et al., 2005). Additional findings have provided conflicting evidence for a role for TSLP in the generation of *in vitro* induced Tregs (iTregs) (Besin et al., 2008; Sun et al., 2007). Furthermore, TSLP has been linked to DC-driven proliferation of Tconvs (Watanabe et al., 2004). Due to these links between TSLP and T cell homeostasis, we assessed the role of each of these processes in the Treg increases in our MC903 topical treatment model.

To first look at the generation of thymically derived Tregs (tTregs) with MC903 topical treatment, we assessed Treg percentages within the thymus of mice treated with EtOH or 2nmol MC903 topically for 7 days. We observed no increase in the percentage of Tregs in the thymus of mice treated with MC903 (Fig. 2.3A). To further assess the contribution of tTreg production with topical MC903 treatment, we utilized a mouse model that allows one to visualize cells that were recently produced in the thymus. RAG2-GFP mice express GFP under the RAG2 promoter and when T cells express RAG2 during development GFP is produced, marking the cells in the periphery that are recent thymic emigrants (Boursalian et al., 2004). When RAG2-GFP mice were treated with EtOH or MC903 topically, we found no difference in the percentage of GFP<sup>+</sup> Tregs in the spleen at day 8 (Fig. 2.3B). Further suggesting that in this topical treatment model, the observed Treg percentage increases are not being driven by greater tTreg production.

While tTreg production did not change in our treatment model, the production of peripherally derived Tregs (pTreg) may have been altered with MC903 treatment. Analysis of helios and neuropilin-1 expression on Tregs in the spleen showed no difference in the expression profiles of these markers between Tregs from EtOH and MC903 treated mice, suggesting no change in the generation of pTregs with topical MC903 treatment (Fig. 2.3C). To further test the contribution of pTregs to the observed Treg increases, we utilized an adoptive transfer model to track the generation of pTregs. Congenically disparate Tconvs and Tregs were co-transferred into a lymphoreplete host and 3 days later the

host mice were treated with EtOH or MC903 topically. We found that of the recovered donor Foxp3<sup>+</sup> cells at the end of the treatment, that there was no increased contribution from the transferred Tconvs to the Foxp3<sup>+</sup> T cells with MC903 treatment compared to EtOH (Fig. 2.3 D-E). This demonstrates that pTreg generation does not account for the Treg percentage increases with topical MC903 treatment. In fact, we observed that TSLP decreased the ability to generate *in vitro* iTregs, suggesting that it is unlikely that conversion of Tconv to Foxp3<sup>+</sup> pTregs occurred in the presence of the large amount of TSLP in the serum of MC903 treated mice (Fig. 2.3F).

Tregs are a subset of cells with a fairly high proliferative capacity and TSLP has been linked to proliferation of Tconvs, so we tested the role that proliferation played in the increases in Tregs with topical MC903 treatment (Watanabe et al., 2004). To measure the proliferation of the Treg population with topical MC903, on the final day of MC903 treatment, mice were fed Bromodeoxyuridine (BrdU) in their drinking water for 3 days, at the end of which BrdU uptake by Tregs was determined by flow cytometry. We found that there was significantly more BrdU incorporation in Tregs from MC903 treated animals in comparison to EtOH treatment (Fig. 2.4A-B). When this result is considered with the data showing the lack of new tTreg and pTreg production, it suggests that the increase in Treg numbers with MC903 treatment was likely a result of the increased proliferation of existing Tregs.

Tregs can be divided into subsets known as central and effector, with the central subset being quiescent and the effector subset being more proliferative (Smigiel et al., 2014b; a). Along with the observed increase in proliferation, we found that the resulting Tregs in MC903 treated mice exhibited an effector phenotype through other markers as well. They were CD62L<sup>LO</sup> and had greater expression of a number of other markers including CTLA4, ICOS, and CD39 (Fig. 2.4C-D). Despite the effector phenotype of the Tregs in MC903 mice, they were no more functional on a per-cell basis as tested by an *in vitro* Tconv proliferation suppression assay (Fig. 2.4E).

Stimulation through the TCR has also been shown to be important in Treg proliferation, so we performed experiments in which TCR/MHC-II interactions were lost in the periphery to determine if MC903 was able to drive Treg expansion without TCR stimulation. The K14 mouse has limited MHC-II expression, with the gene for MHC-II being driven by the K14 promoter. This leads MHC-II expression in the thymus, but little exposure to TCR stimulation in the periphery due to lack expression of MHC-II in APCs (Laufer et al., 1996). In the K14 mouse, CD4<sup>+</sup> Tcells, including Tregs, are able to develop but do not encounter TCR stimulation within the periphery, providing an environment in which we could determine the requirement for TCR stimulation in Treg increases with MC903 treatment. We treated WT and K14 mice with EtOH or MC903 topically for 7 days. On day 8, we analyzed the Treg percentage in the spleen and in the dLN of mice. We found that within the spleen, both WT and K14 mice exhibited an increase in Treg percentages with MC903 treatment (Fig. 2.4F). In

contrast, in the dLN K14 mice did not have increases in Treg percentages to the same extent as WT mice (Fig. 2.4G).

Together, these data demonstrate that Treg increases with MC903 treatment are established through proliferation of existing Tregs as opposed to production of tTreg or pTregs. Despite comparable function *in vitro*, these resulting Tregs in MC903 treated mice are found to be more effector-like. Interestingly, the local Treg increases may be more dependent on TCR signals than the systemic increases in the spleen.

### **Tregs increase with MC903 treatment independent of a Th2 response**

Topical MC903 treatment has been studied for its ability to cause an immune response in the skin of mice that models the Th2-driven atopic dermatitis (AD). This model has been shown to be dependent on the ability of keratinocytes in the skin to produce TSLP and that production of this cytokine drives the development of Th2 inflammation characterized by increased IgE, production of Th2 cytokines such as IL-4 and IL-13 and increased levels of eosinophils (Li et al., 2009). Due to this known effect of MC903, it is important to understand if the Treg increases that we have seen with topical MC903 are in response to and dependent on the presence of the developing Th2 inflammation. While the 7-day treatment model used here is a shorter course of treatment than that used to model AD, there is still some inflammation present in the skin as evidenced by thickening of the ear skin during MC903 treatment (Fig. 2.5A). We therefore

wanted to examine the ability of topical MC903 to drive Treg increases in an environment with decreased Th2 inflammation after MC903 treatment.

AD is characterized by an increase in the levels of Th2 cells and decreases in Th1 cells (Harskamp and Armstrong, 2013). Current strategies to treat this condition target the important Th2 cytokine, IL-4, and neutralization of this cytokine has in fact shown to be beneficial in lessening the symptoms of AD (Montes-Torres et al., 2015). We therefore sought to analyze Treg increases in response to topical MC903 in IL-4 KO mice, which should have similar decreases in the AD symptoms.

We treated WT and IL-4 KO mice with EtOH or 2nmol/ear MC903 topically for 7 days and followed ear thickness over the course of treatment as a measure of inflammation. Additionally, Treg percentages were measured in the spleen at day 8. We found that IL-4 KO mice had significantly less ear thickening with MC903 treatment than that found with WT treated mice (Fig. 2.5A). Despite the partial decrease in the AD inflammation, we observed a comparable increase in Treg percentages in the spleen at day 8 between both WT and IL-4 KO mice that were treated with MC903. (Figure 2.5B) These data suggest that the Treg increases are not solely a response to Th2 inflammation as they are the same in the presence of lessened AD, and that the mechanism of increase likely occurs through a pathway independent of the Th2 inflammation.

## **Tregs are increased with topical MC903 indirectly through effects of TSLP on dendritic cells**

Expression of the TSLP cytokine is largely restricted to epithelial cells, while expression of the TSLP receptor has been found in many immune cell types, including Tregs (He and Geha, 2010). To determine if the proliferating Tregs required a direct TSLP interaction, we co-transferred  $5 \times 10^6$  MACS-purified CFSE-labeled WT C57BL/6 and TSLPR KO CD4<sup>+</sup> T cells into a congenically disparate WT host and treated the mice with EtOH or 2nmol MC903 topically (Fig. 2.6A). Both WT and TSLPR KO Tregs diluted CFSE to a similar extent with EtOH treatment and both populations had significant increases in proliferation with MC903 treatment (Fig. 2.6B-C). Additionally, the WT and the TSLPR KO cells had equivalent increases in Treg percentages with MC903 treatment in comparison to EtOH treated mice (Fig. 2.6D). These data suggest that the proliferating Tregs do not require a direct TSLP interaction, and that the effect of TSLP on Treg proliferation is likely through another cell type.

It has been appreciated that both *in vivo* and *in vitro*, Treg proliferation is largely dependent on dendritic cells (DCs) (Yamazaki et al., 2003; Tarbell et al., 2006; Fehervari and Sakaguchi, 2004; Zou et al., 2010). This proliferation is stimulated by both antigen dependent and independent interactions between Tregs and DCs (Zou et al., 2010). To determine if DCs were playing a role in the expansion of Tregs with topical MC903 treatment, we employed a model in which mice were lacking DCs. CD11c-Cre and ROSA-DTA mice were crossed to create



CD11c-DTA mice which results in the deletion of CD11c<sup>HI</sup> cells, and therefore the majority of DCs. CD11c-DTA and WT littermates were treated with EtOH or MC903 topically for 7 days and on day 8, Tregs were assessed in the spleen and locally within the skin of the mice. While WT mice showed significant Treg increases in both these locations, there was no increase in the proportion of Tregs in the CD11c-DTA mice (Fig. 2.6E-F). This suggested the requirement for DCs in this model of MC903 induced Treg expansion.

To further understand the role of DCs in this model and to determine if the DC-driven Treg increases were dependent on the ability of DCs to respond to TSLP, we transferred  $2-5 \times 10^6$  CD11c<sup>+</sup> MACS-purified WT DCs into TSLPR KO mice at day 0 and day 4 of EtOH and MC903 treatment and measured the ability of the WT DCs to rescue the Treg expansion that was lost in TSLPR KO mice. We found that there was a small but significant increase in the proportion of Tregs with MC903 topical treatment in the dLN of TSLPR KO mice that received WT DCs (Fig. 2.6G). Together, these experiments demonstrate that there is a requirement in the topical MC903 treatment model for TSLP to act on DCs in order to stimulate increases in Tregs.

In addition to the requirement of DCs *in vivo*, we found that DCs alone were able to stimulate Treg proliferation *in vitro* in the presence of TSLP. More dramatically, TSLP was able to strongly amplify Treg proliferation by DCs in the presence of IL-2 (Fig. 2.7A). This effect was dependent on the expression of TSLPR on DCs, but was independent of expression on Tregs. Altogether, these

data suggest that TSLP-sensing DCs are an integral intermediary in the Treg increases observed in the topical MC903 treatment model.

The ability of DCs to drive Treg proliferation is linked to the expression of costimulatory molecules on the surface of DCs. Topical treatment with MC903 increased the expression of costimulatory receptors on DCs, including CD80, CD86, and PD-L1 by day 4 of treatment *in vivo* (Fig. 2.8A-B). Additionally, DCs unregulated these receptors with *in vitro* treatment with 50 ng/ml TSLP for 24 hours (Fig 2.8C). To test the role of this phenotypic change on the ability of DCs to promote TSLP driven Treg proliferation, we blocked the costimulatory interactions *in vitro* with 20 ug/ml anti PD-L1 antibody or CTLA4-Ig. Blockade of PD-L1 interactions did not alter the ability of DCs to stimulate Treg proliferation, while CD80/CD86 blockade with CTLA4-Ig partially reduced Treg proliferation by both TSLP as well as IL-2 (Fig. 2.8D). Indicating that there is little need for PD-L1/PD-1 interactions between DCs and Tregs for the increased proliferation, while there may still be a requirement for direct contact between DCs and Tregs through CD80/CD86.

### **Topical MC903 treatment decreases the incidence of diabetes in NOD mice**

Multiple studies have shown that there is a link between the incidence of diabetes in the nonobese diabetic (NOD) model of type 1 diabetes and the number of Tregs. Increases in Tregs delay the onset of disease, while depletion of Tregs accelerates disease progression (Salomon et al., 2000; Lepault and

Gagnerault, 2000; Szanya et al., 2002; Besin et al., 2008). This strong link between Treg number and autoimmune diabetes led us to test the ability of topical MC903 treatment to affect diabetes incidence in NOD mice.

We treated female NOD mice with MC903 with a modified, chronic treatment model. Mice were given EtOH or MC903 (2nmol/ear) three times a week, every other week. This allowed for continual treatment of the mice over a prolonged period. In NOD mice treated with MC903, we found that Tregs in the peripheral blood remained elevated in comparison the EtOH treated mice (Fig. 2.9A). Incidence of diabetes was determined by two consecutive readings of  $\geq 250$ mg/dl blood glucose level. NOD mice receiving topical MC903 treatment had a decreased incidence of diabetes in comparison to EtOH treated mice (Fig. 2.9B). These data demonstrate that the topical treatment with MC903 has the ability to decrease the rate of diabetes in NOD mice and that this is associated with a peripheral increase in Tregs.

## Discussion

Results presented here describe the ability of local skin responses to alter systemic Treg numbers and in turn, to alter the progression of autoimmunity in the NOD mouse model of T1D. We show that topical treatment to the ears of mice with the vitamin D<sub>3</sub> analogue, MC903, led to systemic two-fold increases in Tregs throughout the animal. As had previously been observed, we found that TSLP was increased in the serum of mice that had been treated topically with MC903. Related to this, signaling through the TSLPR was absolutely required for the observed increases in Tregs.

This increase required topical treatment, as i.p. injection did not lead to any increase in Tregs. The lack of increases with i.p. administration suggests that it is unlikely that the MC903 topical treatment is causing any Treg changes due to the compound passing through the skin to the bloodstream. Furthermore, leakiness of the system is not expected with MC903 treatment because the compound was created to be retained in the skin and to have a very short half-life if it were to make it to the circulation. Due to previous findings that vitamin D<sub>3</sub> can have direct effects on the immune system, we tested this possibility in our model. We observed similar increases in Tregs in VDR KO and WT BM chimeras. Supporting the conclusion that MC903-driven Treg increases did not require hematopoietic expression of vitamin D receptor.

The peripheral Treg population is maintained through a combination of production of new Tregs and proliferation of existing Tregs. Phenotypic analysis

and transfer studies elucidated that the increases in Tregs with MC903 treatment were not occurring due to an increase in the production of either tTregs or pTregs, This was further supported by the observation that addition of TSLP to *in vitro* iTreg generation assays led to decreases in the induction of Foxp3 expression in Tconvs. The increases in Tregs observed were occurring primarily due to a large increase in the proliferation of existing Tregs. In agreement with literature on subsets of Tregs, the proliferating Tregs found in MC903 mice exhibited an effector phenotype with decreased expression of CD62L and increases in a number of phenotypic markers. Despite this effector phenotype of the Tregs, they were no more suppressive of Tconv proliferation on a per-cell basis in an *in vitro* assay of Treg function.

Despite the known ability of topical MC903 to drive Th2 immune activation, Treg increases did not occur solely as a response to this effect. Decreasing the Th2 AD response through genetic knockout of the important Th2 cytokine IL-4 did not abrogate the Treg increases observed. Further suggesting that the Treg increases with topical MC903 are through an alternate pathway, and not solely a response to the inflammation. Additionally, due to differences observed in the requirement for TCR stimulation in local versus systemic Treg increases in the K14 mouse model, there is a possibility that local Th2 responses in the skin play a larger role and in a different pathway to drive the Treg increases than what is working systemically. More should be done to determine if the local response in this model is driving antigen specific Treg expansion to fully

understand the role of TCR in the local expansion of Tregs with topical MC903 treatment.

Surprisingly, despite the ability of Tregs to express the TSLPR, the requirement of TSLP for Treg proliferation with MC903 topical treatment was not direct, as Tregs lacking TSLPR still proliferated in response to MC903. In fact, it appeared that the TSLP signaling requirement fell on dendritic cells. Without the presence of DCs, the increase in Treg numbers with MC903 topical treatment was lost. Furthermore, WT DCs were able to partially rescue the ability of MC903 to drive Treg increase in TSLPR KO mice. Addition of TSLP to *in vitro* co-cultures of Tregs and DCs demonstrated an ability of TSLP to drive Treg proliferation, requiring the expression of TSLPR on DCs, but not on Tregs.

With MC903 topical treatment, DCs unregulated the PD-L1 surface receptor associated with a non-inflammatory environment and CD80/CD86, which are implicated in the ability of DCs to stimulate Treg proliferation. The change in DC receptor expression was partially expected due to previous studies demonstrating that TSLP treated DCs upregulate these surface markers and adopt a noninflammatory phenotype (Hanabuchi et al., 2012; He and Geha, 2010; Rimoldi et al., 2005; Liu et al., 2007). The *in vitro* Treg proliferation driven by TSLP and DCs did not require PD-L1/PD-1 interactions, as anti PD-L1 blockade did not alter the proliferative capacity. CD80/CD86 may still be required, as blockade of these receptors partially reduced the TSLP driven Treg proliferation.

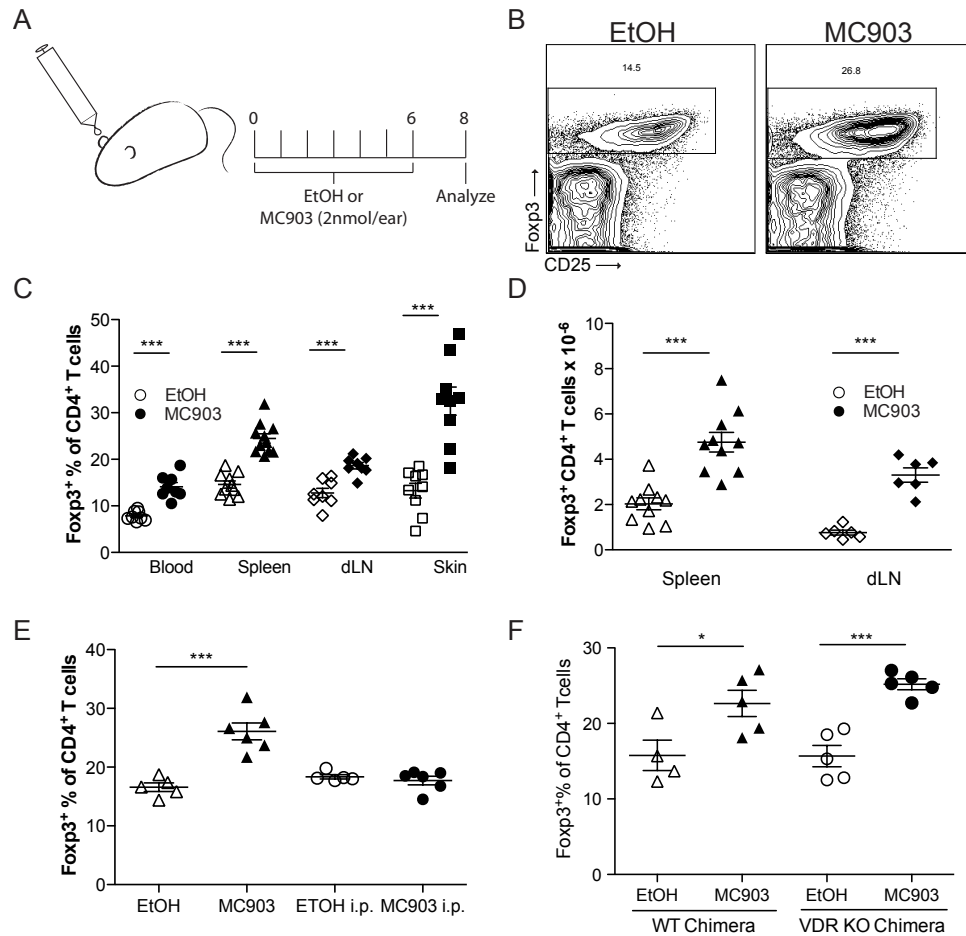
The NOD mouse model of T1D has been shown to be affected by alterations in Treg numbers. We found that topical treatment of NOD mice with MC903 led to long term increases in Treg percentages during the course of treatment. Correlated with this, we observed a reduction in diabetes incidence from 85-100% in EtOH treated mice to 40-50% in MC903 treated mice. While this effect was striking, it is not yet clear if the increases in Tregs are part of the protection. There are possibilities that the increased level of TSLP in the serum of mice treated with MC903 could lead to other effects resulting in protection from diabetes for a subset of these mice. Further studies would have to be performed to determine the complete spectrum of effects of topical MC903 in the NOD mouse.

Taken together, these data present a model in which topical MC903 is able to increase Treg percentages through the production of TSLP and interactions between DCs and Tregs (Fig 2.10). This suggests a compelling potential for the simple topical treatment with vitamin D<sub>3</sub> analogues to create a systemic immunosuppressive effect. Vitamin D<sub>3</sub> as an immunomodulatory treatment has not lived up to the expectations that previous work had suggested, possibly due to the lack of recognition that the method of administration is incredibly important in the efficacy of vitamin D<sub>3</sub> therapy. This is particularly of importance as we begin to recognize the strong ability of local tissue responses, in places such as the skin, to alter the immune cells outside of the immediate environment. There is great potential in creating therapies with such ease of

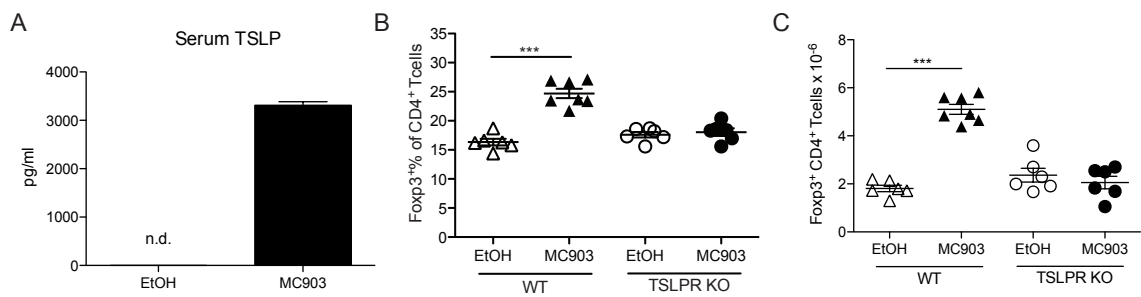
administration by taking advantage of the biology in the skin to produce the intermediary cytokines needed for systemic immune alterations.



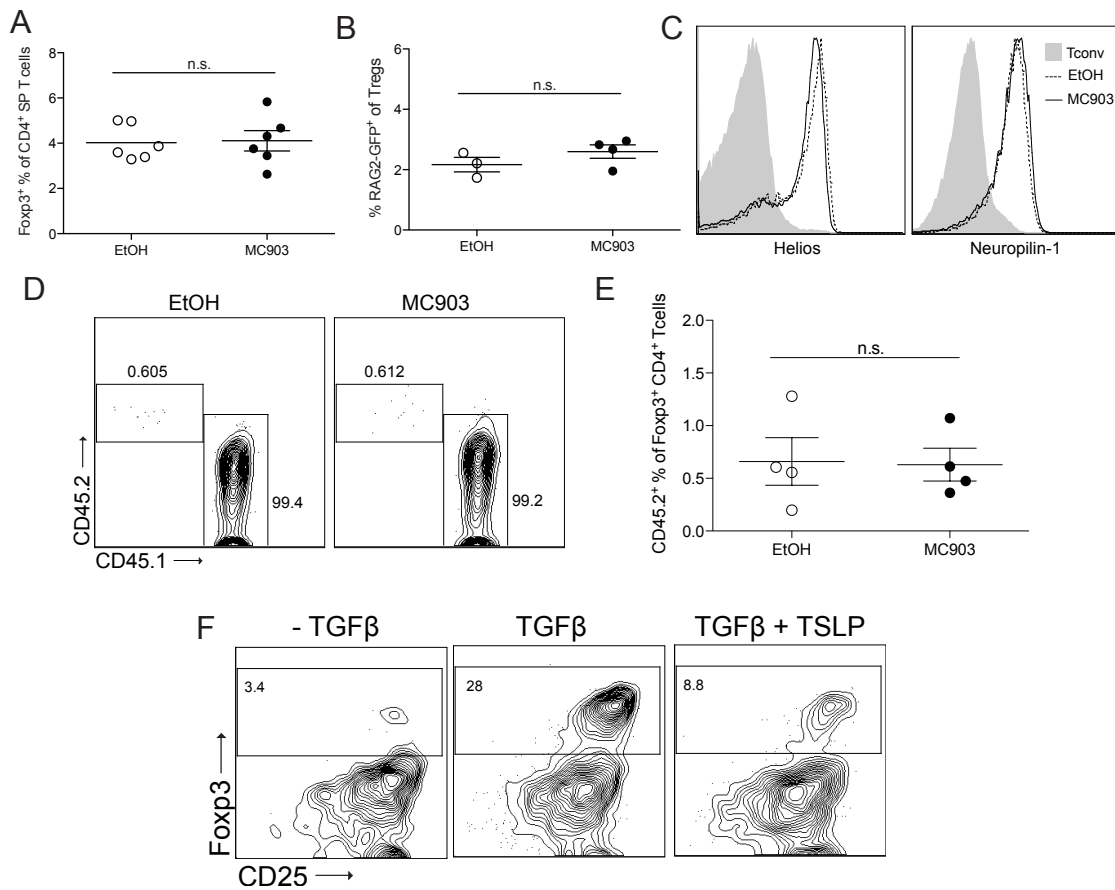
## Figures



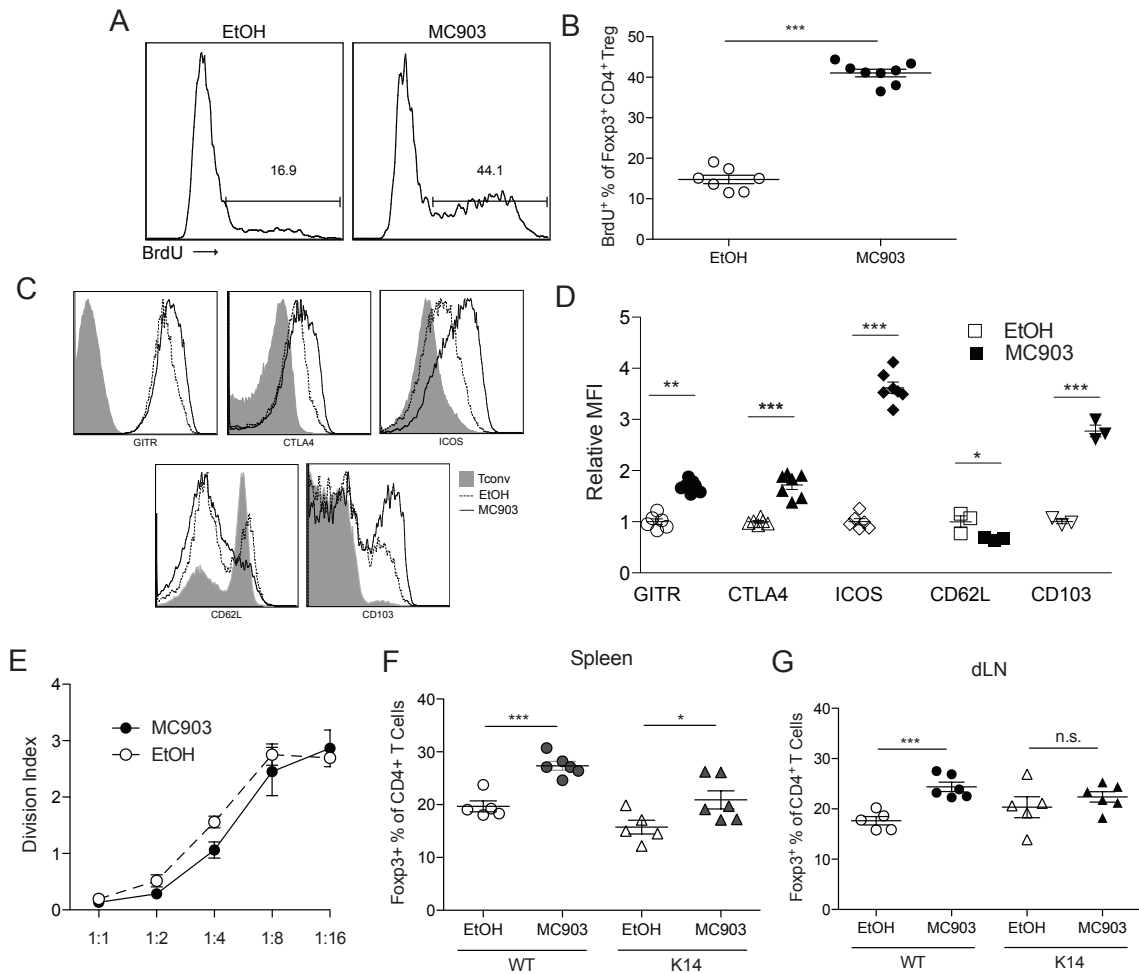
**Figure 2.1. Topical MC903 treatment increases Treg numbers independent of VDR expression on hematopoietic cells.** A. C57BL/6 mice were treated with Ethanol (EtOH) vehicle or MC903 (2nmol/ear) once daily for 7 days. On day 8, mice were euthanized and analyzed. B. Representative flow plots of live CD4<sup>+</sup> cells from spleens of EtOH and MC903 treated mice at day 8. C. Treg percentages of total CD4<sup>+</sup> cells in blood, spleen, skin-draining lymph nodes (dLN) and skin at day 8. Summary data plotted as mean  $\pm$  SEM (n=6-10/group from 4 independent experiments) D. Total Treg numbers in the spleen and dLN at day 8. Summary data plotted as mean  $\pm$  SEM (n=6-10/group from 3 independent experiments). E. Treg percentages at day 8 from mice treated with EtOH or MC903 (2nmol/ear) topically or through intraperitoneal injection (i.p.; 4nmol MC903 or equivalent EtOH volume in 500 $\mu$ L PBS). Summary data plotted as mean  $\pm$  SEM (n=5-6/group from 2 independent experiments) F. WT and VDR KO bone marrow chimeras were created by transferring 4x10<sup>6</sup> bone marrow cells into lethally irradiated C56BL/6.SJL hosts. 9-10 weeks post-transfer, chimeras were treated with EtOH or MC903 (2nmol/ear). Treg percentages were measured on day 8 after treatment in the spleen. Summary data plotted as mean  $\pm$  SEM (n=4-5/group from 2 independent experiments) \*  $p < 0.05$  and \*\*\* $p < 0.001$  by unpaired, two tailed Student's *t* test.



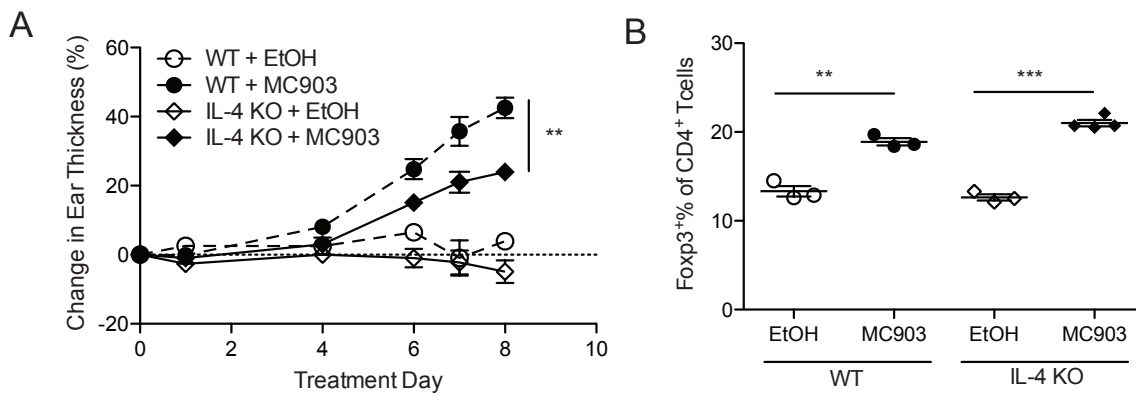
**Figure 2.2. Treg increases are dependent on TSLPR signaling.** A. Serum TSLP measured by ELISA on day 3 of EtOH and MC903 (2nmol/ear) topical treatment. Summary data of one representative experiment plotted as mean  $\pm$  SEM B. Treg percentages measured in the spleen at day 8 after EtOH or MC903 (2nmol/ear) treatment of WT and TSLPR KO mice. Summary data plotted as mean  $\pm$  SEM (n=6-7/group from 2 independent experiments) C. Total Treg numbers in the spleen at day 8 after EtOH or MC903 (2nmol/ear) treatment of WT and TSLPR KO mice. Summary data plotted as mean  $\pm$  SEM (n=6-7/group from 2 independent experiments) \*\*\*p<0.001 by unpaired, two tailed Student's *t* test.



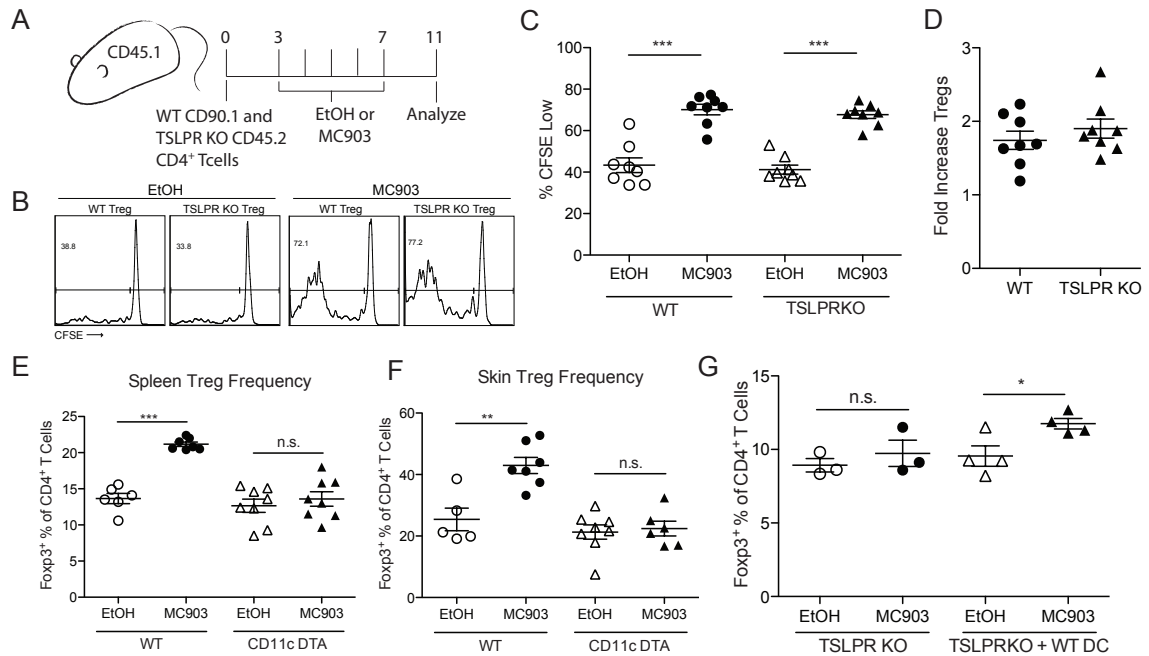
**Figure 2.3. Treg increases do not occur due to tTreg or pTreg generation.** A. Treg percentages in the thymus at day 8 after EtOH or MC903 (2nmol/ear) treatment. Summary data plotted as mean  $\pm$  SEM (n=6/group from 2 independent experiments) B. RAG2-GFP reporter mice were treated with EtOH or MC903 (2nmol/ear) for 7 days. On day 8, the percentage of RAG2-GFP<sup>+</sup>CD4<sup>+</sup>CD25<sup>HI</sup> Tregs was measured in the spleen. C. Helios and neuropilin-1 staining of Tregs in the spleen of mice on day 8 after EtOH or MC903 (2nmol/ear) treatment. D. FACS-sorted Tconvs (CD45.2<sup>+</sup>Foxp3.GFP<sup>-</sup>) and Tregs (CD45.1<sup>+</sup>Foxp3.GFP<sup>+</sup>) were transferred into lymphoreplete C57BL/6.Thy1.1 WT mice. Mice were treated with EtOH or MC903 (2nmol/ear) topically 3 days after cell transfer for 7 days. Representative flow plots gated on live, CD4<sup>+</sup> Foxp3-GFP<sup>+</sup> cells showing Tregs derived from Tconv (CD45.2<sup>+</sup>) and Treg (CD45.1<sup>+</sup>) transferred cells. E. Percent of Tregs derived from pTreg formation in EtOH and MC903 treated mice. Summary data of one representative experiment plotted as mean  $\pm$  SEM (n=4/group) F. FACS-sorted DCs and CFSE labeled Tconvs were plated at a 1:1 ratio (15,000-20,000 each/well) for 96 hours and iTreg generation was measured in the presence and absence of TSLP in vitro. Statistical analysis was performed using two-tailed unpaired Student's *t* test.



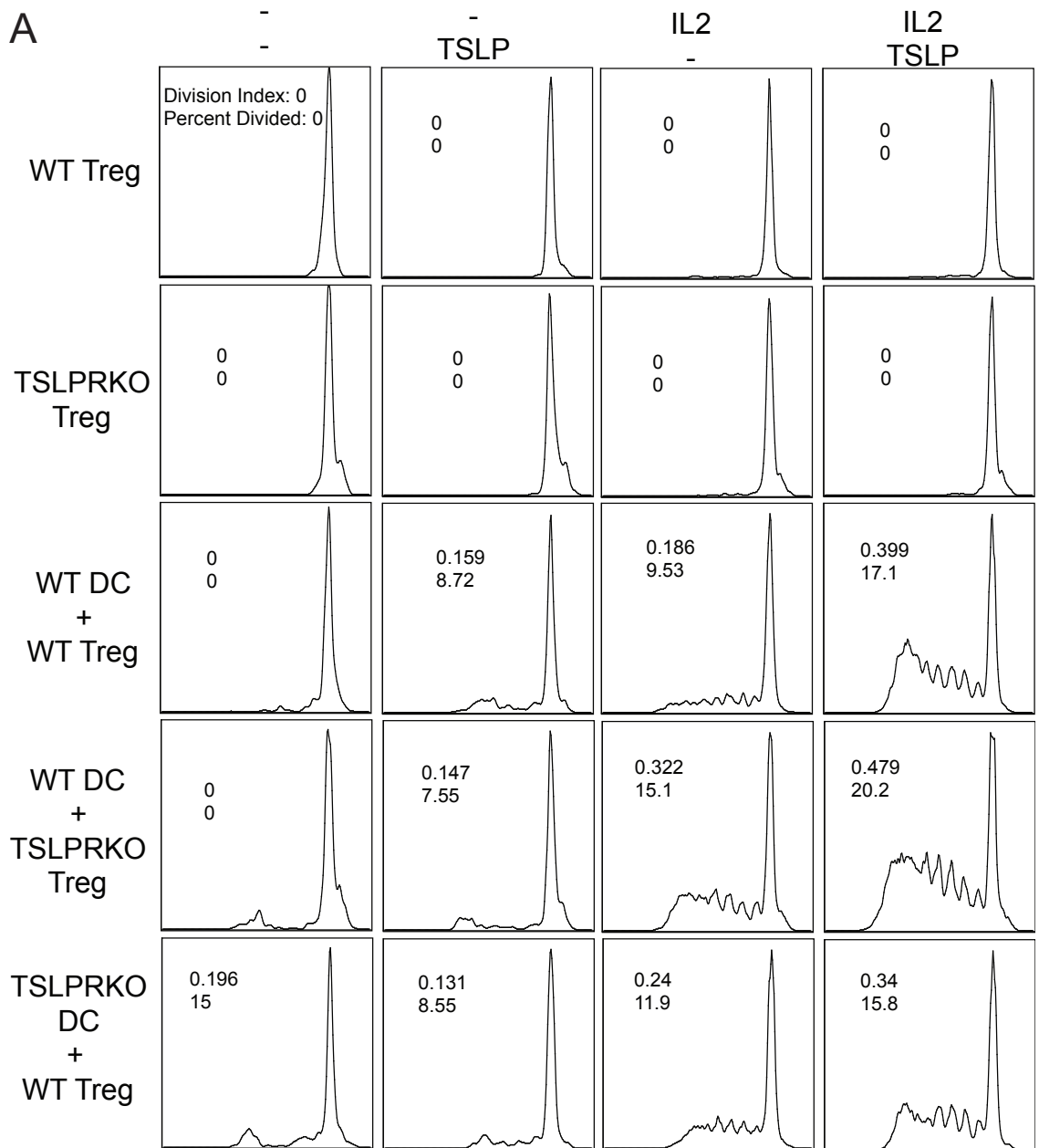
**Figure 2.4. Tregs are proliferative and gain an effector phenotype in MC903 treated mice.** A. EtOH and MC903 (2nmol/ear) treated mice were given BrdU in one injection and in the drinking water from day 6-9 after treatment. BrdU incorporation was measured in Tregs in the spleen at day 9. B. Percent of Tregs incorporating BrdU at day 9. Summary data plotted as mean  $\pm$  SEM (n=7-8/group of two independent experiments) C. Phenotypic surface markers were measured on Tregs in the spleen at day 8 after EtOH or MC903 (2nmol/ear) treatment. D. Summary data of the MFI relative to EtOH on Tregs in the spleen at day 8 after treatment. Summary data plotted as mean  $\pm$  SEM (n=3-7/group) E. *in vitro* suppression assay of Tconv proliferation by Tregs from EtOH and MC903 treated mice. One representative experiment plotted as mean  $\pm$  SEM. F. WT and K14 mice were treated with EtOH or MC903 (2nmol/ear) for 7 days. Treg percentages were measured in the spleen at day 8. Summary data plotted as mean  $\pm$  SEM (n=5-6/group of two independent experiments). G. WT and K14 mice were treated with EtOH or MC903 (2nmol/ear) for 7 days. Treg percentages were measured in the draining lymph node at day 8. Summary data plotted as mean  $\pm$  SEM (n=5-6/group of two independent experiments). \*  $p < 0.05$ . \*\*  $p < 0.01$  and \*\*\* $p < 0.001$  by unpaired, two tailed Student's *t* test.



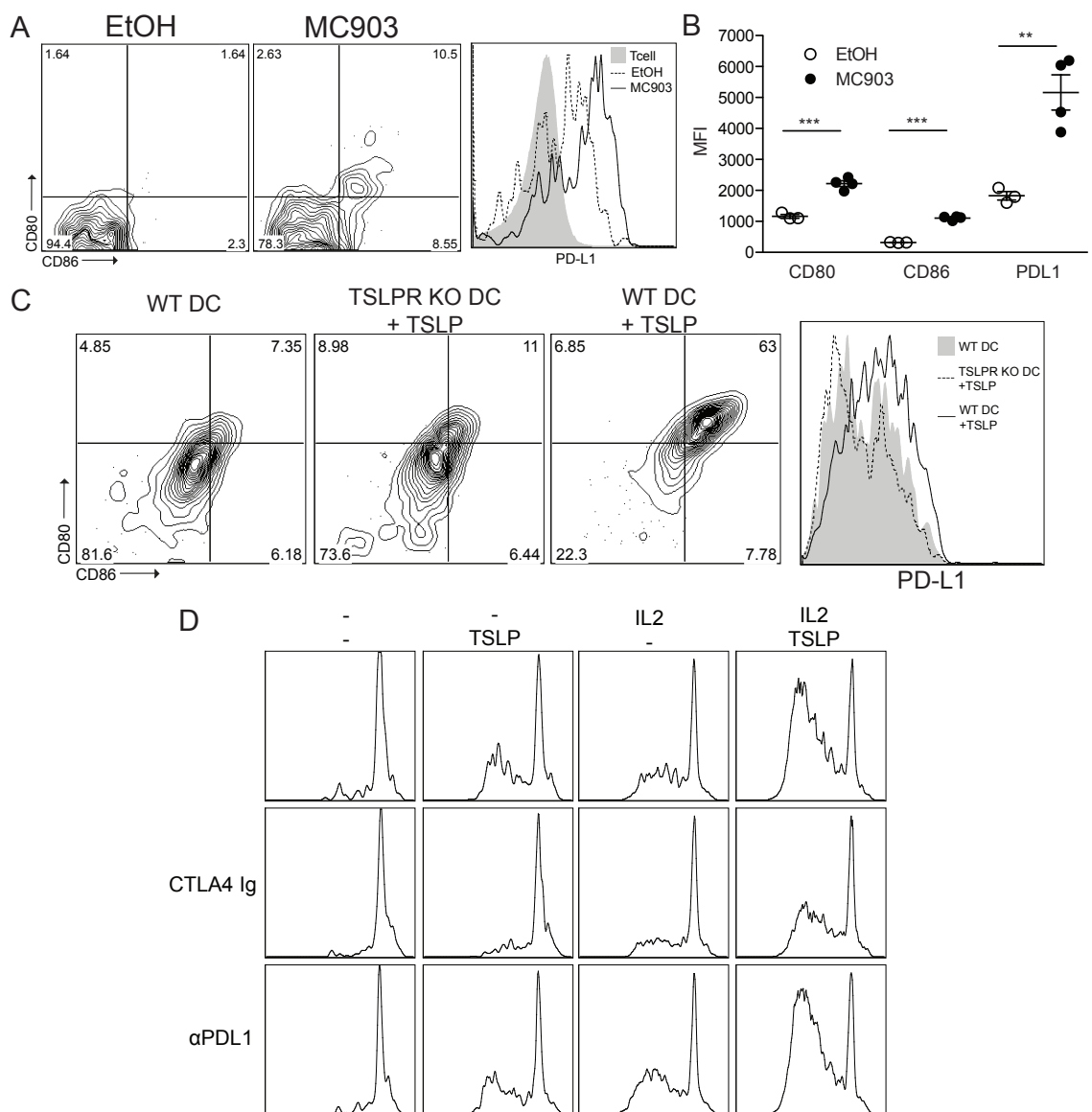
**Figure 2.5. Treg increases occur independently of the Th2 response to topical MC903.** A. WT and IL-4 KO mice were treated with EtOH or MC903 (2nmol/ear) for 7 days. Ear thickness was measured over the course of the experiment. Representative data are plotted as percent change in ear thickness from baseline (n=3-4/group, one representative experiment). B. WT and IL-4 KO mice were treated with EtOH or MC903 (2nmol/ear) for 7 days. Treg percentages were measured in the spleen on day 8. Representative data are plotted as mean  $\pm$  SEM (n=3-4/group, one representative experiment). \*\*  $p < 0.01$  and \*\*\* $p < 0.001$  by unpaired, two tailed Student's *t* test.



**Figure 2.6. Tregs are increased with topical MC903 treatment indirectly through TSLP and dendritic cells.** A.  $5 \times 10^6$  MACS-sorted WT CD90.1<sup>+</sup> and TSLPR KO CD45.2<sup>+</sup> CD4<sup>+</sup> T cells were CFSE labeled and transferred into lymphoreplete WT CD45.1<sup>+</sup> hosts. Mice were treated with EtOH or MC903 (2nmol/ear) from day 3-7 and analyzed on day 11. B. Representative CFSE dilution plots of CD4<sup>+</sup> Foxp3<sup>+</sup> T cells of WT and TSLPR KO origin in dLN of EtOH and MC93 treated mice at day 11. C. Summary data of the percent of Tregs in the dLN at day 11 that are CFSE low. Plotted as mean  $\pm$  SEM (n=8/group from 2 independent experiments) D. Fold increase in Treg percentage (% Treg in MC903 treated/average % Treg in EtOH treated) for both WT and TSLPR KO Tregs. Summary data plotted as mean  $\pm$  SEM (n=8/group from 2 independent experiments) E. WT and CD11c-DTA mice were treated with EtOH or MC903 (2nmol/ear) and Tregs were measured in the spleen at day 8. Summary data plotted as mean  $\pm$  SEM (n=6-8/group from two independent experiments) F. WT and CD11c-DTA mice were treated with EtOH or MC903 (2nmol/ear) and Tregs were measured in the skin at day 8. Summary data plotted as mean  $\pm$  SEM (n=5-8/group from two independent experiments) G. WT dendritic cells or PBS as a control were transferred into TSLPR KO mice on day 0 and day 4 of EtOH or MC903 (2nmol/ear) treatment. Treg percentages were measured in the dLN at day 8. Summary data of one representative experiment plotted as mean  $\pm$  SEM (n=3-4/group). \*  $p < 0.05$ . \*\*  $p < 0.01$  and \*\*\* $p < 0.001$  by unpaired, two tailed Student's *t* test.

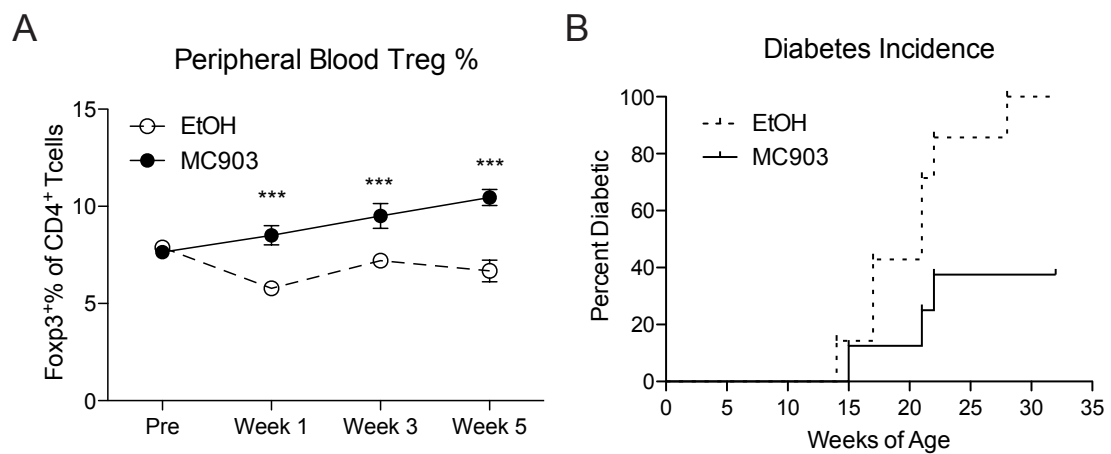


**Figure 2.7. TSLP stimulates Treg proliferation *in vitro* through dendritic cells. A.** WT or TSLPR KO Tregs were CFSE labeled and co-cultured with or without dendritic cells (1:1 15,000-20,000 each/well) from either WT or TSLPR KO mice for 96 hours with TSLP (50ng/ml) and/or IL-2 (50U/ml). Division index and percent divided were measured through CFSE dilution using FlowJo.

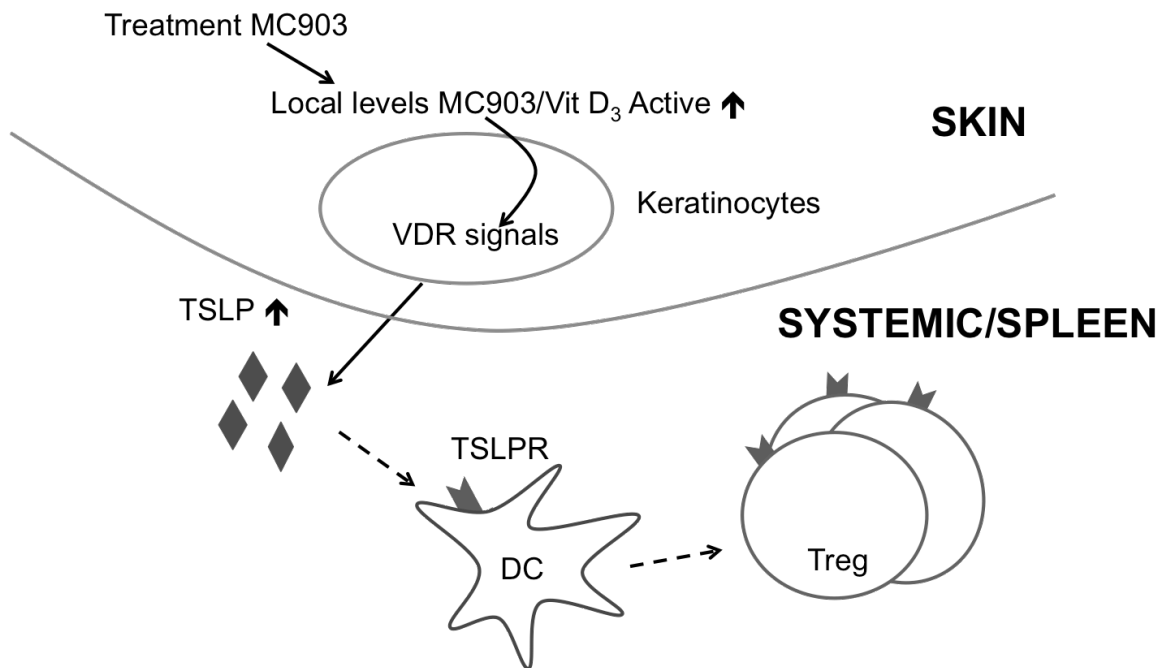


**Figure 2.8. TSLP stimulates upregulation of DC costimulatory receptors.** A. Representative flow plots showing CD80, CD86 and PDL1 on the surface of dendritic cells at day 4 of EtOH or MC903 (2nmol/ear) treatment. B. Summary data of surface MFI of CD80, CD86 and PDL1. Representative experiment plotted as mean  $\pm$  SEM (n=3/group) C. Representative flow plots showing CD80, CD86 and PDL1 on the surface of dendritic cells cultured *in vitro* with and without TSLP (50ng/ml) for 24 hr D. WT Tregs were co-cultured with WT dendritic cells (1:1 15,000-20,000 each/well) for 96 hours in the presence or absence of costimulatory blockade with CTLA4-Ig or anti PD-L1 (both at 20 $\mu$ g/ml). \*\*  $p < 0.01$  and \*\*\* $p < 0.001$  by unpaired, two tailed Student's *t* test.





**Figure 2.9. Long-term topical MC903 treatment increases Tregs in NOD mice and decreases the incidence of diabetes.** A. Female NOD mice were treated with EtOH or 2nmol/ear MC903 three times a week every other week beginning at 5 weeks of age. Treg percentage in the peripheral blood was measured every two weeks. Summary data from one representative experiment plotted as mean  $\pm$  SEM ( $n=7-8/\text{group}$ ) \*\*\* $p<0.001$  by unpaired, two tailed Student's  $t$  test. B. Percent diabetic (two consecutive blood glucose readings  $\geq 250$  mg/dl) over time of EtOH and MC903 treated NOD mice of one representative experiment ( $n=7-8/\text{group}$ ).  $p=0.015$  by Mantel-Cox test.



**Figure 2.10. Model of topical MC903 mediated Treg increases.** Data here suggest that topical MC903 increases Treg percentages through the production of TSLP by keratinocytes in the skin leading to systemic TSLP levels that alter interactions between DCs and Tregs to drive the expansion through proliferation of Tregs.

## **CHAPTER III:**

### **Results**

#### **TCR signaling by conventional CD4<sup>+</sup> T cells is required for optimal maintenance of peripheral regulatory T cell numbers**

##### **Summary**

To maintain immune tolerance, regulatory T cell (Treg) numbers must be closely indexed to the number of conventional T cells (Tconvs) so that an adequate Treg:Tconv ratio can be maintained. Two factors important in this process are the cytokine interleukin-2 (IL-2) and T cell receptor (TCR) stimulation by major histocompatibility complex class II (MHC-II). Here, we report that in addition to TCR stimulation of Tregs themselves, the maintenance of Tregs also requires TCR signaling by Tconvs. We found that Tconvs produce IL-2 in response to self-peptide-MHC-II complexes and that Tconvs possessing more highly self-reactive TCRs express more IL-2 at baseline. Furthermore, selective disruption of TCR signaling in Tconvs led to a trend towards decreased expression of IL-2 and attenuated their ability to maintain Treg numbers. These data suggest that in order to maintain an adequate Treg:Tconv ratio, Tregs are continuously indexed to self-peptide-MHC-II-induced TCR signaling of Tconvs. These results have implications in attempts to modulate immune tolerance, as Treg numbers adjust to the self-reactivity, and ultimately IL-2 production by the T cells around them.

## Introduction

Development of immunological tolerance to self is an essential biologic process to prevent over-activation of the immune system and resulting autoimmunity. This tolerance is effectively achieved through selection of T lymphocytes with low affinity to self-antigens as well as the creation and maintenance of Foxp3<sup>+</sup> regulatory T cells (Treg)s, a subset of T cells with suppressive function. Failure to develop Tregs results in the development of a widespread, fatal autoimmune disease (Sakaguchi et al., 1995; Fontenot et al., 2003). The maintenance of the peripheral Treg population is achieved through a combination of survival and proliferation attained by signaling through the cytokine receptor for interleukin 2 (IL-2) and through the T cell Receptor (TCR) (Hogquist et al., 1994; Fisson et al., 2003; Hedrick, 2012; Setoguchi et al., 2005; Bhandoola et al., 2002; Gavin et al., 2001).

Despite the homeostatic requirement for IL-2 and the expression of the high affinity IL-2 receptor (CD25) on Tregs, these cells do not produce this cytokine themselves (Setoguchi et al., 2005). Instead, Treg maintenance and/or development depends on IL-2 produced by other TCRab<sup>+</sup> T cells (Almeida et al., 2006). In fact, it has been shown that the number of Tregs surviving in the periphery is directly indexed to the number of CD4<sup>+</sup> conventional T cells (Tconv)s able to produce IL-2 (Almeida et al., 2006). However, the mechanism by which IL-2 is produced by Tconvs in the steady state to maintain Tregs is not known. Since TCR stimulation drives IL-2 production in T cells, we hypothesized that

Treg numbers might be indexed to TCR signaling by Tconvs (as a readout of activation state), rather than to the absolute numbers of Tconvs.

To test this notion, we hereby examined the role of TCR signaling in Tconvs at the steady state for both their ability to produce IL-2 as well as their capacity to maintain the Treg population. Through in vivo and in vitro approaches, we find that Tconvs produce IL-2 through sub-activating TCR stimulation by self-peptide MHC class II (MHC-II) complexes. Furthermore, the selective attenuation of TCR signaling in Tconvs results in decreased IL-2 production and an impairment in Treg maintenance. Our data suggest that Treg numbers are indexed to TCR signaling by Tconvs, both from sub-threshold self-antigens in the steady state as well as foreign, activating antigens in an immune response.

## Results

### Tconvs produce IL-2 in response to self peptide-MHC-II complexes

We hypothesized that Tconvs produce IL-2 in the steady state due to interactions of their TCR with self-peptide MHC-II complexes. To test this hypothesis, we first tested the ability of self-peptide MHC-II complexes to stimulate TCR-mediated IL-2 production in an in vitro system (Fig 3.1A). When co-cultured with syngeneic DCs for 96 hours, naïve WT Tconvs ( $CD4^+CD45RB^{hi}CD25^-$ ) produced IL-2 in response to syngeneic WT DCs but not when the DCs were derived from MHC-II KO mice (Fig 3.1B). Next, we disrupted TCR signaling in response to MHC-II ligation by using T cells from mice with a Y→F mutation in Y145 (Y145F) of the adaptor molecule SLP-76, which leads to decreased TCR-mediated PLC $\gamma$ 1 activation (Jordan et al., 2008). Co-culture of naïve Y145F Tconvs with syngeneic DCs for 96 hours showed significantly decreased IL-2 production compared to WT Tconvs (Fig 3.1B). Together, these data suggest that self-peptide MHC-II complexes induce IL-2 production in a TCR/MHC-II signaling-dependent manner.

To test the role of TCR/self MHC-II peptide complex interactions in IL-2 production in vivo, T cells possessing high affinity TCRs were compared to T cells with low affinity TCRs against self MHC-II peptide complexes. The expression level of CD5 on T cells correlates with TCR affinity to self MHC-II peptide complexes, which is established during thymic selection and maintained in the periphery (Azzam et al., 1998). Recent work has shown that Tconvs with

higher affinity for self-peptide MHC-II, as detected by the amount of CD5 expression, have a greater level of proximal TCR signals in the form of TCR  $\zeta$ -chain phosphorylation (Mandl et al., 2012). Consistent with our hypothesis, we have found that Tconvs with high CD5 expression (top 20%) have a significantly elevated amount of IL-2 mRNA expression in comparison to Tconvs with low CD5 expression (bottom 20%) (Fig 3.1C-D). This suggests that steady-state IL-2 production by Tconvs correlates with their TCR affinity for self-peptide MHC-II complexes.

### **TCR signaling by Tconvs is required for maintenance of the Tconv:Treg ratio**

To test if the baseline TCR interaction with self-peptide MHC-II complexes was important in maintaining Treg numbers, we utilized an adoptive transfer system in which expression of MHC-II was lacking in hematopoietic cells. In this model, lethally irradiated CD45.1<sup>+</sup> WT hosts were reconstituted with CD45.2<sup>+</sup> WT or MHC-II KO bone marrow (BM) and adoptively transferred with CD90.1<sup>+</sup> WT CD4<sup>+</sup> T cells (Fig 3.2A). We found that the CD90.1<sup>+</sup> Tregs adoptively transferred into MHC-II KO BM chimeric mice were unable to maintain their numbers in the peripheral blood to the same extent as WT BM chimeric mice 20 days post transfer (Fig 3.2B). Linked to this, CD90.1<sup>+</sup> Tconvs in the MHC-II KO BM chimeric mice expressed significantly lower IL-2 mRNA in comparison to Tconvs from WT BM chimeric mice (Fig 3.2C). Furthermore, we utilized IL-2/anti IL-2

antibody immune complexes (IL-2 IC) to determine if the lack of IL-2 was playing a role in the maintenance of Tregs in this model (Webster et al., 2009). Indeed, we found that treatment with IL-2 IC partially restored Treg percentages in the peripheral blood of the MHC-II KO BM chimeric mice, suggesting that the lack of IL-2 contributes to the failure to maintain Tregs in the absence of MHC-II/TCR interactions (Fig 3.2B).

In MHC-II KO BM chimeric mice, Tregs and Tconvs both lose MHC-II/TCR interactions. Thus, the lack of TCR signaling by Tregs could also contribute to defective maintenance in this model. In order to more fully test whether the baseline TCR signaling ability of Tconvs alone was important in the maintenance of Tregs in vivo, it was necessary to utilize a second system whereby TCR signaling was attenuated in Tconvs but not in Tregs. To accomplish this task, we designed an adoptive transfer model in which TCR signals could be inducibly decreased specifically in Tconvs (Fig 3.3A). We utilized a Tamoxifen-inducible system in which a WT loxp-flanked SLP-76 allele is deleted upon treatment, leaving either a single WT SLP-76 allele (cSLP76) or a SLP-76 Y145F mutant allele (cY145F) (Wu et al., 2011). Tconv from either cSLP76 or cY145F mice were mixed with WT Tregs at a 4:1 ratio and adoptively transferred into T cell-deficient TCR $\beta/\delta$  KO mice. We waited for T cell reconstitution and steady state to be reached, i.e., until the peripheral blood CD4<sup>+</sup> T cells reached a constant percentage of lymphocytes (~8-10 weeks). The mice were then treated with Tamoxifen to induce deletion of the loxp-flanked WT SLP-76 allele (Fig 3.3A). 5 weeks after Tamoxifen treatment, Tconvs from peripheral lymphoid organs were



analyzed for IL-2 expression. The CD45.2<sup>+</sup>CD4<sup>+</sup> Tconvs from the spleen of the cY145F adoptive transfer showed a trend towards decreased IL-2 mRNA expression in comparison to the Tconvs from the cSLP76 transfer (Fig 3.3B). Longitudinal analysis of the Treg percentage (of CD4<sup>+</sup> T cells) in the peripheral blood showed a significant decrease in mice transferred with cY145F compared to cSLP76 Tconvs starting at week 3, which was sustained through week 12 (Fig 3.3C). Moreover, at week 12 post-Tamoxifen treatment, we found that CD45.1<sup>+</sup> WT Tregs made up a smaller fraction of the total CD4<sup>+</sup> T cell pool in the spleens of mice with Tconvs from cY145F compared to cSLP76 mice (Fig 3.3D-E). This was observed despite finding variable reconstitution levels of Tconvs between mice within each group, further suggesting that the primary effect of this manipulation in our model was the ability of Treg numbers to be indexed to the TCR signaling capacity of Tconvs (data not shown). Together, these data suggest that TCR signaling by Tconvs is important for steady state IL-2 production, which correlates with their ability to maintain Tregs.

## Discussion

Our data provided here in this manuscript support a model by which Treg numbers are indexed to IL-2 produced by Tconvs through TCR stimulation by self peptide/MHC-II complexes. We examined IL-2 production from Tconvs in vivo using quantitative PCR, since direct measurement of IL-2 protein production in a naïve mouse is difficult to measure, as steady state IL-2 most likely remains local to the secondary lymphoid organs. Still, antibody neutralization of IL-2 demonstrates that a functionally significant amount of IL-2 plays a role in maintenance of peripheral Tregs (Setoguchi et al., 2005). Using CD5 as a measure of TCR affinity for self peptide/MHC-II complexes showed that high TCR affinity of Tconvs correlated with increased IL-2 mRNA expression. Moreover, an acute decrease in TCR signaling by Tconvs through the Y145F mutation showed a trend towards decreased IL-2 mRNA expression. The latter analysis may not have reached statistical significance due to the nature of the Tamoxifen-inducible system, which results in incomplete deletion of the loxp-flanked WT SLP-76 allele. Thus, the contamination of Tconvs that have not deleted SLP-76 may have contributed to higher IL-2 mRNA expression in this setting. Further support of this concept was found in the observation of significantly decreased IL-2 mRNA in Tconvs transferred into an MHC-II deficient environment. This was associated with a decrease in Treg proportions that was partially rescued after treatment with IL-2 IC.

The ability of Tconvs to produce IL-2 in response to TCR stimulation has long been appreciated as a result of signals provided by an activating, often foreign, antigen. The potential for Tconvs to produce IL-2 at baseline, or from non-activating ligands has not been studied despite evidence for its existence, primarily from observations of normal percentages of Tregs in germ free mice (Min et al., 2007; Östman et al., 2006). The absence of foreign antigen in germ free mice suggests that the IL-2 required for Treg maintenance is not produced through stimulation of the TCR on Tconvs by foreign peptides presented on MHC-II. Therefore, we propose that baseline IL-2 production is a result of TCR interactions with self-peptide MHC-II complexes.

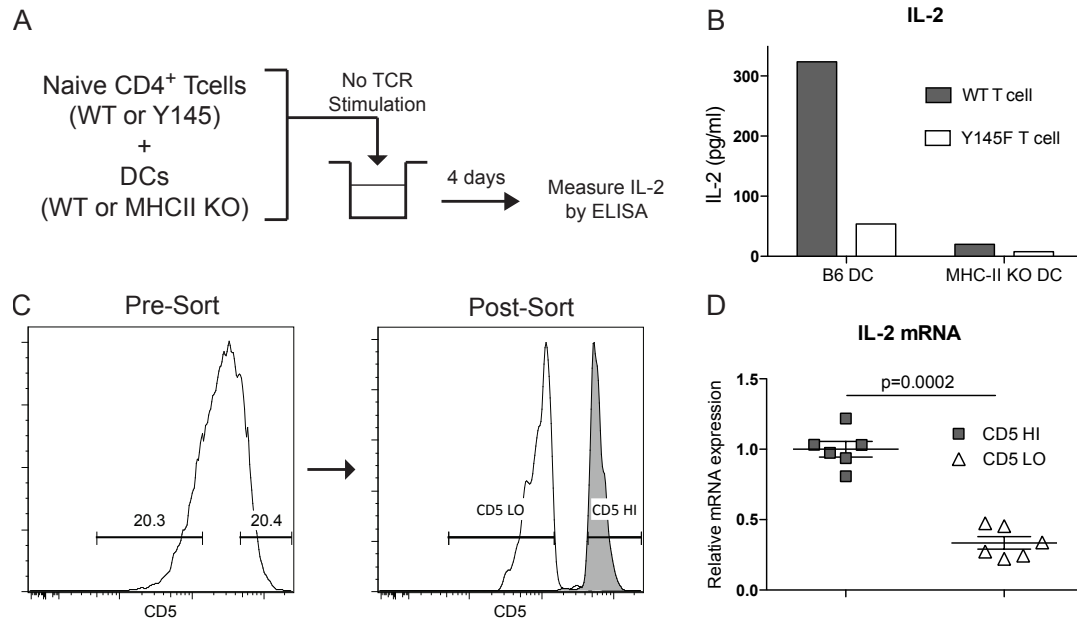
Tregs are characterized to be part of either central or effector subsets, which are distinguished by varying surface receptor phenotypes, proliferative capacity, function, and dependence on IL-2 (Smigiel et al., 2014a; Campbell and Koch, 2011; Siegmund, 2005). Given that IL-2 was dependent on TCR signaling by Tconvs, one would predict that the Tregs remaining in the cY145F adoptive transfer would be enriched for the IL-2-independent and highly proliferative effector Treg subset. However, we could not test this in our adoptive transfer model, since in all locations observed (spleen, LN, gut, mLN) the WT Treg had an effector phenotype, regardless of whether the adoptively transferred Tconv were of cSLP76 and cY145F origin (data not shown). This phenotype was likely acquired during the lymphopenic expansion that occurred prior to the SLP-76 deletion, which precluded the analysis of the Treg subsets that remained.

Despite the caveat of initial lymphopenic expansion for reconstitution of the T cell-deficient mouse, our adoptive transfer model corrected for a number of concerns involved in testing the role of TCR signaling by Tconvs in Treg maintenance. First, the SLP-76 protein was altered only in Tconvs, allowing for all Treg-intrinsic homeostatic factors to remain intact. Second, deletion of SLP-76 using the Tamoxifen-inducible cre-lox system negated the concern that Tconvs with decreased TCR signaling would develop differently than the WT controls, potentially leading to an altered TCR repertoire or cytokine production downstream of TCR stimulation. Finally, in order to minimize any confounding factors associated with lymphopenic expansion, we deleted the loxp-flanked SLP-76 allele only after the transferred cells of both types (cSLP76 and cY145F) fully expanded and reached a steady state.

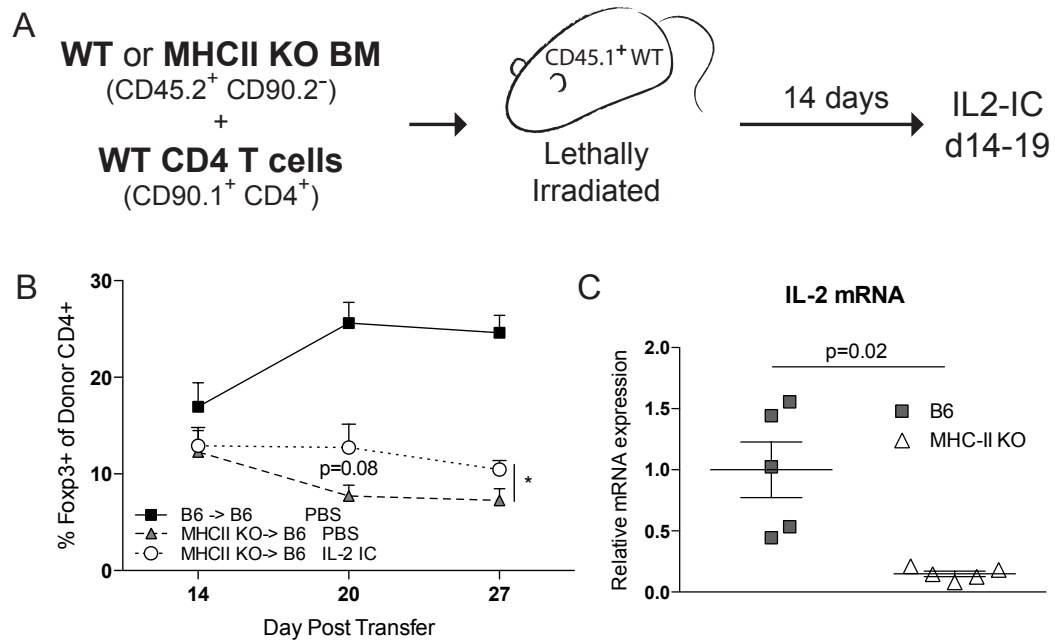
The results of these studies demonstrate a previously unrecognized role of TCR affinity of Tconvs to self-peptide MHC-II complexes in the maintenance of Tregs. These are novel findings because they link the size of the Treg population not only to activated T cells producing high levels of IL-2, but to the broad level of self-reactivity found in the Tconv pool. It is reasonable then to propose that thymic positive selection of CD4<sup>+</sup> Tconvs, which creates a population of Tconvs with low affinity to self-peptide MHC-II complexes, allows Tregs to be appropriately indexed to Tconvs in the steady state for immune tolerance. Overall, these observations are important in the understanding of autoimmunity because they demonstrate a potential avenue in which self-tolerance mechanisms can fail. Additionally, they provide important considerations in

formulating Treg-based immunotherapies, as the transfer of Tregs paired with inhibition of TCR signaling might actually lessen the ability of the transferred Tregs to maintain sufficient numbers to be effective over time.

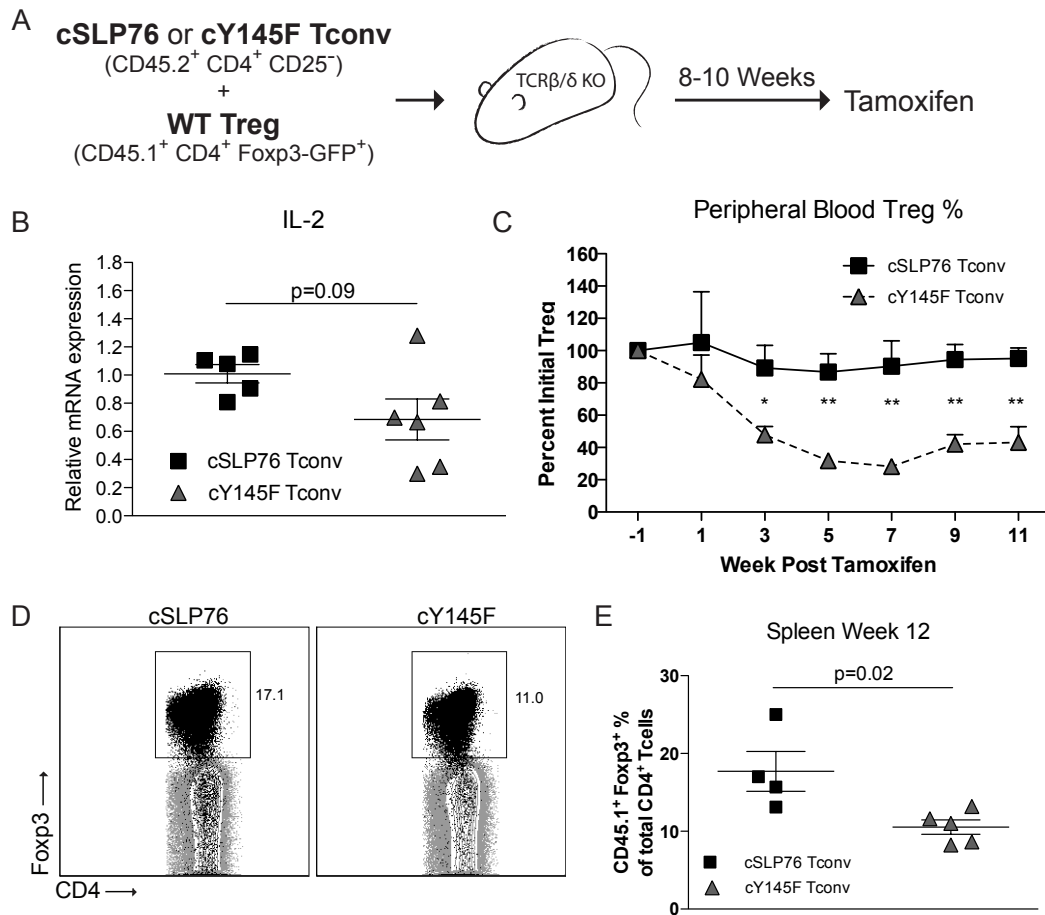
## Figures



**Figure 3.1. IL-2 is induced by stimulation of CD4<sup>+</sup> Tconv by self peptide-MHC-II complexes** A. Naïve T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup>) from WT or Y145F mice were FACS-sorted and co-cultured at a 1:1 ratio with DCs from either WT or MHC-II KO mice with no added TCR stimulation. B. 96 hours later, IL-2 content in the supernatant was assessed by ELISA. One representative of two experiments is shown. C. Sorting strategy for upper and lower 20% of CD5 expressing (CD5<sup>hi</sup> and CD5<sup>lo</sup>, respectively) Tconv (CD4<sup>+</sup>GFP<sup>-</sup>) cells from C57BL/6 Foxp3.GFP reporter mice is shown. D. IL-2 mRNA expression in the CD5<sup>hi</sup> and CD5<sup>lo</sup> Tconv populations, plotted as mean ± SEM of 6 mice from two individual experiments is shown. Statistical analysis was performed using two-tailed paired Student's *t* test.



**Figure 3.2. Lack of IL-2 Production in MHCII deficient environment leads to decreased Treg percentages** A. T cell depleted bone marrow from WT or MHCII KO mice (both CD45.2<sup>+</sup>) was acquired through MACS-sorting and transferred with MACS-sorted CD4<sup>+</sup> T cells (CD90.1<sup>+</sup>) into lethally irradiated WT (CD45.1<sup>+</sup>) hosts. IL-2 IC (0.25ug IL-2 and 1.25ug  $\alpha$ IL-2 mAB) or PBS control were given from day 14-19. B. Treg percentages were measured in the peripheral blood over time after cell transfer to irradiated recipients. Summary data plotted as the mean  $\pm$  SEM (n=12 per group) from 3 independent experiments. C. IL-2 mRNA was measured from the CD90.1<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup> FACS-sorted Tconvs from the spleens of WT or MHCII KO chimeras at day 21 post transfer. Summary data plotted as the mean  $\pm$  SEM (n=5 per group) of 2 independent experiments. \*  $p < 0.05$  or as noted by unpaired, two tailed Student's *t* test.



**Figure 3.3. Selective ablation of TCR signaling in CD4<sup>+</sup> Tconvs leads to decreased Treg numbers** A. FACS-sorted CD45.2<sup>+</sup> Tconvs (CD4<sup>+</sup>CD25<sup>-</sup>) from conditional WT SLP-76 (cSLP76) or conditional Y145F mutant SLP-76 (cY145F) mice were mixed with CD45.1<sup>+</sup> WT Tregs and transferred into TCR β/δ KO mice at a 4:1 ratio. After 8-10 weeks, the mice were treated with Tamoxifen. B. IL-2 mRNA was assessed in FACS-sorted splenic CD45.2<sup>+</sup>CD4<sup>+</sup> T cells at week 5 post-Tamoxifen treatment. Summary data from two independent experiments are represented by mean ± SEM with n=5-6/group. C. The change in percentage of peripheral blood CD45.1<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs was plotted against time post-Tamoxifen treatment. One representative experiment is plotted as the mean ± SEM (n=4-6 per group) of 3 independent experiments. D. Representative flow plots of total CD4<sup>+</sup> T cells from mouse spleens at 12 weeks post-Tamoxifen treatment is shown. The number in each plot represents the proportion of CD45.1<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> cells of total CD4<sup>+</sup> T cells. E. A summary graph depicts mean %CD45.1<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> cells of total CD4<sup>+</sup> T cells ± SEM of n=4-6/group. \* *p*<0.05 and \*\* *p*<0.01 or as noted by unpaired, two tailed Student's *t* test.



## CHAPTER IV: DISCUSSION

### Overview

Although a number of the factors important in Treg homeostasis have been revealed through extensive study of these cells since their discovery, there is still much to unveil about the cell types and factors that have the potential to alter Treg numbers. This thesis sought to further elucidate the biological influences important in peripheral Treg maintenance through both looking at novel tissue interactions as well as further understanding known links between Tregs and Tconvs.

While interactions between Tregs and other immune cells have been the focus of many of the studies on Treg homeostasis, work by a number of labs has begun to appreciate the complex interface between non-immune cells and cells of the immune system in local tissues (Saenz et al., 2008). Particularly, sites that interface with the environment, such as the skin, gut and lungs provide important sensors that can dictate nearby immune responses (Belkaid and Naik, 2013; Naik et al., 2012; Monticelli et al., 2015; Belkaid and Artis, 2013). What has become apparent in recent years is that these local, non-immune responses have the ability to not only modify the immediate immune environment, but can in fact cause widespread alterations in the systemic immune response (Siracusa et al., 2011; Lee et al., 2011b; Wu et al., 2010). While many of these tissue specific

studies have focused on enhancement of effector T cell responses, we sought to determine if local responses within the skin had the ability to alter systemic tolerance through increasing the immunosuppressive cell type of Tregs.

We found that a simple treatment of the skin was able to produce wide-reaching effects on the immune system through increases in Treg numbers. Production of the cytokine TSLP, which canonically has been associated with type 2 helper (Th2) T cell responses, is crucial for the Treg increases and likely works through altering DCs to be more tolerogenic. In addition to the clarification of how Treg numbers increase with this treatment, we also observed general tolerance induction within the NOD diabetes mouse model, with treatment of the skin leading to a reduction in diabetes incidence. These results add to the recently appreciated, yet complex understanding of the role of TSLP in Treg biology.

A second cytokine that is profoundly important in Treg homeostasis is IL-2. IL-2 has been studied for its tolerogenic properties since it was first observed that this cytokine is not solely immunostimulatory (Malek and Bayer, 2004). The source of this critical cytokine for Treg maintenance has been determined to be Tconv, but the ability of this cell type to make IL-2 in the steady state, without overt immune activation, has been an accepted, yet unclear process (Almeida et al., 2006). In the work presented within this thesis, we have observed that TCR signals specifically within the Tconv population are important for IL-2 production without full activation of the cells. In turn, this Tconv-specific TCR signaling is

required for the maintenance of Tregs. Recent intriguing findings from the laboratory of Dr. Ron Germain have begun to further illuminate this process of IL-2 production and the following discussion sections will describe our current findings in relation to these recently published results (Liu et al., 2015).

Together, these findings have advanced our understanding of the complex networks that maintain tolerance within the immune system through homeostasis of the crucially immunosuppressive cells, Tregs. Here we will discuss the immunologic and clinical implications of these discoveries as well as outline the questions that remain to be studied.

### **Skin mediated control of systemic regulatory T cell numbers**

There is a recognized importance of non-immune cells within local tissue to produce factors critical in the immune response. In particular, epithelial cells (ECs) have been linked to the production of a number of cytokines that are known to play roles in immune activation (Saenz et al., 2008; Belkaid and Artis, 2013). In the lungs, ECs are important in producing signals to limit the inflammation associated with infections as well as trigger tissue repair through the immune system (Snelgrove et al., 2011). Similarly, within the intestine, ECs produce critical cytokines after induction of colitis inflammation to trigger a cascade of immunologic signals for repair (Monticelli et al., 2015; García-Miguel et al., 2013). Furthermore, intestinal signals driven by the presence of segmented filamentous bacteria (SFB) have been found to be important in the activation of

not only local responses, but also can have effects on EAE and autoimmune arthritis in sites far removed from SFB exposure (Lee et al., 2011b; Wu et al., 2010). It has been found recently that the skin also possesses the ability to alter immune responses in more than just the local tissue. Treatment of the skin of mice with the vitamin D<sub>3</sub> analogue, MC903, leads to the production of the cytokine TSLP by the keratinocytes in the skin and leads to the hematopoiesis of basophils from the bone marrow (Siracusa et al., 2011; Zhang et al., 2009).

Work in this thesis sought to understand the ability of the local skin response to MC903 to control the number of Tregs found systemically. Despite its traditionally Th2-driving role, TSLP has been linked to Tregs through a number of findings. Global TSLPR KO does not lead to decreases in Tregs, yet there has been work supporting an enhancement of tTreg generation with TSLP (Watanabe et al., 2005; Mazzucchelli et al., 2008). Additionally, there have been studies suggesting no need for TSLP signals in iTreg generation, yet other observations demonstrate an enhancement of this process with TSLP treatment (Besin et al., 2008; Sun et al., 2007). Due to the noteworthy, albeit contradictory, links between TSLP and Tregs, and the large amounts of TSLP generated by keratinocytes with topical MC903 treatment, we hypothesized that the skin would be able to stimulate increases in the Treg population through TSLP production (Li et al., 2009). We found that with topical MC903 treatment, TSLP was significantly increased in the serum of mice, and that there were widespread increases in Treg number as well as Treg percentage of CD4<sup>+</sup> T cells. The possibility of this effect being mediated by the passing of MC903 through the skin and entering the

circulation was ruled out through administering MC903 systemically with an intraperitoneal injection. We found that only the topical treatment lead to increases in Tregs, further supporting the role of skin tissue in altering Treg numbers and adding this ability to the already known effects that the skin can have on the immune system.

Vitamin D<sub>3</sub> has been studied as a compound with immunosuppressive effects. It has been shown to be able to interact with many different immune cell types, including T cells, B cells, DCs and macrophages (Mora et al., 2008; Gorman et al., 2010; Penna and Adorini, 2000). Our studies have shown that this direct effect of Vitamin D<sub>3</sub> on the immune system is not at play in our model. Generation of Vitamin D receptor (VDR) KO BM chimeras demonstrated that VDR was not required on cells of the hematopoietic system to drive increases in Tregs with MC903 treatment; establishing here, as well as agreeing with previously published data, that the effects of topical MC903 on the immune system are driven primarily by the production of TSLP within the skin.

### **Role of TSLP in expansion of regulatory T cells**

As would be suggested by previous studies as well as the results already discussed here, we found that the ability of topical MC903 treatment to increase Tregs was mediated through TSLP. This was demonstrated by our observation that topical MC903 treatment of TSLPR KO mice did not result in any increases in Tregs. Drawing from known Treg homeostasis mechanisms as well as

previous studies looking at TSLP effects on Tregs, there are a number of potential pathways by which this cytokine could be driving Treg increases.

One potential mechanism for the Treg increases is the initiation of more tTreg generation within the thymus. Human studies have shown that TSLP production by Hassall's corpuscles drive the production of Tregs through conditioning thymic DCs (Watanabe et al., 2005). While mice do not have Hassall's corpuscles in the thymus to drive this process, work has shown that *in vitro*, TSLP is able to increase the development of Foxp3<sup>+</sup> Tregs from CD4 single positive thymocytes (Lee et al., 2007). We found that in our topical MC903 treatment model there was no increase in Tregs within the thymus, supporting, but not decisively showing, that there was no enhancement of tTreg generation after MC903 treatment.

To begin to better understand this, we took advantage of a mouse model that allows us to visualize recently generated cells of the adaptive immune system. A mouse that expresses GFP-tagged RAG2 protein is a reliable reporter for recently produced T cells. Tregs in this mouse that have newly developed in the thymus will express high levels of the RAG2-GFP reporter due to the expression of RAG2 during formation of the TCR (Boursalian et al., 2004). Studies we have performed using this RAG2-GFP reporter mouse have shown no increase in RAG2-GFP<sup>HIGH</sup> Tregs in mice treated with MC903, further suggesting that topical MC903 treatment is not driving tTreg generation.

A role for TSLP in the *in vitro* generation of iTregs has been studied by a number of groups. It was initially observed that bone marrow derived DCs were able to induce Foxp3 expression in a greater number of naïve Tconvs if the DCs developed in conditions containing TSLP (Besin et al., 2008). The specific role that these TSLP-DCs played in generating a greater number of iTregs was not fully clear, as they also observed greater expansion of Tregs after conversion. This role for TSLP in DC-mediated iTreg generation was further confused by the observation of another group showing no need for TSLP responsiveness on DCs to generate iTregs (Sun et al., 2007). While these studies focus on the production of iTregs specifically, our work has shown through adoptive transfer experiments that *in vivo* generation of pTregs is not enhanced with MC903 treatment. Furthermore, in *in vitro* iTreg generation assays, addition of TSLP to the culture led to a decrease in the ability of naïve Tconvs to up-regulate Foxp3 expression, further suggesting that the presence of systemic TSLP in the serum would not be inducing pTreg generation in our model. In agreement with these analyses, Tregs in MC903 treated mice did not develop any changes in the level or percentage of cells expressing either helios or neuropilin-1, suggesting no shift in the contribution of pTregs to the peripheral Treg pool.

These conclusions allowed us to rule out novel Treg production, whether it is through tTreg or pTreg generation, as the source of Treg increases and led us to the hypothesis that the expansion of the Treg population is due to an increase in Treg proliferation. It has been shown that TSLP has the ability to expand naïve CD4<sup>+</sup> Tconvs through proliferation driven by DCs, so we tested the ability of

Tregs to respond similarly (Watanabe et al., 2004). We observed an increase in the incorporation of the thymidine analogue Bromodeoxyuridine (BrdU) by Tregs with MC903 treatment, suggesting an increase in proliferation of Tregs. Furthermore, adoptive transfer of CFSE labeled Tregs showed large amounts of proliferation with topical MC903 treatment, supporting this conclusion. Related to this, the Tregs in MC903 treated mice exhibited an effector phenotype that is characteristic of proliferating Tregs.

One additional factor that may lead to increases in Treg numbers that still needs to be tested is the ability of this treatment to increase Treg survival. Because this subset of T cells has such a high turnover, with Tregs constantly dying by apoptosis, an increase in survival of the Tregs could add to the effects we observe with increased Treg proliferation (Tai et al., 2013; Barron et al., 2010). The anti-apoptotic Bcl-2 family member, Mcl-1, has been shown to be important in driving the survival of Tregs (Pierson et al., 2013). It will be of interest to determine if there is any change in expression of this important survival factor in Tregs with MC903 treatment.

With the knowledge of proliferation as the driving process resulting in the increased percentage of Tregs, it was important to consider the Treg effect in the context of MC903 topical treatment. This treatment was first studied because it was observed that it is a useful model of atopic dermatitis (AD) in mice (Li et al., 2006). The Th2 inflammation present in AD could drive the expansion of Tregs as a response to the immune activation in this model. This was not completely



the case, as we observed decreased inflammation in IL-4 KO compared to WT mice with MC903 treatment with a similar increase in Tregs in both genotypes. Suggesting the ability of MC903 to cause Treg increases was not linked directly to the amount of Th2 inflammation, and that in fact there may be a separate pathway driving the Treg increases unrelated to the level of Th2 inflammation.

### **Dendritic cells are intermediates to TSLP driven regulatory T cell proliferation**

Intriguingly, we found that TSLP driven Treg expansion did not require TSLPR expression on the proliferating cells. This suggested that the TSLP requirement for Treg proliferation was dependent on an additional intermediate factor. The ability of DCs to control Treg homeostasis has been long appreciated (Swee et al., 2009; Darrasse-Jèze et al., 2009; Zou et al., 2010). Moreover, a number of studies have shown that DCs express TSLPR and respond to TSLP treatment *in vitro* (Hanabuchi et al., 2012; Zhang and Zhou, 2012). Because of this, we sought to determine if DCs were the potential intermediate between TSLP production and Treg increases. We observed no increases in Treg numbers with MC903 treatment in mice lacking DCs, suggesting a requirement for this cell type in the TSLP-driven Treg proliferation. Additionally, we found a partial rescue of Treg increases with the injection of WT DCs into TSLPR KO mice, further supporting the conclusion that DCs, through their ability to signal through the TSLPR, are able to stimulate Treg increases.

Co-culture of DCs and Tregs *in vitro* further demonstrated that TSLP was able to drive Treg proliferation as well as drastically augment IL-2 stimulated proliferation. In agreement with our *in vivo* studies, this was dependent on TSLPR expression on DCs but not Tregs. DCs are known to be able to control both Treg maintenance as well as overall immune tolerance through providing costimulation with CD80, CD86, and PD-L1 surface receptors to both Tconvs and Tregs (Bar-On et al., 2011; Fife and Bluestone, 2008; Francisco et al., 2009). We observed that with MC903 treatment *in vivo* as well as TSLP treatment *in vitro*, that DCs up-regulated the expression of these key costimulatory molecules. Further analysis through blocking these pathways *in vitro* showed that PD1/PD-L1 interaction was not required for TSLP induced Treg proliferation, while CD80/CD86 interactions were required for optimal proliferation. The overarching accepted importance of DCs and the costimulation they provide in Treg maintenance is further supported by the observations we have made here.

Apart from the costimulatory cell contact provided by DCs, MHC-II/TCR interactions have also been implicated in Treg maintenance (Gavin et al., 2001; Bhandoola et al., 2002). Here we have shown preliminary work performed to determine the precise role of TCR mediated signals in the Treg proliferation after MC903 treatment. The K14 mouse is an ideal model to test this idea due to its limited MHC-II expression, with the gene for MHC-II being driven by the K14 promoter. This leads to development of CD4<sup>+</sup> T cells including Tregs through MHC-II expression in the thymus, but little exposure to TCR stimulation in the periphery due to lack expression of MHC-II in APCs (Laufer et al., 1996). Using

this model of decreased MHC-II/TCR interactions, we have found that there is no requirement for TCR stimulation for the systemic Treg increases seen in the spleen of MC903 treated mice. Further studies should be done to fully determine if TCR stimulation is required in this model, due to the contrasting data in the dLN of MC903 treated mice.

The loss of the Treg increases in the dLN of K14 mice suggests that there may be a different pathway leading to the increase in Tregs seen locally with MC903 treatment versus the increases seen in the spleen and blood of treated mice. The dependence on TCR signals for MC903 driven Treg increases within the dLN may signal the expansion of antigen specific Tregs. Tregs within the skin and skin draining LN have a different TCR repertoire than those found in the spleen, making it likely that these Tregs could be more dependent on TCR-specific signals for their proliferation (Lathrop et al., 2008). If this is the case, further studies should be done to determine if the local Treg increases are dependent on the TCR specificity, or if it is potentially only dependent on syngeneic TCR/MHC-II interactions. Alongside this, it would be of interest to further assess the need for TCR signals in the expansion of Tregs within the systemic organs including the spleen and blood of MC903 treated mice using genetic models. If there is truly a different pathway leading to Treg proliferation locally versus systemically, it would give more insight into the processes that are important in control Treg expansion in response to inflammation as well as in response to interactions with tolerogenic DCs.

## **Skin mediated protection from type 1 diabetes**

Changes in Treg numbers have been implicated in both the prevention and the enhancement of the progression of type 1 diabetes (T1D) in the NOD mouse model. Depletion of Tregs leads to a significantly faster onset of disease as measured by blood glucose level (Salomon et al., 2000). Alternatively, increasing Tregs through either adoptive transfer or treatment with agents to increase Tregs *in vivo* leads to protection from diabetes (Lepault and Gagnerault, 2000; Szanya et al., 2002; Besin et al., 2008). As MC903 treatment results in comparable increases in Tregs as were observed in these studies, we sought to determine if this skin mediated control would have the potential to limit incidence of T1D. Topical MC903 treatment of NOD mice led to sustained Treg increases in the peripheral blood of mice during the treatment period. Results showed that control treated NOD mice reached near 100% incidence of diabetes, while only about 40% of mice that received topical MC903 developed overt diabetes, as measured by blood glucose level.

Even though the observation of decreased incidence correlated with the increases in Tregs, more work will have to be performed in order to determine if there is a causal relationship between the increases in Tregs and protection from diabetes. The systemic TSLP induction with this topical MC903 model has the potential to drive other effects within the NOD mouse. TSLP has traditionally been studied as a Th2 driving cytokine and the role that it plays in creating a Th2 environment within the NOD mouse must be assessed. There has been work

demonstrating protection from diabetes with a skewing of the immune response away from the Th1 inflammation that drives diabetes by enhancing the Th2 response (Wang et al., 1998; Rabinovitch, 1994; Walker and Herrath, 2015). Further studies in the lab will investigate and draw apart the role of both Treg increases, as well as potential Th2 skewing in the MC903-mediated protection of NOD mice. This will be performed by removing the ability of the treatment to enhance either of these pathways through depletion and genetic manipulation. The ability or inability of MC903 to protect against diabetes in each setting will add to our knowledge on how this topical treatment is leading to suppression of overt autoimmunity.

### **TCR requirements for regulatory T cell maintenance**

In Chapter III of this thesis, the focus shifted from the novel skin mediated control of Tregs to studies aimed at better understanding the currently appreciated roles of Tconv and IL-2 in Treg homeostasis. The possibility that the accepted role of TCR stimulation was in fact for the production of IL-2 by Tconvs and not solely for direct stimulation of Tregs had not been completely investigated. Our studies on this subject importantly linked the baseline TCR signal strength with the ability of Tconvs to produce IL-2, both through *in vitro* analysis of syngeneic MHC-II/TCR interactions as well as looking at IL-2 production in Tconvs with greater reactivity to self. These findings link the need

for TCR signaling in the production of the low levels of IL-2 in response to self-peptide MHC-II complexes.

In a transfer model in which hematopoietic cells were lacking MHC-II expression, we observed drastic decreases in Treg percentages and a near complete loss of IL-2 production by Tconvs. Treatment with IL-2 immune complexes led to a partial rescue of the Treg percentage, suggesting that the lack of IL-2 production in this transfer setting was in part responsible for the loss of Treg maintenance. Due to the fact that this model did not fully distinguish between the loss of TCR signals in Tconvs and Tregs, we utilized a second, more specific model. This approach created a targeted decrease in TCR signaling ability solely within Tconvs. With this one modification, we found decreases in the percentage of Tregs, demonstrating a role for TCR signals in Tconvs for the peripheral maintenance of Tregs. Furthermore, this modification led to a trend towards decreased IL-2 production by Tconvs with lower TCR signaling ability. Together, suggesting a role for IL-2 as a mediator of the indexing of Tregs to the TCR signaling capacity of Tconvs.

A revealing study was published recently from the Germain lab showing that Tconvs can be found in lymphoid tissue interacting with DCs expressing self antigens in order to produce IL-2 (Liu et al., 2015). Tregs are found in this cluster of immune cells as well, with high levels of phosphorylated STAT5 indicating they are utilizing the IL-2 produced by this Tconv/DC interaction. These findings suggest that there is a subset of self-reactive Tconvs that produce IL-2 in

response to syngeneic contacts with DCs in the steady state of a healthy mouse. This is similar to the *in vitro* findings in which we observed IL-2 production to self-peptide MHC-II complexes as well as supports our finding that Tconvs with greater self-reactivity make more IL-2 in the steady state. The Tconvs that take part in this process are described by Liu *et. al.* as being “proto-effector” T cells which are critical to maintain the Tregs that are then reciprocally pivotal in suppressing the stimulation of these Tconvs into full auto-reactive effector cells (Liu et al., 2015; Amado et al., 2013). This all leads to a model in which the immune system is poised at a highly regulated point between auto-reactivity and tolerance dependent on both TCR signals as well as production and competition for IL-2.

Future work on this topic should look at the TCR repertoire of the IL-2 producing Tconvs to determine if these cells truly possess self-reactive TCRs. The existence of Tconvs that escape central tolerance mechanisms to exist in the periphery may in fact play a role in this process of IL-2 production. The Tconvs that have been thought to be a mistake in their escape from the negative selection may in fact be more likely to become the “proto-effector” Tconvs that play a role in Treg maintenance. Clarification of these findings will add to our understanding of the complex and slightly precarious nature of the Treg/Tconv balance.

## **Implications for regulatory T cell therapeutics**

The potential for Treg therapy to be used in human patients is critically dependent on the ability to create stable, functional Tregs that survive in the patient. Current *ex vivo* expansion techniques have been hampered by the inability to create Tregs that survive long term after transfer (Rossetti et al., 2014). The most promising Treg based therapies involve treatment with small molecules and proteins that drive the expansion of pre-existing Tregs. The primary example of this has been the use of low dose IL-2 therapy. The efficacy of IL-2 has been exhibited in the setting of GVHD, with robust increases in Foxp3<sup>+</sup> Tregs as well as reductions in chronic GVHD manifestations (Koreth et al., 2011; Matsuoka et al., 2013). Furthermore, low dose IL-2 therapy has been successful in lessening symptoms associated with chronic hepatitis C virus (HCV) infection (Saadoun et al., 2011; Schlaak et al., 2002). Human T1D patients have also demonstrated increases in Tregs with low dose IL-2 therapy resulting in decreases in the activation of effector T cells (Rosenzweig et al., 2015). With these promising findings, future studies must be performed to optimize the dose in each setting for Treg-specific expansion (Pham et al., 2016).

In a similar manner to low dose IL-2 treatment, MC903 topical application expands pre-existing Tregs in the host, making it a promising method to suppress immune responses. MC903, also known as calcipotriol and marketed as “Dovonex”, is currently used as a treatment for autoimmune psoriasis. Despite its established efficacy in treating psoriasis, the mechanism of action is unclear.



Further studies would need to be performed to test the hypothesis, but there is potential that treatment of psoriatic lesions may lead to TSLP production and expansion of Tregs for suppression of the autoimmune response in human psoriasis patients. If Tregs are observed to expand systemically in human patients, as they have been found to do in mouse models, it would be useful to determine the ability of MC903 treatment to work as a therapy for diseases that have been shown to benefit from systemic Treg increases, such as GVHD, chronic HCV, and in particular, T1D. The potential for topical MC903 to extend past treating just skin disorders is highly desirable as a therapy due to the low cost and ease of treatment. As we begin to better understand the ability of skin tissue to instruct immune responses, we open doors to be able to provide simpler therapies that require less invasive procedures.

## **Conclusions**

In total, this work has expanded our knowledge on the extrinsic factors important in regulating Treg numbers. We have demonstrated the ability of the skin to alter the systemic maintenance of Tregs by driving proliferation through DCs that sense increased levels of TSLP. Furthermore, we have illuminated additional details associated with the complex requirements of TCR signals and IL-2 in the indexing of Tregs to Tconv numbers. Together with additional published knowledge on Treg homeostasis, this information will be critical in understanding the environment that best maintains the most important

immunosuppressive cell type, the Treg. This significance is highlighted by the fact that the skin mediated control of Tregs proved to be able to prevent autoimmunity from developing in the NOD mouse model of T1D. While much more needs to be determined in order to translate these observations to clinical impact, the importance of understanding these mechanisms of tolerance is clear.

## **APPENDIX I:**

### **List of Abbreviations**

#### **A**

APC: antigen presenting cell

#### **B**

BrdU: bromodeoxyuridine

BM: bone marrow

#### **C**

CFSE: carboxyfluorescein succinimidyl ester

CTLA4: cytotoxic T-lymphocyte-associate protein 4

cSLP76: conditional WT SLP76 expressing cells

cY145F: conditional Y145F SLP76 expressing cells

#### **D**

DC: dendritic cell

dLN: skin-draining lymph node

#### **E**

EAE: experimental autoimmune encephalomyelitis

ELISA: enzyme-linked immunosorbent assay

EtOH: ethanol

## **F**

FACS: fluorescence activated cell sorting

Foxp3: forkhead box P3

## **G**

GFP: green fluorescent protein

## **H**

HA: hemagglutinin

## **I**

i.p.: intraperitoneal

IL-2: interleukin 2

IL-2 IC: interleukin 2 cytokine/anti-interleukin-2 antibody complexes

iTreg cell: in vitro induced regulatory T cell

## **K**

KO: knock out

## **L**

Lck: lymphocyte specific protein tyrosine kinase

LAT: linker of activated T cells

LN: lymph node

## **M**

MACS: magnetic activated cell sorting

MHC-II: major histocompatibility complex class II

MFI: mean fluorescence intensity

## **N**

NOD: non-obese diabetic

## **P**

PBS: phosphate buffered saline

pTreg: peripherally-derived regulatory T cell

## **S**

SEM: standard error of the mean

SLP-76: Src homology 2 domain-containing leukocyte protein of 76 kD

STAT5: signal transducer and activator of transcription 5

## **T**

T1D: type 1 diabetes

Tconv: conventional CD4<sup>+</sup> T cell

TCR: T cell receptor

TGF $\beta$ : transforming growth factor beta  
Treg: regulatory CD4<sup>+</sup> T cell  
TSLP: thymic stromal lymphopoietin  
tTreg: thymically-derived regulatory T cell

## **V**

VDR: vitamin D receptor

## **W**

WT: wild type

## **Y**

Y145F: SLP76.Y145F mutant  
YFP: yellow fluorescent protein

**APPENDIX II:**  
**Materials and Methods**  
**Chapter II**

**Mice**

TSLPR KO mice were a generous gift from Dr. Warren Leonard. K14 mice were a generous gift from Dr. Terri Laufer. CD11cDTA, C56BL/6.Thy1.1, C56BL/6 Foxp3.GFP and C57BL/6.SJL Foxp3.GFP reporter mice are maintained in our animal facility. All other mice were purchased from The Jackson Laboratory or Charles River. Mice were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

**MC903 treatment**

MC903 (Tocris Cat# 2700) was dissolved in 100% EtOH. Mice were treated on both ears with 2nmol or EtOH vehicle for 5-7 days. MC903 and EtOH treatment intraperitoneally consisted of injecting 4nmol MC903 or an equivalent volume of EtOH in 500 $\mu$ L PBS for 5 days.

## **Flow cytometry, cell sorting, and data analysis**

Antibodies for flow cytometry were purchased from eBioscience (San Diego, CA), BD Bioscience (San Jose, CA), or Tonbo Bioscience (San Diego, CA). Flow cytometry was performed with an LSR II, FACSCanto, or a FACSCalibur. Cell sorting was performed with a FACS Aria cell sorter (BD Biosciences) or MACS Cell Separation (Miltenyi Biotec; San Diego, CA). Data were analyzed with FlowJo software (TreeStar) and Prism (GraphPad).

## **Tissue Processing**

Ear skin tissue was obtained by separating the dermal sheets of the ear and digesting in 250 µg/ml LiberaseTL (Roche, Cat# 5401020001) and 10 µg/ml DNase (Roche, Cat# 10104159001) for 90 min at 37°C. Digestion was quenched with complete media and tissue was mashed through cell strainers and washed twice for flow cytometry.

## **VDR KO chimeras**

C57BL/6.SJL mice were lethally irradiated with a split dose of 11 Gy and reconstituted with  $4 \times 10^6$  MACS-purified T cell-depleted (CD90.2) bone marrow of either C57BL/6 or VDR KO origin. 9-10 weeks post reconstitution, mice were treated with EtOH or MC903 (2nmol/ear) topically for 7 days.



### **Serum TSLP ELISA**

Serum was collected from mice on day 3 of EtOH and MC903 (2nmol/ear) topical treatment. ELISA was performed using 2 µg/ml capture antibody (R&D Cat #MAB5551) and 0.1 µg/ml detection antibody (R&D Cat #BAF555) and read by TMB reaction.

### **pTreg adoptive transfer**

FACS-sorted Tconvs (CD45.2<sup>+</sup>Foxp3.GFP<sup>-</sup>) and Tregs (CD45.1<sup>+</sup>Foxp3.GFP<sup>+</sup>) were transferred into lymphoreplete C57BL/6.Thy1.1 WT mice at a Tconv:Treg ratio of 3:1 (approximately 1.5x10<sup>6</sup> Tconv: 0.5x10<sup>6</sup> Treg). Three days post transfer, mice were treated with EtOH or MC903 (2nmol/ear) topically from day 3-9 and analyzed on day 11.

### ***in vitro* iTreg assay**

FACS-sorted DCs (Dump (CD3ε,CD19,DX5)<sup>-</sup>CD11c<sup>+</sup>IAbB<sup>+</sup>) and Tconvs (CD4<sup>+</sup>Foxp3.GFP<sup>-</sup>) and were plated at a 1:1 ratio of 15,000-20,000 each/well for 96 hours. Culture medium consisted of MEM-a with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES, and 1x10<sup>-5</sup>M 2-mercaptoethanol and contained anti-CD3 (1 µg/ml), IL-2 (100 U/ml), TGFβ (1 ng/ml), and TSLP (50 ng/ml).

### ***in vivo* BrdU treatment**

Mice were treated with EtOH or MC903 (2nmol/ear) topically from day 0-6. On the final day of topical treatment, mice were administered BrdU with an initial bolus of BrdU (2 mg in 200 ml PBS) i.p. and given drinking water containing BrdU (1 mg/mL) until the time of sacrifice 3 days later.

### ***in vitro* suppression assay**

Tregs were FACS-sorted (CD90.2<sup>+</sup>CD8 $\alpha$ <sup>-</sup>Foxp3.GFP<sup>+</sup>) from EtOH and MC903 (2nmol/ear) topically treated WT C57BL/6.SJL Foxp3.GFP mice at day 8 and Tconvs (CD4<sup>+</sup>GFP<sup>-</sup>) from WT C57BL/6 Foxp3.GFP reporter mice. The Tconvs were CFSE-labeled and cultured at various ratios with 15,000 Tregs/well in the presence of irradiated T cell–depleted feeder cells and soluble anti-CD3 (1  $\mu$ g/ml). CFSE labeling was performed by resuspending cells with PBS containing CFSE (5 mM) at 37°C followed by continuous shaking for 9 min. The reaction was then immediately quenched with 100% FBS, and the cells were washed before culture. CFSE dilution of Tconvs was assessed by flow cytometry after 4 d in culture.

### **CFSE labeled Tcell adoptive transfer**

MACS-sorted CD4<sup>+</sup> T cells from WT C57BL/6.SJL or TSLPR KO (CD45.2<sup>+</sup>) mice were CFSE labeled and 5 x 10<sup>6</sup> cells of each type were co-transferred into WT

C57BL/6.Thy1.1 host mice. Mice were treated with EtOH or MC903 (2nmol/ear) topically from day 3-7 and analyzed on day 11.

### **WT DC adoptive transfer**

WT DCs were obtained from spleens of mice subcutaneously injected 8-10 days prior with FLT3L-expressing EL4 cells.  $2-5 \times 10^6$  DCs were injected i.v. at day 0 and day 4 of treatment. Mice were treated with EtOH or MC903 (2nmol/ear) topically from day 0-6 and analyzed on day 8.

### ***in vitro* DC/Treg co-cultures**

FACS-sorted DCs (Dump(CD3 $\epsilon$ ,CD19,DX5)<sup>-</sup>CD11c<sup>+</sup>IAbB<sup>+</sup>) and CFSE labeled Tregs (CD90.2<sup>+</sup>CD8 $\alpha$ <sup>-</sup>Foxp3.GFP<sup>+</sup>) and were plated at a 1:1 ratio of 15,000-20,000 each/well for 96 hours. Culture medium included combinations of IL-2 (50 U/ml) and TSLP (50 ng/ml). CTLA4 Ig and anti-PDL1 were used at 20  $\mu$ g/ml.

### **NOD T1D model**

Female NOD mice were treated with EtOH or MC903 (2nmol/ear) topically three times a week, every other week, beginning between 5 and 12 weeks of age. Development of diabetes was determined by two consecutive blood glucose readings  $\geq 250$ mg/dl.

## Chapter III

### Mice

Y145F knock-in mice, SLP-76<sup>flox/Y145F</sup> conditional mutant (cY145F), and SLP-76<sup>flox/+</sup> conditional heterozygous (cSLP76) mice were generated as previously described (Jordan et al., 2008; Wu et al., 2011) and bred in our facility. TCR $\beta$ / $\delta$  KO, I-Ab $\beta$  KO (MHC-II KO), C57BL/6 CD90.1, C57BL/6 Foxp3.GFP and C57BL/6.SJL Foxp3.GFP reporter mice were purchased from The Jackson Laboratory or Charles River and were bred and maintained in our animal facility. Mice were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

### Flow cytometry, cell sorting, and data analysis

Antibodies for flow cytometry were purchased from eBioscience (San Diego, CA), BD Bioscience (San Jose, CA), or Tonbo Bioscience (San Diego, CA). Flow cytometry was performed with an LSR II, FACSCanto, or a FACSCalibur. Cell sorting was performed with a FACS Aria cell sorter (BD Biosciences) or MACS Cell Separation (Miltenyi Biotec; San Diego, CA). Data were analyzed with FlowJo software (TreeStar) and Prism (GraphPad).

### **In vitro co-cultures and IL-2 detection**

FACS-sorted Naïve Tconvs ( $CD4^+CD25^-CD45RB^{hi}$ ) from either C57BL/6 or SLP76.Y145F mice were co-cultured with  $CD11c^+$  MACS-sorted DCs at a 1:1 ratio in 200  $\mu$ l T cell media (MEM- $\alpha$  with 10% FBS, 1% penicillin/ streptomycin, 10 mM HEPES, and  $1 \times 10^{-5}$  M 2-mercaptoethanol) with mouse GM-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ). Culture supernatant was collected at 96 h and analyzed by ELISA for IL-2 production.  $CD11c^+$  DCs were sorted from spleens of mice subcutaneously injected 8-10 days prior with FLT3L-expressing EL4 cells. IL-2 was detected using the Mouse IL-2 ELISA Ready-SET-Go! kit (eBioscience).

### **CD5 high and low Tconv sort, RNA extraction, and Quantitative PCR**

Tconvs were FACS sorted using a FACSAria sorting on  $CD4^+CD25^-$  T cells and then on CD5 levels (highest and lowest 20%). RNA was isolated using QIAshredder columns paired with the RNeasy minikit (QIAGEN; Germantown, MD). Expression of IL-2 mRNA was measured by real-time PCR (Applied Biosystems StepOnePlus Real-Time PCR System; Carlsbad, CA) using SYBR Green Master Mix (Applied Biosystems) on 1000-cell equivalents of cDNA template and 100nM primer concentration. The oligonucleotides used to amplify the template DNA were //2 fwd, 5'-AGCAGCTGTTGATGGACCTA-3'; //2 rev, 5'-

CGCAGAGGTCCAAGTTCAT-3'; 18S fwd, 5'-TCAAGAACGAAAGTCGGAGG-3';  
18S rev, 5'-GGACATCTAAGGGCATCACA-3'.

### **Irradiation, Reconstitution and IL-2 IC treatment**

C57BL/6.SJL mice were lethally irradiated with a split dose of 11 Gy and reconstituted with  $5 \times 10^6$  MACS-purified T cell-depleted (CD90.2) bone marrow of either C57BL/6 or MHC-II KO origin. At the same time as the bone marrow transfer, the mice received  $5 \times 10^6$  MACS-purified CD4<sup>+</sup> T cells. At day 14 post transfer, mice were treated with IL-2 immune complexes (0.25ug IL-2 and 1.25ug αIL-2 mAB) or PBS for 5 days. Treg percentages were measured in the peripheral blood at day 14, 20 and 27 post transfer.

### **Adoptive transfers and Tamoxifen administration**

Tconvs (CD45.2<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup>) were FACS-sorted from spleens of SLP-76<sup>flox/Y145F</sup> conditional mutant (cY145F) and SLP-76<sup>flox/+</sup> conditional heterozygous (cSLP76) mice. Tconvs from either source were transferred in a 4:1 ratio with FACS-sorted WT Tregs (CD45.1<sup>+</sup>CD4<sup>+</sup>GFP<sup>+</sup>) from C57BL/6.SJL Foxp3.GFP reporter mice into TCRβ/δ KO mice. For deletion of the loxp-flanked SLP-76 allele 8-10 weeks after cell transfer, mice were orally given 200 μg/g body weight of Tamoxifen in corn oil every day for 5 days. Mice were bled weekly to measure circulating Tconvs and Tregs for 12 weeks. Spleens were dissociated and set in erythrocyte lysis

buffer (140mM NH<sub>4</sub>Cl, 17 mM Tris pH 7.5) for 2 minutes. Cells were then filtered through 70 micron nylon mesh to obtain a single cell suspension for flow cytometry staining. Treg percentages were assessed as CD4<sup>+</sup>CD45.1<sup>+</sup>Foxp3<sup>+</sup> percent of total CD4<sup>+</sup> T cells.

## BIBLIOGRAPHY

- Almeida, A.R.M., B. Zaragoza, and A.A. Freitas. 2006. Indexation as a novel mechanism of lymphocyte homeostasis: the number of CD4+CD25+ regulatory T cells is indexed to the number of IL-2-producing cells. *J Immunol.* 177:192–200.
- Almeida, A.R.M., N. Legrand, M. Papiernik, and A.A. Freitas. 2002. Homeostasis of Peripheral CD4+ T Cells: IL-2R and IL-2 Shape a Population of Regulatory Cells That Controls CD4+ T Cell Numbers. *The Journal of Immunology.* 169:4850–4860. doi:10.4049/jimmunol.169.9.4850.
- Amado, I.F., J. Berges, R.J. Luther, M.P. Mailhe, S. Garcia, A. Bandeira, C. Weaver, A. Liston, and A.A. Freitas. 2013. IL-2 coordinates IL-2-producing and regulatory T cell interplay. *Journal of Experimental Medicine.* 210:2707–2720. doi:10.1371/journal.pone.0017423.
- Anderson, M.S., E.S. Venanzi, L. Klein, Z. Chen, and S.P. Berzins. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science.* doi:10.1126/science.1077464.
- Anderson, M.S., E.S. Venanzi, Z. Chen, S.P. Berzins, C. Benoist, and D. Mathis. 2005. The Cellular Mechanism of Aire Control of T Cell Tolerance. *Immunity.* 23:227–239. doi:10.1016/j.immuni.2005.07.005.
- Apostolou, I., and H. Von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* doi:10.1084/jem.20040249.
- Arstila, T.P., A. Casrouge, V. Baron, J. Even, J. Kanellopoulos, and P. Kourilsky. 1999. A direct estimate of the human alphabeta T cell receptor diversity. *Science.* 286:958–961.
- Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* 184:387–396.
- Aschenbrenner, K., L.M. D'Cruz, E.H. Vollmann, M. Hinterberger, J. Emmerich, L.K. Swee, A. Rolink, and L. Klein. 2007. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat Immunol.* 8:351–358. doi:10.1038/ni1444.
- Azzam, H.S., A. Grinberg, K. Lui, H. Shen, E.W. Shores, and P.E. Love. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J. Exp. Med.* 188:2301–2311.
- Bar-On, L., T. Birnberg, K.-W. Kim, and S. Jung. 2011. Dendritic cell-restricted CD80/86 deficiency results in peripheral regulatory T-cell reduction but is not associated with lymphocyte hyperactivation. *Eur. J. Immunol.* 41:291–298. doi:10.1002/eji.201041169.
- Baron, U., S. Floess, G. Wieczorek, K. Baumann, A. Grützkau, J. Dong, A. Thiel, T.J. Boeld, P. Hoffmann, M. Edinger, I. Türbachova, A. Hamann, S. Olek, and J. Huehn. 2007. DNA demethylation in the humanFOXP3 locus discriminates



- regulatory T cells from activated FOXP3<sup>+</sup> conventional T cells. *Eur. J. Immunol.* 37:2378–2389. doi:10.1002/eji.200737594.
- Barron, L., H. Doms, K.K. Hoyer, W. Kuswanto, J. Hofmann, W.E. O'Gorman, and A.K. Abbas. 2010. Cutting Edge: Mechanisms of IL-2-Dependent Maintenance of Functional Regulatory T Cells. *The Journal of Immunology.* 185:6426–6430. doi:10.4049/jimmunol.0903940.
- Belkaid, Y., and D. Artis. 2013. Immunity at the Barriers. *Eur. J. Immunol.* 43:3096–3097. doi:10.1002/eji.201344133.
- Belkaid, Y., and S. Naik. 2013. Compartmentalized and systemic control of tissue immunity by commensals. *Nat Immunol.* 14:646–653. doi:10.1038/ni.2604.
- Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27:20–21. doi:10.1038/83713.
- Besin, G., S. Gaudreau, M. Menard, C. Guindi, G. Dupuis, and A. Amrani. 2008. Thymic Stromal Lymphopoietin and Thymic Stromal Lymphopoietin-Conditioned Dendritic Cells Induce Regulatory T-Cell Differentiation and Protection of NOD Mice Against Diabetes. *Diabetes.* 57:2107–2117. doi:10.2337/db08-0171.
- Bhandoola, A., X. Tai, M. Eckhaus, H. Auchincloss, K. Mason, S.A. Rubin, K.M. Carbone, Z. Grossman, A.S. Rosenberg, and A. Singer. 2002. Peripheral expression of self-MHC-II influences the reactivity and self-tolerance of mature CD4(+) T cells: evidence from a lymphopenic T cell model. *Immunity.* 17:425–436.
- Bikle, D.D. 2011. Vitamin D Regulation of Immune Function. 86. 1st ed. Elsevier Inc. 21 pp.
- Bilate, A.M., and J.J. Lafaille. 2012. Induced CD4<sup>+</sup>Foxp3<sup>+</sup>Regulatory T Cells in Immune Tolerance. *Annual review of immunology.* 30:733–758. doi:10.1146/annurev-immunol-020711-075043.
- Boismenu, R., and W.L. Havran. 1994. Modulation of epithelial cell growth by intraepithelial gamma delta T cells. *Science.* 266:1253–1255.
- Bouneaud, C., P. Kourilsky, and P. Bousso. 2000. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity.* 13:829–840.
- Boursalian, T.E., J. Golob, D.M. Soper, C.J. Cooper, and P.J. Fink. 2004. Continued maturation of thymic emigrants in the periphery. *Nat Immunol.* 5:418–425. doi:10.1038/ni1049.
- Boyman, O., and J. Sprent. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol.* 12:180–190. doi:10.1038/nri3156.
- Boyman, O., M. Kovar, M.P. Rubinstein, C.D. Surh, and J. Sprent. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science.*

311:1924–1927. doi:10.1126/science.1122927.

- Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27:68–73. doi:10.1038/83784.
- Brusko, T., C. Wasserfall, K. McGrail, R. Schatz, H.L. Viener, D. Schatz, M. Haller, J. Rockell, P. Gottlieb, M. Clare-Salzler, and M. Atkinson. 2007. No Alterations in the Frequency of FOXP3+ Regulatory T-Cells in Type 1 Diabetes. *Diabetes.* 56:604–612. doi:10.2337/db06-1248.
- Burmeister, Y., T. Lischke, A.C. Dahler, H.W. Mages, K.P. Lam, A.J. Coyle, R.A. Kroczek, and A. Hutloff. 2008. ICOS Controls the Pool Size of Effector-Memory and Regulatory T Cells. *The Journal of Immunology.* 180:774–782. doi:10.4049/jimmunol.180.2.774.
- Cabello-Kindelan, C., A. de la Barrera, T.R. Malek, and A.L. Bayer. 2014. In vivo environment necessary to support transplanted donor mouse T regulatory cells. *Am. J. Transplant.* 14:1032–1045. doi:10.1111/ajt.12650.
- Campbell, D.J., and M.A. Koch. 2011. Phenotypical and functional specialization of FOXP3. *Nat Rev Immunol.* 11:119–130. doi:10.1038/nri2916.
- Chatila, T.A., F. Blaeser, N. Ho, H.M. Lederman, C. Voulgaropoulos, C. Helms, and A.M. Bowcock. 2000. JM2, encoding a fork head–related protein, is mutated in X-linked autoimmunity–allergic dysregulation syndrome. *J. Clin. Invest.* 106:R75–R81. doi:10.1172/JCI11679.
- Chen, C., E.A. Rowell, R.M. Thomas, and W.W. Hancock. 2006. Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. *Journal of Biological ....*
- Chen, W., W. Jin, N. Hardegen, K.-J. Lei, L. Li, N. Marinos, G. McGrady, and S.M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 198:1875–1886. doi:10.1084/jem.20030152.
- Cheng, G., A. Yu, and T.R. Malek. 2011. T-cell tolerance and the multi-functional role of IL-2R signaling in T-regulatory cells. *Immunological Reviews.* 241:63–76. doi:10.1111/j.1600-065X.2011.01004.x.
- Costello, E.K., C.L. Lauber, M. Hamady, N. Fierer, J.I. Gordon, and R. Knight. 2009. Bacterial community variation in human body habitats across space and time. *Science.* 326:1694–1697. doi:10.1126/science.1177486.
- Darrasse-Jèze, G., S. Deroubaix, H. Mouquet, G.D. Victora, T. Eisenreich, K.-H. Yao, R.F. Masilamani, M.L. Dustin, A. Rudensky, K. Liu, and M.C. Nussenzweig. 2009. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *Journal of Experimental Medicine.* 206:1853–1862. doi:10.1084/jem.20090746.
- Ehrenstein, M.R. 2004. Compromised Function of Regulatory T Cells in Rheumatoid Arthritis and Reversal by Anti-TNF Therapy. *Journal of Experimental Medicine.*

- 200:277–285. doi:10.1084/jem.20040165.
- Fehervari, Z., and S. Sakaguchi. 2004. Control of Foxp3+ CD25+CD4+ regulatory cell activation and function by dendritic cells. *International Immunology*. 16:1769–1780. doi:10.1093/intimm/dxh178.
- Fife, B.T., and J.A. Bluestone. 2008. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunological Reviews*. 224:166–182. doi:10.1111/j.1600-065X.2008.00662.x.
- Fisson, S., G. Darrasse-Jeze, E. Litvinova, F. Septier, D. Klatzmann, R. Liblau, and B.L. Salomon. 2003. Continuous Activation of Autoreactive CD4+ CD25+ Regulatory T Cells in the Steady State. *Journal of Experimental Medicine*. 198:737–746. doi:10.1084/jem.20030686.
- Floess, S., J. Freyer, C. Siewert, U. Baron, S. Olek, J. Polansky, K. Schlawe, H.-D. Chang, T. Bopp, E. Schmitt, S. Klein-Hessling, E. Serfling, A. Hamann, and J. Huehn. 2007. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*. 5:e38. doi:10.1371/journal.pbio.0050038.
- Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 4:330–336. doi:10.1038/ni904.
- Francisco, L.M., V.H. Salinas, K.E. Brown, V.K. Vanguri, G.J. Freeman, V.K. Kuchroo, and A.H. Sharpe. 2009. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *Journal of Experimental Medicine*. 206:3015–3029. doi:10.1016/j.molimm.2007.08.013.
- García-Miguel, M., M.J. González, R. Quera, and M.A. Hermoso. 2013. Innate Immunity Modulation by the IL-33/ST2 System in Intestinal Mucosa. *BioMed Research International*. 2013:1–13. doi:10.1073/pnas.0906988106.
- Garza, K.M., S.S. Agersborg, E. Baker, and K.S.K. Tung. 2000. Persistence of Physiological Self Antigen Is Required for the Regulation of Self Tolerance. *The Journal of Immunology*. 164:3982–3989. doi:10.4049/jimmunol.164.8.3982.
- Gavin, M.A., S.R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2001. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nat Immunol*. 3:33–41. doi:10.1038/ni743.
- Gershon, R.K., and K. Kondo. 1970. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology*. 18:723–737.
- Gorman, S., M.A. Judge, and P.H. Hart. 2010. Journal of Steroid Biochemistry and Molecular Biology. *Journal of Steroid Biochemistry and Molecular Biology*. 121:247–249. doi:10.1016/j.jsbmb.2010.02.034.
- Gratz, I.K., and D.J. Campbell. 2014. Organ-specific and memoryTreg cells: specificity, development, function, and maintenance. 1–17. doi:10.3389/fimmu.2014.00333/abstract.
- Grice, E.A., H.H. Kong, S. Conlan, C.B. Deming, J. Davis, A.C. Young, NISC Comparative Sequencing Program, G.G. Bouffard, R.W. Blakesley, P.R. Murray,

- E.D. Green, M.L. Turner, and J.A. Segre. 2009. Topographical and temporal diversity of the human skin microbiome. *Science*. 324:1190–1192. doi:10.1126/science.1171700.
- Guo, F., C. Iclozan, W.K. Suh, C. Anasetti, and X.Z. Yu. 2008. CD28 Controls Differentiation of Regulatory T Cells from Naive CD4 T Cells. *The Journal of Immunology*. 181:2285–2291. doi:10.4049/jimmunol.181.4.2285.
- Hahn, S.A., I. Bellinghausen, B. Trinschek, and C. Becker. 2015. Translating Treg Therapy in Humanized Mice. *Front Immunol*. 6:623. doi:10.3389/fimmu.2015.00623.
- Hanabuchi, S., N. Watanabe, and Y.-J. Liu. 2012. TSLP and immune homeostasis. *Allergol Int*. 61:19–25. doi:10.2332/allergolint.11-RAI-0394.
- Harskamp, C.T., and A.W. Armstrong. 2013. Immunology of atopic dermatitis: novel insights into mechanisms and immunomodulatory therapies. *Semin Cutan Med Surg*. 32:132–139. doi:10.12788/j.sder.0018.
- He, R., and R.S. Geha. 2010. Thymic stromal lymphopoietin. *Ann. N. Y. Acad. Sci*. 1183:13–24. doi:10.1111/j.1749-6632.2009.05128.x.
- Hedrick, S.M. 2012. Positive Selection in the Thymus: An Enigma Wrapped in a Mystery. *The Journal of Immunology*. 188:2043–2045. doi:10.4049/jimmunol.1200077.
- Herman, A.E., G.J. Freeman, D. Mathis, and C. Benoist. 2004. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J. Exp. Med*. 199:1479–1489. doi:10.1084/jem.20040179.
- Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell*. 76:17–27.
- Hori, S., T. TAKAHASHI, and S. Sakaguchi. 2003. Control of Autoimmunity by Naturally Arising Regulatory CD4+ T Cells. *In Advances in Immunology*. Elsevier. 331–371.
- Horwitz, D.A. 2008. Regulatory T cells in systemic lupus erythematosus: past, present and future. *Arthritis Res Ther*. 10:227. doi:10.1186/ar2511.
- Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol*. 162:5317–5326.
- Jiang, Q., H. Su, G. Knudsen, W. Helms, and L. Su. 2006. Delayed functional maturation of natural regulatory T cells in the medulla of postnatal thymus: role of TSLP. *BMC Immunol*. 7:6. doi:10.1186/1471-2172-7-6.
- Jordan, M.S., A. Boesteanu, A.J. Reed, A.L. Petrone, A.E. Hohenbeck, M.A. Lerman, A. Najj, and A.J. Caton. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2:301–306. doi:10.1038/86302.

- Jordan, M.S., J.E. Smith, J.C. Burns, J.-E.T. Austin, K.E. Nichols, A.C. Aschenbrenner, and G.A. Koretzky. 2008. Complementation In Trans of Altered Thymocyte Development in Mice Expressing Mutant Forms of the Adaptor Molecule SLP76. *Immunity*. 28:359–369. doi:10.1016/j.immuni.2008.01.010.
- Josefowicz, S.Z., C.B. Wilson, and A.Y. Rudensky. 2009. Cutting Edge: TCR Stimulation Is Sufficient for Induction of Foxp3 Expression in the Absence of DNA Methyltransferase 1. *The Journal of Immunology*. 182:6648–6652. doi:10.4049/jimmunol.0803320.
- Josefowicz, S.Z., L.-F. Lu, and A.Y. Rudensky. 2012. Regulatory T Cells: Mechanisms of Differentiation and Function. *Annual review of immunology*. doi:10.1146/annurev.immunol.25.022106.141623.
- Kawabe, Y., and A. Ochi. 1991. Programmed cell death and extrathymic reduction of Vbeta8+ CD4+ T cells in mice tolerant to Staphylococcus aureus enterotoxin B. *nature*. 349:245–248. doi:10.1038/349245a0.
- Khattari, R., T. Cox, S.-A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol*. 4:337–342. doi:10.1038/ni909.
- Kim, H.P., J. Kelly, and W.J. Leonard. 2001. The basis for IL-2-induced IL-2 receptor alpha chain gene regulation: importance of two widely separated IL-2 response elements. *Immunity*. 15:159–172.
- Kim, J.K., M. Klinger, J. Benjamin, Y. Xiao, D.J. Erle, D.R. Littman, and N. Killeen. 2009. Impact of the TCR Signal on Regulatory T Cell Homeostasis, Function, and Trafficking. *PLoS ONE*. 4:e6580. doi:10.1371/journal.pone.0006580.s006.
- Kim, J.M., and A. Rudensky. 2006. The role of the transcription factor Foxp3 in the development of regulatory T cells. *Immunological Reviews*. 212:86–98. doi:10.1111/j.0105-2896.2006.00426.x.
- Kissmeyer, A.M., and L. Binderup. 1991. Calcipotriol (MC 903): pharmacokinetics in rats and biological activities of metabolites. A comparative study with 1,25(OH)<sub>2</sub>D<sub>3</sub>. *Biochem. Pharmacol*. 41:1601–1606.
- Knoechel, B., J. Lohr, E. Kahn, J.A. Bluestone, and A.K. Abbas. 2005. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J. Exp. Med*. 202:1375–1386. doi:10.1084/jem.20050855.
- Knutson, J.C., L.W. LeVan, C.R. Valliere, and C.W. Bishop. 1997. Pharmacokinetics and systemic effect on calcium homeostasis of 1 alpha,24-dihydroxyvitamin D<sub>2</sub> in rats. Comparison with 1 alpha,25-dihydroxyvitamin D<sub>2</sub>, calcitriol, and calcipotriol. *Biochem. Pharmacol*. 53:829–837.
- Kojima, A., and R.T. Prehn. 1981. Genetic susceptibility to post-thymectomy autoimmune diseases in mice. *Immunogenetics*. 14:15–27.
- Koreth, J., K.-I. Matsuoka, H.T. Kim, S.M. McDonough, B. Bindra, E.P. Alyea, P. Armand, C. Cutler, V.T. Ho, N.S. Treister, D.C. Bienfang, S. Prasad, D. Tzachanis, R.M. Joyce, D.E. Avigan, J.H. Antin, J. Ritz, and R.J. Soiffer. 2011.

- Interleukin-2 and regulatory T cells in graft-versus-host disease. *N. Engl. J. Med.* 365:2055–2066. doi:10.1056/NEJMoa1108188.
- Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M.C. Nussenzweig, and H. Von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol.* 6:1219–1227. doi:10.1038/ni1265.
- Lahl, K., C. Loddenkemper, C. Drouin, J. Freyer, J. Arnason, G. Eberl, A. Hamann, H. Wagner, J. Huehn, and T. Sparwasser. 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *Journal of Experimental Medicine.* 204:57–63. doi:10.1084/jem.20061852.
- Lathrop, S.K., N.A. Santacruz, D. Pham, J. Luo, and C.-S. Hsieh. 2008. Antigen-specific peripheral shaping of the natural regulatory T cell population. *Journal of Experimental Medicine.* 205:3105–3117. doi:10.1084/jem.20081359.
- Laufer, T.M., J. DeKoning, J.S. Markowitz, D. Lo, and L.H. Glimcher. 1996. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature.* 383:81–85.
- Lechler, R., J.G. Chai, F. Marelli-Berg, and G. Lombardi. 2001. The contributions of T-cell anergy to peripheral T-cell tolerance. *Immunology.* 103:262–269.
- Lee, H.-M., J.L. Bautista, and C.-S. Hsieh. 2011a. Thymic and Peripheral Differentiation of Regulatory T Cells. *Regulatory T-Cells.* 112:25–71. doi:10.1016/B978-0-12-387827-4.00002-4.
- Lee, J.-H., L.-C. Wang, Y.-T. Lin, Y.-H. Yang, D.-T. Lin, and B.-L. Chiang. 2006. Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus. *Immunology.* 117:280–286. doi:10.1111/j.1365-2567.2005.02306.x.
- Lee, J.-Y., Y.-M. Lim, M.-J. Park, S.-Y. Min, M.-L. Cho, Y.-C. Sung, S.-H. Park, H.-Y. Kim, and Y.-G. Cho. 2007. Murine thymic stromal lymphopoietin promotes the differentiation of regulatory T cells from thymic CD4+CD8–CD25– naïve cells in a dendritic cell-independent manner. *Immunol Cell Biol.* 86:206–213. doi:10.1038/sj.icb.7100127.
- Lee, Y.K., J.S. Menezes, Y. Umesaki, and S.K. Mazmanian. 2011b. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences.* 108 Suppl 1:4615–4622. doi:10.1073/pnas.1000082107.
- Lemire, J.M., J.S. Adams, R. Sakai, and S.C. Jordan. 1984. 1 alpha,25-dihydroxyvitamin D3 suppresses proliferation and immunoglobulin production by normal human peripheral blood mononuclear cells. *J. Clin. Invest.* 74:657–661. doi:10.1172/JCI111465.
- Lepault, F., and M.C. Gagnerault. 2000. Characterization of Peripheral Regulatory CD4+ T Cells That Prevent Diabetes Onset in Nonobese Diabetic Mice. *The Journal of Immunology.* 164:240–247. doi:10.4049/jimmunol.164.1.240.
- Li, M., P. Hener, Z. Zhang, K.P. Ganti, D. Metzger, and P. Chambon. 2009. Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for

- generating an atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. *J. Invest. Dermatol.* 129:498–502. doi:10.1038/jid.2008.232.
- Li, M., P. Hener, Z. Zhang, S. Kato, D. Metzger, and P. Chambon. 2006. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. *Proc Natl Acad Sci USA.* 103:11736–11741. doi:10.1073/pnas.0604575103.
- Liang, S., P. Alard, Y. Zhao, S. Parnell, S.L. Clark, and M.M. Kosiewicz. 2005. Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J. Exp. Med.* 201:127–137. doi:10.1084/jem.20041201.
- Lindley, S., C.M. Dayan, A. Bishop, B.O. Roep, M. Peakman, and T.I.M. Tree. 2005. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. *Diabetes.* 54:92–99.
- Lio, C.-W.J., and C.-S. Hsieh. 2008. A two-step process for thymic regulatory T cell development. *Immunity.* 28:100–111. doi:10.1016/j.immuni.2007.11.021.
- Lio, C.W.J., L.F. Dodson, C.M. Deppong, C.-S. Hsieh, and J.M. Green. 2010. CD28 Facilitates the Generation of Foxp3- Cytokine Responsive Regulatory T Cell Precursors. *The Journal of Immunology.* 184:6007–6013. doi:10.4049/jimmunol.1000019.
- Littorin, B., P. Blom, A. Schölin, H.J. Arnqvist, G. Blohmé, J. Bolinder, A. Ekbom-Schnell, J.W. Eriksson, S. Gudbjörnsdottir, L. Nyström, J. Östman, and G. Sundkvist. 2006. Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS). *Diabetologia.* 49:2847–2852. doi:10.1007/s00125-006-0426-x.
- Liu, Y.-J., V. Soumelis, N. Watanabe, T. Ito, Y.-H. Wang, R. de Waal Malefyt, M. Omori, B. Zhou, and S.F. Ziegler. 2007. TSLP: An Epithelial Cell Cytokine that Regulates T Cell Differentiation by Conditioning Dendritic Cell Maturation. *Annual review of immunology.* 25:193–219. doi:10.1146/annurev.immunol.25.022106.141718.
- Liu, Z., M.Y. Gerner, N. van Panhuys, A.G. Levine, A.Y. Rudensky, and R.N. Germain. 2015. Immune homeostasis enforced by co-localized effector and regulatory T cells. *Nature.* 528:225–230. doi:10.1038/nature16169.
- Lohmann, T., R.D. Leslie, and M. Londei. 1996. T cell clones to epitopes of glutamic acid decarboxylase 65 raised from normal subjects and patients with insulin-dependent diabetes. *J. Autoimmun.* 9:385–389. doi:10.1006/jaut.1996.0052.
- Malek, T.R., and A.L. Bayer. 2004. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol.* 4:665–674. doi:10.1038/nri1435.
- Maloy, K.J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat Immunol.* 2:816–822. doi:10.1038/ni0901-816.
- Mandl, J.N., J.P. Monteiro, N. Vrisekoop, and R.N. Germain. 2012. T Cell-Positive

- Selection Uses Self-Ligand Binding Strength to Optimize Repertoire Recognition of Foreign Antigens. *Immunity*. 38:263–274. doi:10.1016/j.immuni.2012.09.011.
- Marson, A., K. Kretschmer, G.M. Frampton, E.S. Jacobsen, J.K. Polansky, K.D. Macisaac, S.S. Levine, E. Fraenkel, H. Von Boehmer, and R.A. Young. 2007. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature*. 445:931–935. doi:10.1038/nature05478.
- Matsuoka, K.-I., J. Koreth, H.T. Kim, G. Bascug, S. McDonough, Y. Kawano, K. Murase, C. Cutler, V.T. Ho, E.P. Alyea, P. Armand, B.R. Blazar, J.H. Antin, R.J. Soiffer, and J. Ritz. 2013. Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci Transl Med*. 5:179ra43. doi:10.1126/scitranslmed.3005265.
- Matzinger, P., and T. Kamala. 2011. PErsPEcTIVEs. *Nature Publishing Group*. 11:221–230. doi:10.1038/nri2940.
- Mazzucchelli, R., J.A. Hixon, R. Spolski, X. Chen, W.Q. Li, V.L. Hall, J. Willette-Brown, A.A. Hurwitz, W.J. Leonard, and S.K. Durum. 2008. Development of regulatory T cells requires IL-7R stimulation by IL-7 or TSLP. *Blood*. 112:3283–3292. doi:10.1182/blood-2008-02-137414.
- McKeever, U., J.P. Mordes, and D.L. Greiner. 1990. Adoptive transfer of autoimmune diabetes and thyroiditis to athymic rats.
- Meehan, M.A., R.H. Kerman, and J.M. Lemire. 1992. 1,25-Dihydroxyvitamin D3 enhances the generation of nonspecific suppressor cells while inhibiting the induction of cytotoxic cells in a human MLR. *Cell. Immunol.* 140:400–409.
- Min, B., A. Thornton, S.M. Caucheteux, S.-A. Younes, K. Oh, J. Hu-Li, and W.E. Paul. 2007. Gut flora antigens are not important in the maintenance of regulatory T cell heterogeneity and homeostasis. *Eur. J. Immunol.* 37:1916–1923. doi:10.1002/eji.200737236.
- Miyara, M., Z. Amoura, C. Parizot, C. Badoual, K. Dorgham, S. Trad, D. Nochy, P. Debre, J.C. Piette, and G. Gorochov. 2005. Global Natural Regulatory T Cell Depletion in Active Systemic Lupus Erythematosus. *The Journal of Immunology*. 175:8392–8400. doi:10.4049/jimmunol.175.12.8392.
- Montes-Torres, A., M. Llamas-Velasco, A. Pérez-Plaza, G. Solano-López, and J. Sánchez-Pérez. 2015. Biological Treatments in Atopic Dermatitis. *JCM*. 4:593–613. doi:10.3390/jcm4040593.
- Monticelli, L.A., L.C. Osborne, M. Noti, S.V. Tran, D.M.W. Zaiss, and D. Artis. 2015. IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin–EGFR interactions. *Proceedings of the National Academy of Sciences*. 112:10762–10767. doi:10.1073/pnas.1509070112.
- Mora, J.R., M. Iwata, and U.H. von Andrian. 2008. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol*. 8:685–698. doi:10.1038/nri2378.
- Moran, A.E., K.L. Holzapfel, Y. Xing, N.R. Cunningham, J.S. Maltzman, J. Punt, and K.A. Hogquist. 2011. T cell receptor signal strength in Treg and iNKT cell



- development demonstrated by a novel fluorescent reporter mouse. *Journal of Experimental Medicine*. 208:1279–1289. doi:10.1084/jem.20110308.
- Morgan, M.E., J.H.M. van Bilsen, A.M. Bakker, B. Heemskerk, M.W. Schilham, F.C. Hartgers, B.G. Elferink, L. van der Zanden, R.R.P. de Vries, T.W.J. Huizinga, T.H.M. Ottenhoff, and R.E.M. Toes. 2005. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Human Immunology*. 66:13–20. doi:10.1016/j.humimm.2004.05.016.
- Morrissey, P.J., K. Charrier, S. Braddy, D. Liggitt, and J.D. Watson. 1993. CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+ T cells. *J. Exp. Med.* 178:237–244.
- Mucida, D., N. Kutchukhidze, A. Erazo, M. Russo, J.J. Lafaille, and M.A. Curotto de Lafaille. 2005. Oral tolerance in the absence of naturally occurring Tregs. *J. Clin. Invest.* 115:1923–1933. doi:10.1172/JCI24487.
- Muller, K., N.J. Kriegbaum, B. Baslund, O.H. Sorensen, M. Thymann, and K. Bentzen. 1995. Vitamin D3 metabolism in patients with rheumatic diseases: low serum levels of 25-hydroxyvitamin D3 in patients with systemic lupus erythematosus. *Clinical Rheumatology*. 14:397–400.
- Naik, S., N. Bouladoux, C. Wilhelm, M.J. Molloy, R. Salcedo, W. Kastenmuller, C. Deming, M. Quinones, L. Koo, S. Conlan, S. Spencer, J.A. Hall, A. Dzutsev, H. Kong, D.J. Campbell, G. Trinchieri, J.A. Segre, and Y. Belkaid. 2012. Compartmentalized Control of Skin Immunity by Resident Commensals. *Science*. 337:1115–1119. doi:10.1126/science.1225152.
- Nishizuka, Y., and T. Sakakura. 1969. Thymus and reproduction: sex-linked dysgenesis of the gonad after neonatal thymectomy in mice. *Science*. 166:753–755.
- Ono, M., H. Yaguchi, N. Ohkura, I. Kitabayashi, Y. Nagamura, T. Nomura, Y. Miyachi, T. Tsukada, and S. Sakaguchi. 2007. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature*. 446:685–689. doi:10.1038/nature05673.
- Osborne, B.A., S.W. Smith, Z.G. Liu, K.A. McLaughlin, L. Grimm, and L.M. Schwartz. 1994. Identification of genes induced during apoptosis in T lymphocytes. *Immunological Reviews*. 142:301–320.
- Östman, S., C. Rask, A.E. Wold, S. Hultkrantz, and E. Telemo. 2006. Impaired regulatory T cell function in germ-free mice. *Eur. J. Immunol.* 36:2336–2346. doi:10.1002/eji.200535244.
- Pacholczyk, R., H. Ignatowicz, P. Kraj, and L. Ignatowicz. 2006. Origin and T Cell Receptor Diversity of Foxp3+CD4+CD25+ T Cells. *Immunity*. 25:249–259. doi:10.1016/j.immuni.2006.05.016.
- Palmer, E. 2003. Cell death and immunity: Negative selection — clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol.* 3:383–391.

doi:10.1038/nri1085.

- Penhale, W.J., A. Farmer, R.P. McKenna, and W.J. Irvine. 1973. Spontaneous thyroiditis in thymectomized and irradiated Wistar rats. *Clinical & Experimental Immunology*. 15:225–236.
- Penhale, W.J., P.A. Stumbles, C.R. Huxtable, R.J. Sutherland, and D.W. Pethick. 1990. Induction of diabetes in PVG/c strain of rats by manipulation of the immune system. *Autoimmunity*. 7:169–179.
- Penhale, W.J., W.J. Irvine, and J.R. Inglis. 1976. Thyroiditis in T cell-depleted rats: suppression of the autoallergic response by reconstitution with normal lymphoid cells. *Clinical and experimental ....*
- Penna, G., and L. Adorini. 2000. 1,25-Dihydroxyvitamin D3 Inhibits Differentiation, Maturation, Activation, and Survival of Dendritic Cells Leading to Impaired Alloreactive T Cell Activation. *The Journal of Immunology*. 164:2405–2411. doi:10.4049/jimmunol.164.5.2405.
- Perez, V.L., L. Van Parijs, A. Biuckians, X.X. Zheng, T.B. Strom, and A.K. Abbas. 1997. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity*. 6:411–417.
- Pham, M.N., M.G. von Herrath, and J.L. Vela. 2016. Antigen-Specific Regulatory T Cells and Low Dose of IL-2 in Treatment of Type 1 Diabetes. *Front Immunol*. 6:1007. doi:10.1016/j.clim.2015.05.023.
- Pierson, W., B. Cauwe, A. Policheni, S.M. Schlenner, D. Franckaert, J. Berges, S. Humblet-Baron, S. Schönfeldt, M.J. Herold, D. Hildeman, A. Strasser, P. Bouillet, L.-F. Lu, P. Matthys, A.A. Freitas, R.J. Luther, C.T. Weaver, J. Dooley, D.H.D. Gray, and A. Liston. 2013. Antiapoptotic Mcl-1 is critical for the survival and niche-filling capacity of Foxp3+ regulatory T cells. *Nat Immunol*. 14:959–965. doi:10.1038/ni.2649.
- Powrie, F., and D. Mason. 1990. OX-22high CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22low subset. *J. Exp. Med*. 172:1701–1708.
- Powrie, F., M.W. Leach, S. Mauze, L.B. Caddle, and R.L. Coffman. 1993. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *International Immunology*. 5:1461–1471.
- Putheti, P., A. Pettersson, M. Soderstrom, H. Link, and Y.M. Huang. 2004. Circulating CD4+CD25+ T regulatory cells are not altered in multiple sclerosis and unaffected by disease-modulating drugs. *Journal of Clinical Immunology*. 24:155–161.
- R Walker, M., D.J. Kasprovicz, V.H. Gersuk, A. Bènard, M. Van Landeghen, J.H. Buckner, and S.F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25– T cells. *J. Clin. Invest*. 112:1437–1443. doi:10.1172/JCI19441.
- Rabinovitch, A. 1994. Perspectives in diabetes. Immunoregulatory and cytokine

imbalances in the pathogenesis of IDDM. Diabetes.

- Reichel, H., H.P. Koeffler, A. Tobler, and A.W. Norman. 1987. 1 alpha,25-Dihydroxyvitamin D3 inhibits gamma-interferon synthesis by normal human peripheral blood lymphocytes. *Proc Natl Acad Sci USA*. 84:3385–3389.
- Richards, D.M., M. Delacher, Y. Goldfarb, D. Kägebein, A.-C. Hofer, J. Abramson, and M. Feuerer. 2015. Treg Cell Differentiation: From Thymus to Peripheral Tissue. 136. 1st ed. Elsevier Inc. 31 pp.
- Rigby, W.F., B. Yirinec, O.R. L., and M.W. Fanger. 1987. Comparison of the effects of 1,25-dihydroxyvitamin D3 on T lymphocyte subpopulations. *Eur. J. Immunol.* 17:563–566.
- Rigby, W.F., T. Stacy, and M.W. Fanger. 1984. Inhibition of T lymphocyte mitogenesis by 1,25-dihydroxyvitamin D3 (calcitriol). *J. Clin. Invest.* 74:1451–1455. doi:10.1172/JCI111557.
- Rimoldi, M., M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G.M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno. 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol.* 6:507–514. doi:10.1038/ni1192.
- Rosenzweig, M., G. Churlaud, R. Mallone, A. Six, N. Dérian, W. Chacara, R. Lorenzon, S.A. Long, J.H. Buckner, G. Afonso, H.-P. Pham, A. Hartemann, A. Yu, A. Pugliese, T.R. Malek, and D. Klatzmann. 2015. Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients. *J. Autoimmun.* 58:48–58. doi:10.1016/j.jaut.2015.01.001.
- Rossetti, M., R. Spreafico, S. Saidin, C. Chua, M. Moshref, J.Y. Leong, Y.K. Tan, J. Thumboo, J. van Loosdregt, and S. Albani. 2014. Ex Vivo-Expanded but Not In Vitro-Induced Human Regulatory T Cells Are Candidates for Cell Therapy in Autoimmune Diseases Thanks to Stable Demethylation of the FOXP3 Regulatory T Cell-Specific Demethylated Region. *The Journal of Immunology.* 194:113–124. doi:10.4049/jimmunol.1401145.
- Rudensky, A. 2005. Foxp3 and dominant tolerance. *Philosophical Transactions of the Royal Society B: Biological Sciences.* 360:1645–1646. doi:10.1038/83707.
- Saadoun, D., M. Rosenzweig, F. Joly, A. Six, F. Carrat, V. Thibault, D. Sene, P. Cacoub, and D. Klatzmann. 2011. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N. Engl. J. Med.* 365:2067–2077. doi:10.1056/NEJMoa1105143.
- Saenz, S.A., B.C. Taylor, and D. Artis. 2008. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. *Immunological Reviews.* 226:172–190. doi:10.1111/j.1600-065X.2008.00713.x.
- Sakaguchi, S., K. Fukuma, K. Kuribayashi, and T. Masuda. 1985. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J. Exp. Med.*

161:72–87.

- Sakaguchi, S., K. Wing, and M. Miyara. 2007. Regulatory T cells – a brief history and perspective. *Eur. J. Immunol.* 37:S116–S123. doi:10.1002/eji.200737593.
- Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* 155:1151–1164.
- Sakaguchi, S., T. Takahashi, and Y. Nishizuka. 1982. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J. Exp. Med.* 156:1577–1586.
- Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell.* 133:775–787. doi:10.1016/j.cell.2008.05.009.
- Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity.* 12:431–440.
- Schlaak, J.F., C. Schramm, K. Radecke, K.-H.M. zum Büschenfelde, and G. Gerken. 2002. Sustained suppression of HCV replication and inflammatory activity after interleukin-2 therapy in patients with HIV/hepatitis C virus coinfection. *J. Acquir. Immune Defic. Syndr.* 29:145–148. doi:10.1007/s40261-014-0189-y.
- Schwartz, R.H. 2003. T Cell Anergy. *Annual review of immunology.* 21:305–334. doi:10.1146/annurev.immunol.21.120601.141110.
- Semana, G., R. Gausling, R.A. Jackson, and D.A. Hafler. 1999. T cell autoreactivity to proinsulin epitopes in diabetic patients and healthy subjects. *J. Autoimmun.* 12:259–267. doi:10.1006/jaut.1999.0282.
- Seneschal, J., R.A. Clark, A. Gehad, C.M. Baecher-Allan, and T.S. Kupper. 2012. Human Epidermal Langerhans Cells Maintain Immune Homeostasis in Skin by Activating Skin Resident Regulatory T Cells. *Immunity.* 36:873–884. doi:10.1016/j.immuni.2012.03.018.
- Serreze, D.V., K. Hamaguchi, and E.H. Leiter. 1989. Immunostimulation circumvents diabetes in NOD/Lt mice. *J. Autoimmun.* 2:759–776.
- Setoguchi, R., S. Hori, T. TAKAHASHI, and S. Sakaguchi. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201:723–735. doi:10.1084/jem.20041982.
- Shen, S., M.I. Chuck, M. Zhu, D.M. Fuller, C.O. Yang, and W. Zhang. 2010. The importance of LAT in the activation, homeostasis, and regulatory function of T cells. *Journal of Biological ....*
- Siegmund, K. 2005. Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo. *Blood.* 106:3097–3104.

doi:10.1182/blood-2005-05-1864.

- Siracusa, M.C., S.A. Saenz, D.A. Hill, B.S. Kim, M.B. Headley, T.A. Doering, E.J. Wherry, H.K. Jessup, L.A. Siegel, T. Kambayashi, E.C. Dudek, M. Kubo, A. Cianferoni, J.M. Spergel, S.F. Ziegler, M.R. Comeau, and D. Artis. 2011. TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. *Nature*. 477:229–233. doi:10.1038/nature10329.
- Smigiel, K.S., E. Richards, S. Srivastava, K.R. Thomas, J.C. Dudda, K.D. Klonowski, and D.J. Campbell. 2014a. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *Journal of Experimental Medicine*. 211:121–136. doi:10.4049/jimmunol.1200507.
- Smigiel, K.S., S. Srivastava, J.M. Stolley, and D.J. Campbell. 2014b. Regulatory T-cell homeostasis: steady-state maintenance and modulation during inflammation. *Immunological Reviews*. 259:40–59. doi:10.1111/imr.12170.
- Snelgrove, R.J., A. Godlee, and T. Hussell. 2011. Airway immune homeostasis and implications for influenza-induced inflammation. *Trends in Immunology*. 32:328–334. doi:10.1016/j.it.2011.04.006.
- Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annual review of immunology*. 21:139–176. doi:10.1146/annurev.immunol.21.120601.141107.
- Strasser, A., and M. Pellegrini. 2004. T-lymphocyte death during shutdown of an immune response. *Trends in Immunology*. 25:610–615. doi:10.1016/j.it.2004.08.012.
- Sugihara, S., Y. Izumi, T. Yoshioka, H. Yagi, T. Tsujimura, O. Tarutani, Y. Kohno, S. Murakami, T. Hamaoka, and H. Fujiwara. 1988. Autoimmune thyroiditis induced in mice depleted of particular T cell subsets. I. Requirement of Lyt-1 dull L3T4 bright normal T cells for the induction of thyroiditis. *J Immunol*. 141:105–113.
- Sun, C.M., J.A. Hall, R.B. Blank, N. Bouladoux, M. Oukka, J.R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *Journal of Experimental Medicine*. 204:1775–1785. doi:10.1084/jem.188.2.287.
- Suri-Payer, E., A.Z. Amar, A.M. Thornton, and E.M. Shevach. 1998. CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J Immunol*. 160:1212–1218.
- Swee, L.K., N. Bosco, B. Malissen, R. Ceredig, and A. Rolink. 2009. Expansion of peripheral naturally occurring T regulatory cells by Fms-like tyrosine kinase 3 ligand treatment. *Blood*. 113:6277–6287. doi:10.1182/blood-2008-06-161026.
- Szanya, V., J. Ermann, C. Taylor, C. Holness, and C.G. Fathman. 2002. The Subpopulation of CD4+CD25+ Splenocytes That Delays Adoptive Transfer of Diabetes Expresses L-Selectin and High Levels of CCR7. *The Journal of Immunology*. 169:2461–2465. doi:10.4049/jimmunol.169.5.2461.
- Taguchi, O., K. Kontani, H. Ikeda, T. Kezuka, M. Takeuchi, T. Takahashi, and T.

- Takahashi. 1994. Tissue-specific suppressor T cells involved in self-tolerance are activated extrathymically by self-antigens. *Immunology*. 82:365–369.
- Tai, X., B. Erman, A. Alag, J. Mu, M. Kimura, G. Katz, T. Guinter, T. McCaughtry, R. Etzensperger, L. Feigenbaum, D.S. Singer, and A. Singer. 2013. Foxp3 Transcription Factor Is Proapoptotic and Lethal to Developing Regulatory T Cells unless Counterbalanced by Cytokine Survival Signals. *Immunity*. 38:1116–1128. doi:10.1016/j.immuni.2013.02.022.
- Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol*. 6:152–162. doi:10.1038/ni1160.
- Takeda, M., T. Yamashita, N. Sasaki, K. Nakajima, T. Kita, M. Shinohara, T. Ishida, and K.I. Hirata. 2010. Oral Administration of an Active Form of Vitamin D3 (Calcitriol) Decreases Atherosclerosis in Mice by Inducing Regulatory T Cells and Immature Dendritic Cells With Tolerogenic Functions. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 30:2495–2503. doi:10.1161/ATVBAHA.110.215459.
- Tang, Q., J.Y. Adams, C. Penaranda, K. Melli, E. Piaggio, E. Sgouroudis, C.A. Piccirillo, B.L. Salomon, and J.A. Bluestone. 2008. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity*. 28:687–697. doi:10.1016/j.immuni.2008.03.016.
- Tang, Q., K.J. Henriksen, E.K. Boden, A.J. Tooley, J. Ye, S.K. Subudhi, X.X. Zheng, T.B. Strom, and J.A. Bluestone. 2003. Cutting Edge: CD28 Controls Peripheral Homeostasis of CD4+CD25+ Regulatory T Cells. *The Journal of Immunology*. 171:3348–3352. doi:10.4049/jimmunol.171.7.3348.
- Tarbell, K.V., S. Yamazaki, and R.M. Steinman. 2006. The interactions of dendritic cells with antigen-specific, regulatory T cells that suppress autoimmunity. *Semin Immunol*. 18:93–102. doi:10.1016/j.smim.2006.01.009.
- Thornton, A.M., P.E. Kory, D.Q. Tran, E.A. Wohlfert, P.E. Murray, Y. Belkaid, and E.M. Shevach. 2010. Expression of Helios, an Ikaros Transcription Factor Family Member, Differentiates Thymic-Derived from Peripherally Induced Foxp3+ T Regulatory Cells. *The Journal of Immunology*. 184:3433–3441. doi:10.4049/jimmunol.0904028.
- van Etten, E., and C. Mathieu. 2005. Immunoregulation by 1,25-dihydroxyvitamin D3: Basic concepts. *The Journal of Steroid Biochemistry and Molecular Biology*. 97:93–101. doi:10.1016/j.jsbmb.2005.06.002.
- Verginis, P., K.A. McLaughlin, K.W. Wucherpfennig, H. Von Boehmer, and I. Apostolou. 2008. Induction of antigen-specific regulatory T cells in wild-type mice: visualization and targets of suppression.
- Viglietta, V. 2004. Loss of Functional Suppression by CD4+CD25+ Regulatory T Cells in Patients with Multiple Sclerosis. *Journal of Experimental Medicine*. 199:971–979. doi:10.1084/jem.20031579.

- Walker, L.S.K., and A.K. Abbas. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol.* 2:11–19. doi:10.1038/nri701.
- Walker, L.S.K., and M. von Herrath. 2015. CD4 T cell differentiation in type 1 diabetes. *Clinical & Experimental Immunology.* 183:16–29. doi:10.1111/cei.12672.
- Wang, B., A. Gonzalez, P. Höglund, J.D. Katz, and C. Benoist. 1998. Interleukin-4 deficiency does not exacerbate disease in NOD mice. *Diabetes.*
- Watanabe, N., S. Hanabuchi, V. Soumelis, W. Yuan, S. Ho, R. de Waal Malefyt, and Y.-J. Liu. 2004. Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4+ T cell homeostatic expansion. *Nat Immunol.* 5:426–434. doi:10.1038/ni1048.
- Watanabe, N., Y.-H. Wang, H.K. Lee, T. Ito, Y.-H. Wang, W. Cao, and Y.-J. Liu. 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature.* 436:1181–1185. doi:10.1038/nature03886.
- Webster, K.E., S. Walters, R.E. Kohler, T. Mrkvan, O. Boyman, C.D. Surh, S.T. Grey, and J. Sprent. 2009. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *Journal of Experimental Medicine.* 206:751–760. doi:10.1126/science.180605.
- Wieczorek, G., A. Asemissen, F. Model, I. Turbachova, S. Floess, V. Liebenberg, U. Baron, D. Stauch, K. Kotsch, J. Pratschke, A. Hamann, C. Loddenkemper, H. Stein, H.D. Volk, U. Hoffmuller, A. Grutzkau, A. Mustea, J. Huehn, C. Scheibenbogen, and S. Olek. 2009. Quantitative DNA Methylation Analysis of FOXP3 as a New Method for Counting Regulatory T Cells in Peripheral Blood and Solid Tissue. *Cancer Research.* 69:599–608. doi:10.1158/0008-5472.CAN-08-2361.
- Wildin, R.S., F. Ramsdell, J. Peake, F. Faravelli, J.L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, F.D. Bricarelli, G. Byrne, M. McEuen, S. Proll, M. Appleby, and M.E. Brunkow. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* 27:18–20. doi:10.1038/83707.
- Williams, L.M., and A.Y. Rudensky. 2007. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol.* 8:277–284. doi:10.1038/ni1437.
- Wong, J., D. Mathis, and C. Benoist. 2007. TCR-based lineage tracing: no evidence for conversion of conventional into regulatory T cells in response to a natural self-antigen in pancreatic islets. *Journal of Experimental Medicine.* 204:2039–2045. doi:10.1016/S1074-7613(00)80561-0.
- Wu, G.F., E. Corbo, M. Schmidt, J.E. Smith-Garvin, M.J. Riese, M.S. Jordan, T.M. Laufer, E.J. Brown, and J.S. Maltzman. 2011. Conditional deletion of SLP-76 in mature T cells abrogates peripheral immune responses. *Eur. J. Immunol.* 41:2064–2073. doi:10.1002/eji.201040809.

- Wu, H.-J., I.I. Ivanov, J. Darce, K. Hattori, T. Shima, Y. Umesaki, D.R. Littman, C. Benoist, and D. Mathis. 2010. Gut-Residing Segmented Filamentous Bacteria Drive Autoimmune Arthritis via T Helper 17 Cells. *Immunity*. 32:815–827. doi:10.1016/j.immuni.2010.06.001.
- Wu, Y., M. Borde, V. Heissmeyer, M. Feuerer, A.D. Lapan, J.C. Stroud, D.L. Bates, L. Guo, A. Han, S.F. Ziegler, D. Mathis, C. Benoist, L. Chen, and A. Rao. 2006. FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT. *Cell*. 126:375–387. doi:10.1016/j.cell.2006.05.042.
- Xing, Y., and K.A. Hogquist. 2012. T-cell tolerance: central and peripheral. *Cold Spring Harb Perspect Biol*. 4. doi:10.1101/cshperspect.a006957.
- Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R.M. Steinman. 2003. Direct Expansion of Functional CD25+ CD4+ Regulatory T Cells by Antigen-processing Dendritic Cells. *Journal of Experimental Medicine*. 198:235–247. doi:10.1084/jem.20030422.
- Zehn, D., and M.J. Bevan. 2006. T Cells with Low Avidity for a Tissue-Restricted Antigen Routinely Evade Central and Peripheral Tolerance and Cause Autoimmunity. *Immunity*. 25:261–270. doi:10.1016/j.immuni.2006.06.009.
- Zhang, R., A. Huynh, G. Whitcher, J. Chang, J.S. Maltzman, and L.A. Turka. 2013. An obligate cell-intrinsic function for CD28 in Tregs. *J. Clin. Invest*. doi:10.1172/JCI65013DS1.
- Zhang, Y., and B. Zhou. 2012. Functions of thymic stromal lymphopoietin in immunity and disease. *Immunol Res*. 52:211–223. doi:10.1007/s12026-012-8264-z.
- Zhang, Z., P. Hener, N. Frossard, S. Kato, D. Metzger, M. Li, and P. Chambon. 2009. Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma. *Proceedings of the National Academy of Sciences*. 106:1536–1541. doi:10.1073/pnas.0812668106.
- Zheng, Y., S. Josefowicz, A. Chaudhry, X.P. Peng, K. Forbush, and A.Y. Rudensky. 2010. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 463:808–812. doi:10.1038/nature08750.
- Zheng, Y., S.Z. Josefowicz, A. Kas, T.-T. Chu, M.A. Gavin, and A.Y. Rudensky. 2007. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*. 445:936–940. doi:10.1038/nature05563.
- Ziegler, S.F. 2007. FOXP3: not just for regulatory T cells anymore. *Eur. J. Immunol*. 37:21–23. doi:10.1002/eji.200636929.
- Zou, T., A.J. Caton, G.A. Koretzky, and T. Kambayashi. 2010. Dendritic Cells Induce Regulatory T Cell Proliferation through Antigen-Dependent and -Independent Interactions. *The Journal of Immunology*. 185:2790–2799. doi:10.4049/jimmunol.0903740.