PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF T CELLS AND
FOXP3+ T REGULATORY CELLS IN INFLAMMATORY BOWEL DISEASE: STEPS
TOWARDS T REGULATORY CELL THERAPY IN MUCOSAL DISEASE

by

Megan Elizabeth Himmel

B.Sc., The University of British Columbia, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

June 2012

© Megan Elizabeth Himmel, 2012
ABSTRACT

Because of their potent suppressive capacity and critical role in the normal function of the human immune system, T regulatory cells (Tregs) have long been considered candidates for the therapeutic treatment of autoimmune and chronic inflammatory diseases. However, the clinical implementation of these cells has proven challenging in practice, in part due to a lack of knowledge surrounding this T cell subset. Specifically, an evaluation of the unique functions of individual Treg cell lineages, along with a comprehensive investigation of the non-suppressive capacities of these cells, including chemokine production, is necessary. Furthermore, in the application of Treg cellular therapy in mucosal diseases such as inflammatory bowel disease, the identification of putative antigens that can be targeted by Tregs is warranted. To these aims, I evaluated the phenotypic and functional characteristics of Helios⁺ and Helios⁻ Treg subsets, with the knowledge that expression of Helios, an Ikaros family transcription factor, may differentiate natural, thymic derived Tregs from their in vivo peripherally induced counterparts. I found that Helios positive and negative Treg subsets expressed similar Treg markers and displayed a similar capacity for suppression and plasticity. However, these Tregs did differ in terms of cytokine/chemokine production as well as methylation state of the FOXP3 Treg-specific demethylated region. Furthermore, total populations of FOXP3⁺ Tregs were evaluated for chemokine expression; I found that Tregs produce significant quantities of CXCL8 and other acute phase chemokines, and are able to attract inflammatory cells of the innate immune system. In addition, FOXP3 expression enhances CXCL8 production, likely because of its ability to bind the CXCL8 gene promoter. To evaluate putative antigens that can be targeted by Treg therapy in inflammatory bowel disease, I assessed the role of flagellin in disease. Flagellin exacerbates colitic disease in mice in a TLR5 independent manner and flagellin-specific T cells can be identified in patients with CD. Collectively, these findings bring us closer to the effective application of Treg cellular therapy in the setting of mucosal disease.
PREFACE

The introduction (Chapter 1): This chapter is based on an extensive literature review, and I wrote all sections. Sections referring to T regulatory cell therapy in mucosal disease are drawn in part from a review article written and researched by myself. M.K. Levings and T.S. Steiner supervised me in the preparation of the aforementioned review article. Y. Yao contributed to sections of the review article not used in this thesis. This is a version of the following article:

Megan E. Himmel, Yu Yao, Theodore S. Steiner, and Megan K. Levings. Regulatory T cell therapy for Inflammatory Bowel Disease: more questions than answers. *Immunology*. In press.

Chapter 2
I was the primary contributor to this work and developed the experimental plan, carried out the majority of experiments, analyzed results, and prepared all figures. I generated Treg cell clones from 4 donors and carried out all experiments using these clones including: cytokine analysis, suppression assays, plasticity assays, and flow cytometry experiments using *ex vivo* CD4+ T cells described in this thesis. R. Garcia performed all work done with *in vitro* induced Tregs, including Helios FACS analysis, suppression assays, and plasticity assays, under my supervision. K. Macdonald and R. Garcia assisted in generating pyrosequencing results. M.K. Levings and T.S. Steiner aided in the experimental design and supervised this research.

Chapter 3
I was the primary contributor to this work, developing the experimental plan, analyzing all results, and preparing the manuscript in its entirety. I performed phenotypic analysis of CXCL8+ Tregs, assays to assess chemokine production by Tregs, developed neutrophil recruitment assays, and isolated RNA from cells for chemokine PCR arrays. S. Crome and S. Ivison aided in experimental design and initial experiments that lead to the genesis of this project, and S. Ivison contributed to chemotaxis assay experiments found in both the published manuscript and this thesis. S. Patterson aided in setting up chemokine PCR arrays. M.K. Levings and T.S. Steiner assisted in the experimental design of this project, supervised this research, and aided in the preparation of the manuscript. C. Piccirillo assisted in experimental feedback and critical editing of the manuscript. Note: sections of manuscript eventually published were incorporated into appropriate sections of the thesis to meet UBC thesis requirements. Figures and text were reprinted with permission of the European Journal of Immunology. This is a version of the following article:

DOI: 10.1002/eji.201040459

Chapter 4
The work in mouse models was performed in collaboration with Dr. Sabine Ivison. I assisted in experimental design and contributed equally to all experiments described, including administering flagellin enemas, collecting tissues, weighing mice, and analyzing samples. I was responsible for histology and CBA data in its entirety, including conducting experiments, analyzing data, and generating figures for the manuscript. I assisted in discussions and data analysis for the manuscript; however Dr. Ivison was responsible for leading data analysis.
discussions and generating the text of the manuscript. P. Wark and G. Hardenberg assisted in weighing mice and collecting/processing endpoint samples. A. Kifayet was responsible for generating all flagellin used in this work and providing technical support when necessary. I developed the method to identify flagellin specific T cells in humans with technical advice from Dr. Nabila Seddeki and Dr. John Zaunders. IBD patient and inflammatory control samples were sent to us by Dr. Ernest Seidman. Dr. T.S. Steiner, and Dr. M.K. Levings supervised the research conducted and aided in the preparation of the manuscript. Note: sections of manuscript published were incorporated into appropriate sections of the thesis to meet UBC thesis requirements. Figures and text were reprinted with permission of Inflammatory Bowel Disease. This is a version of the following article:

DOI: 10.1002/ibd.21097

Work performed under approval by the Clinical Research Ethics Board (UBC CREB NUMBER: C03-0062)

Animal work performed under approval by UBC Animal Care and Use Committee (UBC APPLICATION NUMBER: A05-1867, A06-0107)
TABLE OF CONTENTS

ABSTRACT .................................................................................................................................. ii
PREFACE ..................................................................................................................................... iii
TABLE OF CONTENTS ..............................................................................................................v
LIST OF TABLES..................................................................................................................... vii
LIST OF FIGURES................................................................................................................... viii
LIST OF SYMBOLS AND ABBREVIATIONS........................................................................ix
ACKNOWLEDGEMENTS ......................................................................................................... x
DEDICATION ............................................................................................................................ xii

1 INTRODUCTION ......................................................................................................................1
  1.1 Human FOXP3+ T regulatory cell subsets ................................................................. 2
    1.1.1 Natural thymus-derived FOXP3+ T regulatory cells ........................................ 3
    1.1.2 Induced FOXP3+ T regulatory cells ................................................................. 5
    1.1.3 Identification of T regulatory cell subsets in humans ........................................ 6
  1.2 T regulatory cell therapy .............................................................................................. 8
  1.3 Inflammatory bowel disease ...................................................................................... 9
    1.3.1 The role of T cells in IBD ................................................................................ 10
    1.3.2 T regulatory cells in IBD: what is known ...................................................... 12
    1.3.3 Steps toward Treg therapy in IBD ................................................................. 14
    1.3.4 Flagellin as a putative antigen involved in IBD ............................................ 16
  1.4 Synopsis of research questions .................................................................................. 18

2 HELIOS+ AND HELIOS- T REGULATORY CELL SUBSETS ARE PHENOTYPICALLY AND FUNCTIONALLY SIMILAR AND HAVE AN EQUAL POTENTIAL FOR PLASTICITY ......................................................................................... 21
  2.1 Introduction ................................................................................................................... 21
  2.2 Materials and methods ............................................................................................... 25
  2.3 Both FOXP3+Helios+ and FOXP3+Helios- Treg subsets can be identified in human peripheral blood ............................................................... 30
  2.4 Helios expression can be induced in both Helios+ and Helios- Treg subsets through T cell receptor activation ................................................................. 31
  2.5 Helios+ and Helios- Tregs express similar Treg subset markers, but differ in cytokine and chemokine expression ................................................................. 33
  2.6 Helios+ and Helios- Treg subsets exhibit similar suppressive capacities .............. 35
  2.7 Helios+ and Helios- Treg subsets display an equal potential for plasticity .......... 36
  2.8 Helios+ and Helios- Treg subsets both exhibit a demethylated FOXP3 TSDR region .... 39
  2.9 Helios- cells are readily identified within the naïve FOXP3+ Treg subset and may not represent in vivo induced Tregs ................................................... 40
  2.10 In vitro induced Tregs differ from Helios- Treg clones in plasticity potential ....... 41
  2.11 Conclusions .................................................................................................................. 43
LIST OF TABLES

Chapter 3
Table 3.1 Mean percentage ± SD of CXCL8, IFN-γ, and IL-17 producing populations in total CD4+ T cell (Tconv) and FOXP3+ T cell (Treg) populations from 3 independent experiments... 56
Table 3.2 Range of chemokine production by ex vivo stimulated CD45RA+ and CD45RA- Tregs from 3 independent donors ................................................................. 58

Chapter 4
Table 4.1 Frequency of CD4+CD3−CD14−CD25−OX40+ T cell responders in response to flagellin stimulation in patients with CD, UC, inflammatory controls, and healthy controls ................. 81
LIST OF FIGURES

Chapter 2

Figure 2.1 Isolation of Helios$^+$ and Helios$^-$ FOXP3$^+$ Treg clones from human peripheral blood .......................................................... 31
Figure 2.2 Evaluating Helios expression with T cell activation .......................................................... 33
Figure 2.3 Expression of Treg markers, cytokines, and chemokines by Helios$^+$ Tregs clones, Helios$^-$ Treg clones, and Tconv cell clones .................................................................................. 35
Figure 2.4 Suppressive capacity of Helios$^+$ and Helios$^-$ Treg clones .................................................................................. 36
Figure 2.5 Plasticity of Helios$^+$ and Helios$^-$ Treg clones .................................................................................. 38
Figure 2.6 Methylation status of the FOXP3 TSDR in Helios$^+$ and Helios$^-$ Treg clones ............. 39
Figure 2.7 Analysis of naïve Treg cell markers in Helios$^+$ and Helios$^-$ FOXP3$^+$ Treg populations ...................................................................................................................................................... 41
Figure 2.8 Helios expression, suppressive capacity, and plasticity potential of ex vivo induced Tregs ............................................................................................................................................. 43

Chapter 3

Figure 3.1 Analysis of CXCL8 expression by CD4$^+$ CD25$^{hi}$ FOXP3$^+$ Tregs ................................ 54
Figure 3.2 Analysis of CXCL8, IFN-$\gamma$, and IL-17 expression in naïve CD4$^+$ CD45RA$^+$ CD25$^+$ Treg and CD4$^+$ CD45RA$^+$ CD25$^-$ T conv cells ........................................................................................................ 55
Figure 3.3 FOXP3 regulation of CXCL8 gene expression .................................................................................. 57
Figure 3.4 Chemokine expression by ex vivo human CD4$^+$ T cells at the protein level .................. 59
Figure 3.5 Chemokine and chemokine related protein expression by FOXP3$^+$ Tregs compared to Tconv cells at the mRNA level .................................................................................................................................................. 59
Figure 3.6 Treg induction of neutrophil chemotaxis .................................................................................. 60

Chapter 4

Figure 4.1 Stimulation of murine TLR5-expressing HeLa cells with WT flagellin versus 2H3 .. 72
Figure 4.2 Survival and weight loss in DSS-colitic mice treated with WT FliC versus 2H3 ...... 74
Figure 4.3 Histological analyses of colons from DSS-colitic mice treated with WT FliC versus 2H3 .................................................................................................................................................. 74
Figure 4.4 Quantification of colonic cytokine production ............................................................................ 75
Figure 4.5 Quantification of (A) IgG1 and (B) IgG2a/b in DSS colitis treated mice with and without flagellin .................................................................................................................................................. 77
Figure 4.6 DSS-colitis and FliC-mediated exacerbation in TLR5$^{-/-}$ versus WT C57BL/6 mice. 78
Figure 4.7 Antigen specific T cell identification in human peripheral blood using markers of activation .................................................................................................................................................. 79
Figure 4.8 Correlation of flagellin reactive T cells with anti-flagellin antibodies in human serum .................................................................................................................................................. 83
Figure 4.9 Anti-FliC and anti-Fla2 antibody titres in human serum of IBD patients and controls .................................................................................................................................................. 83
**LIST OF SYMBOLS AND ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
<td></td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>Chemokine ligand</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>Chemokine receptor</td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
<td></td>
</tr>
<tr>
<td>δ</td>
<td>Delta</td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulfate</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
<td></td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
<td></td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
<td></td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
<td></td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
<td></td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
<td></td>
</tr>
<tr>
<td>IPEX</td>
<td>Immune dysregulation, polyendocrinopathy, enteropathy, X-linked</td>
<td></td>
</tr>
<tr>
<td>iTreg</td>
<td>Induced T regulatory cell</td>
<td></td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
<td></td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein-1 alpha</td>
<td></td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
<td></td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural T regulatory cell</td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>TBET</td>
<td>T box transcription factor 21 (protein)</td>
<td></td>
</tr>
<tr>
<td>Tconv</td>
<td>T conventional cell</td>
<td></td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
<td></td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
<td></td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
<td></td>
</tr>
<tr>
<td>Tr1</td>
<td>T regulatory type 1 cell</td>
<td></td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
<td></td>
</tr>
<tr>
<td>TSDR</td>
<td>Treg-specific demethylated region</td>
<td></td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
<td></td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I will be forever grateful to Dr. Megan Levings for her mentorship, advice (both academic and personal), and friendship. She has been an inspiration for me in every way and has encouraged me in all of my pursuits. I was extremely lucky to have Dr. Levings as my supervisor, and will always strive to follow in her example. In addition to her guidance, I would like to thank Dr. Theodore Steiner, my co-supervisor, who has been equally as supportive and an excellent mentor. Dr. Steiner is an incredible physician, researcher, and friend, and has enabled my success. In addition to my supervisors, I am incredibly grateful for my thoughtful and supportive Supervisory Committee: Dr. Laura Sly, Dr. Colby Zaph, and Dr. Kevan Jacobson. It has been a pleasure to work with this fantastic group of researchers who have always pointed me in the right direction and offered helpful insights.

I am very fortunate to have had the opportunity to work with a diverse, intelligent, and extraordinary group of people both at Jack Bell Research Centre and the Child & Family Research Institute. I am incredibly thankful for the friendship of my colleagues Adele Wang and Alicia McMurchy who helped me navigate my way through graduate school and provided support in every facet of my life. I would also like to express gratitude to Dr. Sarah Crome and Dr. Sabine Ivison who provided mentorship, experimental technique instruction, and manuscript preparation advice. I would like to thank Dr. Paul Orban for critical reading of several manuscripts and my fellow colleagues who provided friendship and thoughtful discussions: Kate Macdonald, Jana Gillies, Gijs Hardenberg, Jon Han, Maggie Yao, and Jessica Huang. In addition, I am thankful to Lisa Xu who provided flow cytometry and FACS technical support. I would like to particularly thank Dr. Scott Patterson, Kristen Reipas, and Matthew Gold for their academic advice, but most importantly their friendship and countless lunches throughout my graduate career.
The studies described in this thesis were made possible by blood donated through various avenues, and thus I would like to thank the many blood donors, the Vancouver General Hospital Cell Seperator Unit, the various nurses involved in blood draws, and Dr. Raewyn Broady for her endless work to insure this program continues. In addition, our collaborator, Dr. Ernest Seidman, recruited our IBD patient cohort and generously sent blood to us from Montreal.

My research has been supported by grants from the Canadian Institute for Health Research, the Crohn’s and Colitis Foundation of Canada, and the Michael Smith Foundation for Health Research.

Finally, I would like to express my deepest gratitude to my parents who have provided nothing but love and support throughout my life. Their belief that I can achieve anything has motivated me to push beyond even my own expectations. In addition to my parents, I would like to thank my twin sister Jennifer and brother Michael for setting a prime example of how to be a hard working individual, as well as for lending an ear when necessary. In addition to my family, I am exceptionally grateful for my best friend and partner, Ryan Thoren, who has provided incredible support and love throughout this process.
To my parents for whole-heartedly believing that I can do anything
and to Ryan for his unwavering and unconditional support.
1 INTRODUCTION

T regulatory cells (Tregs) play a critical role in establishing and maintaining immune homeostasis, as well as limiting chronic inflammatory responses directed against pathogens and environmental factors (Vignali et al., 2008). Classically found within the CD4+ T cell pool, Tregs actively suppress immune responses initiated by cells of both the innate and adaptive immune system that are directed at self and foreign, non-pathogenic antigens (Vignali et al., 2008; Maloy et al., 2005). The importance of Tregs in immune regulation has been well established by a number of studies using mouse models: with a lack of functional Tregs due to a spontaneous mutation in scurfy mice, or with depletion of Tregs from wild-type adult mice, a fatal autoimmune-type disease develops (Brunkow et al., 2001; Sakaguchi et al., 1995); in the presence of Tregs, autoimmunity can be prevented or even cured (Powrie et al., 1994; Powrie et al., 1993; Mottet et al., 2003). Studies in humans have corroborated these results, where mutations leading to a loss of Treg function, known as IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), manifests as severe autoimmunity with multi-organ involvement and often leads to death (Le Bras and Geha, 2006). Enhancement of Treg function in the clinical setting promotes tolerance to transplanted allografts (Graca et al., 2002) as well as the prevention of chronic inflammatory responses (Riley et al., 2009). Because of their potent suppressive capacity and critical role in the normal function of the human immune system, Tregs have long been considered candidates for the therapeutic treatment of autoimmune and chronic inflammatory diseases. However, the clinical implementation of these cells has proven challenging in practice, in part due to a lack of knowledge surrounding this T cell subset. Indeed, with recent work demonstrating the presence of a number of different types of Tregs, as well as the propensity for these cells to re-program in an inflammatory milieu, termed “plasticity”,
further investigation into the fundamental phenotype and function of T suppressor cells is warranted in order to improve clinical success in the establishment of Treg mediated tolerance.

1.1 Human FOXP3\(^+\) T regulatory cell subsets

Since the discovery of “suppressor” cells in the 1970’s (Gershon and Kondo, 1970), much research has focused on specifically identifying and characterizing the subset of cells capable of mediating immune suppression. And although many cells have been found to be suppressive, including CD8\(^+\) regulatory T cells, natural killer T cells, and \(\gamma\delta\) T cells, it is specifically the CD4\(^+\) T cell lineage expressing high levels of the IL-2 receptor alpha chain CD25 that have come into focus due to their essential role in maintaining immune homeostasis (Sakaguchi et al., 1995; Zwar et al., 2006; Shevach, 2009). These cells, now termed Tregs, account for 5-10% of peripheral CD4\(^+\) T cells, and when transferred into immune-deficient nude mice suffering from CD25-depleted CD4\(^+\) T cell mediated autoimmunity, are able to suppress inflammation in a dose-dependent manner (Sakaguchi et al., 1995). Further work has corroborated these results, demonstrating that even in established inflammatory disease, transfer of CD25-expressing Tregs potently ameliorates inflammation and cures autoimmunity (Mottet et al., 2003). In both mice and humans, these cells have also been shown to constitutively express the lineage-defining transcription factor Forkhead box P3 (FOXP3) (Hori et al., 2003) which is necessary for their function (Allan et al., 2008a). Furthermore, numerous studies have shown that FOXP3-expressing Tregs can be divided into two distinct subsets: naturally occurring Tregs (nTregs) which develop in the thymus via central tolerance mechanisms, and peripherally-induced Tregs (iTregs) which differentiate under tolerogenic conditions in peripheral lymphoid tissues (Curotto de Lafaille and Lafaille, 2009; Horwitz et al., 2008a). Both subsets of FOXP3\(^+\) Tregs display similar phenotypes, including an inability to produce effector cytokines such as IL-2, IFN-\(\gamma\), or IL-4, and are believed to suppress other cells in a contact-dependent manner that has
not been well characterized (Shevach, 2009; Horwitz et al., 2008a). Of note, a third distinct subset of Tregs, referred to as Type 1 regulatory (Tr1) cells, have been identified. However these cells do not constitutively express FOXP3 and are induced in the periphery in the presence of IL-10 and/or specialized subsets of antigen presenting cells (Roncarolo et al., 2006). In contrast to FOXP3\(^+\) Tregs, there is currently no known lineage-defining transcription factor for Tr1 cells, and they are identified solely on the basis of their cytokine production profile (IL-10\(^+\)IL-4\(^-\)IFN-\(\gamma\)\(^{low}\)) as well as their IL-10-dependent, contact-independent suppression of immune responses (Roncarolo et al., 2006). Because of the fundamental differences between the suppressive mechanisms of FOXP3\(^+\) Tregs and Tr1 cells, as well as the inherent difficulty in studying in vivo differentiated Tr1 cells, since they lack a lineage specific marker, most research in the field of regulatory T cells has focused on the former FOXP3-expressing subsets.

1.1.1 Natural thymus-derived FOXP3\(^+\) T regulatory cells

FOXP3 expressing natural Tregs are produced in the thymus as a functionally distinct subset of T cells (Sakaguchi et al., 2006). Upon egress from the thymus, these cells express the naïve T cell marker CD45RA, and continue to do so until they encounter their cognate antigen and become activated in the periphery (Valmori et al., 2010). Their T cell receptor repertoire, the antigens that are recognized by the T cell receptor (TCR), is diverse and most similar to that of conventional thymus-derived T effector cells (Sakaguchi et al., 2006). Typically, nTregs recognize auto/self antigens presented in the thymus during T cell development, and require high affinity MHC-peptide interactions as well as strong CD28 mediated co-stimulation for their generation (Horwitz et al., 2008a). Furthermore, this generation is not dependent on the growth enhancing cytokines interleukin-2 (IL-2) or TGF-\(\beta\), both essential in iTreg formation, as evidenced by the presence of nTregs in the thymus of mice deficient for these factors (Horwitz et al., 2008a). However, IL-2 and TGF-\(\beta\) are required for the continued maintenance and survival
of nTregs in vivo, as well as for the stable expression of FOXP3 once in the periphery (Fontenot et al., 2005; Marie et al., 2005).

Following their generation in the thymus, nTregs traffic throughout the body and mediate suppression in both lymphoid and peripheral tissues when activated through their TCR (Shevach, 2009). Once activated, these cells do not require TCR restimulation or matching of major histocompatibility complex (MHC) to mediate suppression, and can even influence other cells within their vicinity to adopt a suppressive program, termed infectious tolerance (Shevach, 2009; Kendal and Waldmann, 2010). Suppression has been shown to be contact dependent and relies, in part, on the inhibitory cytokines IL-10, IL-35, and TGF-β (Vignali et al., 2008). Of note, it is likely that in order for soluble factors to mediate suppressive effects, a gradient must be established, which can only occur when nTregs are in close proximity to their target cells. Apart from cytokines, nTregs have been shown to suppress via a variety of other mechanisms, both soluble and otherwise, including: metabolic disruption by IL-2 consumption, cytolysis mediated by granzyme and/or perforin production, and inhibition of dendritic cell maturation and function via expression of inhibitory surface receptors such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Vignali et al., 2008; Shevach, 2009). In addition, tumor necrosis factor-alpha (TNF-α) signaling through the type II tumor necrosis factor receptor (TNFR-2) has been shown in mice to be particularly important for the suppressive effects of nTregs, but interestingly not iTregs (Housley et al., 2011). This suggests there may be a fundamental difference between these subsets in terms of suppression. Overall, although the exact mechanisms of suppression employed in various in vivo settings is unclear, it is likely that nTregs are able to tailor their response based on the inflammatory milieu encountered.
1.1.2 Induced FOXP3+ T regulatory cells

Early evidence to suggest that FOXP3+ Tregs can be generated outside of the thymus originated from experiments demonstrating that injection of polyclonal CD4+CD25- T cells into lymphopenic mice led to the generation of cells that had acquired CD25, CTLA-4, and FOXP3 expression, as well as suppressive activity (Curotto de Lafaille and Lafaille, 2009; Furtado et al., 2002; Curotto de Lafaille et al., 2004). Studies since then have shown that Tregs can be readily generated in the periphery in both mouse and humans, and that these cells represent a distinct subset of FOXP3+ Tregs, termed iTregs (Curotto de Lafaille and Lafaille, 2009). Along with their generation in vivo, iTregs can be generated in vitro when conventional CD4+ T cells are cultured in the presence of TGF-β and IL-2, along with TCR stimulation (Chen et al., 2003). This has allowed for the study of this specific Treg subset, and in particular an evaluation of differences between iTregs and nTregs. For example, iTregs are highly dependent on IL-2 for their generation, whereas nTregs only require IL-2 for their long-term maintenance (Shevach, 2009). Furthermore, unlike nTregs, which require CD28 mediated co-stimulatory signals, iTreg generation is more dependent on the upregulation of CTLA-4 (Zheng et al., 2006). In fact, CD28 costimulation has been shown to actually inhibit generation of iTregs (Benson et al., 2007). In addition, the iTreg TCR repertoire typically recognizes non-pathogenic, but non-self, antigens such as those found in allergens or commensal microbiota (Shevach, 2009). This suggests that iTregs may be generated in the periphery to control chronic inflammation directed against non-harmful antigens that are encountered throughout the human lifespan.

In terms of suppression, initial studies of in vitro iTregs suggested that this subset was less suppressive then their nTreg counterparts (Horwitz et al., 2008a). However, murine in vivo iTregs have been shown to be effective suppressors of disease (Horwitz et al., 2008a). Regardless of their potency, iTregs likely employ similar suppressive mechanisms as nTregs, although some nuances in their function do exist. For example, unlike nTregs, iTregs do not...
depend on TNF-α signaling for their effects (Housley et al., 2011) nor can their suppressive capacity be blocked by the presence of the Th17 associated cytokine IL-6 (Zheng et al., 2008). Furthermore, due to their dependence on the upregulation of CTLA-4, it can be hypothesized that this subset likely relies more heavily on inhibition of dendritic cell maturation.

Concerning the comparison of nTregs and iTregs, it is important to note that the majority of these studies have been conducted in mice or with iTregs that have been induced in vitro. Because of a lack of markers to identify in vivo iTregs in humans, a true comparison of these distinct Treg lineages has not been possible. These studies are necessary since evidence in mice suggests that iTregs may behave very differently in the in vivo setting as opposed to in in vitro assays. This is highly relevant to the development of Treg cellular therapy as although all Tregs are ultimately defined by their ability to suppress immune responses, nTregs and iTregs may differ in their biology and thus each may have distinct advantages as therapies for mucosal diseases.

1.1.3 Identification of T regulatory cell subsets in humans

In order to appropriately compare the biological function of nTregs and iTregs in humans and assess their unique advantages in a cellular therapy setting, identification of distinct markers that permit the isolation of individual subsets is necessary. This feat has proven challenging, as even markers to identify the Treg lineage as a whole have not been effectively identified. This is because, in humans, Treg associated markers such as CD25, FOXP3, glucocorticoid induced tumor necrosis factor receptor (GITR), and CTLA-4 are upregulated in conventional T cells upon activation (Allan et al., 2007) making it difficult to distinguish Tregs from activated effector cells particularly in a disease setting. Recently, a method has been described that allows for identification of Tregs subsets based on the methylation state of CpG residues in an evolutionarily conserved region in the FOXP3 locus. This region is located upstream of FOXP3
exon 1 and is referred to as the Treg cell-specific demethylated region (TSDR). Natural FOXP3+ Tregs are demethylated at all residues in this region allowing for potent FOXP3 gene expression, whereas *in vitro* iTregs display only partial demethylation (Floess *et al*., 2007). Because iTregs are still able to express FOXP3 at the protein level, partial methylation of the TSDR region suggests that this subset, although able to function as suppressor cells in the short term, may represent a less stable Treg lineage. However, of note, *in vitro* iTregs are generated in a system that lacks the environmental cues that may be encountered *in vivo*, and these “artificial” iTregs may not necessarily share the same characteristics of *in vivo* generated human iTregs. Indeed, when *in vivo* iTregs in mice were examined for TSDR methylation state, it was found that these cells exhibit similar methylation patterns to natural Tregs (Polansky *et al*., 2008). This supports the idea that, in humans, *in vivo* iTregs may also display similar methylation patterns to that of nTregs, and this technique will not be useful for subset identification. However, without additional markers for the differentiation of nTreg and iTreg subsets, it will be difficult to conduct these studies.

Along with the methylation state of the FOXP3 TSDR, a recent report has described expression of Helios, an Ikaros family transcription factor, as a marker of natural, thymic derived FOXP3+ Tregs and not of iTregs (Thornton *et al*., 2010). In this study, Helios was expressed in 70% of FOXP3+ Tregs in both mice and humans, and was restricted to cells originating from the thymus (Thornton *et al*., 2010). Importantly, *in vitro* iTregs and *in vivo* iTregs in mice were found to be FOXP3+ but Helios- in all cases. However, since this report, a number of groups have disputed the use of Helios as a marker of nTregs, particularly in humans (Akimova *et al*., 2011). This is because Helios can be induced by T cell activation, much like other markers expressed by Tregs, and is associated with proliferation (Akimova *et al*., 2011). Overall, the use of Helios as an identifying marker for human natural and induced Tregs is controversial, and further investigation into this is warranted.
1.2 T regulatory cell therapy

Once markers of FOXP3+ Treg subsets are identified, and the basic biology of this cellular subset as a whole is explored, the next step will be to apply our knowledge to develop a cellular therapy regimen that can be used in chronic inflammatory settings such as transplantation and autoimmune diseases. In terms of Treg cellular therapy, the hope is that boosting this natural mechanism of tolerance will offer a replacement for the broad-spectrum immunosuppressive drugs that are often ineffective and carry the risk of promoting cancer and/or infections. In practice, Treg cellular therapy involves removal of Tregs from patient or donor peripheral blood, expansion of the populations, and injection of these expanded cells into the patient. Indeed, this regimen is already being tested in the context of allogeneic hematopoietic stem cell transplantation (HSCT) for hematological malignancies. These patients are at a high risk for life-threatening graft versus host disease (GVHD) and/or mortality, making this an appropriate setting for early trials of experimental therapies. In a Phase I/II clinical trial conducted in 2010, 23 patients with advanced or high-risk hematologic malignancy received an infusion of nTregs isolated from partially HLA-matched umbilical chord blood (Brunstein et al., 2010). These patients showed a reduced incidence of grade II-IV acute GVHD compared to historical controls with no deleterious affect on infection risk, relapse, or early mortality (Brunstein et al., 2010).

Further Phase I/II clinical trials are continuing to evaluate whether infusion of Tregs might ameliorate GVHD following HSCT (Edinger and Hoffmann, 2011; McMurchy et al., 2011; Engelhardt et al., 1998; Di Ianni et al., 2011; Brunstein et al., 2010) and these trials have so far shown that infusion of Tregs is safe and possibly efficacious. As work continues in this field and clinical regimens are improved, we can begin to consider a variety of other scenarios in which Treg therapy may be beneficial. One such setting would be that of inflammatory bowel disease.
(IBD). This is because pioneering studies performed by the Powrie group initially showed the benefit of Treg adoptive transfer in a mouse model of intestinal inflammation, with Tregs preventing T cell driven colitis (Powrie et al., 1994; Powrie et al., 1993). In later studies by the same group, it was shown that Tregs can in fact cure previously established colitis in mice (Mottet et al., 2003). This suggests that the use of Treg cellular therapy in the context of IBD may be effective, and further investigation into the etiology of this disease would be beneficial to comprehensively evaluate this strategy.

1.3 Inflammatory bowel disease

Crohn’s disease (CD) and ulcerative colitis (UC), the two most common forms of inflammatory bowel disease (IBD), are idiopathic immune mediated inflammatory disorders characterized by chronic relapsing inflammation of the intestinal tract, which causes a range of symptoms including abdominal pain, severe diarrhea, rectal bleeding, and weight loss or poor weight gain (Cho, 2008; Strober et al., 2007). IBD most commonly emerges in young adulthood, although can present at any age, and is highly prevalent in developed regions of the world including Canada (Loftus, 2004). A report from the Crohn’s and Colitis Foundation of Canada (CCFC) in 2008 estimates that 1 out of 160 people in Canada have IBD, and over 700 million health care dollars are spent each year for the care of these individuals (CCFC, 2008). CD and UC are in part distinguished by the tissues affected: CD can affect any region of the gastrointestinal tract in a discontinuous and transmural fashion; whereas UC is restricted to the surface lining of the colon, contiguous with the rectum (Cho, 2008; Strober et al., 2007). In addition, CD tends to be associated with the development of obstructive stricturing, known as fistulæ, whereas UC is not (Strober et al., 2007). Although the specific causes of IBD are difficult to pinpoint, it is believed that a variety of factors play a role, including the environment, genetic factors, and the microbial communities that naturally colonize the gut (Khor et al., 2011).
Current treatment regimens, including anti-inflammatory and immunosuppressive agents, are not curative and only reduce the intestinal inflammation associated with disease (Strober et al., 2007). It is because of this that new strategies, such as Treg cellular therapy, for the treatment and cure of this disease are necessary. However, first we must understand the role of T cells in IBD and whether boosting immunoregulation specifically in the gut would be beneficial in this setting.

1.3.1 The role of T cells in IBD

Although the etiology of IBD is unclear, both CD and UC are believed to be T-cell driven processes. Evidence for this can be drawn from studies showing that transfer of CD4+ T cells alone from normal mice into immune deficient mice (which lack Tregs) leads to severe Th1 cell driven colitis (Powrie et al., 1993; Mottet et al., 2003). Along with T cell transfer models of colitis, Tconv cells have also been shown to contribute to a number of other mouse models of IBD including the disease observed in IL-10 deficient and STAT4 transgenic mice (Maynard and Weaver, 2009). In humans, correlative data suggests that CD is driven by exaggerated Th1 and Th17 responses, since inflamed lesions contain increased levels of Th1 and Th17 associated cytokines including IFN-γ, TNF-α, IL-12, IL-17 and IL-18 (Bouma and Strober, 2003; Xavier and Podolsky, 2007; Eastaff-Leung et al., 2010; Pene et al., 2008; Brand, 2009). In contrast, UC is associated with a Th2 cytokine profile, and patients have high levels of IL-13 in the mucosa compared to CD patients or healthy controls (Brand, 2009; Heller et al., 2005; Danese and Fiocchi, 2011). Studies using anti-CTLA4 antibodies for the treatment of refractory melanoma have also shown that when the ability to suppress T cell activation through CTLA4 engagement is blocked, IBD-like intestinal inflammation results (Lord et al., 2011).

Gene wide association studies (GWAS) have also provided evidence to suggest that T cells play a role in propagating IBD. For example, an allele found in the IL23R gene has been
associated with significant protection from the development of CD (Xavier and Rioux, 2008). Although the exact mechanism of this protection is unknown, IL-23 does play an important role in the generation and maintenance of Th17 cells (Xavier and Rioux, 2008). In addition, a non-synonymous amino acid change of alanine to threonine at position 197 in \textit{ATG16L1} is also associated with the development of CD (Xavier and Rioux, 2008). ATG5, a protein required for lymphocyte development and signaling, is known to form a complex with ATG16L1 and thus it may be that individuals carrying the risk allele may have altered lymphocyte function.

In addition to GWAS, the success of a variety of treatment regimens for IBD has provided insight into the mechanisms contributing to disease. In particular, monoclonal antibodies directed against tumor necrosis factor-alpha (TNF-\(\alpha\)), a T cell derived cytokine, have proven effective in ameliorating inflammation in both CD and UC (Osterman and Lichtenstein, 2007). Further support for the role of TNF-\(\alpha\) producing T cells in IBD can be drawn from studies showing that mouse T cells engineered to over-express TNF-\(\alpha\) \textit{in vivo} are sufficient to cause IBD-like intestinal inflammation (Kontoyiannis \textit{et al.}, 2002). Antibodies that bind the \(\text{p40}\) subunit of IL-12, neutralizing the T cell derived cytokines IL-12 and IL-23, are also effective in the treatment of CD, again demonstrating the importance of T cells in IBD (Mannon \textit{et al.}, 2004). More recently, both allogeneic and autologous stem cell transplants in patients with haematological malignancy along with IBD have resulted in disease inactivity despite the discontinuation of immunosuppressive treatments (Hawkey, 2000). This discovery has lead to the establishment of Phase I/II clinical trials using stem cell transplantation specifically for the treatment of CD, with the idea that this regimen reduces disease severity by eliminating autoreactive T cells (Swenson and Theise, 2010).

Although T cells are thought to propagate inflammation in IBD, it is likely that this disease is not due to a primary T cell dysfunction. Instead, a number of factors contribute to the initiation of T cell driven pathology in IBD including: breakdown of the intestinal mucosal
barrier, dysregulation of the endoplasmic reticulum (ER) stress response, and primary innate immune cell dysfunction. Specifically, the intestinal barrier is known to play a key role in mediating immune homeostasis, and indeed is often disrupted in patients with IBD (Laukotter et al., 2008). Because intestinal epithelial cells promote tolerance and Treg induction through secretion of the vitamin A metabolite retinoic acid and the cytokines thymic stromal-lymphopoietin (TSLP) and TGF-β, a disruption in their function can lead to dysregulated T cell responses (Khor et al., 2011). In addition, UC patients often exhibit a defect in the ER stress response pathway in colonic epithelial cells (Khor et al., 2011; Treton et al., 2011). This leads to an increase in IL-23 production, which can induce IL-17 secretion from CD4+ T cells and Th17 cell development (Goodall et al., 2010). Defects in innate immunity may also contribute to dysregulated T cells responses. CD patients have reduced neutrophil function and gross impairment of macrophage inflammatory cytokine secretion (Smith et al., 2009); this deficiency in the primary innate immune response has been hypothesized to allow for impaired clearance of ingressed gut bacteria that leads to secondary T cell driven chronic inflammation (Smith et al., 2009). Impaired clearance of bacteria is further implicated in the initiation and/or pathogenesis of IBD as gene-wide association studies (GWAS) have found that alleles found in genes responsible for bacterial recognition and clearance, such as NOD2, IL-23 receptor, and ATG16L1, are associated with disease (Khor et al., 2011). Thus, although in most cases T cell dysfunction is unlikely to be the initiating cause of IBD (Saleh and Elson, 2011), there is substantial evidence that dysregulated T helper cell responses perpetuate the disease and the cycle that leads to chronic inflammation.

1.3.2 T regulatory cells in IBD: what is known

Along with aberrant T cell responses, there is evidence that simply lacking Tregs leads to intestinal inflammatory disease. Patients with genetic mutations in FOXP3 who have non-
functional or absent Tregs always have severe intestinal inflammation associated with lymphocytic infiltration of the intestinal mucosa (McMurchy et al., 2011; Bacchetta et al., 2006). Similarly, mice lacking FOXP3+ Tregs (Fontenot et al., 2005), or the ability to suppress via Treg-derived cytokines such as IL-10 (Huber et al., 2011), IL-35 (Collison et al., 2007), and in some cases TGF-β (Konkel and Chen, 2011), are not protected from colitis. In the more common forms of IBD, however, there is little evidence to suggest that patients simply lack Tregs in the circulation and/or the affected tissues. Maul et al. found that although both CD and UC patients had decreased Treg populations in the peripheral blood during active disease, Treg numbers in intestinal tissue biopsies were not substantially different from those in patients with other inflammatory diseases (Maul et al., 2005). Other studies corroborate these results, and in most cases show a consistent expansion of Tregs in both inflamed and non-inflamed sections of the gut in adult and pediatric patients with IBD (Reikvam et al., 2011; Holmen et al., 2006; Yu et al., 2007). Notably, the majority of these studies did not take into consideration the possibility that FOXP3 expression can also identify activated effector T cells (Roncarolo and Gregori, 2008) or the possibility that FOXP3-expressing cells can trans-differentiate into inflammatory cytokine producing cells that may or may not retain their suppressive function (Zhou et al., 2009). Thus the question of whether or not Tregs are numerically deficient in IBD is unclear and will await re-investigation using more comprehensive panels of cell surface markers and cytokines as they are developed.

There is also little evidence to support the possibility that intestinal Tregs are dysfunctional in IBD since Tregs isolated from the intestinal mucosa of IBD patients are suppressive in vitro (Maul et al., 2005; Holmen et al., 2006). On the other hand, there is evidence that Tregs from inflamed colonic tissue undergo apoptosis more readily than Tregs found in non-inflamed tissue, possibly rendering the Tregs less effective (Veltkamp et al., 2011). It is important to note, however, that the functional Treg assays in these studies were performed
using non-specific antigen stimulation in conditions lacking many of the cytokines that would be found in the inflamed intestinal environment. Moreover, to date only suppression of T cell responses has been examined, and the possibility that Tregs from IBD patients may lack the ability to suppress other cell types, such as antigen presenting cells or B cells, has yet to be investigated. Thus whether or not the inflamed mucosal environment renders Tregs dysfunctional and hence what would happen to Tregs—i.e. would they remain suppressive—if they were administered as a cellular therapy remains unknown. An answer to this will in part require further investigation into the basic biology of Tregs and the potential for plasticity of known Treg subsets.

If the inflamed intestine has a normal number of Tregs, which, at least in vitro, appear to be functional, then why are they unable to suppress inflammation? In other autoimmune diseases, including type 1 diabetes and multiple sclerosis, there is extensive evidence suggesting that the defect in immune regulation lies within the effector cell/inflammatory environment and not the Tregs themselves (Long and Buckner, 2011). In IBD the question of whether effector T cells show abnormal resistance to suppression has not yet been comprehensively studied but there are some studies suggesting this may be the case; in colitic mice and humans, effector T cells can be resistant to Tregs if they become insensitive to TGF-β-mediated suppression (Monteleone et al., 2001; Fantini et al., 2009). However, in order to fully evaluate the phenotype of colitogenic T cells and their potential for Treg mediated suppression, we must first develop better methods to identify these cells. Once this is established, we can begin to explore the potential for Treg mediated cellular therapy in the setting of inflammatory bowel disease.

### 1.3.3 Steps toward Treg therapy in IBD

The intestinal mucosal tissues pose a unique challenge for the maintenance of immune homeostasis. Representing the largest mucosal surface area in the body, these tissues are in direct
contact with the external environment and must simultaneously maintain tolerance to commensal bacteria and/or food and the ability to eliminate pathogens (Izcue and Powrie, 2008). Furthermore, the gut must be permeable to allow for nutrient absorption but must also maintain a tight barrier against pathogens. The gut has thus developed a complex immune network that can process and respond to an enormous number of stimuli at one time. This network includes intestinal epithelial cells, macrophages, B cells, dendritic cells, conventional T cells, and Tregs, with the latter believed to be critical for the maintenance of intestinal immune homeostasis (Izcue et al., 2009). Under normal physiological conditions, compared to all other tissues, the intestinal lamina propria has the greatest proportion of CD4+ Tregs (Maynard et al., 2007), which are thought to be primarily specific for antigens in food and commensal flora (Saleh and Elson, 2011). Since CD and UC are both T cell driven diseases, it logically follows that increasing appropriate Treg activity in the gut should help restore the balance of suppression in inflamed tissues. However, in order to properly investigate this therapeutic option, an understanding of basic Treg function and identification of the appropriate Treg subset to employ in this strategy is necessary. In addition, recent work in the field of transplantation and autoimmunity has shown that antigen specific Tregs are much more effective at preventing graft rejection or diabetes than are polyclonal populations (McMurchy et al., 2011). Significantly fewer antigen-specific Tregs are required to mediate potent suppression, and the delivery of antigen-specific cells decreases the risk of global immunosuppression and the possibility of increased risk of infection and cancer. Notably, antigen-specific Tregs can prevent colitis, as demonstrated by the adoptive transfer of ovalbumin(OVA)-specific Tregs (Zhou et al., 2004) or OVA-specific Tr1 cells (Groux et al., 1997) into OVA-TCR-Tg mice fed ovalbumin. However, since ovalbumin is unlikely to be a disease-driving antigen in IBD, the question of whether OVA-specific Tregs would be effective at suppressing established effector responses directed at pathogenic antigens...
remains outstanding. Thus, when considering the use of Treg cellular therapy in the context of IBD, one must also consider putative antigens that could be targeted by this strategy.

1.3.4 Flagellin as a putative antigen involved in IBD

In order to develop antigen-specific Treg therapy in IBD at least some of the dominant antigens that perpetuate effector T cell responses in the intestine need to be identified. This will not only allow for effective Treg targeting, but also enable evaluation of antigen-specific colitogenic T cells for their capacity to be suppressed. Using T cell clones isolated from IBD patients, Duchmann et al. found that many of the clones were specific for commensal gut flora, including species of Enterobacteriaceae, Bacteroides, and Bifidobacterium (Duchmann et al., 1999). Corroborating these data, Cong et al. found that T cells specific for enteric bacterial flora drive disease in spontaneously colitic C3H/HeJ Bir mice (Cong et al., 1998). It was subsequently demonstrated that bacterial flagellin, a protein present on all flagellated bacteria including commensal species found in the gut, is a dominant antigen in these mice. Flagellin is the major structural protein of bacterial flagella, the organelles of motility. In mammals, flagellin interacts with Toll-like receptor 5 (TLR5) leading to MyD88-dependent activation of NF-κB and MAP kinases, and production of pro-inflammatory cytokines and chemokines (Khan et al., 2004; Hayashi et al., 2001; Gewirtz et al., 2001). It can also bind the innate pattern recognition receptor NLRC4 (Ipaf) leading to IL-1β and IL-18 processing via the inflammasome, although this pathway is less well characterized and its effects can be partially blocked by TLR5 signalling (Vijay-Kumar et al., 2010; Carvalho et al., 2011). The flagellin protein itself consists of highly conserved N- and C-terminal domains (termed C1 and C2, respectively) that form a hairpin structure with an intervening hypervariable domain. TLR5 agonist activity resides in the conserved domains (Donnelly and Steiner, 2002; Smith et al., 2003; Murthy et al., 2004). While the in vivo expression pattern of TLR5 remains controversial due to its low copy number in cells
and a lack of sensitive detection antibodies, many human and murine cell types respond vigorously to flagellin, including intestinal epithelial cells and professional immune cells, such as T cells (Bambou et al., 2004; Mizel et al., 2003; Uematsu et al., 2006; Maaser et al., 2004; Vijay-Kumar et al., 2008; Crellin et al., 2005). Like other TLR agonists, flagellin also acts at the interface between innate and adaptive immunity, providing a co-stimulatory signal to dendritic cells (DCs) that facilitates their maturation and antigen presentation to T- and B-lymphocytes in a pro-inflammatory rather than tolerogenic fashion (Didierlaurent et al., 2004; Sanders et al., 2006; Sanders et al., 2008; Vicente-Suarez et al., 2009; Bates et al., 2009). Indeed, TLR5 stimulation is enough to protect mice from *Clostridium difficile* (*C. difficile*) infection likely via these pro-inflammatory effects which delay *C. difficile* growth and toxin production (Jarchum et al., 2011). However, unlike other TLR agonists, flagellin is a protein and thus it not only acts via TLR5, but also has many antigenic epitopes recognized by the adaptive immune system.

Following the identification of anti-flagellin antibodies in colitic mice, responses to flagellin were examined in human subjects (Lodes et al., 2004). These studies found that antibodies to CBir1, the flagellin found to be dominant in colitic mice, as well as Fla2, a flagellin derived from *Lachnospiraceae* bacteria and closely related to CBir1, are present in about half of patients with CD. Since this discovery, the presence of CBir1 antibodies has become an important serodiagnostic tool for CD and is part of commercially licensed antibody testing panels for aiding in IBD diagnosis and prognosis prediction (Prometheus® IBD Serology 7, Prometheus® Crohn’s Prognostic, and Prometheus® IBD sgi Diagnostic Test; Prometheus Therapeutics and Diagnostics, San Diego, CA). Because CBir1 and Fla2 are both found on commensals present in the normal gut environment, one could hypothesize that perhaps antibodies directed against these antigens may be harmful and even propagate the inflammation observed in IBD. The strongest evidence for this possibility comes from one study showing that mice hyper-immunized with *Salmonella* flagellin (FliC), or infused with anti-FliC antibodies
from other immunized mice, were hyper-susceptible to chemical colitis induced by dextran sodium sulfate (DSS). This effect required expression of the neonatal Fc receptor (FcRn) on myeloid cells, suggesting that anti-flagellin antibodies are bound and internalized by antigen-presenting cells (APCs) bearing FcRn, leading to enhanced processing and antigen presentation of flagellin to T cells, resulting in a worsening of colitis (Kobayashi et al., 2009). Together, these observations actually suggest that it is activation of flagellin-reactive T cells downstream of anti-flagellin antibodies that may be more critical to the pathogenesis of CD. Therefore, further investigation into the role flagellin plays in IBD and in particular the T cells that recognize flagellin is important and may allow for the effective development of antigen targeted Treg cell therapy for this disease.

1.4 Synopsis of research questions

In order to take Treg cellular therapy from bench to bedside, a number of considerations are necessary. First, we must better understand differences in natural and induced Treg subsets, and identify markers that can be used to isolate these subsets from human peripheral blood. I hypothesized that the transcription factor Helios would indeed be useful in differentiating nTregs and iTregs in humans, and that there would be a difference in their ability to trans-differentiate in polarizing conditions. To this aim, I evaluated Helios⁺ and Helios⁻ Treg subsets isolated from human peripheral blood for their phenotype, suppressive capacity, and plasticity potential. In addition, I examined human CD4⁺ T cell populations in order to determine whether Helios can be used as a marker to differentiate nTregs from in vivo iTregs in humans. I also evaluated Helios expression, suppressive capacity, and potential for plasticity in human in vitro iTregs in order to determine if Helios⁻ Treg clones are representative of in vivo iTregs.

A second consideration in the translation of Treg therapy is the basic biological function of Tregs. Although the body of literature concerning these cells is growing, there is much to be
determined and an in-depth understanding of the normal function of Tregs is key to their effective use in a clinical setting. These studies were driven by the hypothesis that human FOXP3+ Tregs have the capacity to secrete chemokines and consequently recruit immune cells. To this aim, I investigated the capacity of Tregs to produce chemokines. In particular, I focused on the production of CXCL8, a chemokine that potently attracts neutrophils, as well as the relationship between FOXP3 and CXCL8 gene expression. Following these studies, I performed a comprehensive evaluation of chemokine production by both Tregs and Tconv cells at the protein and mRNA level to elucidate any differences in these subsets. I also evaluated the biological function of Treg derived chemokines by determining if Tregs can induce neutrophil chemotaxis. With a better understanding of the non-suppressive biological functions of Tregs, including chemokine production, we can begin to appreciate the role this lineage may play in the complete immune response and apply this to cellular therapy.

To further apply Treg cellular therapy to disease, a basic understanding of what antigens are driving the inflammatory response is beneficial. This allows for an antigen-targeted therapy that foregoes the complications encountered when using globally immunosuppressive treatments. Because pioneering studies in Treg adoptive cell therapy were conducted in a mouse model of intestinal inflammation, and these studies demonstrated the effective use of this strategy even in established disease, I theorized that human IBD may be an appropriate next-step for this technology. Consequently, I evaluated flagellin, a bacterial product reported to play a role in IBD, as an antigen in this disease. Flagellin has been shown to exacerbate inflammation in an animal model of colitis, however the mechanism of this is unclear. Accordingly, I examined if flagellin mediated exacerbation of disease is dependent on its TLR5 stimulatory capacity or its antigenic properties, hypothesizing that its role as an antigen is what drives inflammation in IBD. If it is indeed the antigenic properties that propagate the immune response, Treg therapy targeting this antigen may be particularly beneficial. Following mouse studies of
flagellin, I sought to identify flagellin-reactive T cells specifically in patients with CD. This was based on reports that 50% of CD patients have detectable levels of anti-flagellin antibodies in their sera, and the hypothesis that these antibodies are likely indicative of the presence of anti-flagellin T cells. To this end, I used a novel method to test for flagellin-reactive T cells in peripheral blood samples from IBD patients and healthy controls. In addition, I sought to correlate the presence of flagellin-specific T cells with disease, as well as with anti-flagellin antibody levels.

The FOXP3+ Treg lineage may someday be a powerful tool in the establishment of immune tolerance in the clinical setting, and thus steps to better understand this T cell subset are warranted. Indeed, with these studies I hope to further our knowledge concerning the basic mechanisms by which these cells function, the individual subsets that contribute to immune homeostasis, and the antigens that are best targeted by Treg cellular therapy. Although there are still many unknowns and theoretical risks, it is the hope that delivery of Tregs will indeed be able to one day reset intestinal immunity and lead to the long-lasting cure of IBD.
2 HELIOS+ AND HELIOS- T REGULATORY CELL SUBSETS ARE PHENOTYPICALLY AND FUNCTIONALLY SIMILAR AND HAVE AN EQUAL POTENTIAL FOR PLASTICITY

2.1 Introduction

A major hindrance to the field of Treg cell study is the inability to identify these cells based on a set of defined markers that are unique to the Treg lineage and are not dependent on the life-cycle or activation state of these cells. Indeed, the potential for Tregs as a therapeutic option in transplantation and inflammatory disease may never be fully realized until these suppressor cells can be robustly isolated in a manner that ensures purity and viability. To this end, a number of groups have focused on identifying markers, which, in conjunction with FOXP3, can be used to further define the Treg lineage explicitly, and differentiate these cells into subsets based on phenotypic and functional characteristics. Furthermore, because in vitro studies have shown that nTregs and iTregs differ in the suppressive mechanisms that they employ and in antigen repertoire, it may become increasingly important to effectively differentiate these subsets for further study and clinical use. For example, it may be found that iTregs are most effective at suppressing inflammation driven by foreign antigens, such as in food allergy, as opposed to nTregs which may be more advantageous in the setting of autoimmune diseases driven by autoantigens, such as diabetes. Although a body of knowledge does exist regarding the phenotype and function of iTregs, these data have been primarily generated from mouse models or with human cells generated in vitro. To date, human in vivo iTregs and nTregs have not been directly compared in order to discover differences in their phenotype and/or function. With a deeper understanding of similarities and differences between induced and natural FOXP3+ Tregs in humans, clinical use of these cells may be tailored more appropriately. Thus, investigation into markers that discriminate between human natural or in vivo induced
Tregs specifically, ultimately allowing for a more thorough investigation of their distinctive properties, is warranted.

Recently, Shevach et al. has suggested that the Ikaros family transcription factor Helios, normally involved in lymphoid development and regulation (Rebollo and Schmitt, 2003), is preferentially expressed by both mouse and human thymus-derived natural FOXP3+ Tregs, and not by iTregs (Thornton et al., 2010). This is an important finding— if it holds true— as this could be the first protein to specifically differentiate the nTreg and iTreg subsets. However in contrast to this study, Verhagen et al. have since reported that culture of CD4+ mouse splenocytes in the presence of IL-2, TGF-β, and autologous APCs leads to the generation of FOXP3+ Tregs that co-express Helios (Verhagen and Wraith, 2010). The authors of the original study have since corroborated these results but also suggested that work with neonatal mice, who lack FOXP3+ Helios- cells until day 7 of life, and evidence from mice exposed to recall antigens in the absence of adjuvant, who generate only Helios-FOXP3+ Tregs, supports their original findings (Gottschalk et al., 2010; Thornton and Shevach, 2010). Furthermore, they argue that in vitro differentiation assays are not representative of the in vivo setting and thus Helios is still a valid marker of natural thymus derived Tregs. Other groups have further added to the controversy, as although Helios can directly bind the FOXP3 promoter as well as promote the suppressive function of Tregs (Getnet et al., 2010), it is also upregulated upon T cell activation and expressed at varying levels depending on cellular division status, suggesting it may not be a suitable marker to distinguish nTreg and iTreg subsets (Akimova et al., 2011). Overall, further confirmation is necessary to determine whether Helios can be used as a marker to discriminate between human in vivo iTregs and nTregs.

Once a marker that differentiates human nTregs from in vivo iTregs has been identified, it will be important to evaluate these subsets for differences in phenotypic and functional characteristics including (but not limited to): suppressive capacity, cytokine expression, and
stability. Furthermore, studies that have identified the existence of Helios⁺ and Helios⁻ Treg subsets have not gone on to characterize the functional properties of these cells, and regardless of whether Helios is a natural Treg marker or not, it is important to evaluate any differences between these subsets in order to fully realize their clinical potential. In particular, the ability of each subset to exhibit plasticity— the trans-differentiation of one cell subset to another— is of specific interest as Tregs have been shown to be capable of converting into T helper-like cytokine producing cells when cultured with inflammatory cytokines. Specifically, Hafler et al. have shown that FOXP3⁺ Tregs are capable of adopting a Th1-like phenotype when cultured in IL-12, and these cells secrete IFN-γ as well as exhibit reduced suppressive capacity (Dominguez-Villar et al., 2011). In addition, “Th1-like Tregs” can be identified in patients with relapsing-remitting multiple sclerosis (RRMS) at a greater frequency then in healthy controls (Dominguez-Villar et al., 2011), suggesting that “plastic” Tregs may play a role in disease. Other groups have supported these findings by showing that IFN-γ producing Tregs are also elevated in patients with type 1 diabetes (McClymont et al., 2011), and in an in vivo mouse setting, lethal infection with Toxoplasma gondii can lead to the breakdown of Treg function and promote IFN-γ production from this subset, possibly leading to increased pathology (Oldenhove et al., 2009).

In addition to conversion into Th1-like cells, Tregs are also capable of converting to a Th17 type phenotype in both mice and humans (Xu et al., 2007; Koenen et al., 2008). In mice, this conversion is dependent on IL-6 (Xu et al., 2007), whereas in humans Th17 plasticity is likely dependent on IL-2 and IL-1β (Valmori et al., 2010), with epigenetic modifications occurring (Koenen et al., 2008). Importantly, when investigating natural versus in vitro induced Tregs, a difference in the ability to trans-differentiate into Th17 cells was found (Horwitz et al., 2008a); namely, in mice nTregs are able to convert to Th17 type cells in the presence of TGF-β and IL-6, whereas iTregs were resistant to the effects of these cytokines (Horwitz et al., 2008a). If a similar difference is found in the human setting, it may be appropriate to hypothesize that
iTregs may be more beneficial as a cellular therapy due to their propensity to remain Tregs. However, it is important to note that although Tregs can convert to IL-17 producing cells, in humans, there is controversy as to whether they actually lose their suppressive capacity (Koenen et al., 2008; Beriou et al., 2009). This suggests that even if nTregs are less stable in an inflammatory milieu, they may still mediate suppression and contribute to disease remediation.

Along with plasticity, a second possible difference in nTregs versus iTregs is the methylation state of the Treg cell-specific demethylated region, which is found within the promoter region of the FOXP3 locus. The level of methylation in this region inversely correlates with FOXP3 expression and the ability to maintain suppressive capacity in long-term culture (Curotto de Lafaille and Lafaille, 2009). In thymus-derived natural FOXP3+ Tregs, this region has been reported to be completely demethylated allowing for high FOXP3 expression. In contrast, in vitro iTregs display partial methylation of this region (Curotto de Lafaille and Lafaille, 2009). However, in mice, in vivo iTregs have been reported to be more similar to nTregs in this respect, with a fully demethylated TSDR region (Curotto de Lafaille and Lafaille, 2009; Polansky et al., 2008). If a marker for the isolation of in vivo iTregs is identified, it will be important to evaluate the methylation state of the FOXP3 TSDR region in this subset in order to infer the stability of FOXP3 expression and suppressive capacity. These characteristics will be important when evaluating these cells for their cellular therapy potential.

Because of the outstanding questions surrounding Helios+ and Helios− Treg subsets, as well as their potential use in the comparison of human in vivo iTregs to nTregs, my work has focused on isolating Treg cell clones from both subsets. Following the identification of FOXP3+Helios+ and FOXP3+Helios− Treg cell clones, I characterized these subsets based on marker expression, cytokine and chemokine expression, suppressive capacity, and plasticity potential. I hypothesized that Helios would indeed be useful in differentiating natural and
induced Tregs in humans, and that there would be a difference in their ability to trans-
differentiate in cells that produce pro-inflammatory cytokines.

2.2 Materials and methods

Cell purification. Peripheral blood was obtained from healthy volunteers following written informed consent. All studies were approved by the University of British Columbia Clinical Research Ethics Board. CD4+ T cells for ex vivo flow cytometry and as responder cells were isolated from Ficoll separated PBMC’s using negative selection (EasySep, Stem Cell Technologies). CD4+CD25hiCD45RA+ Tregs (naïve Tregs) and CD4+CD25−CD45RA+ T cells (naïve T conventional cells) were isolated from enriched CD4+ T cells (negative selection), stained with CD4, CD25, and CD45RA (all eBioscience), and then sorted on a FACS Aria with gates set using fluorescence minus one controls. Purity based on CD25 expression (BD Biosciences) was greater than 95%. For induced Treg experiments, CD4+CD25−CD45RA+ T conv cells and CD4+CD25hiCD45RA+ Tregs were obtained from enriched CD4+ T cells (negative selection) stained with CD4, CD25, CD45RO, and CD45RA (all eBioscience) on a FACS aria, by first gating on CD4+CD45RO− cells and then on CD25−CD45RA+ (naïve T conventional cells) or CD25hiCD45RA+ cells (naïve Tregs).

T cell cloning. CD4+ T cells purified from PBMC’s using negative selection were stained with CD4, CD25, CD14, and CD45RA (all eBioscience). Naïve Tregs (CD4+CD14−CD25hiCD45RA+) and naïve Tconv cells (CD4+CD14−CD25−CD45RA+) were then purified using a FACS Aria, with gates set using fluorescence minus one controls. Gates were set stringently (naïve Tregs: gated less then 1.5% of total CD4+, naïve Tconv: gated less then 10% of total CD4+) to ensure purity. Sorted Tregs and Tconv cells were subsequently cloned at 1 cell/well (Treg) or 0.1
cell/well (Tconv) in 96-well round bottom plates in the presence of an allogeneic feeder-cell mixture containing $5 \times 10^5$ PBMCs/ml (irradiated 7,000 RADS) isolated from two separate donors, $5 \times 10^4$ JY cells/ml (an Epstein Barr Virus-transformed lymphoblastoid cell line; irradiated 10,500 RADS), and 0.05 ug/ml Phytohemagglutinin (PHA; Roche). All cultures were performed in complete media (X-Vivo 15 [Cambrex] supplemented with 5% pooled AB human serum [Cambrex], penicillin/streptomycin [Invitrogen] and GlutaMAX [Invitrogen]) with rhIL-2 (100 U/ml; Chiron). Media was replenished every 2-3 days, and after 8 days, one 96-well plate from each cloning was pulsed overnight with $[^3]H$thymidine in order to determine the total number of wells with proliferating cells (the cloning efficiency). At day 14, growing wells were picked and restimulated with an allogeneic feeder-cell mixture consisting of $1 \times 10^6$ irradiated PBMCs/ml from two separate donors, $10^5$ JY cells/ml, 0.1ug/ml PHA, and 600 U/ml IL-2. Medium was again replenished every 2-3 days. Clones were then screened for FOXP3 and Helios expression on Day 10. Helios$^+$ Tregs are defined as having a FOXP3 MFI > 1400 and a Helios MFI >1200, Helios$^-$ Tregs as having a FOXP3 MFI > 1400 and a Helios MFI < 750, and Tconv cells as having a FOXP3 MFI < 800 and a Helios MFI < 300. Helios MFI cutoffs were set in order to compare frequency of Helios$^+$ and Helios$^-$ Treg clones between donors, and based on the observed natural clustering of Helios$^+$ Treg clones. Clones were restimulated as above every 14 days with media replenished every 2-3 days. Clones were used for experiments between days 10 and 14 after restimulation (i.e. in the resting phase).

**In vitro induced Tregs.** CD4$^+$ T cells were enriched from PBMCs using negative selection (RosetteSep, Stemcell Technologies). CD4$^+$CD25$^-$CD45RO$^-$CD45RA$^+$ naïve T cells were isolated by negative selection with CD45RO and CD25 microbeads or by sorting using a FACS Aria, gating on CD4$^+$CD45RO$^-$ cells and then CD25$^-$CD45RA$^+$ cells within that population. Naïve Tregs (CD4$^+$CD25$^{hi}$CD45RA$^+$) were isolated in parallel either by magnetic bead positive
selection for CD25 (Miltenyi microbeads) or by sorting using a FACS Aria, gating on CD4^+CD25^+CD45RO^-CD45RA^+ cells to act as controls. Purified naïve T cells and Tregs were stimulated with L cells (irradiated, 7,500 RADS) at a 1:1 L cell: T cell ratio, and incubated with anti-CD3 (OKT3) at 100ng/ml in complete media containing rhIL-2 (300U/ml). Neutral controls are defined as naïve T cells cultured with L cells, anti-CD3, and IL-2. Induced Tregs are defined as naïve T cells cultured with L cells, anti-CD3, and IL-2, with the addition of TGF-β (10ng/ml) and Rapamycin (109nM). nTregs are defined as naïve Tregs cultured with L cells, anti-CD3, and IL-2, with the addition of Rapamycin (109nM). Media was replenished every 2-3 days and cells were restimulated with L cells and appropriate cytokines on Day 7. Suppressive capacity, cytokine production, and plasticity were assessed at day 6-7 (resting cells), with withdrawal of rhIL-2 for 24h before assays.

**Surface and intracellular flow cytometry.** Analysis of CD4 (eBioscience; Clone 3T4), CD25 (eBioscience; Clone M-A251), FOXP3 (eBioscience; Clone 259D/C7), CD45RA (eBioscience; Clone HI100), CD45RO (BD Biosciences; Clone UCHL1), CD39 (eBioscience; Clone eBioA1), CD31 (eBioscience; Clone WM-59), CCR7 (eBioscience; Clone 3D12), CD62L (eBioscience; Clone DREG-56), Helios (Biolegend; 22F6), Tbet (eBioscience; Clone eBio4B10), CTLA-4 (BD; Clone BNI3), CXCL8 (eBioscience; Clone G265-8), IFN-γ (eBioscience; Clone 4SB3), and Granzyme A (BD Biosciences; Clone CB9) was performed either on ex vivo CD4^+ T cells, induced Tregs, or expanded Treg or Tconv clones. Prior to intracellular staining, cells were stimulated with PMA (10ng/ml) and Ca^{2+} ionophore (500ng/ml) (both Sigma-Aldrich) for 6h with the addition of Brefeldin A (10ug/ml, Sigma-Aldrich) after 2h as described (Broady *et al.*, 2009). Samples were read on a FACSCanto (BD Biosciences) and analyzed using FlowJo Software Version 8.7. Gates for FOXP3^+ cells were set based on fluorescence minus one controls and for cytokines on unstimulated, but stained, samples.
**Cytokine and chemokine production.** T cell clones (5 x 10⁵ /ml) were activated with anti-CD3/anti-CD28-coated beads at 1:8 cell:bead ratio (Invitrogen) for 72h in complete media containing rhIL-2 (100U/ml). Concentrations of IFN-γ and MIP-1α in supernatants were determined using CBA Flex Set according to the manufacturer’s instructions (BD Biosciences).

**Nucleic acid extraction and analysis of DNA methylation.** DNA was extracted from T cell clones and expanded T cells using the DNeasy Blood and Tissue Kit (Qiagen). DNA methylation was determined at the FOXP3 Treg-specific demethylated region (TSDR) within the FOXP3 promoter (Baron et al., 2007). Bisulphite conversion was performed as described (Reiss et al., 2007; Cohen et al., 2011), and converted DNA was used as a template for PCR with PyroMark DNA polymerase (Qiagen). Methylation was analyzed by pyrosequencing. Pyrosequencing primers were designed using PyroMark Q24 software (Qiagen). A total of 45 cycles of PCR were performed, and PCR products were prepared for the pyrosequencing reaction using PyroMark reagents (Qiagen). The pyrosequencing reactions were performed on a Biotage Pyromark Q96 MD Pyrosequencer (Qiagen), and the levels of methylation for each CpG dinucleotide were quantitated using Pyro Q-CpG software (Biotage). A representative 10 of 15 CpG’s within the TSDR region were analyzed.

**Suppression assays.** To test for suppressive capacity of T cell clones and expanded Tregs, CD4⁺ T cells (negative selection) were plated at 16,000 cells/well (in 200ul) in 96-well round-bottom plates, and stimulated with anti-CD3/anti-CD28-coated beads at 1:8 cell:bead ratio in complete medium. Treg and Tconv cell clones or expanded iTregs and nTregs were added in decreasing amounts, starting at a ratio of 1:2 Treg:CD4⁺ T cell. rhIL-2 (100U/ml; Chiron) was added to suppressor cells alone to assess viability. Proliferation was assessed at day 6, after addition of
[\textsuperscript{3}H]thymidine for 16 hours. For \textit{in vitro} induced Treg experiments, suppressive capacity was determined by combining Tregs with freshly isolated CFSE labeled PBMC’s and assessing CD8\textsuperscript{+} T cell proliferation by CFSE dilution on Day 4. Data are expressed as the percent suppression of proliferation compared to CD4\textsuperscript{+} responder cells (Treg clone suppression assays) or CD8\textsuperscript{+} responder cells (\textit{in vitro} iTreg experiments) alone.

\textbf{In vitro polarization of T cells.} To determine the effects of culturing cells in discordant cytokine conditions, 1 x 10\textsuperscript{5} T cell clones or cultured T cells were expanded in neutral conditions or Th1-polarizing conditions. Neutral conditions contained L cells (irradiated 7,500 RADS) at a 1:1 ratio of L cells: T cells, rhIL-2 (1000U/ml), and anti-CD3 (soluble, 100ng/ml). Th1-polarizing conditions contained all components of neutral conditions with the addition of 10ng/ml IL-12 (BD Biosciences). T cells were incubated for 5 days, and then stimulated with PMA (10ng/ml) and Ca\textsuperscript{2+} ionophore (500ng/ml) (both Sigma-Aldrich) for 5h with the addition of Brefeldin A (10ug/ml, Sigma-Aldrich) after 2h as described (Broady \textit{et al.}, 2009). Cells were then stained with CD4, FOXP3, IFN-\gamma, and Tbet and analyzed for IFN-\gamma and Tbet expression using a FACSCanto. Gates were set based on neutral conditions and unstained controls.

\textbf{Statistical analysis.} All analysis for statistically significant differences was performed using the student’s unpaired, 2-tailed \textit{t}-test. \textit{p} values less than 0.05 (indicated by *) were considered significant. All cultures were performed in triplicate and error bars represent the SD unless otherwise indicated.
2.3 Both FOXP3⁺Helios⁺ and FOXP3⁺Helios⁻ Treg subsets can be identified in human peripheral blood

In order to confirm previous findings that human FOXP3⁺ Tregs could be further subdivided into Helios⁺ and Helios⁻ populations, I performed FACS analysis on *ex vivo* CD4⁺ T cells isolated from human peripheral blood. Indeed, within the naïve Treg population (CD4⁺FOXP3⁺CD45RA⁺), both Helios⁺ and Helios⁻ subsets were identified at an approximate ratio of 3:2 Helios⁺:Helios⁻ (n=7) (Figure 2.1A). A small percentage (< 5%, n=7) of naïve Tconv cells (CD4⁺FOXP3⁻CD45RA⁺) were also found to express Helios (Figure 2.1A), which is in accordance with previous findings in humans (Thornton *et al.*, 2010). In order to further analyze these populations for both phenotypic and functional characteristics, FACS sorting was used to isolate pure populations of CD25⁺CD45RA⁺ naïve Tregs and CD25⁻CD45RA⁺ naïve Tconv cells from human peripheral blood CD4⁺ T cells. Gates were set stringently to ensure purity of both populations, and this method consistently results in purities based on CD25 expression of >95% (Figure 2.1B). Following isolation the resulting cells were cloned using limiting dilution assays, as described in Materials and Methods. After clonal expansion, Treg and Tconv cell clones were analyzed for FOXP3 and Helios expression and found to fall into one of three subsets: Helios⁺ Tregs, Helios⁻ Tregs, and Tconv cells (Figure 2.1C). Figure 2.1D shows a representative plot of Helios versus FOXP3 mean fluorescence intensity (MFI) for all clones isolated from a single donor. Categorization of clones was based on FOXP3 and Helios MFI, and Helios⁺ Tregs were found to cluster in all donors. Helios⁻ clones were defined as having a high FOXP3 MFI but falling outside the Helios⁺ Treg cluster. A summary of the frequency of Helios⁺ and Helios⁻ Treg clones from two donors is shown in Figure 2.1E. On average, 89 ± 2% and 11 ± 2% of viable clones were Helios⁺ and Helios⁻ Tregs, respectively. Although this frequency varies from what is expected based on the frequency seen in *ex vivo* CD4⁺ T cells, this discrepancy is likely due to the stringent gating protocols used for sorting, as well as the strict cut-offs in FOXP3 and Helios
MFI used to define each group. Overall, these data demonstrate that Helios$^+$ and Helios$^-$ Treg subsets exist in human peripheral blood, and can be clonally expanded for further analysis.

Figure 2.1 Isolation of Helios$^+$ and Helios$^-$ FOXP3$^+$ Treg clones from human peripheral blood

(A) Naïve CD45RA$^+$FOXP3$^+$ Tregs and CD45RA$^+$FOXP3$^-$ Tconv cells were identified in a population of *ex vivo* enriched CD4$^+$ T cells and analyzed for Helios expression via intracellular staining. Percentages reported are an average of 7 independent experiments. (B) CD4$^+$CD25$^-$CD45RA$^+$ (naïve Tregs) and CD4$^+$CD25$^+$CD45RA$^+$ (naïve Tconv) cells were sorted into pure populations from human peripheral blood CD4$^+$ T cells. Stringent sorting gates were set based in part on FMO controls to ensure purity. Sorted populations were subsequently cloned by limiting dilution and categorized into three populations based on Helios and FOXP3 expression, represented in (C). (D) All viable clones from a single donor plotted on a Helios MFI vs. FOXP3 MFI scatterplot. Helios$^+$ Treg clones are defined as having a Helios MFI > 1200, whereas Helios$^-$ Treg clones have a Helios MFI < 750. Clones with a Helios MFI >750 but <1200 were excluded from analysis. (E) The mean percentage of total clones that expressed Helios (Helios$^+$ Treg) and did not express Helios (Helios$^-$ Treg) from two separate donors, with error bars representing the standard deviation from the mean.

2.4 Helios expression can be induced in both Helios$^+$ and Helios$^-$ Treg subsets through T cell receptor activation

Following isolation of Helios$^+$ and Helios$^-$ Treg clones from human peripheral blood, I sought to determine if Helios expression in these clones was stable, or if it could be altered with
T cell activation; this is because Akimova et al. have suggested that Helios can be upregulated upon stimulation of T cells, and thus is not a good marker of thymic derived Tregs alone (Akimova et al., 2011). To this end, Treg and Tconv cell clones were stimulated with allogeneic feeder cells to induce activation and analyzed for Helios expression on Day 0, 5, 7 and 12 (representative plot shown in Figure 2.2A and summarized in Figure 2.2B). I found that all populations were able to upregulate Helios expression upon activation, with maximal expression reached between Day 5 and Day 7. Helios− Treg clones had a 5 ± 2 fold increase in Helios expression between Day 0 and Day 7, which was significantly greater than the 2.4 ± 0.4 fold increase observed in Helios+ Treg clones in this same time frame (n=2 and n=5, respectively, p=0.04) (Figure 2.2C). Tconv cells also upregulated Helios expression, with a 2.8 ± 0.9 fold change between Day 0 and Day 7. Interestingly, although Helios− Treg clones demonstrated a greater fold change in Helios expression, the total Helios MFI at Day 7 never reached the level expressed by Helios+ Treg clones at rest (i.e Helios MFI of activated Helios− Treg clones was < 1200). These data suggest that although TCR activation does lead to Helios upregulation, two distinct populations of Tregs can be differentiated on the basis of Helios expression intensity.
Figure 2.2 Evaluating Helios expression with T cell activation
(A) A representative clone from each subset (Helios⁺ Treg, Helios⁻ Treg, and Tconv) was activated in the presence of allogeneic feeder cells and then evaluated at Day 0, Day 5, Day 7, and Day 12 for Helios and FOXP3 expression. (B) The change in Helios MFI at Day 0, 5, 7, and 12 is plotted, with each line representing an individual clone. Solid lines are Helios⁺ Treg clones (n=5), thin dotted lines are Helios⁻ Treg clones (n=2), and thick dotted lines are Tconv clones (n=2). (C) The fold increase in Helios MFI is represented for clones shown in (B). Helios⁺ Treg fold increase in MFI vs. Helios⁻ Treg fold increase in MFI is significant, p=0.04.

2.5 Helios⁺ and Helios⁻ Tregs express similar Treg subset markers, but differ in cytokine and chemokine expression

Because Helios expression may allow us to differentiate human in vivo iTregs from thymically derived nTregs and because Helios expressing Tregs have never been phenotypically or functionally evaluated, I aimed to carry out these studies in Treg cell clones. I first sought to determine if Helios⁺ or Helios⁻ Treg clones vary in their expression of previously reported Treg associated proteins including FOXP3, CD39 (an ectoenzyme that degrades adenosine triphosphate (ATP) to adenosine monophosphate (AMP) that is expressed on highly suppressive FOXP3⁺ Treg subsets (Borsellino et al., 2007)) and cytotoxic T lymphocyte antigen-4 (CTLA-4) that is also expressed on suppressive FOXP3⁺ Tregs (Takahashi et al., 2000). I found that both Helios⁺ and Helios⁻ Treg clones express high levels of FOXP3, based on MFI, as well as CD39
and CTLA-4, and that there was no significant difference between subsets (Figure 2.3A). These data suggest that Helios$^+$ and Helios$^-$ Treg clones cannot be further differentiated into previously defined Treg subsets based on expression of these proteins.

Along with proteins that aid in Treg cell function, clones were evaluated for cytokine and chemokine expression either by intracellular cytokine staining or by Cytokine Bead Arrays following TCR stimulation. Although chemokine expression by human FOXP3$^+$ Tregs has not been widely reported in the literature, our group has shown these cells are capable of producing a variety of inflammatory chemokines (explored further in Chapter 3), and thus this attribute was examined in this study. Interestingly, Helios$^-$ Treg clones were found to produce significantly more macrophage inflammatory protein (MIP)-1$\alpha$, an acute inflammatory chemokine that potently attracts and activates neutrophils, as well as IFN-$\gamma$, when compared to Helios$^+$ Treg clones (MIP-1$\alpha$: 2,236±1027pg/ml compared to 584±258pg/ml, respectively, $p=0.01$ and IFN-$\gamma$: 3,815±1851pg/ml compared to 596±212pg/ml, respectively, $p=0.006$, $n \geq 3$) (Figure 2.3B). As expected, Tconv cell clones expressed greater amounts of MIP-1$\alpha$ and IFN-$\gamma$ compared to Treg clones (MIP-1$\alpha$: 12,942±4458pg/ml, IFN-$\gamma$: 11,513±2149pg/ml). In contrast, the Treg clone subsets did not differ in their ability to produce Granzyme A, which is reported to be expressed by Tregs and function in suppression (Czystowska et al., 2010), or CXCL8, also a neutrophil chemoattractant and shown to be expressed by Tregs in Chapter 3 of this thesis (Figure 2.3B). Of note, neither Treg nor Tconv cell clones produced detectable amounts of IL-17 (data not shown, evaluated by intracellular cytokine staining). Overall, increased expression of MIP-1$\alpha$ and IFN-$\gamma$ suggests that the Helios$^-$ Treg subset may be more inflammatory or “Th1-like” then Helios$^+$ subsets and may exhibit decreased suppressive capacity or an increased potential for plasticity under certain conditions.
Figure 2.3 Expression of Treg markers, cytokines, and chemokines by Helios+ Tregs clones, Helios- Treg clones, and Tconv cell clones

(A) Helios+ Treg clones, Helios- Treg clones, and Tconv cell clones were evaluated for expression of the Treg markers FOXP3 (MFI), CD39, and CTLA-4 by flow cytometry. (B) Helios+ Treg clones, Helios- Treg clones, and Tconv cell clones were evaluated for MIP-1α and IFN-γ expression after stimulation with anti-CD3/anti-CD28 beads for 72 hours by Cytokine Bead Array. MIP-1α: p=0.01, IFN-γ: p=0.006. Clones were evaluated for Granzyme A and CXCL8 expression by intracellular staining following stimulation with PMA/Ionomycin for 6 hours, with the addition of Brefeldin A at 2 hours. n≥3 clones for all groups.

2.6 Helios+ and Helios- Treg subsets exhibit similar suppressive capacities

Because Helios- Treg clones were found to produce greater amounts of inflammatory chemokines and cytokines, i.e. MIP-1α and IFN-γ, I hypothesized that this subset would exhibit a reduced suppressive capacity compared to Helios+ Treg clones. This hypothesis was generated in part by literature suggesting that “Th1-like” Tregs isolated from patients with autoimmune disease are less suppressive then cytokine-negative Tregs (Dominguez-Villar et al., 2011). Using in vitro suppression assays, I evaluated the ability of Treg clones to suppress CD4+ T cell proliferation at varying ratios of Tregs:CD4+ T cells. I found that both Helios+ and Helios- Treg clones were able to suppress T cell proliferation in a similar manner at all ratios tested (average
% suppression at 1:2 was 98 ± 4% and 98 ± 2%, respectively, at 1:4 was 93 ± 20% and 98 ± 2%, respectively, and at 1:8 was 80 ± 40% and 70 ± 30%, respectively, n=4) (Figure 2.4A and summarized in Figure 2.4B). Although this data is not in line with published studies on IFN-γ producing Tregs, other groups have found that cytokine producing Tregs do exhibit potent suppressive activity (McClymont et al., 2011; Beriou et al., 2009). Furthermore, our clones were not cultured in, or recently exposed to, an inflammatory milieu and thus may represent a fundamentally different population compared to previously described cytokine producing Tregs.

Figure 2.4 Suppressive capacity of Helios$^+$ and Helios$^-$ Treg clones
To test for suppressive capacity, Helios$^+$ and Helios$^-$ Treg clones were stimulated with anti-CD3/anti-CD28-coated beads in the presence of CD4$^+$ responder T cells at ratios of 1:2, 1:4 and 1:8 Treg:CD4$^+$ T cell. Proliferation of responder cells was assessed at day 6, after addition of $[^3]$H]thymidine for 16 hours. Data are expressed as the percent suppression of proliferation compared to CD4$^+$ responder cells. (A) Percent suppression at all Treg:CD4$^+$ T cell ratios. Each line indicates an individual clone, with closed circles representing Helios$^+$ Treg clones and open circles representing Helios$^-$ Treg clones. (B) Percent suppression for all Helios$^+$ and Helios$^-$ Treg clones is summarized at each ratio of Treg:CD4$^+$ T cell, n=4.

2.7 Helios$^+$ and Helios$^-$ Treg subsets display an equal potential for plasticity
In order to determine if Helios$^+$ or Helios$^-$ Tregs differ in their ability to transdifferentiate into IFN-γ producing cells in Th1 conditions, I cultured clones in IL-12 containing media for 5 days and then analyzed IFN-γ expression levels by intracellular staining. Both Helios$^+$ and Helios$^-$ Treg clones displayed similar expression of IFN-γ after culture in IL-12
(mean IFN-γ production was 50 ± 20% and 40 ± 20%, respectively), compared to neutral conditions (mean IFN-γ production was 6 ± 3% and 4 ± 5%, respectively) (representative shown in Figure 2.5A, summarized in Figure 2.5B, n=8). Furthermore, Helios+ and Helios− Treg clones displayed an equal potential for plasticity when the increase in IFN-γ expression in IL-12 conditions was normalized to that of neutral conditions (Helios+ Treg: 40 ± 20% and Helios− Tregs: 34 ± 20%, n=8) (Figure 2.5C). As expected, Tconv cell clones displayed high levels IFN-γ production and this was further enhanced by IL-12, illustrating the effectiveness of the Th1-polarizing cytokines. Of note, by intracellular staining, Helios− Treg clones did not produce more IFN-γ than Helios+ Treg clones under neutral conditions. This is contradictory to previous results showing that, by cytokine bead array, these subsets differ significantly in terms of IFN-γ production. This discrepancy is common as often intracellular cytokine staining is not necessarily representative of protein production, however both methods are valid and useful in the appropriate contexts (Haining, 2012).

Along with IFN-γ production, I analyzed clones in these assays for the expression of Tbet, a transcription factor expressed by Th1 cells and upregulated in Th1 polarizing conditions (Szabo et al., 2000). I found that both Helios+ and Helios− Treg clones upregulated Tbet expression in a similar manner when cultured in IL-12 (Neutral conditions: average Tbet MFI was 1600 ± 400 and 1300 ± 400 for Helios+ and Helios− clones, respectively; IL-12 conditions: average Tbet MFI was 4000 ± 1000 and 3000 ± 800 for Helios+ and Helios− clones, respectively; Fold change in MFI between neutral and IL-12 conditions: 3 ± 1 and 2.0 ± 0.3 for Helios+ and Helios− clones, respectively) (representative experiment shown in Figure 2.5D, average MFI summarized in Figure 2.5E, and average fold change in MFI summarized in Figure 2.5F, n=4). Tbet MFI was similar in Treg and Tconv cell clones, and although unexpected, this may be attributed to the ubiquitous nature of Tbet expression in T cell subsets (Cohen et al., 2011) and
Tregs (Koch et al., 2009). Of note, clones were also placed in Th17 polarizing conditions containing IL-6, IL-1β, and IL-23 (Cohen et al., 2011), but in our hands these conditions did not induce IL-17 production (data not shown).

Figure 2.5 Plasticity of Helios+ and Helios- Treg clones
Helios+ Treg clones, Helios- Treg clones, and Tconv cell clones were stimulated with anti-CD3 and L cells in IL-2 containing media alone (neutral) or media containing 10 ng/ml IL-12 for 5 days and then analyzed for IFN-γ production by intracellular staining following PMA/Ionomycin stimulation for 5 hrs. (A) Clones from each subset were analyzed by intracellular staining for FOXP3 and IFN-γ expression. (B) A summary of the percentage of cells expressing IFN-γ in each subset. (C) The propensity to convert to IFN-γ producing cells was calculated by subtracting the percent of IFN-γ producing cells in neutral conditions from the percent of IFN-γ producing cells in IL-12 conditions. (D) A representative figure of Tbet expression in each clonal subset cultured in neutral and IL-12 containing conditions, analyzed by intracellular staining. (E) A summary of Tbet expression as measured by Tbet MFI in clonal subsets. (F) The fold increase in Tbet MFI with IL-12 treatment compared to neutral conditions. n≥8 for IFN-γ data, and n≥4 for Tbet data.
2.8 Helios⁺ and Helios⁻ Treg subsets both exhibit a demethylated FOXP3 TSDR region

Because FOXP3 expression and Treg lineage stability are closely associated with a demethylated FOXP3 TSDR, I isolated DNA from Helios⁺ and Helios⁻ Treg clones from two separate donors and used pyrosequencing to determine methylation state at 10 CpG sites in this region. The percent methylation of 10 CpG loci examined is displayed on a heat map in Figure 2.6. As expected based on previous literature and FOXP3 expression levels measured in clones by flow cytometry, I found that on average, Tconv clones were 1.6 ± 0.2 fold and 2.5 ± 0.2 fold more methylated at all 10 CpG sites in the FOXP3 TSDR region compared to Helios⁺ and Helios⁻ Treg clones, respectively. Interestingly, when comparing Treg clone subsets, I found that Helios⁺ Treg clones are significantly more methylated than Helios⁻ Treg clones at all CpG sites examined (p<0.0001). However, this result is difficult to interpret as FOXP3 MFI and plasticity potential are similar between clone subsets, and thus the biological consequence of this degree of difference in FOXP3 TSDR methylation is unknown.

Figure 2.6 Methylation status of the FOXP3 TSDR in Helios⁺ and Helios⁻ Treg clones
DNA was isolated from Helios⁺ Treg clones, Helios⁻ Treg clones, and Tconv cell clones and analyzed by bisulphate pyrosequencing to determine the methylation state of 10 CpG residues found within the FOXP3 TSDR locus. Each box represents a CpG residue, and percent methylation is the average of 3 replicates. Clones were isolated from two separate donors.
2.9 **Helios\(^{-}\) cells are readily identified within the naïve FOXP3\(^{+}\) Treg subset and may not represent *in vivo* induced Tregs**

Helios has been identified as a putative marker of nTregs, and thus could be useful in the isolation of *in vivo* induced FOXP3\(^{+}\) Tregs in humans. However, the exclusive expression of this marker by FOXP3\(^{+}\) nTregs is controversial and further investigation is warranted, particularly in the human setting. Following isolation of distinct subsets of Helios\(^{+}\) and Helios\(^{-}\) Tregs from human peripheral blood, I aimed to determine if our Helios\(^{-}\) Treg clones were representative of peripherally induced Tregs. If this were the case, I hypothesized that Helios\(^{-}\) Tregs would not be present in the naïve CD45RA expressing FOXP3\(^{+}\) Treg subset (Mackay, 1999), as these cells should display a mature phenotype if induced by antigen in the periphery. To this end, I used flow cytometry to examine enriched CD4\(^{+}\) T cells isolated from human peripheral blood, gating on the naïve CD45RA\(^{+}\)CD62L\(^{-}\)CCR7\(^{+}\)FOXP3\(^{+}\) Treg population (Sallusto *et al.*, 1999) (**Figure 2.7A**). Surprisingly, I found up to 38% of these naïve Tregs did not express Helios suggesting that not all Helios\(^{-}\) Tregs are peripherally derived. Furthermore, Helios\(^{-}\) cells were also present in the CD4\(^{+}\)CD45RA\(^{+}\)CD31\(^{+}\)FOXP3\(^{+}\) Treg fraction (**Figure 2.7B**); because CD31 is thought to be a marker of cells that have recently emigrated from the thymus (Kimmig *et al.*, 2002), the presence of Helios\(^{-}\) cells in this fraction further suggests that this subset is not exclusively derived from the periphery, and thus may not represent *in vivo* iTregs.
Enriched CD4⁺ T cells were stimulated with PMA/Ionomycin for 6 hours, with the addition of Brefeldin at 2 hours. Cells were analyzed by intracellular staining. (A) Expression of FOXP3 and Helios in naïve CD4⁺CD45RA⁺CCR7⁺CD62L⁺ T cells. A summary of Helios⁺ and Helios⁻ cells within the FOXP3⁺ populations is represented in the table, n=3. (B) Expression of FOXP3 and Helios in naïve CD4⁺CD45RA⁺CD31⁺ recent thymic emigrants and naïve CD4⁺CD45RA⁺CD31⁻ populations of T cells. A summary of Helios⁺ and Helios⁻ cells within each population is represented in the table, n=2. Gates are as indicated.

2.10 In vitro induced Tregs differ from Helios⁻ Treg clones in plasticity potential

To determine if Helios⁻ Treg clones bear any resemblance to in vitro iTregs, and to further examine if a lack of Helios expression can be used as a marker for the identification of in vivo FOXP3⁺ iTregs, I cultured human peripheral blood naïve CD4⁺ T cells (sorted from magnetic bead enriched CD4⁺ T cells, sorting scheme shown in Figure 2.8A) in Treg differentiation conditions and then examined these cells for Helios expression, suppressive capacity, and plasticity potential. In a representative experiment, after 5 days in the presence of...
TGF-β and rapamycin, iTregs were 55.4% FOXP3+ and mostly Helios− (Figure 2.8B). However, 17.9% of FOXP3+ cells were Helios+, much more then in the neutral control, again suggesting that Helios expression can be induced outside of the thymus and may not be useful in the differentiation of iTregs from nTregs. Control nTregs, which were cultured alongside iTregs in conditions containing rapamycin, maintained their FOXP3 expression (83.1% at 5 days), and were mostly Helios+. To assess suppressive capacity of \textit{in vitro} iTregs and nTregs, suppression assays were set up and proliferation of PBMC responder cells in culture with Tregs was measured by CFSE dilution. With this method, I found that both iTregs and nTregs displayed an equal ability to suppress CD8+ T cell proliferation at varying ratios (Figure 2.8C). To determine the potential of \textit{in vitro} iTregs and nTregs to produce IFN-γ in Th1 polarizing conditions, cells were stimulated for 5 days in media with or without IL-12. I found that unlike Helios+ and Helios− Treg clones, which displayed equal plasticity potential, \textit{in vitro} iTregs produced more IFN-γ in IL-12 conditions compared to nTregs or control cells (Figure 2.8D). This further suggests that \textit{in vitro} iTregs are not the same population as Helios− Treg clones.
Figure 2.8 Helios expression, suppressive capacity, and plasticity potential of ex vivo induced Tregs

(A) Naïve CD4^+CD45RO^-CD45RA^+CD25^+ Tregs and CD4^+CD45RO^-CD45RA^+CD25^- Tconv cells were isolated by flow cytometry. Gates were set based on FMO controls. (B) CD4^+CD45RO^-CD45RA^+CD25^- Tconv cells were cultured in neutral conditions containing L cells, rhIL-2, and anti-CD3 (control cells) or in neutral conditions with the addition of TGF-β and Rapamycin (iTregs) and analyzed for FOXP3 and Helios expression by intracellular staining on Day 7. Naïve CD4^+CD45RO^-CD45RA^+CD25^- Tregs (nTregs) were cultured in neutral conditions with the addition of Rapamycin and analyzed in parallel. (C) To test for suppressive capacity, iTregs and nTregs were cultured with CFSE labeled PBMC’s for 4 days. Proliferation of CD8^+ responder cells was assessed on Day 4 by CFSE dilution and percent suppression was calculated. (D) To assess plasticity potential, control cells, iTregs, and nTregs were cultured in neutral conditions containing L cells, anti-CD3, and rhIL-2 or in neutral conditions with the addition of 10ng/ml IL-12 for 5 days. IFN-γ expression was assessed on Day 5 after stimulation with PMA/Ionomycin for 5 hours with the addition of Brefeldin A at 2hrs.

2.11 Conclusions

This study provides the first evaluation of Helios^+ and Helios^- Treg subsets at a clonal level, and offers insight into the potential of Helios as a marker for the differentiation of human nTregs and in vivo iTregs. Indeed, based on the identification of both Helios^- and Helios^- cells within the FOXP3^+ Treg population by flow cytometry, I was able to perform limiting dilution assays on pure populations of sorted CD4^+CD25^hi Tregs to isolate clones that could be
categorized based on Helios expression. Following their isolation, Helios$^+$ and Helios$^-$ Treg clones were evaluated for the propensity to upregulate Helios with T cell activation. This investigation was initiated because of reports that Helios is associated with activated and proliferating T cells, rather than expressed in a lineage of Tregs alone (Akimova et al., 2011). Although I found that Helios is indeed upregulated upon TCR engagement, Helios$^-$ Treg clones never expressed Helios at levels greater than those expressed by resting Helios$^+$ Treg clones. Furthermore, as Tregs become less activated, their Helios expression returns to original expression levels. Thus, distinct populations can still be differentiated based on Helios expression, and further investigation into the phenotype and function of each subset was warranted. To this end, I first evaluated expression of Treg associated markers, and found that Helios$^+$ and Helios$^-$ Treg subsets did not differ in FOXP3 MFI, CD39, or CTLA-4. However, these clonal subsets did differ in terms of cytokine and chemokine production: Helios$^-$ Treg clones produced more MIP-1$\alpha$ and IFN-$\gamma$ than Helios$^+$ Treg clones. This suggested that perhaps Helios$^-$ Treg clones displayed a more inflammatory phenotype and may be less suppressive or unstable in terms of their Treg committment. Both suppression and stability were consequently examined, but it was found that Helios$^-$ Treg clones were identical to Helios$^+$ Treg clones in both these respects. Furthermore, Helios$^-$ Treg clones actually displayed a less methylated FOXP3 TSDR region compared to Helios$^+$ Treg clones, indicative of more stable FOXP3 expression and lineage commitment. Moreover, Helios$^-$ cells can be readily identified within naive (CD45RA$^+$CD31$^+$CCR7$^+$CD62L$^+$) FOXP3$^+$ Tregs, a finding that is inconsistent with the notion that Helios$^-$ Tregs are peripherally induced. In addition, when compared to in vitro iTregs, Helios$^-$ Treg clones differed in terms of plasticity potential. Collectively, these results suggest that Helios$^-$ Treg clones may not be inflammatory type Tregs or have characteristics akin to in vivo iTregs as previously suggested. Overall, these results further our knowledge concerning the unique characteristics of Helios$^+$ and Helios$^-$ Treg cell subsets, regardless of their identities as
natural or *in vivo* induced Tregs, and may be beneficial in the development of Treg cellular therapy for the clinical treatment of a variety of inflammatory diseases.
3 HUMAN CD4+FOXP3+ T REGULATORY CELLS EXPRESS CHEMOKINES AND RECRUIT INFLAMMATORY IMMUNE CELLS

3.1 Introduction

The role of Tregs in maintaining tolerance and limiting human disease is not trivial and likely extends beyond the suppressive capacities of these cells. For example, studies have shown that a large number of resident Tregs can be found in the skin in a homeostatic state (Matsushima and Takashima, 2010; Sather et al., 2007) and these cells express a variety of TLRs that can recognize pathogen derived products (Himmel et al., 2008). Upon TLR engagement, Treg function can be modified to result in enhancement or dampening of the immune response, depending on the type of triggering interaction (Himmel et al., 2008). This suggests that Tregs may be able to perform front-line functions such as pathogen detection in the early stages of an immune response, and modify their role accordingly. Along with pathogen detection, Rudensky et al. have shown that during viral infection in mice, both Tconv cells and FOXP3+ Tregs display identical kinetics as they accumulate at the site of infection and become activated (Lund et al., 2008). Importantly, in the absence of Tregs in this model, NK cells, CD4+ T cells, and plasmacytoid DC’s show reduced trafficking to the site of infection (Lund et al., 2008). These data demonstrate that early accumulation/retention of Tregs at the site of infection is critical for immune cell recruitment, and that Tregs do not simply mediate suppression at the end of an immune response. In addition, this work also demonstrates that in order to fully realize their potential in a cellular therapy setting, further investigation into the non-suppressive functional aspects of Tregs is critical.

In order for Tregs to mediate front-line effects such as pathogen detection and immune cell recruitment, they must already be present in the target organs at the time of insult or be equipped to rapidly traffic to the site. Tregs are known to express a variety of chemokine
receptors that target them to both lymphoid and non-lymphoid tissues throughout the body in order to fulfill this requirement (Campbell and Koch, 2011). For example, Tregs migrating to the skin express the alpha E integrin chain CD103 and CC-chemokine receptor 4 (CCR4), and generate carbohydrate ligands via the \( \alpha-(1,3) \)-fucosyltransferase VII enzyme that are recognized by the endothelial adhesion receptors P-selectin and E-selectin (Campbell and Koch, 2011). For specific gut homing, Tregs express the integrin \( \alpha 4 \beta 7 \) and CC-chemokine receptor 9 (CCR9) (Campbell and Koch, 2011; Stenstad et al., 2006). Tregs are unable to prevent skin-specific autoimmunity or gut associated diseases such as colitis in the absence of any of these molecules, suggesting that appropriate Treg trafficking is critical to their effective function (Campbell and Koch, 2011). In addition to peripheral tissue migration, Tregs must be equipped to traffic into lymph nodes where their suppressive effects are often mediated on Tconv cells that have migrated from peripheral tissues upon activation. CC-chemokine receptor 7 (CCR7) and CD62L, also known as L-selection, are critical for lymph node homing, and again a lack of these molecules can lead to abrogated Treg function, as demonstrated in models of colitis and allograft rejection (Schneider et al., 2007; Zhang et al., 2009).

Along with tissue specific homing, chemokine receptor expression can also influence the migration of Tregs to varying types of inflammatory responses. For example, in a T helper 17 (Th17) cell mediated model of inflammatory experimental autoimmune encephalomyelitis (EAE), Tregs are recruited by specific expression of CC-chemokine receptor 6 (CCR6) after exposure to the effector cytokine IL-17 (Yamazaki et al., 2008). Alternatively, in an environment containing IFN-\( \gamma \), Tregs expressing CXC-chemokine receptor 3 are recruited preferentially to dampen T helper 1 (Th1) cell mediated inflammation (Santodomingo-Garzon et al., 2009). In all cases, it is the timely homing of Tregs to the appropriate sites via chemokine receptor expression that allows for the various functions of this subset to be carried out during an immune response.
Along with chemokine receptor expression, the corresponding chemokines that bind these receptors must be present in target tissues to facilitate immune cell recruitment. Indeed, chemokine expression is essential in recruiting immune cells out of circulation to mount an immune response, and is a first-line response in the orchestration of an immune attack. Thus, the ability to produce chemokines is an important role, and often performed by cells of both the innate and adaptive immune system including intestinal epithelial cells, antigen presenting cells such as macrophages and dendritic cells, and Tconv cells (Rot and von Andrian, 2004). Furthermore, murine studies have provided both direct and indirect evidence to suggest that Tregs can also produce chemokines (Lund et al., 2008; Nguyen et al., 2011). However, in humans the capacity of Tregs to express chemokines has not been directly evaluated, in part due to earlier findings demonstrating that they are unable to secrete typical T cell derived cytokines such as IFN-γ and TGF-β (Allan et al., 2008b). This is an important question for the context of Treg therapy as understanding the simple biology of these cells, including capacity to secrete chemokines and recruit other immune cells, will greatly contribute to our ability to effectively design cellular therapy protocols.

With evidence drawn from mouse studies and the ubiquitous expression of chemokine receptors on Tregs, I hypothesized that human FOXP3+ Tregs have the capacity to secrete chemokines and consequently recruit immune cells. Because Tregs are present in peripheral tissues in the early stages of an immune challenge, and neutrophils, an innate immune cell type, are often recruited as a first line defense, I first focused on the ability of these cells to produce CXCL8 (also known as IL-8), a potent neutrophil chemoattractant. After a complete investigation of CXLC8 production and its biological consequences, I next expanded my studies to evaluate the capacity of human ex vivo FOXP3+ Tregs to produce a variety of homeostatic and inflammatory type chemokines beyond CXCL8. With these studies, I aimed to define a new role
for human FOXP3+ Tregs in orchestrating the immune response and carrying out functions beyond their suppressive capacity.

3.2 Materials and methods

**Cell purification.** Peripheral blood was obtained from healthy volunteers following written informed consent. All studies were approved by the University of British Columbia Clinical Research Ethics Board. CD4+ T cells were purified by negative selection (EasySep, Stem Cell Technologies), followed by magnetic bead sorting for CD25 over two columns (Miltenyi Biotec) (Allan et al., 2007). CD4+CD25hi cells (referred to as Tregs) were sorted from PBMCs or enriched CD4+ T cells (negative selection) on a FACS Aria as CD4+CD14- cells, followed by gating on the top 3% or less of CD25 bright cells. To isolate naive and memory Tregs, PBMCs were sorted after staining with antibodies for CD4, CD25, CD14, and CD45RA (all eBioscience). Naive Tconv cells were defined as CD25-CD45RA+ cells, memory Tconv cells as CD25-CD45RA- cells, naïve Tregs as CD25hiCD45RA+ and memory Tregs as CD25hiCD45RA-.

Purity based on CD25 expression (BD Biosciences) was >85% or >95% for magnetically separated and sorted Tregs, respectively. FACS sorted CD4+CD25hi Tregs contained less than 0.1% contaminating CD11c+, CD14+, CD19+, or CD56+ cells and were >99% TCRab+, excluding the possibility that contaminating monocytes contributed significantly to measured chemokine production.

**Determination of cytokine and chemokine production.** Magnetic bead sorted T cells (5x10⁵/ml) or FACS sorted T cells (1x10⁶/ml) were activated with anti-CD3/anti-CD28-coated beads at a 1:8 cell:bead ratio (Invitrogen) for 72 hours in complete media. Concentrations of CXCL8, IFN-γ, and IL-17 in supernatants were determined using capture ELISAs or a CBA Flex Set.
according to the manufacturer's instructions (BD Biosciences). The chemokine secretion profile, including Eotaxin, CXCL1, CXCL9, CXCL10, CCL2, CCL3, CCL4, CCL5, CCL7, and CCL8, was determined using a human Chemokine Ten-Plex Luminex bead array kit (Invitrogen, Cat. # LHC6001) according to the manufacturer's instructions and analyzed using a Bio-Plex 200 Luminex machine (Biorad).

**Chemokine PCR arrays.** FACS sorted CD4⁺CD14⁻CD25hi Tregs and CD4⁺CD14⁻CD25⁻ Tconv cells (both at 1x10⁶/ml) were stimulated with anti-CD3/anti-CD28-coated beads at a 1:8 cell:bead ratio (Invitrogen) for 48 hours in complete media containing 100U/ml IL-2. Cells were collected and RNA was isolated using E.Z.N.A Total RNA Isolation Kit (Omega Bio-Tek, Inc.) according to manufacturer’s directions. cDNA was generated from 200ng of total RNA using the qScript cDNA SuperMix kit (Quanta Biosciences) according to manufacturer’s directions. PCR array analysis was performed on equal amounts of cDNA from each sample using RT² Profiler PCR Array System (Human Chemokines and Receptors PCR Array, Cat #PAHS-022E-1) on the ABI 7500 Real Time PCR Machine using the RT² real-time SYBR Green PCR master mix (SA Biosciences). PCR Array was carried out using manufacturer’s instructions in a total volume of 10ul per well.

**Intracellular staining.** Analysis of CD4 (Clone 3T4), CD25 (Clone M-A251), FOXP3 (Clone 259D/C7), CXCL8 (Clone G265-8), IFN-γ (Clone 4SB3), and IL-17 (Clone eBio64/Dec17) production was performed either on ex vivo CD4⁺ T cells or sorted and expanded (Levings et al., 2001) naive and memory T cell subsets. Prior to intracellular staining, cells were stimulated with PMA (10ng/ml) and Ca²⁺ ionophore (500ng/ml) (both Sigma-Aldrich) for 6 h with the addition of Brefeldin A (10ug/ml, Sigma-Aldrich) after 2 h as described (Broady et al., 2009). Samples were read on a FACSCanto (BD Biosciences) and analyzed using FlowJo Software Version 8.7.
Gates for FOXP3\(^+\) cells were set based on fluorescence minus one controls and for cytokines on unstimulated, but stained, samples.

**Transduction of CD4\(^+\) T cells with FOXP3.** The production of lentivirus and transduction of T cells has been previously described (Allan et al., 2008c). Control DNGFR\(^-\)-transduced T cells and FOXP3-transduced T cells were purified (>90% based on surface NGFR expression) and expanded in rhIL-2-containing media (100U/ml, Chiron) (Allan et al., 2008c). T cells in the resting phase (10-13 days after activation) were washed and rested in IL-2 free media overnight, and stimulated with anti-CD3/anti-CD28-coated beads at a 1:8 cell:bead ratio for 72 hours.

**CXCL8 promoter luciferase assay.** The CXCL8 promoter (region -1793 to +49; 1,842 bp) was amplified from human genomic DNA and cloned into pGL3. Jurkat cells were transiently transfected as described (Bacchetta et al., 2006) with pGL3 or pGL3-CXCL8 and a renilla luciferase reporter vector (pRL-TK), in the presence or absence of FOXP3. After 24h cells were stimulated with PMA (10ng/ml) and Ca\(^{2+}\) ionophore (500ng/ml) for 6 h. Luciferase activity was measured using a luminometer (EG&G Burthold) and a Dual Luciferase Reporter Assay System (Promega). All values were normalized to renilla luciferase activity and expressed relative to unstimulated controls.

**Neutrophil recruitment assays.** Supernatants (235ml) from FACS-sorted CD4\(^+\)CD25\(^-\) Tconv and CD4\(^+\)CD25\(^hi\) Treg cells cultured at 1x10\(^6\)/ml for 72 hours with anti-CD3/anti-CD28-coated beads at a 1:8 cell:bead ratio in complete medium, but with serum replaced by 1% human serum albumin, were added to the lower chamber of a transwell plate (Corning HTS 96well transwell, 3.0mm pore size). Neutrophils were isolated using a Ficoll separation followed by a 6% dextran gradient, and 100,000 cells were added to the upper chamber of the transwell plate. In some
cases, anti-CXCL8 mAb (2A2, 150mg/ml, BD Biosciences) was added to lower chamber for 1 hour at 37°C prior to neutrophil addition. This amount of mAb neutralized migration in response to at least 8 ng/ml of CXCL8 (data not shown). Dilutions ranging from 200 pg/ml to 100 ng/ml of rhCXCL8 (eBiosciences) were added to the lower chamber as a positive control. After 30 minutes of incubation at 37°C, 50,000 surfactant-free white sulfate latex beads (4.9mm, Dynamics) were added to lower chamber supernatants, and the number of neutrophils that had migrated to the lower chamber per 10,000 beads were counted by flow cytometry based on FSC and SSC parameters.

**Statistical analysis.** All analysis for statistically significant differences was performed using the student’s paired, 2 tailed t-test. $p$ values less than 0.05 (indicated by *) were considered significant. All cultures were performed in triplicate and error bars represent the SD unless otherwise indicated.

### 3.3 Human CD4⁺CD25hi Tregs produce CXCL8

As Tregs are present in the early stages of an immune response, I investigated whether they may have the capacity to influence the recruitment of innate immune cells such as neutrophils via production of chemokines. I initially focused on CXCL8, which is made by a variety of leukocytes and signals through CXCR1 and CXCR2, since this is a strong chemoattractant for neutrophils (Engelhardt *et al.*, 1998; Rot and von Andrian, 2004). CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were isolated using magnetic separation, stimulated with anti-CD3/anti-CD28-coated beads and levels of secreted CXCL8 in supernatants were determined. As shown in **Figure 3.1A**, CD4⁺CD25⁺ T cells produced similar levels of CXCL8 compared to CD4⁺CD25⁻ T cells, with an average of 2 ± 2 ng/ml of CXCL8 and 0.7 ± 0.8 ng/ml of CXCL8, respectively (n=4, p=0.17).
Recent studies have demonstrated that a significant proportion of Tregs have the capacity to produce IL-17 (Beriou et al., 2009; Voo et al., 2009; Fletcher et al., 2009; Miyara et al., 2009) and Th17 cells are known to produce CXCL8 (Crome et al., 2010; Pelletier et al., 2009). To exclude the possibility that the source of CXCL8 was contaminating Th17 cells and/or IL-17-secreting FOXP3+ T cells which are present in populations sorted solely on the basis of CD25, I FACS-sorted CD25hi cells, referred to as Tregs, into naïve (CD45RA+) and memory (CD45RA-) subsets, since CD45RA+CD25hi Tregs do not contain IL-17-secreting cells (Miyara et al., 2009). I found that both Tconv cells (defined as FACS sorted CD4+CD25-) and Treg cells produced CXCL8 at similar concentrations (Figure 3.1B&C) even in the absence of TCR activation, suggesting that like endothelial cells, T cells may have preformed stores of CXCL8 (Wolff et al., 1998) that are released by the shearing stress of cell sorting. Notably, CXCL8 production by CD25- and CD25hi T cells was not restricted to cells with a naïve (CD45RA+) or memory (CD45RA-) phenotype. Similar results were obtained when cells were stimulated in the presence of exogenous IL-2 (data not shown). In parallel, I analyzed production of IFN-γ or IL-17 and confirmed that the CD25hiCD45RA- Tregs produce a significant amount of IL-17, as expected, and that neither CD25hiCD45RA- nor CD25hiCD45RA+ Tregs produced IFN-γ (Figure 3.1B). These findings indicate that CD4+CD25hi Tregs produce CXCL8 irrespective of whether they are naïve or memory cells and that this is not the result of contaminating IL-17-secreting cells.
Figure 3.1 Analysis of CXCL8 expression by CD4+CD25hiFOXP3+ Tregs

(A) CD4+CD25+ and CD4+CD25− T cells were purified by magnetic separation, stimulated with anti-CD3/anti-CD28-coated beads (5x10^5/ml) and supernatants were collected at 72 hours and analyzed for CXCL8 by ELISA. Dots represent values from individual donors (n=4). (B&C) CD4+CD25hi Tregs and CD4+CD25− Tconv cells were sorted into naïve (RA+) and memory (RA−) cells and stimulated at 1x10^6 cells/ml for 72 hours with anti-CD3/anti-CD28-coated beads. Concentrations of CXCL8, IFN-γ, and IL-17 in supernatants were analyzed using CBA, with error bars representing standard deviation. Open circles in (C) indicate that CXCL8 levels were greater than the upper detection limits of the standard curve (>12.2ng/ml). Data are representative of 3 to 4 independent experiments. © European Journal of Immunology, Himmel et al. 2011, reprinted by permission.

Isolation of cells on the basis of CD25, even in conjunction with other markers such as CD45RA, does not necessarily result in a homogenous population of FOXP3+ cells. Therefore to further confirm that Tregs produce this chemokine, CXCL8 production was analyzed by intracellular staining. Ex vivo CD4+ T cells were stimulated with PMA/ionomycin for 6 hours and CXCL8 producing cells were detected in both the FOXP3+ and FOXP3− populations (representative experiment in Figure 3.2A, summarized in Figure 3.2B). On average, 28 ± 1% (n=4, average±SEM) of stimulated CD4+FOXP3− T cells and 25 ± 4% (n=4) of stimulated CD4+FOXP3+ T cells were CXCL8+ (Figure 3.2B). To further confirm these data, as well as to determine the cytokine profile of these CXCL8+ T cells, naïve and memory Tconv and Treg cells were sorted, expanded, and analyzed by intracellular staining (sorting scheme shown in Figure 3.2C, representative intracellular staining plot shown in Figure 3.2D). On average 13 ± 2% of FOXP3+CD45RA+ Tregs and 20 ± 3% of FOXP3+CD45RA− Tregs expressed CXCL8 (Figure 3.2E). When analyzing cytokine production from these subsets, it was found that neither the CD45RA−CXCL8+ nor the CD45RA−CXCL8+ Treg populations co-expressed significant levels
of IFN-γ or IL-17, further confirming that it is indeed naturally occurring FOXP3+ Treg cells that express CXCL8 (Figure 3.2F). A summary of CXCL8, IFN-γ, and IL-17 expression from expanded populations is seen in Table 3.1. Of note, a significant level of FOXP3 expression is seen in expanded T conv cells; this is expected as activated T cells have been shown to upregulate FOXP3 upon TCR stimulation (Allan et al., 2007).

Figure 3.2 Analysis of CXCL8, IFN-γ, and IL-17 expression in naïve CD4+CD45RA+CD25+ Treg and CD4+CD45RA−CD25+ T conv cells
(A) The proportion of CXCL8+ positive cells in FOXP3+ and FOXP3− populations was analyzed in gated ex vivo unstimulated CD4+ T cells. A summary of findings for 3 independent experiments is shown in (B). Populations were sorted and expanded for 10 days then restimulated to analyze for cytokine/chemokine expression. Gating strategies are shown in (C). Expanded populations were analyzed for FOXP3 and CXCL8 expression (D), and a summary of 3 independent experiments is shown in (E). (F) The proportion of CXCL8, IFN-γ, and IL-17 in gated FOXP3+ for Tregs or the total populations for Tconv cells was analyzed. © European Journal of Immunology, Himmel et al. 2011, reprinted by permission.
Table 3.1 Mean percentage ± SD of CXCL8, IFN-γ, and IL-17 producing populations in total CD4⁺ T cell (Tconv) and FOXP3⁺ T cell (Treg) populations from 3 independent experiments

<table>
<thead>
<tr>
<th></th>
<th>Mean % of Total CD4⁺ T cells ± SD</th>
<th>Mean % of FOXP3⁺ T cells ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tconv</td>
<td>Treg</td>
</tr>
<tr>
<td></td>
<td>CD45RA⁺</td>
<td>CD45RA⁻</td>
</tr>
<tr>
<td>CXCL8⁺IFN-γ⁺</td>
<td>1 ± 1</td>
<td>31 ± 10</td>
</tr>
<tr>
<td>CXCL8⁺IFN-γ⁻</td>
<td>25 ± 6</td>
<td>12 ± 7</td>
</tr>
<tr>
<td>CXCL8⁺IFN-γ⁻</td>
<td>5 ± 1</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>CXCL8⁺IL-17⁺</td>
<td>0.2 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>CXCL8⁺IL-17⁻</td>
<td>25 ± 6</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>CXCL8⁺IL-17⁺</td>
<td>0.4 ± 0.2</td>
<td>10 ± 7</td>
</tr>
</tbody>
</table>

3.4 FOXP3 directly regulates CXCL8 gene expression

To ask whether FOXP3 directly regulates CXCL8 production I investigated whether inducing over-expression of FOXP3, which is known to reprogram Tconv cells into Tregs (Allan et al., 2008c), modulates CXCL8 production. CD4⁺ T cells were transduced with a FOXP3 expressing lentivirus or a control virus and then assessed for production of CXCL8. I found that FOXP3 transduced T cells produced significantly more CXCL8 compared to control transduced cells, with the expected parallel suppression of IFN-γ production (Figure 3.3A). To determine if FOXP3 can directly bind the CXCL8 promoter region and enhance protein expression, I generated a CXCL8-promoter reporter construct that resulted in luciferase activity when activated. I found that FOXP3 can directly transactivate the CXCL8 promoter as evidenced by enhanced luciferase activity in transiently transfected cells when FOXP3 was present (Figure
3.3B). Together, these data conclusively demonstrate that FOXP3\textsuperscript{+} cells produce CXCL8 and indicate that FOXP3 directly regulates CXCL8 gene expression.

Figure 3.3 FOXP3 regulation of CXCL8 gene expression

(A) CD4\textsuperscript{+}CD25\textsuperscript{-}CD45RA\textsuperscript{+} T cells were transduced with either control or FOXP3 lentivirus. Transduced cells were purified, allowed to rest, then re-stimulated with anti-CD3/anti-CD28-coated beads for 72 hours. Supernatants were analyzed for CXCL8, IFN-\(\gamma\), and IL-17. Data represent mean \(\pm\) SD; \(p<0.05\) for unstimulated and stimulated FOXP3-transduced cells, compared to control transduced cells, using the student’s paired t-test. (B) Jurkat cells were transfected with a pGL3 CXCL8-promoter reporter construct along with empty vector or a FOXP3. Luciferase activity was measured and the fold increase in activity in stimulated cells over unstimulated controls was calculated. Data are the average of 2 independent experiments with error bars representing the standard deviation between experiments. © European Journal of Immunology, Himmel \textit{et al.} 2011, reprinted by permission.

3.5 Tregs produce both CC and CXC family chemokines

Having demonstrated that FOXP3\textsuperscript{+} Tregs produce CXCL8, I investigated whether they may produce other chemokines. To determine chemokine production at the protein level, naive and memory Tregs and Tconv cells were sorted and stimulated with anti-CD3/anti-CD28-coated beads for 72 hours and supernatants were analyzed using a multiplex bead array. I found that Tregs secreted significant amounts of a number of chemokines, including those involved in the acute phase response, such as CCL2, CCL3, CCL4, CCL5, CCL7, and CXCL10 (Figure 3.4 & Table 3.2). Neither Tregs nor Tconv cells produced significant levels of CCL8, CCL11, CXCL1, or CXCL9. In general, both naïve and memory Tregs displayed a similar chemokine expression profile to that of Tconv. To further investigate any differences in the chemokine expression
profile of Tregs and Tconv, and to determine chemokine production at the mRNA level, a PCR array was conducted on mRNA isolated from sorted and activated populations of CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconv cells. I found that compared to Tconv cells, the chemokines/chemokine receptors and chemokine related proteins TREM1, TNFSF14, MMP7, CXCL8, CSF3, CCR5, CCL7, CCL13, and CCL11 were all expressed at greater levels (>3 fold) in Tregs (Figure 3.5). Interestingly, many of these chemokines/receptors and chemokine related proteins are involved in monocyte and neutrophil recruitment (Charo and Ransohoff, 2006). Of note, several chemokines and chemokine related proteins were more highly expressed in Tconv cells compared to Tregs, including CXCL12, CXCL6 and matrix metalloproteinase-2 (MMP-2). Overall, these data demonstrate that in addition to CXCL8, Tregs produce a variety of chemokines that are known to mediate the trafficking of immune cells such as monocytes, dendritic cells, and T cells to sites of inflammation.

Table 3.2 Range of chemokine production by ex vivo stimulated CD45RA⁺ and CD45RA⁻ Tregs from 3 independent donors

<table>
<thead>
<tr>
<th></th>
<th>CCL2 (ng/ml)</th>
<th>CCL3 (ng/ml)</th>
<th>CCL4 (ng/ml)</th>
<th>CCL5 (pg/ml)</th>
<th>CCL7 (pg/ml)</th>
<th>CXCL10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45RA⁺ Treg</td>
<td>0.3-2.8</td>
<td>5.0-14.9</td>
<td>3.1-12.8</td>
<td>0.4-3.1</td>
<td>25.0-109.3</td>
<td>11.3-33.1</td>
</tr>
<tr>
<td>CD45RA⁻ Treg</td>
<td>0.5-3.3</td>
<td>0.8-18.9</td>
<td>0.9-16.0</td>
<td>0.9-1.2</td>
<td>9.9-140.5</td>
<td>3.5-9.3</td>
</tr>
</tbody>
</table>
Figure 3.4 Chemokine expression by *ex vivo* human CD4⁺ T cells at the protein level
Cells were sorted by flow cytometry based on surface expression of CD4, CD25, and CD45RA and stimulated for 72 hrs with anti-CD3/anti-CD28-coated beads. Supernatants were analyzed using a Luminex Chemokine 10-plex kit. Data shown are the mean ± SD of 3 independent experiments. © European Journal of Immunology, Himmel et al. 2011, reprinted by permission.

Figure 3.5 Chemokine and chemokine related protein expression by FOXP3⁺ Tregs compared to Tconv cells at the mRNA level
mRNA was extracted from sorted and stimulated populations of CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconv cells from a single donor and analyzed for chemokine expression using a Human Chemokines and Receptors PCR Array kit. Green bars represent chemokines that are upregulated in Tregs compared to Tconv cells, and red bars represent chemokines that are downregulated in Tregs compared to Tconv cells (n=1).
3.6 Supernatants from Tregs attract neutrophils

I next asked whether the chemokines produced by Tregs are biologically active and investigated whether they could recruit neutrophils. Supernatants from Tconv and Treg cells that were activated with anti-CD3/anti-CD28-coated beads for 72 hours were added to the bottom of transwells and assayed for their ability to recruit neutrophils. In 4 independent experiments supernatants from both Tregs and Tconv cells significantly stimulated the migration of neutrophils compared to medium alone (Figure 3.6A). Moreover, addition of neutralizing anti-CXCL8 mAbs to the T cell-derived supernatants significantly decreased neutrophil migration (Figure 3.6B). Neutrophil recruitment, however, was not completely blocked in the presence of anti-CXCL8 mAbs, likely due to the presence of other chemokines that can recruit neutrophils, such as CCL3 and CCL4. These data indicate that the CXCL8 produced by Tregs is functional and contributes to the recruitment of innate immune cells in vitro.

Figure 3.6 Treg induction of neutrophil chemotaxis
(A) CD4⁺CD25⁺ Treg and CD4⁺CD25⁻ Tconv were sorted by flow cytometry and left unstimulated or stimulated with anti-CD3/anti-CD28-coated beads in serum-free media for 72 hrs and the fold migration compared to that stimulated by medium alone was calculated. In (B), an aliquot of supernatants from stimulated cells was incubated for 1 hour with a neutralizing anti-CXCL8 mAbs prior to initiation of the neutrophil recruitment assay. Data from 4 independent experiments are shown in (A) and one representative experiment of 4 is shown in (B), with error bars in (B) representing experimental triplicates. © European Journal of Immunology, Himmel et al. 2011, reprinted by permission.
3.7 Conclusions

Although Tregs are classically defined by their inability to produce typical T cell derived cytokines, the capacity of these cells to produce chemokines has not been specifically determined to date. This study is the first broad examination of both CC and CXC family chemokine expression by human Tregs. I found that human FOXP3+ Tregs are capable of producing CXCL8, a potent neutrophil chemoattractant, and that CXCL8 producing Tregs are not restricted to a naïve or memory phenotype. Furthermore, CXCL8 producing Tregs do not co-express IFN-γ or IL-17, suggesting they are not memory T cells or recently described “inflammatory-type” Tregs. In order to determine the relationship between the Treg lineage defining transcription factor FOXP3 and CXCL8, I used FOXP3 transduction of cells to show that FOXP3 alone can enhance expression of this chemokine. Using a CXCL8 promoter luciferase construct, I determined that FOXP3 can directly bind the CXCL8 promoter region to regulate its expression. Furthermore, along with CXCL8, Tregs can produce a variety of chemokines involved in the acute inflammatory response, including CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES), all of which bind the chemokine receptor CCR5. The chemokines produced by Tregs are biologically active as they allow for neutrophil recruitment, and this recruitment is dependent, in part, on the production of CXCL8 specifically. Overall, this work suggests that chemokine production may be a normal function of classically suppressive Tregs, and indeed may play a role in allowing these cells to mediate their effects, suppressive or otherwise. In particular, because Tregs seem to produce a number of acute phase chemokines that are able to recruit inflammatory immune cells such as neutrophils, it may be that Tregs assist in orchestrating an early immune response in peripheral tissues. This would be a novel role for Tregs, which are classically defined in terms of the adaptive immune response, and demonstrate a close link with innate immunity. With this new knowledge concerning chemokine production
by Tregs, we take steps towards a deeper understanding of their basic biological function in the human immune system, and consequently towards a better translation into the clinical setting.
4 BACTERIAL DERIVED FLAGELLIN AS A DISEASE ASSOCIATED ANTIGEN IN INFLAMMATORY BOWEL DISEASE

4.1 Introduction

As the body of literature concerning the basic biology of Tregs grows, we must begin to consider the opportunities for bench-to-bedside translation of this cellular subset. One such setting that may benefit from the development of Treg therapy is that of inflammatory bowel disease. IBD is characterized by chronic, relapsing inflammation of the intestinal tract (Khor et al., 2011). Although the precise cause of this inflammation is unknown, one hypothesis suggests that IBD occurs in genetically predisposed individuals and is thought to result from an inappropriate activation of CD4+ T cells by components of commensal bacteria, along with concomitant loss of Treg suppressor activity. Specifically, effector T cell profiles in patients with CD are known to be skewed toward Th1 and Th17 type responses, in contrast to the Th2 profile seen in UC patients (Sartor, 2006). Indeed, macrophages from CD patients have increased IL-12 production, leading to Th1 cell differentiation and IFN-γ production, and T cell nuclear extracts from CD patient disease lesions contain high levels of the Th1-associated signaling transcription factors STAT4 and Tbet (Parronchi et al., 1997). IFN-γ-secreting lymphocytes are abundant in the gut environment, particularly at the onset of CD, and likely contribute substantially to disease pathogenesis. Along with Th1 cells, Th17 cells are implicated in CD. Intestinal tissues from CD patients have increased expression of IL-23 and IL-17, both hallmarks of Th17 cells (Sartor, 2006), and the IL-12p40 subunit shared with IL-12 and IL-23 is over-expressed in mouse colitis models (Brand, 2009).

In addition to aberrant effector T cell responses, dysregulation of Tregs is also thought to contribute to CD. A significant number of experimental colitis models result from a defect in Tregs (Izcue et al., 2009), with the most compelling data coming from the T cell transfer model.
of colitis pioneered by Powrie and colleagues (Powrie et al., 1994). In this model, transfer of naïve T cells in the absence of Tregs into immunodeficient mice results in colitis, whereas co-transfer of Tregs protects from disease. Moreover, transfer of Tregs after disease induction cures established colitis (Mottet et al., 2003). Correlative data suggest that defective Tregs also contribute to IBD in humans (Himmel et al., 2008). Perhaps the strongest evidence comes from the study of patients with IPEX, a primary immunodeficiency caused by mutations in FOXP3, who have defective Tregs and uniformly suffer from intestinal inflammation (Gambineri et al., 2008). Thus, restoring Treg function in IBD offers the potential to restore intestinal immune homeostasis, however, similar to effector T cells, nothing is known about what antigens Tregs respond to in IBD.

Identification of antigens that drive inflammation in IBD is an important consideration as this will allow for specific evaluation of the T cells that propagate disease, as well as provide a target for antigen specific Treg therapy. Previous reports have shown that flagellin, a bacterial protein present on all flagellated bacteria including many commensals hosted within the gut, can exacerbate disease in a mouse model of inflammatory bowel disease (Rhee et al., 2005). However, little is know about the nature of the anti-flagellin immune response, and if it is more dependent on the TLR5 stimulatory effect of flagellin or its antigenic properties. Furthermore, although the humoral response against flagellin has been studied in mice and humans, and shown to be dependent on T cells in mice (Sanders et al., 2006), characterization of flagellin-reactive T cells in human CD remains limited. In the only report from human patients, Shen et al. found increased T cell proliferative responses to CBir1 flagellin in peripheral blood as well as IFN-γ-producing cells by ELISPOT from intestinal tissue in CD patients but not healthy controls (Shen et al., 2008). However, these cells were not characterized further, and hence their antigen specificity (as opposed to, for example, to a nonspecific proliferative effect of TLR5 activation) has never been determined.
In order to further characterize the anti-flagellin immune response observed in IBD, we first evaluated the mechanism by which flagellin exacerbates the symptoms observed in mice treated with dextran sodium sulfate (DSS), a chemical that induces an IBD-like disease including weight loss, rectal bleeding, and colonic inflammation. Specifically, we sought to determine if this effect is due to the antigenic properties of flagellin or its ability to activate TLR5 signalling pathways. Using similar methodology to that used by Rhee et al., we compared the *E. coli* H18 flagellin (FliC, derived from enteroaggregative strain 042) to the insertional transposon mutant FliC-2H3, which is unable to signal through TLR5 but retains the putative antigenic sites of wild-type flagellin, in the exacerbation of DSS-induced colitis (Donnelly and Steiner, 2002). Following these studies on the mechanisms of flagellin-mediated exacerbation of colitis, I sought to utilize a novel method to identify flagellin reactive T cells in peripheral blood samples from patients with CD, hypothesizing that patients with anti-flagellin antibodies will also have detectable levels of anti-flagellin T cells in their sera. With these studies, we hope to further our knowledge concerning the role of flagellin in the pathogenesis of IBD, as well as identify this bacterial component as a putative antigen for targeting in Treg cellular therapy for this disease.

4.2 Materials and methods

**Mice and reagents.** Six to eight-week-old male C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). TLR5<sup>−/−</sup> mutants of C57BL/6 mice (Uematsu *et al.*, 2006) were a generous gift from Alan Aderem (Institute for Systems Biology, Seattle, WA); both TLR5<sup>−/−</sup> mice and the WT C57BL/6 mice were bred in our facility and used at 6–8 weeks of age. The H18 flagellin from enteroaggregative *E. coli* strain 042 was expressed in pCR-NT-T7-Topo (Invitrogen, La Jolla, CA) in BL21 (DE3) pLysS cells, purified by metal affinity chromatography as previously described (Donnelly and Steiner, 2002), and passed repeatedly...
over a polymyxin B agarose column (Sigma, St. Louis, MO) according to the manufacturer's instructions until a limulus test (MJS BioLynx, Brockville, ON) for endotoxin was negative. 2H3 flagellin was purified in the same way. All oligonucleotides were obtained from Operon (Huntsville, AL).

**Cell culture.** HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in Eagle's minimal essential medium (Stemcell Technologies, Vancouver, BC) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine (Stemcell), 1 mM sodium pyruvate (Stemcell), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Sigma). Cells were passaged 2–3 times per week and seeded at 10^5/well in 24-well polystyrene dishes for transient transfections.

**Cloning and expression of Salmonella enterica serovar Typhimurium flagellin (StFliC).** Genomic DNA from *S. enterica*, serovar Typhimurium kindly provided by B. Finlay (UBC) was amplified with *fliC*-specific primers: 5'-CAGTAGTTCGAATTAACGCAGTAAAGAGAGGAC and 5'-GGATCCATGGCACAAGTCATTAAT-3'. The polymerase chain reaction (PCR) fragment was cloned into the BamHI and BstBI sites of pCR-NT-T7 (Invitrogen). A clone that had been verified by sequencing analysis was transformed into BL21 (DE3) pLysS cells for expression of the protein; protein was isolated as described above for the *E. coli* flagellin and functionally verified by elicitation of IL-8 production in Caco-2 cells (not shown).

**DSS colitis and administration of enemas.** Acute colitis was induced with 2.5% (w/v) DSS (molecular weight 36–50 kDa; MP Biochemicals, Solon, OH) dissolved in drinking water, which was fed ad libitum for 10 days (Wirtz *et al.*, 2007). Water consumption per mouse per day was
evaluated and found to be similar among the experimental groups. For enemas, 100 µL volumes of 1 µg/100 µL flagellin or phosphate-buffered saline (PBS) were administered daily to isoflurane-anesthetized mice via Intramedic polyethylene tubing (BD Biosciences Pharmingen, San Diego, CA), inserted 4 cm into the rectum, starting from Day 4.

**Clinical assessment of colitis.** Animals were observed daily for morbidity, weight loss, and rectal bleeding, for the duration of the experiment. Mice were euthanized if they demonstrated signs of distress or ≥20% weight loss.

**Histology.** At day 7 post flagellin administration, the entire mouse colon was excised, weighed, measured, and photographed and 0.5 cm segments of proximal, mid, and distal colon were fixed in 10% buffered formalin. The following day the sections were washed and transferred to 70% EtOH. Hematoxylin and eosin-stained thin sections were graded in a blinded fashion by 2 separate investigators. Each section was scored from 0–3 for inflammation, mucosal injury, and submucosal edema, and the scores from the worst 3 colonic sections from each mouse added to achieve a total score from 0 to 27. A separate analysis of the inflammatory infiltrate scored 0–3 per section for amounts of lymphocytes and neutrophils, and the values from all 4 sections were added to obtain a score from 0–12 for each type of infiltrate.

**Expression of murine TLR5 in HeLa cells.** A cDNA clone of murine TLR5 (cat. no. MMM1013-99828756) was purchased from Open Biosystems (Huntsville, AL), amplified with specific primers: mTLR5FP 5'-CTTTGGTACCAGGCATGTCATCAACTTGACCTGCTCATAGG-3' and mTLR5RP: 5'-AGAAGCGGCCGAATGTTGCTATGTTGTTCGCAACTG-3' and the resulting fragment.
was cloned with NotI and KpnI into pcDNA3.1. The mTLR5 clone was transiently transfected into HeLa cells with Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. After 2 days, untransfected and transfected cells were stimulated with varying concentrations of 2H3 or WT FliC for 3 hours and the supernatants subjected to IL-8 determination by enzyme-linked immunosorbent assay (ELISA) using the OptEIA kit from BD Biosciences Pharmingen (San Diego, CA) according to the manufacturer's instructions. All assays were performed in duplicate and data are expressed as mean ± SEM.

Mouse anti-flagellin ELISAs. Mouse blood was taken from the saphenous vein before treatment and by heart puncture directly after euthanizing, allowed to clot at room temperature for 30 minutes, spun at 14,000 rpm for 20 minutes and aliquots of the serum were stored at −80°C. Plates were coated with 1 µg/mL *E. coli* H18 flagellin in 100 mM sodium carbonate/bicarbonate coating buffer (pH 9.6) overnight at 4°C, then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline with 0.1% Tween-20 (PBST) for 2 hours at 37°C. Samples of mouse serum were diluted in blocking buffer starting at 1:25 in 4 rows of 5-fold serial dilution and incubated overnight at 4°C. Secondary antibodies were diluted as follows: 1:5000 for IgG1, 1:2000 for IgG2a and IgG2b (all horseradish peroxidase [HRPO]-conjugated goat-α-mouse from Caltag-Invitrogen, Carlsbad CA) and 1:10,000 for total IgG (HRPO-conjugated goat α-mouse, Sigma). After a further 2-hour incubation at room temperature, plates were washed and bound antibodies were detected with the OptEIA substrate reagent set (BD Biosciences Pharmingen) according to the manufacturer's instructions. Samples from different plates were compared by equalizing with 2 different anti-flagellin monoclonal antibodies obtained from injection of Balb/c mice with the H18 flagellin (Lymphocyte Culture Center, University of Virginia). One relative IgG1 unit was equated with a 1:12,500 dilution of mAb 6A1, while 1 relative IgG2a/2b
unit was defined as equivalent to a 1:1,562,500 dilution of mAb 5D4. All samples were tested in duplicate and expressed in means in standard units ± SD.

**Cytokine bead arrays.** Mouse colons, including the cecum, were dissected from the mice and feces removed. Tissue was flash-frozen at −80°C. Homogenates were made from frozen tissue using a tissue homogenizer in 1 mL PBS with 1/100 dilution of Protease Inhibitor Cocktail (Sigma). Homogenates were spun at 14,000 rpm for 5 minutes and supernatants were removed and aliquotted. Cytokine concentrations of IL-6, MCP-1, IL-10, TNF-α, IFN-γ, and IL-12p70 were assessed using the BD Mouse Inflammation CBA kit per the manufacturer's instructions. Samples were read using a BD FACSCanto and analyzed using the manufacturer's software. Cytokine concentrations were normalized to total protein concentration, which was assessed using the BCA Protein Assay kit (Pierce, Rockford, IL).

**Patient cohort.** The study patients were recruited by Dr. Ernest Seidman at McGill University Health Center in Montreal. The cohort included 11 patients with CD, 2 patients with UC, and 4 patients with intestinal inflammatory conditions other than CD or UC. Nine healthy controls were recruited in Vancouver for this study. Patient blood collected in Montreal was sent overnight and assayed immediately upon arrival (approximately 24 hours after initial draw). Blood collected in Vancouver was assayed the same day as drawn.

**Identification of antigen-specific T cells in humans.** Human peripheral blood was combined with IMDM media at a 1:1 ratio and then incubated at 37°C for 48 hours with or without antigen. Antigens used include *Escherichia coli* H18 FliC flagellin, *Lachnospiraceae* A4 flagellin Fla2, and 2H3 (Donnelly and Steiner, 2002). All assays included a positive control for each donor,
either 10ng/ml anti-CD3 to show T cells were able to proliferate and upregulate activation markers in response to TCR stimulation, or 5ug/ml Tetanus Toxoid (CedarLane). Tetanus toxoid was used as a positive control in some assays as most donors should have been vaccinated against tetanus and thus have tetanus-specific T cells in circulation. After 48 hours, 100ul of whole blood was stained with antibodies for CD4, CD3, CD14, CD25 and CD134 (OX40) for 30 minutes and then red blood cells were lysed using FACS Lysing Solution (BD). Samples were read using a FACS Canto, gating first on CD4+CD3+CD14- T cells, and then analyzing populations for CD25 and OX40 expression. A positive result is considered ≥ 1% CD4+CD3+CD14-CD25+OX40+ responder cells within the CD3+CD4+CD14- T cell population after antigen stimulation. Flagellin responders were characterized as having ≥ 1% CD4+CD3+CD14-CD25+OX40+ responder cells in response to either FliC or Fla2 stimulation (or both).

**Human anti-flagellin ELISAs.** Human blood was left to separate overnight or spun at 14,000rpm for 30 minutes, and then serum aliquots were extracted. Serum was stored at -80°C. Plates were coated with 1 µg/mL *E. coli* H18 flagellin in phosphate-buffered saline (PBS) overnight at 4°C, then blocked with 1% bovine serum albumin (BSA) in PBS for 2 hours at 37°C. Samples of human serum were diluted in blocking buffer starting at 1:50 in 4 rows of 10-fold serial dilution and incubated overnight at 4°C. Anti-human IgG (Fab specific)-peroxidase secondary antibody (Sigma) was diluted to 1:40,000 in PBS and added to wells. After a further 2-hour incubation at room temperature, plates were washed and wells were incubated with Streptavidin-HRP (Roche) diluted in PBS at 1:10,000 for 30 min. Bound antibody-streptavidin-HRP complexes were detected with the OptEIA substrate reagent set (BD Biosciences Pharmingen) according to the manufacturer's instructions. Relative antibody concentrations were
expressed as optical density (OD) readings at 450nm. All serum samples were tested at one time and in duplicate, optical densities are expressed in means ± SD.

**Statistical analyses.** For mouse studies, statistical analyses were performed using SPSS (Chicago, IL) or GraphPad Prism (San Diego, CA). Data are expressed as mean ± SEM. Analysis of variance (ANOVA) was used for intergroup comparisons and t-tests or Mann–Whitney U-test used for 2-sample comparisons of parametric and nonparametric data, respectively. For human studies, statistical analyses were performed using student’s paired, 2-tailed t-tests. Comparison of the frequency of flagellin-specific T cells in CD patients versus healthy controls in our cohort was analyzed using a Fisher’s exact test.

**Ethical considerations.** All animal procedures were approved by the University of British Columbia (UBC) Animal Care and Use Committee in accordance with the Canadian guidelines. All laboratory work was carried out with the approval of the biosafety committee of the University of British Columbia. Peripheral blood was obtained following written informed consent. All studies were approved by the University of British Columbia Clinical Research Ethics Board.

4.3  **Escherichia coli flagellin (FliC), but not 2H3, is a potent stimulator of murine TLR5**

In order to ensure that murine TLR5, like its human homolog, is not stimulated by 2H3, the gene was cloned and expressed in HeLa cells. Cells were then stimulated with a range of wildtype (WT) and 2H3 flagellin concentrations and the IL-8 content of the supernatants determined as a quantification of TLR5 activation. As shown in **Figure 4.1**, WT FliC but not
2H3 is a potent stimulator of murine TLR5 at concentrations between 1 and 100 ng/mL, eliciting between 15 and 30 times the amount of IL-8 produced by unstimulated, transfected HeLa cells.

![IL-8 Produced by Different Treatments](image)

**Figure 4.1 Stimulation of murine TLR5-expressing HeLa cells with WT flagellin versus 2H3**

Cells were stimulated with PBS, flagellin (FliC) or 2H3 for 3 hours, and the IL-8 content of the supernatants was quantified by ELISA. Results are expressed as the fold increase in IL-8 versus unstimulated, TLR5-transfected cells. Shown are the means ± SD of 2 experiments. © Inflammatory Bowel Diseases, Ivison et al. 2010, reprinted by permission.

### 4.4 2H3 and FliC cause a similar decrease of survival and increase of weight loss in colitic mice

It has been reported that administration of intrarectal *S. Typhimurium* flagellin to DSS-treated C3H/HeJ mice significantly increases the severity of colitis (Rhee et al., 2005). In order to determine the importance of TLR5 stimulation in the mechanism driving exacerbation, we repeated this experiment in C3H/HeJ mice using *E. coli* WT FliC and 2H3. Mice received 2.5% DSS in their drinking water for 10 days. Daily enemas of PBS, FliC, or 2H3 were commenced on Day 4, and continued until Day 10. Mice were sacrificed 7 days post flagellin administration. While average survival in PBS-treated mice was 83%, treatment with either FliC or 2H3 reduced survival to 62% and 58%, respectively, although the differences were not statistically significant.
(Figure 4.2A). Similarly, average weight loss was increased by treatment with both types of flagellin (Figure 4.2B). Variance in mouse weight was high and statistical significance was only achieved with 2H3 versus PBS ($P < 0.05$ by Tukey's multiple comparison test). Administration of flagellin or PBS-containing enemas to mice given water instead of DSS did not induce significant weight loss or other signs of colitis (data not shown). Although histological analyses of total colons did not show significant differences between mice treated with PBS and those treated with either FliC or 2H3 flagellin (Figure 4.3A), examination of the composition of inflammatory infiltrates revealed a higher proportion of lymphocytes in the FliC- and 2H3-treated mice (Figure 4.3B). Differences were significant for PBS versus FliC ($P < 0.05$, t-test).

In order to ensure that the previously published results, which showed a greater difference between flagellin-treated and PBS-treated mice than those presented here, were not due to the use of *E. coli* rather than *Salmonella* flagellin, we repeated this experiment with *S. Typhimurium* FliC. While eliciting comparable amounts of IL-8 secretion from in vitro cultures of IECs, *Salmonella* FliC did not cause more weight loss than the *E. coli* flagellin 042 (data not shown).
Figure 4.2 Survival and weight loss in DSS-colitic mice treated with WT FliC versus 2H3
(A) C3H/HeJ mice were given 2.5% DSS with their drinking water for 10 days in total. On day 4 of treatment the administration of daily enemas of PBS, 10 µg/mL FliC, or 10 µg/mL 2H3 began. After 7 days of enemas, both enemas and DSS treatment were discontinued. (A) Survival curve; n=13 for FliC and 2H3 and n=12 for PBS. (B) Percent weight loss beginning from start of enemas; n=23 for FliC and 2H3 and n=21 for PBS. Shown are the means ± SEM. Analysis of weight loss with ANOVA showed significant differences between the means (P < 0.05) while Tukey's multiple comparison test showed 2H3, but not FliC differed from PBS at the indicated days (*P < 0.05). © Inflammatory Bowel Diseases, Ivison et al. 2010, reprinted by permission.

Figure 4.3 Histological analyses of colons from DSS-colitic mice treated with WT FliC versus 2H3
C3H/HeJ mice were given 2.5% DSS with their drinking water for 10 days in total. After 3 days, daily enemas of PBS, 10 µg/mL FliC, or 10 µg/mL 2H3 were administered. After 7 days of enemas, mice were euthanized and hematoxylin and eosin (H&E)-stained thin slices of proximal, mid-, and distal sections of colons were graded for either total damage ((left): inflammation, edema and injury; medians shown) or composition of inflammatory infiltrate (right); n = 9 for PBS, 9 for FliC, and 6 for 2H3. *p < 0.05 by t-test. © Inflammatory Bowel Diseases, Ivison et al. 2010, reprinted by permission.
4.5 Flagellin treatment increases colonic IL-12 in a non-TLR5-dependent fashion

For quantification of differences in degree and polarization of inflammation in flagellin-treated versus non-treated tissue, as well as to assess the contribution of TLR5 stimulation to those differences, the cytokine environment of the colon in PBS-, 2H3-, and FliC-treated C3H/HeJ mice was examined by cytokine bead array (CBA). This enabled simultaneous quantification of TNFα, IFN-γ, IL-12p70, and IL-10 production directly in the inflamed tissue. Analysis of samples from the entire length of the acutely inflamed colon revealed that both FliC- and 2H3-treated mice expressed significantly more colonic IL-12 than did the PBS-treated mice (Figure 4.4, $P < 0.05$, 2-tailed Mann–Whitney $U$-test). Of the four cytokines tested, IL-12 was the only one that exhibited significant differences among the groups. These results suggest that treatment with flagellin increases IL-12 production in the colonic mucosa, and that this polarization toward Th-type immunity is not dependent on TLR5-stimulation.

**Figure 4.4 Quantification of colonic cytokine production**

Mice were given 2.5% DSS with their drinking water for 10 days in total; after 3 days of treatment, enemas of PBS, 1 µg FliC, or 1 µg 2H3 were given daily. After 7 days mice were sacrificed and cytokine concentrations in homogenized colon measured by cytometric bead array (CBA). Cytokines were normalized to total protein amounts. IL-12p70 was the only cytokine to yield statistically significant differences among the groups. *$p < 0.05$ by Mann Whitney U-test. © Inflammatory Bowel Diseases, Ivison et al. 2010, reprinted by permission.
4.6 Both 2H3 and FliC induce a strong IgG response in DSS colitis

As the flagellin-mediated increase in disease is not the result of TLR5 stimulation and downstream innate immune responses, it is likely that the increased damage is mediated instead by adaptive immunity. If this is the case, FliC and 2H3, which induce comparable damage in the DSS-treated mice, should also induce a comparable antibody response. To investigate this, we treated a group of C3H/HeJ mice with DSS and either PBS, flagellin, or 2H3 enemas for 7 days, and then discontinued the DSS and allowed mice to heal for 2 weeks. We then measured anti-flagellin titers in serum. To detect a shift in T-cell polarization, IgG1 was measured as an indication of a Th2 response, while IgG2a and IgG2b were measured as an indication of a Th1 response. While analysis of total IgG in the pretreatment serum revealed no detectable anti-flagellin antibodies (not shown), analysis of post-treatment serum showed that PBS-treated mice developed modest IgG1 and IgG2a/2b responses to flagellin (Figure 4.5A&B). In contrast, 2H3- and FliC-treated mice produced high titers of anti-flagellin IgG1 and IgG2a/2b antibodies. The only significant difference found between the 2 groups was that FliC-treated mice had higher titers of IgG2a/2b antibodies than 2H3-treated mice. Therefore, both proteins are capable of inducing a robust immune response under these conditions, although TLR5 stimulation appears to contribute to IgG2 production.
Figure 4.5 Quantification of (A) IgG1 and (B) IgG2a/b in DSS colitis treated mice with and without flagellin

Mice were given 2.5% DSS with their drinking water for 10 days in total; after 3 days of treatment, enemas of PBS, 1 µg FliC, or 1 µg 2H3 were given daily. After 7 days, enemas and DSS-treatment were stopped. Two weeks later the mice were euthanized and serum anti-flagellin antibodies measured by ELISA. Absorbances were normalized according to monoclonal antiflagellin antibodies as described in Materials and Methods; n = 3 for FliC and PBS, n = 4 for 2H3. Shown are the means ± SEM. *FliC>2H3, P < 0.05, t-test. © Inflammatory Bowel Diseases, Ivison et al. 2010, reprinted by permission.

4.7 Lack of TLR5 increases susceptibility to DSS-colitis and does not prevent increased weight loss after administration of colonic flagellin

The results presented above indicate that TLR5 plays a negligible role in the mechanisms driving DSS colitis. Therefore, we examined the ability of DSS to cause colitis in mice devoid of TLR5. For these experiments, TLR5<sup>−/−</sup> C57BL/6 mice and WT mice of the same strain were given 2.5% DSS for 11 days and monitored for weight loss. As shown in Figure 4.6A&B, mice lacking TLR5 are more susceptible to DSS-colitis as evidenced by weight loss (p < 0.05, 2-tailed t-test) and survival analysis (p < 0.001, log rank [Mantel-Cox] test). If pathology mediated by intrarectal administration of flagellin is independent of TLR5, then exacerbation of colitis by FliC enemas should be similar in both TLR5<sup>−/−</sup> and WT C57BL/6 mice. FliC-treated mice lost significantly more weight early in the experiment (day 5 of DSS) than did the PBS-treated mice.
(8% versus 3%, p < 0.001, t-test), although these differences were not sustained on subsequent days (Figure 4.6C). Notably, a similar pattern of weight loss was observed in TLR5<sup>−/−</sup> mice (Figure 4.6D); after 5 days, FliC-treated versus PBS-treated mice lost 6% versus 0.3% (P < 0.001, t-test). These results support the uncoupling of TLR stimulation from colonic damage that was initially reported in experiments showing increased susceptibility to DSS colitis in MyD88-deficient mice (Strober et al., 2007; Uematsu et al., 2006).

Figure 4.6 DSS-colitis and FliC-mediated exacerbation in TLR5<sup>−/−</sup> versus WT C57BL/6 mice
(A) Weight loss during DSS-colitis. Mice were given 2.5% DSS with their drinking water for 11 days and weighed daily to assess illness. (Bl/6 n = 9, TLR5<sup>−/−</sup> n = 8) *P < 0.05, **P < 0.01 by ANOVA. (B) Survival curve of DSS-colitic mice. P < 0.01, Bl/6 versus TLR5<sup>−/−</sup>, log-rank. (C) Weight loss in DSS-colitic C57BL/6 (WT) mice treated with enemas of PBS (n = 11) or FliC (n = 12). P < 0.05 by ANOVA; *P < 0.05, **P < 0.001 by t-test. (D) Weight loss in DSS-colitic TLR5<sup>−/−</sup> mice treated with enemas of PBS (n = 5) or FliC (n = 5). P < 0.05 by ANOVA, **P < 0.001 by t-test. © Inflammatory Bowel Diseases, Ivison et al. 2010, reprinted by permission.
4.8 Antigen specific T cells can be identified in human peripheral blood using CD25 and CD134 (OX40) as markers of activation

Once we determined that flagellin is able to exacerbate inflammatory responses in a mouse model of IBD, and that this effect is not dependent on its TLR5 stimulatory capacity, I began to evaluate the role of this antigen in human disease. To this end, I adapted a novel technique first described by our collaborators Zaunders et al. (Zaunders et al., 2009) to identify antigen specific T cells in human peripheral blood. This technique involves stimulating peripheral whole blood samples with antigen, and then evaluating CD4+ T cells after 48 hours for the expression of the activation markers CD25 and CD134 (OX40). Only cells that are specifically activated by antigen should begin to express these markers of activation, and thus CD25+OX40+ cells at 48 hours should be antigen-specific T cells. Indeed, with antigen stimulation, CD3+CD4+CD25+OX40+ T cells can be identified in human peripheral blood samples, while these cells are lacking in whole blood samples that were not stimulated with antigen (Figure 4.7A).

Figure 4.7 Antigen specific T cell identification in human peripheral blood using markers of activation
(A) Peripheral blood samples were incubated with and without FliC or Fla2 for 48 hours and analyzed by flow cytometry for CD3+CD4+ T cells expressing CD25 and OX40 after red blood cell lysis. (B) Peripheral blood from a healthy control donor and a patient with CD was left unstimulated or incubated with 1ug/ml FliC or 1ug/ml Fla2 for 48 hours and then analyzed for expression of CD25 and OX40 within the CD3+CD4+ T cell pool.
4.9 Flagellin activated T cells are present in peripheral blood of Crohn’s disease patients at a greater frequency than in ulcerative colitis or healthy controls

With this novel technique for identification of antigen-specific T cells, I next evaluated peripheral blood samples from patients with CD and UC, as well as control samples from patients with other inflammatory conditions (termed “inflammatory controls) and healthy controls for the presence of flagellin-specific T cells. After incubation of peripheral whole blood samples with flagellin, I used flow cytometry to identify cells that had upregulated CD25 and OX40. Both *E.coli* FliC flagellin as well as A4-Fla2 flagellin were tested in this assay. I found that CD4⁺CD3⁺CD14⁻CD25⁺OX40⁺ T cells could indeed be identified in IBD patients after flagellin stimulation (Figure 4.7B). Of note, similar results were obtained when using 2H3, the transposon mutant of flagellin that lacks TLR5 stimulatory effects, suggesting that T cell activation in response to flagellin is dependent on the antigenic properties of this bacterial product alone (data not shown).

When IBD patients and controls were classified as responders (≥1% CD4⁺CD3⁺CD14⁻CD25⁺OX40⁺ T cells in response to FliC or Fla2) or non-responders (<1% CD4⁺CD3⁺CD14⁻CD25⁺OX40⁺ T cells in response to FliC or Fla2), I found that CD patients had the greatest frequency of responders compared to UC patients, inflammatory controls, and healthy controls, and that the difference between CD patients and healthy controls in terms of responders was significant (Fisher’s exact, p=0.024) (Table 4.1). This correlates well with literature suggesting that CD patients have greater frequencies of anti-flagellin antibodies compared to UC or healthy control individuals. Because our cohort had only 2 UC patients, one of which was a responder, it is difficult to speculate the true frequency of flagellin-specific T cells in this disease, and it is likely that with a larger cohort, the frequency may be different than what was observed in our study.
Table 4.1 Frequency of CD4⁺CD3⁺CD14⁻CD25⁻OX40⁺ T cell responders in response to flagellin stimulation in patients with CD, UC, inflammatory controls, and healthy controls

<table>
<thead>
<tr>
<th>Donor Type</th>
<th>Flagellin Responders</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohn’s Disease</td>
<td>7 of 11</td>
<td>64%</td>
</tr>
<tr>
<td>Ulcerative Colitis</td>
<td>1 of 2</td>
<td>50%</td>
</tr>
<tr>
<td>Inflammatory Controls</td>
<td>2 of 4</td>
<td>50%</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>1 of 9</td>
<td>11%</td>
</tr>
</tbody>
</table>

4.10 Anti-flagellin antibodies do not always correlate with anti-flagellin T cells and do not correlate with Crohn’s disease in our cohort

Because levels of anti-flagellin antibodies have been shown to be elevated in patients with CD (Lodes et al., 2004), I investigated if flagellin-specific T cells identified in our cohort correlate with the presence of these antibodies. By plotting antibody titres from responders and non-responders individually, I found that anti-FliC antibodies are indeed elevated in individuals with FliC-specific T cells (p=0.03, comparing responders to non-responders) (Figure 4.8A). However, when anti-Fla2 antibody titres were plotted for Fla2 responders and non-responders, I found there was no correlation between Fla2 responders and high Fla2 antibody titres (Figure 4.8B). Although this is surprising, it may be due to a relatively small cohort or treatment effects. Specifically, blood samples were drawn from our patients at various stages in treatment, and it may be that immunosuppressive therapies used for the treatment of inflammatory symptoms dampened the anti-Fla2 humoral immune response that may have otherwise been detected. Although, it is unclear why anti-FliC antibody titres did correlate with T cell responses while anti-Fla2 titres did not, and this may warrant further investigation into the relationship between treatment regimen, humoral anti-flagellin immune responses, and the anti-flagellin T cell response.
Aside from correlating the anti-flagellin humoral response with anti-flagellin T cell responses, I sought to confirm previously published findings and determine if anti-flagellin antibody titres correlated with disease in our cohort. When antibody titres were grouped according to disease, I found that anti-FliC antibody titres were not elevated in patients with CD, and instead were similar to levels found in UC patients, inflammatory controls, and healthy controls (Figure 4.9A&B). This is not entirely unexpected as elevated anti-flagellin antibodies in the literature have been directed against *Lachnospiraceae* flagellins as opposed to *E. coli* derived flagellin (Lodes *et al.*, 2004). However, when anti-Fla2 antibody titres were measured in our own cohort, I found no significant differences between CD patients and our controls (Figure 4.9C&D). This result is surprising as previous studies have shown that anti-Fla2 antibody titres are elevated in about half of CD patients. Again, it may be that with our small sample size and the possibility of treatment regimens interfering with antibody titres, any differences between disease groups may be masked. Thus, without a full analysis of treatment regimens and an increase in cohort size, it is difficult to draw firm conclusions on the anti-flagellin antibody titres found within our study.
Figure 4.8 Correlation of flagellin reactive T cells with anti-flagellin antibodies in human serum
Antibody titres of all responder individuals regardless of disease class were plotted versus antibody titres from non-responders for FliC (A) and Fla2 (B). Responders are defined as individuals with ≥1% CD3^+CD4^+CD25^+OX40^+ T cells in peripheral blood when stimulated for 48 hours with antigen and non-responders are defined as individuals with <1% CD3^+CD4^+CD25^+OX40^+ T cells in these conditions. For FliC responders: n=8, non-responders: n=16, p=0.03; for Fla2 responders: n=6, non-responders: n=18, non-significant difference.

Figure 4.9 Anti-FliC and anti-Fla2 antibody titres in human serum of IBD patients and controls
(A & C) Anti-FliC and anti-Fla2 antibody titres were measured in OD450 units at dilutions of 1:50, 1:500, 1:5,000 and 1:50,000, respectively. A summary of antibody titres for each group is shown in (B & D) for Flic and Fla2, respectively. Abbreviations: CD= Crohn’s disease, UC= Ulcerative colitis, I.C.= Inflammatory controls, H.C.= Healthy controls. CD n=11, UC n=2, I.C. n=4, and H.C. n=9.
4.11 Conclusions

In order to effectively develop Treg cellular therapy for the treatment of IBD, we must first understand the antigens that lead to aberrant T cell proliferation and drive disease. To this aim, we investigated the role flagellin plays in this disease. Specifically, we found that administration of either *E. coli* derived flagellin (FliC) or a transposon mutant of flagellin that is unable to stimulate TLR5 (2H3) leads to increased weight loss and decreased survival in DSS treated mice. This suggests that the detrimental effects of flagellin in this disease are independent of its TLR5 signaling capacity and more dependent on its antigenic properties. In addition, flagellin administration elicits an increase in colonic IL-12 levels, and this is also independent of TLR5; increased IL-12 levels likely result in an increase in Th1 differentiation, and this could in part contribute to the pathology observed with flagellin administration. Apart from T cell mediated immunity, flagellin also induces a strong humoral response, with an increase in total IgG antibody responses after treatment. When these responses were further evaluated for antibody subclass, it was found that both FliC and 2H3 elicit strong IgG1 and IgG2a/2b responses, indicative of Th2 and Th1 type responses, respectively. However, FliC was found to induce a comparatively stronger IgG2a/IgG2b response compared to 2H3, suggesting that this outcome is dependent on TLR5 stimulation. In order to further examine the uncoupling of TLR5 stimulation and flagellin pathology, we evaluated the effect of flagellin enemas in TLR5\(^{-/-}\) C57BL/6 and WT mice treated with DSS. As expected, we found that flagellin leads to weight loss in both groups, further confirming that TLR5 is not required for the exacerbation of colitis. Of note, TLR5\(^{-/-}\) mice treated with DSS alone lost significantly more weight compared to WT mice undergoing the same treatment during the early stages of DSS exposure, suggesting that TLR5 stimulation actually plays a protective role in this model.

Following our investigation into the mechanisms of flagellin mediated disease exacerbation in mice, I employed a novel technique for the detection of antigen-specific T cells
in human peripheral blood to identify T cells that specifically upregulated activation markers in response to flagellin in an IBD cohort. I found that both FliC- and Fla2-reactive T cells could be identified in peripheral blood samples, with a greater frequency observed in patients diagnosed with CD compared to UC patients, inflammatory controls, and healthy controls. By investigating the humoral response to flagellin in humans, I found that FliC-reactive T cells did correlate well with the presence of anti-FliC antibodies, however this was not found for Fla2-reactive T cells or anti-Fla2 antibodies. Similarly, anti-flagellin antibodies did not correlate with disease, and unlike findings of previously published reports, CD patients did not have elevated levels of *Lachnospiraceae* anti-flagellin antibodies. However, these differences may be attributed to a small cohort size and the effects of immunosuppressive treatment regimens.

These studies provide a comprehensive evaluation of the mechanisms by which flagellin may drive inflammation in IBD, as well as a framework to suggest that flagellin-specific T cells may play a role in disease pathology in humans. Furthermore, work to identify flagellin-specific T cells from patient sera may provide a novel diagnostic marker for patients with CD specifically, which can be used in conjunction with, or in place of, the detection of anti-flagellin antibodies. In addition, with the knowledge that the antigenic properties of flagellin drive disease, I can begin to evaluate the effectiveness of Treg therapies directed against this antigen in the treatment of IBD.
5 CONCLUSIONS

The maintenance of immune homeostasis involves a delicate balance of effector responses necessary for the prevention of pathogenic infections and regulatory mechanisms that limit chronic inflammation and prevent autoimmunity. CD4+FOXP3+ Tregs are a key mediator of immune regulation, and are responsible for inducing tolerance to both self and non-pathogenic foreign antigens. If Tregs are lost through genetic or environmental factors, dysregulated immune responses can lead to autoimmune and inflammatory disease. On the other hand, replacing or enhancing Tregs can restore immune homeostasis, prevent autoimmunity, and facilitate long-term acceptance of foreign cells or tissues. Because of their potent, antigen specific suppressive capacity and expansion potential, FOXP3+ Tregs have long represented promising candidates for immune therapy in a chronic inflammatory setting, ideally replacing classical treatment such as broad-spectrum immunosuppressive drugs that run the inherent risk of promoting cancer or increasing susceptibility to infection. Indeed, Treg therapy is already being employed in the setting of allogeneic hematopoietic stem cell transplantation (HSCT) for hematological malignancies, and it is only a matter of time before trials in the setting of autoimmunity commence. However, Treg therapy is not trivial, and has been fraught with setbacks. In particular, concerns regarding the appropriate subset of Tregs to employ and the potential for Tregs to trans-differentiate into effector cell types in an inflammatory milieu have been raised. Thus, if Treg cellular therapy is to succeed, we must first take a step back and examine some of the basic biological characteristics of the Treg lineage. With this in mind, I investigated the phenotypic and functional characteristics of two Treg cell subsets, as well as the capacity of Tregs to mediate immune cell recruitment by the production of chemokines. Furthermore, I investigated putative antigens that could be targeted by Tregs in IBD in order to effectively translate Treg therapy to this setting.
5.1 Phenotypic and functional characteristics of Helios$^+$ and Helios$^-$ Treg cell subsets

Based on reports that the transcription factor Helios can be used to differentiate natural, thymic derived Treg subsets from in vivo peripherally induced Tregs in mice, I evaluated the validity of this marker for this purpose in the human setting. I first identified Helios$^+$ and Helios$^-$ cells in human peripheral blood populations of naive CD4$^+$CD45RA$^+$FOXP3$^+$ T cells, and found that approximately 70% are Helios$^+$. This is in accordance with previously published literature that found the same percentage of Helios$^+$ cells within the FOXP3$^+$ Treg subset in both mice and humans (Thornton et al., 2010). In order to evaluate the phenotypic and functional characteristics of these subsets, something that had not been done, Helios$^+$ and Helios$^-$ Treg cells were isolated from human peripheral blood by sorting on the Treg lineage markers CD4 and CD25 and then performing limiting dilution assays to obtain FOXP3$^+$ Treg cell clones. I was able to categorize clones based on the expression of FOXP3 and Helios in the resting state and then expand these individual populations for subsequent analysis of phenotype and function. Using this method, I showed that ~85% of FOXP3$^+$ clones isolated were Helios$^+$. Although this is a greater proportion compared to that in ex vivo T cells, this discrepancy is likely due to the stringent sorting gates utilized and possibly differences in viability of the two subsets.

There are conflicting reports regarding the stability of Helios expression in Treg cells: one group argues that Helios can only be acquired in the thymus and its expression is maintained in the periphery (Thornton et al., 2010); in contrast, another group suggests that Helios expression can indeed be acquired and it is T cell activation/proliferation that induces it (Akimova et al., 2011). I found that although both Helios$^+$ and Helios$^-$ Treg clonal subsets upregulate Helios expression upon activation— as indicated by an increase in Helios MFI when measured by intracellular staining at various points of activation— increased Helios expression in Helios$^-$ Treg subsets does not reach levels observed in Helios$^+$ Treg clones. Helios$^-$ Treg clones
do demonstrate a ~2 fold greater increase in Helios expression upon activation, but this is likely an artifact of the maximum biological level of Helios that can be expressed in Helios$^+$ Tregs. When combined with the fact that distinct subsets of cells can be isolated based on Helios expression after T cell expansion, and that Helios expression is maintained through a number of rounds of expansion, these data collectively suggests that Helios expression is stable and likely an inherent property of distinct Treg subsets. These data, however, do not indicate if Helios$^+$ and Helios$^-$ subsets are representative of natural and \textit{in vivo} induced Tregs, respectively.

Evaluation of the phenotype of Helios$^+$ and Helios$^-$ Treg clones demonstrated that these subsets express similar levels of previously described Treg markers including FOXP3, CD39, and CTLA-4, and thus cannot be readily differentiated into further subsets. However, Helios$^+$ and Helios$^-$ clones do differ in their production of MIP-1$\alpha$ and IFN-$\gamma$, with Helios$^-$ Tregs producing significantly more of both proteins. The original work describing Helios positive and negative Treg subsets corroborates these results, as Helios$^-$ Tregs in mice were found to express elevated levels of IL-2, IFN-$\gamma$, and IL-17. IL-17 production by our clonal subsets was tested, but never detected by intracellular staining or cytokine bead array. However, this discrepancy may be due to the origin of our clones, as Tregs in our studies were sorted from naïve CD25$^{hi}$ T cells, shown to lack Th17 precursor populations (Miyara et al., 2009), whereas the previous report examined cytokine production in a mixed population of naive and memory Helios$^-$ Tregs. The increased cytokine/chemokine production by Helios$^-$ Treg clones, and particularly the increased levels of IFN-$\gamma$ secretion, is interesting as IFN-$\gamma$ producing Tregs have been identified in patients with multiple sclerosis (Dominguez-Villar et al., 2011). It may be that the Helios$^-$ Treg population is being specifically identified in this study, although this is only speculative as this group did not include Helios staining in their assays. The subsets of Treg clones did not differ in their production of Granzyme A, suggesting they may both rely on this effector molecule for their suppressive capacities.
In terms of function, I found that Helios\(^+\) and Helios\(^-\) Treg clones display equal abilities to suppress proliferating CD4\(^+\) T cells and trans-differentiate into IFN-\(\gamma\) producing cells when cultured in Th1 conditions. This is the first study comparing these characteristics in Helios Treg subsets, and lends support to the idea that both populations would be effective in the context of cellular therapy. Furthermore, the equal potential to trans-differentiate is particularly important as this provides circumstantial evidence to suggest that Helios\(^+\) and Helios\(^-\) clones are not representative of natural and \textit{in vivo} induced Tregs, respectively. This is because the literature indicates that nTregs and iTregs differ in plasticity potential, with \textit{in vitro} iTregs exhibiting less stable FOXP3 expression and a propensity to express cytokines. Moreover, this was also seen in our own studies, as \textit{in vitro} iTregs express more IFN-\(\gamma\) compared to nTregs when cultured in Th1 conditions. Of note, IFN-\(\gamma\) expression in our studies was expressed as the percent of cells producing this cytokine by IC staining, and not by the concentration of this protein in supernatants, as measured by ELISA. In future studies, IFN-\(\gamma\) expression could be evaluated by ELISA in conjunction with IC staining to further confirm our findings, as IC staining tends to underestimate the amount of IFN-\(\gamma\) produced.

The methylation state of the FOXP3 TSDR is indicative of FOXP3 stability and lineage commitment. A demethylated FOXP3 TSDR is suggestive of long-term FOXP3 expression and has been described in natural FOXP3\(^+\) Tregs. The methylation state of iTregs is controversial, as murine studies have found a difference based on the \textit{in vitro} or \textit{in vivo} origin of these cells (Curotto de Lafaille and Lafaille, 2009). Helios\(^+\) and Helios\(^-\) Treg clones in my studies are less methylated at all CpG loci in the FOXP3 TSDR compared to Tconv clones. However, when comparing Helios\(^+\) and Helios\(^-\) Treg clones to each other, Helios\(^-\) clones are more demethylated. It is difficult to speculate what this means as FOXP3 expression and lineage commitment seem to be equal in both subsets, although this does lend validity to the idea that Helios\(^+\) and Helios\(^-\) Treg subsets are distinct lineages.
To better define the putative origin of Helios- Tregs, I used flow cytometry to examine markers that are indicative of thymic derived versus peripherally induced cells. I found this subset to exist in naïve ex vivo CD4+CCR7+CD62L−CD45RA+FOXP3+ Treg populations. CCR7 and CD62L are both chemokine receptors that are expressed predominantly in naïve, circulating T cells and allow for the migration of these cells into the periphery upon activation. If the Helios- Treg subset indeed represents peripherally induced Tregs, I would expect these cells to display a more mature phenotype as a consequence of their previous activation, with a loss of CCR7 and CD62L. The finding of up to 38% Helios- Tregs in the naïve Treg pool suggests that this subset is not composed exclusively of in vivo iTregs. Moreover, in the examination of naïve Tregs that have recently emigrated from the thymus, I found ~34% of CD4+CD45RA+CD31+ cells did not express Helios. Similar findings are reported in the literature, as half of recent thymic emigrants have been shown to be Helios- (Akimova et al., 2011). Although there is small discrepancy in the precise percentage of Helios- cells identified in the population of recent thymic emigrant Tregs, this is likely due to differences in gating strategies. Regardless, along with reports showing that Helios can indeed be induced in peripheral Tregs in the presence of APCs (Verhagen and Wraith, 2010), these data support the suggestion that Helios is not an exclusive marker of thymic derived Tregs and cannot be used to differentiate in vivo peripherally induced Tregs.

In conclusion, I have shown that Helios+ and Helios- FOXP3+ Treg subsets can be identified and cloned from human peripheral blood. Furthermore, I evaluated phenotypic and functional characteristics of these subsets to show they are equal in terms of Treg lineage marker expression, suppressive capacity, and plasticity potential. However, Helios positive and negative Treg subsets do differ in cytokine/chemokine production as well as in methylation state of the FOXP3 TSDR. Together, these data comprise the first comprehensive study on Treg subsets defined by their Helios expression and suggests that both subsets would be equally beneficial in terms of Treg cellular therapy.
5.2 Chemokine production by FOXP3⁺ T regulatory cells

This study is the first broad examination of both CC and CXC family chemokine expression by human Tregs and an important characterization of Treg function beyond their suppressive capacity. I found that pure populations of TCR-stimulated CD4⁺FOXP3⁺ Tregs produce significant levels of CXCL8, and these levels were comparable to those produced by CD4⁺CD25⁻ Tconv cells. This finding alone is surprising as Tregs are classically defined by their inability to produce effector cytokines, and in particular those that are involved in inflammation. CXCL8 is a key mediator of innate immune activity by promoting the recruitment of neutrophils, a highly inflammatory cell type. Thus, the production of this chemokine by Tregs is counter to current opinion concerning the strict immunosuppressive role of these cells in disease.

Furthermore, naïve CD4⁺CD45RA⁺ and memory CD4⁺CD45RA⁻ Tregs both produce CXCL8 with stimulation, demonstrating that chemokine production is not a unique characteristic of either subset. However, naïve Tregs are capable of producing greater amounts of CXCL8 compared to memory Tregs, as measured by cytokine bead array. This is a particularly important finding as cells within the memory Treg pool have been associated with inflammatory characteristics, secreting IL-17 and expressing the Th17 lineage transcription factor RORgt (Ayyoub et al., 2009); it would be expected that the expression of CXCL8, classically considered to be a chemokine involved in the promotion of inflammation, would be restricted to this population of cells. However, this is not the case, as it is indeed classically defined FOXP3⁺ Tregs that are responsible for CXCL8 production, and not IFN-γ or IL-17 expressing subpopulations. This result, as measured by cytokine bead array testing of supernatants isolated from stimulated and unstimulated populations of Tregs and Tconv cells, was also confirmed by intracellular staining of ex vivo CD4⁺ T cell populations and expanded populations of sorted cells. Intracellular staining was also used to confirm that it is indeed FOXP3⁺ Tregs, and not
contaminating cells within the population such as natural killer cells or conventional T cells, that produce significant amounts of CXCL8.

Using FOXP3 transduced T cells, I investigated the relationship between FOXP3 and CXCL8 expression. I found that transduction of conventional T cells with FOXP3, a technique that is sufficient to generate properties of Treg cells including suppressive capacity, results in the enhancement of CXCL8 production. Moreover, analysis of the CXCL8 promoter region upstream of the CXCL8 gene reveals several sequences reported to be putative FOXP3 binding sites (Koh et al., 2009); by generating a luciferase reporter construct containing the CXCL8 promoter region, I demonstrated that FOXP3 can directly bind and enhance CXCL8 gene transcription. FOXP3 has been shown to regulate a number of genes, including those necessary for the expression of CD25, GITR, Neuropilin-1, and the chemokine receptor CCR4 (Marson et al., 2007), however this is the first evidence to suggests that this transcription factor can enhance chemokine gene expression. This is particularly of interest as FOXP3 primarily acts as a transcriptional repressor of genes associated with T cell proliferation, as opposed to an enhancer.

Along with CXCL8 production, using a Luminex based chemokine array, I demonstrated that pure, sorted populations of naïve and memory Tregs produce a number of other chemokines including CCL2, CCL3, CCL4, CCL5, CCL7, and CXCL10. CCL3 and CCL4, commonly referred to as macrophage inflammatory protein-1a (MIP-1α) and macrophage inflammatory protein-1b (MIP-1β) respectively, are both involved in the acute phase immune response along with CXCL8, and are potent mediators of neutrophil and monocyte recruitment (Charo and Ransohoff, 2006). CCL2, also known as monocyte chemotactic protein-1 (MCP-1), along with CCL7 (monocyte specific chemokine-3, MCP-3) and CXCL10 (interferon gamma induced protein-10) are all involved in the recruitment of monocytes (Charo and Ransohoff, 2006). Collectively, this suggests a role for Tregs in directing the innate immune cell response, although the effect of Tregs on these cells- i.e. if innate immune cell function is enhanced or
repressed once recruitment occurs- is unknown. In addition to secreting innate immune cell chemoattractants, Tregs also produce lymphocyte recruitment factors (CCL5, also known as RANTES, and CXCL10) that are likely involved in allowing Tregs to mediate contact-dependent suppressive effects on conventional T cell populations (Charo and Ransohoff, 2006). Of note, investigations concerning FOXP3 regulation of chemokines apart from CXCL8 were not conducted, but may be beneficial in better understanding the direct relationship between chemokine production and the Treg cell program.

Chemokine production by Tregs is similar to that of Tconv cells, although I sought to pinpoint any differences in order to elucidate if a unique role exists for chemokines produced specifically by Tregs. Using a chemokine and chemokine receptor PCR array, I measured levels of mRNA and identified genes that are differentially expressed in Tregs compared to Tconv cells. I found that a number of chemokine and chemokine related genes are upregulated in Tregs, including: TREM1, TNFSF14, CSF3, CCR5, CCL7, CCL13, and CCL1. In addition, CXCL8 and CCR5, the receptor for MIP-1α and MIP-1β, are also upregulated in this array, further validating our previous findings. Again, a majority of these genes are involved in the activation or recruitment of monocytes, neutrophils, and lymphocytes (Charo and Ransohoff, 2006). Interestingly, a number of these chemokine related genes are actually involved in the inflammatory response, counter to what would be expected in Tregs: TREM-1 is a recently described pathway important for the activation of inflammatory responses in neutrophils and monocytes (Bouchon et al., 2000); TNFSF14 (LIGHT) is a tumor necrosis factor family receptor that enhances the proliferation of T cells (Cohavy et al., 2005); and CSF3 (Colony-stimulating factor 3) promotes the differentiation and function of neutrophils (He et al., 2008). Perhaps Treg functions extend beyond adaptive immune suppression and into orchestration of the inflammatory innate immune response.
Of note, several chemokines and chemokine associated proteins are more highly expressed in Tconv cells compared to Tregs, including CXCL12, CXCL6 and matrix metalloproteinase-2 (MMP-2). MMP-2 has been reported to cleave CD25 and decrease Treg proliferation (Sheu et al., 2001), so it is not surprising this factor is downregulated in these cells. However, CXCL12 and CXCL6 are both involved in the normal recruitment of immune cells (Charo and Ransohoff, 2006) and are expressed at lower levels in Tregs for unknown reasons.

In the assessment of the biological consequence of chemokine production by Tregs, I found that supernatants from stimulated CD4⁺CD25<sup>hi</sup> cells induce neutrophil chemotaxis in a similar manner to CD4⁺CD25<sup>+</sup> Tconv cells. Neutrophil recruitment is reduced by addition of anti-CXCL8 antibodies, demonstrating that chemotaxis is in part dependent on CXCL8 expression. Chemotaxis in the presence of anti-CXCL8 antibodies suggests that chemokines such as CCL3 (MIP-1α) and CCL4 (MIP-1β) may also play a biologically significant role, although this is not conclusively shown. The concept that chemokine production by Tregs is biologically important is supported by the previous finding that human Tregs also make XCL1 (lymphotaxin α), and this C-family chemokine contributes to their suppressive function (Nguyen et al., 2008). Interestingly, other chemokines, such as CCL4, CCL19 and CCL21 can also suppress T cell responses (Ziegler et al., 2007; Joosten et al., 2007), suggesting that chemokine production by Tregs could contribute to their suppressive mechanism of action.

An open question remains as to what the consequence of bringing neutrophils in close proximity to Tregs would be. One study suggested that Tregs may suppress the function of neutrophils by inhibiting reactive oxygen species generation and cytokine production, as well as promoting neutrophil apoptosis and death (Lewkowicz et al., 2006). The validity of these data, however, is unclear as the findings were based on activating Tregs with LPS, not via the TCR, and our group has previously shown that human Tregs do not respond to LPS (Crellin et al., 2005). On the other hand, chemokine production by Tregs could serve to facilitate innate
immunity by recruiting phagocytic cells that are essential for healing of injured tissue. For instance, neutrophils are necessary for effective wound healing (Nathan, 2006). Intriguingly, many of the toxic products of neutrophils, such as arginase and reactive oxygen species, directly suppress T cell activation (Muller et al., 2009). Moreover, Tregs are less sensitive than Tconv cells to oxidative stress-induced cell death and maintain their suppressive activity at H$_2$O$_2$ levels that are lethal for Tconv cells (Mougiakakos et al., 2009), suggesting they are well equipped to withstand the toxic products of innate immune cells. Alternatively, the recruitment of neutrophils and monocytes in the early stages of an immune response may allow for quick and effective resolution of an immune insult with limited involvement of effector T cells. This partly avoids the possibility of chronic inflammation and may be less energetically taxing on the immune system as well as avoids the activation of T cells specific for self or commensal antigens in the acute setting.

In conclusion, I have shown that FOXP3$^+$ Tregs produce CXCL8 along with a variety of other chemokines at both the protein and mRNA level. Furthermore, FOXP3 can directly regulate CXCL8 gene expression, and Treg derived CXCL8 is biologically active. Collectively, these data support a role for Tregs outside of their suppressive capacities alone, a characteristic that must be considered and may be advantageous in the development of Treg therapy for mucosal disease.

5.3 **Flagellin exacerbates colitis independently of TLR5 and is a putative antigen recognized by T cells in inflammatory bowel disease**

The data presented in this chapter indicate that the ability of flagellin to exacerbate damage in DSS colitis is not dependent on TLR5 activation. This would suggest that flagellin-mediated damage is driven by adaptive, rather than innate immunity. A key tool in this evaluation is the transposon mutant of wild-type *E. coli* derived flagellin, 2H3. 2H3 does not
elicit IL-8 (CXCL8) production from HeLa cells expressing TLR5 showing it lacks TLR5 stimulatory capacity.

Using 2H3 in our mouse studies, we demonstrated that the effect of flagellin on colitis is much less pronounced than was reported by Rhee et al. (Rhee et al., 2005). While the survival rates exhibited by DSS- but not flagellin-treated C3H/HeJ mice are comparable in the two studies (83% versus 85%), our flagellin-treated mice survive about 60% of the time, while theirs had only a 26% survival rate. In this study, a histological analyses of colitic colons reveals no statistically significant difference among the groups. Mice treated with S. Typhimurium flagellin also fail to develop increased pathology, indicating that reduced exacerbation is not due to the use of E. coli flagellin in place of the Salmonella-derived protein. The observed differences in flagellin potency to increase pathology may reflect local, facility-linked variations in commensal microbiota as well as general stress levels, food, or other upkeep conditions.

However, despite differences in degree, our results confirm that colonic application of flagellin does exacerbate DSS-colitis and provides evidence that TLR5 is not involved in the mechanism. This is supported by the finding that DSS-colitis actually downregulates TLR expression in colonic tissue (Ortega-Cava et al., 2006), and also by a recent report that an intraperitoneal injection of flagellin administered immediately prior to the start of DSS treatment can actually be protective (Vijay-Kumar et al., 2008). The fact that intrarectal flagellin worsens colitis while intraperitoneal flagellin is protective is likely the consequence of different routes of administration; TLR5 distribution in mouse tissues remains somewhat uncertain due to the lack of high-affinity detection antibodies, but there are reports that expression on the colonic mucosa is low and mainly confined to the cecum (Ortega-Cava et al., 2006). Moreover, there are differences in outcome following TLR5 ligation in different tissue compartments. For example, flagellin lacking the hypervariable domain induces a strong mucosal but a poor systemic antibody response (Nempont et al., 2008). A protective effect for TLR5 is also supported by the
fact that deletion of TLR5 in mice predisposes them to spontaneous colitis (Vijay-Kumar et al., 2007).

The fact that we see a significant increase of illness in the C3H/HeJ mice given flagellin enemas within 7 days suggests that it is unlikely to be antibody-mediated damage. However, as T cells are activated within hours and proliferate within days (Mowat, 2003), they could contribute to the illness seen in our model. Thus, if the findings in this murine model can be extended to those found in humans, the anti-flagellin antibodies seen in CD patients are likely an indication of prior (or ongoing) T-cell activation rather than being direct mediators of damage. This is supported by the following: antibody titers do not correlate with disease severity (Targan et al., 2005), antibody responses to flagellin require T-cell help (Sanders et al., 2006), and adoptive transfer of flagellin-reactive T-cells can drive colitis in susceptible mice (Lodes et al., 2004). Although the ability of DSS to induce acute colitis in mice devoid of T-cells (Axelsson et al., 1996; Dieleman et al., 1994) has led to the postulation that damage in the acute model is largely dependent on innate immunity, experiments in other strains of mice show that, even within the first 6–8 days of DSS-treatment, severity of colitis correlates positively with the presence of T cells in the large intestine (Sund et al., 2005; Araki et al., 2005). Interestingly, our histological analyses of inflammatory infiltrates reveals a tendency of FliC- and 2H3-treated mice to accumulate more lymphocytes in their colons than did PBS-treated mice, further supporting this hypothesis.

The mechanisms of T-cell mediated damage depend on the polarity of the response. Analysis of T-cell polarizing cytokines in the acutely inflamed colon indicates that there are no obvious differences between FliC- and 2H3-treated mice. Generally, TNF-α levels are high in all of the mice, consistent with the severe colitis observed. Interestingly, both FliC- and 2H3-treated mice produce more IL-12 than PBS-treated mice. It is difficult to explain how a non-TLR5-stimulating flagellin could induce as much IL-12 as a wildtype flagellin; this indicates that there
is a TLR5-independent mechanism at work. At present, it is not known if alternative, intracellular flagellin receptors such as Ipaf are significant contributors to mucosal immunity or if 2H3 is capable of binding them. However, a recent report showed that the C-terminal 35 amino acids of Salmonella FliC are required for binding to Naip5 and activating Ipaf (Lightfield et al., 2008); as this region is 20 amino acids downstream of the transposon insertion site in 2H3, the mutation might not prevent Ipaf activation. Therefore, the potential role of Ipaf in DSS colitis may merit further study.

None of the other cytokines analyzed showed significant differences between flagellin and PBS-treated mice, including the classic Th1 and Tr1 cytokines IFN-γ and IL-10. Colitic mice that received PBS enemas exhibit modest anti-flagellin IgG1 and IgG2a/b responses; these are presumably induced by endogenous flagellins and similar findings have been reported by others (Sanders et al., 2006). In contrast, mice receiving enemas containing FliC or 2H3 produce large quantities of anti-flagellin antibodies of both the IgG1 and IgG2 subtypes (associated with Th2 and Th1 responses, respectively). However, 2H3 did produce slightly lower amounts of IgG2a/2b than FliC, indicating that the lack of TLR5 stimulation does have some downstream consequences for Th1-type antibody production. The equivalent ability of FliC and 2H3 to generate antibodies demonstrates that there is enough adjuvant activity in the colon contents to compensate for lack of TLR5-mediated auto-adjuvancy with 2H3. This result, which also confirms the conservation of antigenic regions in 2H3 compared to the wildtype protein, is supported by a recent report showing that TLR5 and even MyD88 are not required for the induction of adaptive immunity by flagellin (Sakaguchi et al., 2006).

Administration of flagellin to DSS-colitic C57BL/6 mice does not exacerbate colitis to the degree observed in the C3H/HeJ mice. Strain differences in the degree and mechanism of chemically induced colitis have been previously reported (Dieleman et al., 1994; Mahler et al., 1998), with C3H/HeJ being more susceptible to DSS than the C57BL/6 strain (Mahler et al.,...
1998). Nevertheless, flagellin enemas do elicit a transient increase in weight loss compared to PBS-treated mice in both the TLR5−/− and WT C57BL/6 strains.

Currently there is a considerable body of literature involving flagellin, TLR5, and colitis that is not easily integrated into a cohesive picture. Although on the one hand there is evidence linking colitis, especially CD, to anti-flagellin adaptive immune responses (Lodes et al., 2004; Rhee et al., 2005; Targan et al., 2005; Sitaraman et al., 2005; Harbord et al., 2006) there is a separate body of literature that suggests that CD is a consequence of downregulated innate immune responses (Nempont et al., 2008; Bardel et al., 2005; Hugot et al., 2001). More recently, the evidence that TLR5 has a protective function in the intestine is also increasing (Strober et al., 2007; Gewirtz et al., 2001; Vijay-Kumar et al., 2007). These results, together with those shown here, highlight the importance of investigating TLR5-independent anti-flagellin immunity in human IBD.

To this end, a novel technique for the identification of flagellin-specific T cells in human IBD patients was employed. Through this, I demonstrate that patients with CD are more likely to have detectable percentages of FliC- or Fla2-reactive T cells in their blood compared to healthy controls. These studies involved a small cohort of IBD patients, and although the sample size from patients with CD and controls was modest but adequate, the analysis of UC was restricted by an extremely small sample size (2 patients).

The technique used for the identification of flagellin-specific T cells is based on the hypothesis that upon recognition of flagellin by its cognate TCR, T cell activation will occur. This is measured by upregulation of CD25 and OX40, both shown to be rapidly expressed following TCR ligation (Allan et al., 2007; Zaunders et al., 2009). This method for antigen-specific T cell detection has been demonstrated and validated in the context of a number of antigens including tetanus toxoid (Zaunders et al., 2009), although its validity for the identification of flagellin-reactive T cells has never been specifically shown. Regardless, this
powerful technique allows us to quickly and efficiently evaluate samples without the need for long-term culture or the risk of compromising viability. Maintenance of viability after identification is important as this will allow for sorting and expansion of antigen specific T cells, and consequently a greater in-depth study of these populations.

Along with the identification of flagellin-specific T cells in CD patients, I demonstrated that anti-flagellin antibodies recognizing both Flic and Fla2 can be detected in patient sera using ELISA. However, although antibodies do correlate with the presence of cognate T cells for FliC, this correlation is not seen for Fla2. Furthermore, contrary to the literature, anti-flagellin antibodies in our sample did not correlate well with disease. These results are all likely influenced by the small cohort recruited for these studies, as well as a lack of discrimination concerning treatment regimen for patients. With an increased cohort size and a comprehensive record and/or analysis of treatment regimens including the use of biological therapies, a more complete assessment of the validity of anti-flagellin antibodies for the diagnosis of CD can be made.

In conclusion, these studies show that flagellin exacerbates colitis via a TLR5 independent mechanism. Furthermore, this exacerbation may be dependent on the adaptive immune response generated by its antigenic properties. Indeed, flagellin-specific T cells are specifically detected in patients with CD, and may be a better measure of disease than serum levels of anti-flagellin antibodies. These data collectively suggest that flagellin may be an ideal antigenic-target for Treg mediated therapy in IBD.

5.4 Challenges of Treg therapy in IBD

Apart from the general phenotype of FOXP3+ Treg subsets and the antigens that drive disease, a number of major questions must be answered before Treg therapy can be contemplated in the context of IBD. For example, if a targeted approach is taken with Tregs that are specific
for disease associated antigens, how would the resultant sudden increase in suppressive mechanisms at the tissue-environment interface affect the risk of infection while preserving a normal balance of commensal flora? Another caveat is the potential for infused Tregs to transdifferentiate and lose their suppressive function. Although expanded Tregs may be suppressive in vitro, the environmental milieu of inflamed mucosal tissues could substantially alter the in vivo function of these cells. For example, in the presence of activated effector T cells secreting inflammatory cytokines, mucosal tissues could preferentially shift Tregs towards Th17-like cells (Zhou et al., 2008). The data generated in this thesis provides some evidence to suggest that Helios+ and Helios− Tregs would be stable in a Th1 inflammatory setting, although investigation into their stability in a Th17-type environment is warranted.

Notably, although Tregs may acquire the ability to make effector cytokines, their suppressive capacity may nevertheless be maintained, circumventing the need to avoid “Th1 conversion” in vivo. Indeed, although CD patients have increased levels of FOXP3−IL-17+ T cells in their inflamed mucosal tissues, these cells retain potent suppressive capacity (Hovhannisyan et al., 2010). Similarly in mice, transfer of FOXP3+ Tregs that recognize microbial antigens into immune deficient animals results in the conversion of these cells into IFN-γ producers, but both their regulatory activity and FOXP3 expression are maintained (Feng et al., 2011). Furthermore, in this thesis, although Helios−FOXP3+ Treg clones produce IFN-γ, they are equally suppressive compared to non-cytokine producing Helios+FOXP3+ Tregs. In the context of cellular therapy, these latter studies and our own work are promising, since they suggest that regardless of the inflammatory environment they encounter, and any transient effector cytokine production, Tregs will remain suppressive.

How do we ensure that therapeutic Tregs travel to the site(s) at which they could be maximally effective? It is currently unclear whether relevant suppression might occur in the local lymph nodes and/or in the intestinal tissue itself. On the one hand, Tregs could be targeted
to the intestinal environment by engineering them to express chemokine receptors that attract them to specific tissues (Campbell and Koch, 2011). On the other hand, it is possible that antigen-specific Tregs would in any case traffic appropriately to the sites where the relevant antigen is concentrated. Thus studies undertaken in this thesis to examine antigens associated with IBD may eventually aid in the development of targeted Treg therapy.

Selection of the best candidates for Treg therapy presents a further problem, since symptom presentation, onset, severity, and treatment response all vary. In mouse models, Tregs are more effective at preventing disease than curing it (Mottet et al., 2003). Treg therapy would likely be most effective in the early stages of disease, but since these patients have many other therapeutic options, it may be difficult to find cohorts in which testing of this therapy can be justified. Furthermore, IBD is a heterogeneous disease and each individual is likely to have distinct disease etiology, microbiota composition, and relevant antigens. It may therefore be challenging to determine standard dosing and delivery schedules, as well as to monitor outcomes.

Animal models of Treg therapy for IBD have relied on transfer of cells into T cell-deficient animals. Will a similar conditioning step be necessary in IBD to make space for the Tregs to engraft and allow their expansion through homeostatic expansion mechanisms? Since IBD is not usually a life-threatening disease would such a pre-conditioning regimen be ethical? Here we will be able to learn from the results of a trial in type 1 diabetes, which is currently enrolling patients, where Tregs will be infused into immunocompetent individuals (http://www.clinicaltrials.gov/ct2/show/NCT01210664).

Another concern for Treg therapy in IBD is the effect on IBD-associated malignancies such as colorectal cancer (Beaugerie, 2012). Long-term suppression of any kind can lead to an increased risk of cancer, and it may be that Treg therapy exacerbates this issue. However, it is likely that the suppression mediated by Tregs will be more targeted compared to the broad-
spectrum immunosuppressants currently used for IBD therapy and thus may actually ease the incidence of associated malignancies. Regardless, a proper benefit-risk ratio must be determined, and indeed it may differ between individuals based on their genetic background and medical history.

Once Treg therapy is administered, what parameters will determine the extent to which treatment has been effective? In contrast to the scenario of transplantation (Sagoo et al., 2010; Newell et al., 2010), there are currently few effective biomarkers of relevant immune status in IBD, and apart from monitoring disease symptoms and crude analysis of T cells from biopsies, there is no way to test if the therapy has re-set immune homeostasis. The efficacy of current therapeutic agents such as anti-TNF-α antibodies will likely set the bar high for Treg therapy, possibly requiring life-long cure with minimal side-effects. Although there are still many unknowns and theoretical risks, it is the hope that delivery of Tregs will indeed be able to reset intestinal immunity that justifies the studies conducted in this thesis.

5.5 Summary and future directions

Herein, I have examined specific subsets and basic functional mechanisms of CD4⁺FOXP3⁺ Tregulatory cells, as well as developed novel methods for the evaluation of putative antigens involved in inflammatory mucosal disease. While the translation of Treg cellular therapy to human disease is not trivial, the data I have generated contribute to our overall understanding of Tregs and proposes new considerations for the application of this therapy in IBD.

By examining human CD4⁺ T cells for Helios expression, I have confirmed that FOXP3⁺ Tregs can be further subdivided into populations that are either Helios⁺ or Helios⁻. These data suggest that the Helios transcription factor may play a functional role in a subset of Tregs, but what this function is remains unknown. Indeed, knockdown of Helios in human
CD4^+CD127^−CD25^{hi} Tregs with small interfering RNA (siRNA) does not reverse Treg cell anergy or impede suppressive function (Thornton et al., 2010). However, the consequence of Helios knockdown by siRNA on the long-term stability of Tregs or the cytokine producing capabilities of these cells was not determined, and these studies would be highly relevant to evaluating the benefit of Helios^+ versus Helios^− Tregs in Treg cellular therapy. In addition, although the scope of studies described in this thesis focused on FOXP3^+ Tregs, it may be interesting to determine if Th17 or Tr1 cells express Helios, either in the resting state or upon activation. These investigations could provide insight into the biological function of the Helios transcription factor, furthering our understanding of its relevance in FOXP3^+ Treg subsets.

I demonstrated that Helios^+ and Helios^− Tregs are similar in suppressive capacity and stability, but differ in cytokine and chemokine production. These data are in line with studies from Shevach et al. showing that FOXP3^+Helios^− T cells in humans produce more IFN-γ than FOXP3^+Helios^+ T cells (Thornton et al., 2010). However, to further our understanding of differences between these subsets, future studies could evaluate the ability of Helios^+ and Helios^− Tregs to produce IL-10 and TGF-β. These cytokines are key mediators of suppression (Horwitz et al., 2008b) and may be beneficial in a Treg therapy setting. Furthermore, because FOXP3^+Helios^− Treg subsets have the propensity to secrete cytokines, it may be that this population relies more heavily on soluble factors to mediate their suppressive effects. This hypothesis could be tested by performing suppression assays in transwell plates to determine if suppression is contact-dependent or by using blocking antibodies to IL-10 or TGF-β.

This work also demonstrated that Helios^+ and Helios^− Treg subsets exhibit similar FOXP3 TSDR demethylation, can be found within the naïve CD4^+ T cell population, and differ from in vitro iTregs in terms of ability to trans-differentiate into IFN-γ producing cells in inflammatory cytokine containing conditions. These data provide indirect evidence to support the hypothesis that Helios^− Tregs are not human in vivo iTregs, as suggested by Shevach et al.
(Thornton et al., 2010). This conclusion has been corroborated by a number of recently published studies emphasizing that Helios can be induced by T cell activation and is not exclusive to the thymus (Akimova et al., 2011; Verhagen and Wraith, 2010). In order to directly assess if FOXP3⁺Helios⁻ Tregs are indeed human in vivo iTregs, it will be necessary to examine human thymocytes for Helios expression. The presence of FOXP3⁺Helios⁻ T cells in the thymus will provide direct evidence that this subset is not exclusively peripherally induced. However, this experiment may be difficult to interpret as in mice between 0.1% and 5% of antigen-mature T cells from the periphery have been demonstrated to re-enter the thymus and persist up to 60 days (Hale and Fink, 2009), and the same may be true for humans. Thus, although trafficking back to the thymus is rare, the existence of a small percentage of FOXP3⁺Helios⁻ Tregs in this organ could be attributed to the re-entry of peripherally induced cells. To rule out this possibility, human neonatal thymocytes could be examined. Mature T cells should not yet exist in neonates and thus the presence of FOXP3⁺Helios⁻ iTregs in the thymus could not be attributed to re-entry from the periphery.

In addition to better understanding Helios⁺ and Helios⁻ Treg subsets, I examined chemokine expression from the FOXP3⁺ Treg population as a whole. I found that FOXP3⁺ Tregs express a variety of chemokines, chemokine receptors, and chemokine related proteins and actively recruit neutrophils. Future work could examine if Tregs are able to attract other innate and adaptive immune cells, including macrophages and T cells. These studies could be performed using recruitment assays similar to those described in Chapter 3, but with total peripheral blood mononucleocytes (PBMC’s) instead of neutrophils alone, to ascertain which cells are preferentially recruited by Tregs. Furthermore, once the cell recruitment capabilities of Tregs are fully realized, studies to determine the biological consequence of Treg chemokine production could be initiated. Specifically, human Tregs could be co-cultured with the cells that they are determined to recruit, and any changes in phenotype or function of these cells could be
evaluated. In addition, the effect of recruited immune cells on Tregs themselves could be examined, including changes in FOXP3 expression, suppressive capacity, or stability. This work would further our knowledge regarding the fundamental role of Tregs in the immune response, and allow us to better realize the potential for Tregs in treating inflammatory diseases.

Direct evidence for the biological role of chemokine production from Tregs would be best ascertained in a mouse model. Although mice do not express CXCL8, they do express a homologue of this chemokine, KC, as well as a number of other chemokines involved in the recruitment of innate and adaptive immune cells. The expression of chemokines could be evaluated in murine Tregs using similar methods to those employed in this thesis, namely: mouse chemokine PCR arrays and CBA analysis of mouse Tregs. Following this, specific knockdown of chemokines in murine Tregs by siRNA targeting could be used to determine the role of chemokines in Treg function. Similarly, chemokine receptor expression by Tregs could also be examined, and Tregs isolated from chemokine receptor knockout mice could be used to evaluate how specific chemokine receptors influence Treg trafficking and function.

In order to use Tregs effectively in the setting of IBD, the antigens that drive disease must be determined. We found that flagellin is able to exacerbate colitis in mice, and that this effect is dependent on its antigenic properties. In these experiments, it may be interesting to test flagellin proteins isolated from a variety of commensal microbes other then those tested to determine if specific bacteria can induce inflammatory immune responses in DSS colitis. Furthermore, although the flagellins tested exacerbated disease in a TLR5 independent manner, it may be that flagellin from other species does indeed rely on this stimulatory pathway.

Along with murine studies examining flagellin responses in IBD, I demonstrated that flagellin-specific T cells could be identified in patients with CD. However, although the antigen-specificity of cells isolated using this method has been confirmed by Zaunders et al. (2009), this will need to be demonstrated specifically for flagellin in our own studies. These experiments
could be carried out by sorting flagellin-specific T cells from human peripheral blood and evaluating these cells for increased proliferation in response to flagellin, compared to a control antigen. Furthermore, sorting of flagellin-specific T cells would allow for the phenotype and function of this T cell subset to be examined. This includes determining if these cells are Th1, Th2, or Th17 skewed, and if flagellin-specific Tregs can be identified in this population. Proliferative and/or suppressive capacity can also be examined, giving insight into the contribution of flagellin-specific T cells to disease.

Studies on the identification of flagellin-specific T cells in humans have thus far been conducted in human peripheral blood, however future studies should focus on identifying these cells in intestinal tissues. This could be achieved by isolating T cells from IBD patient intestinal biopsies obtained through disease screening processes, followed by testing for flagellin specificity by the methods described in this thesis. The flagellin-specific T cells identified in intestinal biopsies may be more relevant to disease and thus their phenotypic and functional characteristics should be examined.

Another consideration to explore in future studies would be the diagnostic potential for methods to identify flagellin-specific T cells. Specifically, because antibodies persist long-term in human sera, it may be that the presence or absence of flagellin-reactive T cells is a better indication of disease status. Furthermore, it would be interesting to determine if current treatments for IBD, such as anti-TNF-α antibody therapy, specifically targets flagellin reactive T cells, and if this perhaps contributes to the effectiveness of this therapy.

With the importance of flagellin as an antigen in IBD demonstrated in this thesis, an appropriate next step would be to begin to consider if flagellin-reactive Tregs would be useful for the treatment of IBD. To this aim, future studies could evaluate the suppressive capacity of flagellin-specific Tregs that are isolated from IBD patient blood and expanded in vitro. Alternatively, a chimeric antigen receptor recognizing flagellin could be generated from human
anti-flagellin antibodies, and transduced into polyclonal Tregs to confer antigen specificity (Sadelain et al., 2009). This strategy has been shown to be effective for targeted T cell therapy of cancer (Porter et al., 2011), but its potential for Treg therapy has not been explored.

Current treatment strategies for IBD rely on the use of non-specific immunosuppressive agents such as steroids and anti-cytokine antibodies; these treatments are not effective in all patients, are non-specific, and never provide a cure. Antigen-specific Treg cellular therapy would, by contrast offer the potential for cure through specific and potent targeting of the response to disease-driving antigens at the site of inflammation. Many animal models have shown that the long-lasting effects of a short dose of Treg cells relies on infectious tolerance – that is, the in vivo generation of new Tregs which ultimately maintain tolerance (Kendal and Waldmann, 2010). Compared to solid organs, the gut is rife with tolerance inducing factors, including TGF-β and retinoic acid (Konkel and Chen, 2011). Therefore the gut may be the optimal site to which to target Tregs with the expectation of inducing a life-long therapeutic effect. In addition, the gut’s capacity for regeneration supports the hope of return to normal homeostasis when chronic inflammation is relieved. With phase I clinical trials using Treg therapy for the treatment of type 1 diabetes currently enrolling participants, Treg cellular therapy for IBD is eagerly anticipated. Major concerns specific to this therapy, however, must first be addressed. Chief amongst these are uncertainties in the basic biological function of Treg subsets. Furthermore, if Treg therapy is to be truly effective in IBD, the antigens that drive disease must be identified. The data presented in this thesis takes steps toward better understanding Helios⁺ and Helios⁻ Treg subsets in terms of their potential for Treg cellular therapy, as well as the capacity of Tregs to perform non-suppressive functions in the immune response, including immune cell recruitment. In addition, the role of flagellin as an antigen in IBD was explored, along with the development of a novel method for flagellin-specific T cell identification in humans. As Treg therapy becomes a bedside reality in the field of transplantation, there is hope
that it will soon also be deployed in the setting of IBD and ultimately prove more effective than the current non-specific immuosuppressive therapies. The studies described in this thesis contribute to the groundwork necessary to allow for the application of Treg therapy in IBD, and ultimately for a long-lasting and effective cure for this disease.
REFERENCES


112


a unique response that is associated independently with complicated Crohn's disease.
Gastroenterology 128, 2020-2028.

171. Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., and
Shevach, E.M. (2010). Expression of Helios, an Ikaros transcription factor family member,
differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol
184, 3433-3441.

Helios, an Ikaros Transcription Factor Family Member, Differentiates Thymic-Derived from
Peripherally Induced FOXP3+ T Regulatory Cells". The Journal of Immunology 185, 1.

173. Treton, X., Pedruzzi, E., Cazals-Hatem, D., Grodet, A., Panis, Y., Groyer, A., Moreau, R.,
affects translation in inactive colon tissue from patients with ulcerative colitis. Gastroenterology
141, 1024-1035.

174. Uematsu, S., Jang, M.H., Chevrier, N., Guo, Z., Kumagai, Y., Yamamoto, M., Kato, H.,
Sougawa, N., Matsui, H., Kuwata, H., Hemmi, H., Coban, C., Kawai, T., Ishii, K.J., Takeuchi,

TH17 cells preferentially differentiate from naive FOXP3+Treg in the presence of lineage-
specific polarizing factors. Proc Natl Acad Sci U S A 107, 19402-19407.

176. Veltkamp, C., Anstaett, M., Wahl, K., Moller, S., Gangl, S., Bachmann, O., Hardtke-
Wolenski, M., Langer, F., Stremler, W., Manns, M.P., Schulze-Osthoff, K., and Bantel, H.
(2011). Apoptosis of regulatory T lymphocytes is increased in chronic inflammatory bowel

transcription factor family member, differentiates thymic-derived from peripherally induced
Foxp3+ T regulatory cells". J Immunol 185, 7129; author reply 7130.

ligation by flagellin converts tolerogenic dendritic cells into activating antigen-presenting cells
that preferentially induce T-helper 1 responses. Immunol Lett 125, 114-118.

Nat Rev Immunol 8, 523-532.

180. Vijay-Kumar, M., Aitken, J.D., Sanders, C.J., Frias, A., Sloane, V.M., Xu, J., Neish, A.S.,

TLR5 or NLRC4 is necessary and sufficient for promotion of humoral immunity by flagellin.
Eur J Immunol 40, 3528-3534.


