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# Induction of interleukin-10 within the T(H)17 effector population using the GPER agonist G-1

Ryan Brunsing

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**Induction of interleukin-10 within the T(H)17 effector  
population using the GPER agonist G-1**

by

**Ryan Lennex Brunsing**

B.S., with majors in Physics and Molecular Biology  
University of California San Diego, 2002

**Dissertation**

Submitted in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy  
In Biomedical Sciences**

The University of New Mexico  
Albuquerque, New Mexico, USA

May 2013

## DEDICATION

The body of work is dedicated to my grandparents. Each was an amazing individual, and the uniqueness of their qualities as people is reflected in the variety of their contributions to my person. All are special, and all are missed. To my father's father, Rex Louis Brunsing, I owe my Honesty and my Intellect. He was a man of great integrity and immense intelligence. His gifts are the foundation of my character, and will serve me as a scientist and professional. To my father's mother, Alice June Pepper Brunsing, I owe my Creativity and my Perspective. She was a woman of unwavering passion and far-reaching insight. Her gifts shape the platform from which I view the world, and will serve me as a leader. To my mother's father, Leonard Edward Byrne, I owe my Kindness and my Freedom of Thought. He was a man who knew how to enjoy life on his terms, and share it freely with the world. His gifts form the core of my spirit, and will serve me as a teacher and mentor. To my mother's mother, Elizabeth Rita Connolly Byrne, I owe my Perseverance and my Perspective. She was a woman of unwavering fortitude and profound humility. Her gifts define the strength of my character, and will serve me as a guiding light when facing the challenges ahead and in judging my fortune. Grandma Byrne was my last living grandparent. She passed away while I was writing this manuscript, and is dearly loved and missed. I told her in the days before she left us that I would dedicate this work to her, so it is her that I honor last.

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University of California San Diego, June 2002

Doctor of Philosophy, Biomedical Sciences

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## **ABSTRACT**

A critical mechanism in immune homeostasis is the ability to stop an ongoing inflammatory response once the inciting agent has been destroyed or neutralized. Failure to do so can lead to autoimmune disease. One mechanism the immune system utilizes to self regulate is the secretion of immunosuppressive cytokines. For example, the cytokine interleukin-10 (IL10) is a potent suppressor of numerous key immune cell populations. Among the cells that secrete IL10 are several subsets of the CD4<sup>+</sup> T cell family. As CD4<sup>+</sup> T cells are commonly found within diseased tissue in the setting of autoimmune disease, medications capable of inducing IL10 expression in local CD4<sup>+</sup> T populations would be of great therapeutic interest. The small molecule G-1, an agonist directed against the membrane-bound estrogen receptor GPER, is known to attenuate the multiple sclerosis-like animal model EAE. However, its effects on

CD4<sup>+</sup> T cell populations were previously unknown. Using cultures of purified CD4<sup>+</sup> T cells, we show that G-1 can elicit ERK-dependent expression of IL10. G-1 treated cultures secreted 3-fold more IL10, with no change in the proinflammatory cytokines IL17A, TNF $\alpha$ , and IFN $\gamma$ . Analysis of Foxp3 and ROR $\gamma$ t expression demonstrated increased percentages of IL10<sup>+</sup> cells in both the T<sub>H</sub>17 (ROR $\gamma$ t<sup>+</sup>) and Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> hybrid T cell compartments. We also show that, in mice, *in vivo* treatment with G-1 leads to increased IL10 secretion from splenocytes. These results demonstrate that G-1 acts directly on CD4<sup>+</sup> T cells, and to our knowledge provide the first example of a synthetic small molecule capable of eliciting IL10 expression in T<sub>H</sub>17 or hybrid T cell populations. While G-1 treatment was not effective in a murine model of colitis, investigations of its effects in other T cell-based disease models are warranted.



# TABLE OF CONTENTS

<b>DEDICATION</b> .....	<b>iii</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>iv</b>
<b>ABSTRACT</b> .....	<b>vi</b>
<b>List of Figures</b> .....	<b>xi</b>
<b>Glossary &amp; Abbreviations</b> .....	<b>xii</b>
<b>Preface</b> .....	<b>xv</b>
<b>Chapter 1 : Introduction</b> .....	<b>1</b>
<b>Section 1.1 : Why do we need an immune system?</b> .....	<b>1</b>
<b>Section 1.2 : Overview of the immune system</b> .....	<b>3</b>
<i>Barrier functions and commensal flora</i> .....	<i>3</i>
<i>Leukocytes</i> .....	<i>8</i>
<i>Innate and adaptive immunity</i> .....	<i>10</i>
<i>T lymphocytes</i> .....	<i>13</i>
<b>Section 1.3 : Development of CD4<sup>+</sup> T lymphocytes</b> .....	<b>16</b>
<i>Helper T cells and the Mossman/Coffman paradigm (T<sub>H</sub>1 &amp; T<sub>H</sub>2 cells)</i> .....	<i>16</i>
<i>T<sub>H</sub>17 cells</i> .....	<i>18</i>
<i>Regulatory T cells (T<sub>REG</sub> cells)</i> .....	<i>24</i>
<i>The T<sub>REG</sub> - T<sub>H</sub>17 axis and T cell-targeted therapy</i> .....	<i>31</i>
<i>Other regulatory T cells (Hybrid T cells and Foxp3<sup>+</sup> IL10<sup>+</sup> cells)</i> .....	<i>34</i>
<b>Section 1.4 : Estrogen</b> .....	<b>37</b>
<i>Estrogens physiological effects</i> .....	<i>37</i>
<i>ER<math>\alpha</math> and ER<math>\beta</math></i> .....	<i>39</i>
<i>GPER (GPR30)</i> .....	<i>40</i>
<b>Section 1.5 : Estrogen and the immune system</b> .....	<b>44</b>
<b>Chapter 2 : The hypothesis</b> .....	<b>49</b>
<b>Section 2.1 : Preliminary findings and figures</b> .....	<b>49</b>
<i>GPER affects cytokine secretion from CD4<sup>+</sup> T cells</i> .....	<i>50</i>
<i>G-1 modulates TGF<math>\beta</math>-induced differentiation of naive CD4<sup>+</sup> T cells</i> .....	<i>51</i>
<i>G-1 enhances differentiation of a CD4<sup>+</sup> IL10<sup>+</sup> IL17<sup>+</sup> T cell population</i> .....	<i>53</i>
<b>Section 2.2 : Development of a hypothesis</b> .....	<b>56</b>
<b>Section 2.3 : Aims of the study</b> .....	<b>57</b>
<b>Chapter 3 : Materials and Methods</b> .....	<b>59</b>
<b>Section 3.1 : Cell sorting with MoFlo and AutoMACS</b> .....	<b>59</b>
<b>Section 3.2 : Culture of CD4<sup>+</sup> T cells</b> .....	<b>59</b>
<b>Section 3.3 : Staining cells for flow cytometry</b> .....	<b>60</b>
<b>Section 3.4 : Flow cytometry</b> .....	<b>62</b>
<b>Section 3.5 : ELISA and Luminex multiplex assays</b> .....	<b>62</b>
<b>Section 3.6 : Immunofluorescence and cryosectioning</b> .....	<b>63</b>
<b>Section 3.7 : Western blots</b> .....	<b>63</b>

Section 3.8 : (q)RT-PCR.....	63
Section 3.9 : T cell-mediated colitis model.....	64
Section 3.10 : Mice .....	65
Section 3.11 : G compounds and estrogen .....	65
Section 3.12 : Administration of compounds in vivo .....	66
Section 3.13 : Statistical analysis.....	67
<b>Chapter 4 : Foxp3 induction .....</b>	<b>68</b>
Section 4.1 : Preface .....	68
Section 4.2 : Introduction.....	68
Section 4.3 : Results .....	71
<i>GPER expression in T cells .....</i>	<i>71</i>
<i>G-1 increases Foxp3 expression in CD4<sup>+</sup> T cells .....</i>	<i>73</i>
<i>G-1-mediated induction of Foxp3 in T<sub>H</sub>17 polarizing conditions.....</i>	<i>75</i>
<i>G-1 induced Foxp3 expression occurs within hybrid T cells .....</i>	<i>77</i>
<i>G-1 treated cells exhibit increased suppressive function ex vivo.....</i>	<i>78</i>
Section 4.4 : Discussion .....	79
Section 4.5 : Conclusions .....	84
Section 4.6 : Figures .....	85
<b>Chapter 5 : ERK-dependent IL10 induction.....</b>	<b>92</b>
Section 5.1 : Preface .....	92
Section 5.2 : Introduction.....	92
Section 5.3 : Results .....	96
<i>G-1 elicits IL10 in CD4<sup>+</sup> cells under T<sub>H</sub>17 polarizing conditions .....</i>	<i>96</i>
<i>Induction of an IL10<sup>+</sup>IL17A<sup>+</sup> and IL10<sup>+</sup>IL17A<sup>-</sup> population by G-1.....</i>	<i>99</i>
<i>ERK signaling is critical for G-1-mediated IL10 expression .....</i>	<i>100</i>
<i>IL10-induction occurs within a hybrid T cell population.....</i>	<i>101</i>
<i>Splenocytes from GPER<sup>-/-</sup> mice produce less IL17A and IL10.....</i>	<i>102</i>
<i>G-1 exhibits unexpected activity in GPER<sup>-/-</sup> cells.....</i>	<i>106</i>
Section 5.4 : Discussion .....	107
Section 5.5 : Conclusions .....	117
Section 5.6 : Figures .....	118
<b>Chapter 6 : Data from the T cell-mediated colitis model .....</b>	<b>131</b>
Section 6.1 : Preface .....	131
Section 6.2 : Introduction.....	131
Section 6.3 : Results .....	135
<i>Treatment of T cells with G-1 in culture does not alter colitis.....</i>	<i>135</i>
<i>Systemic treatment with G-1 does not inhibit colitis .....</i>	<i>136</i>
<i>G-1 augments wasting disease when given therapeutically .....</i>	<i>138</i>
<i>G-1 has distinct effects on the mucosal &amp; systemic immune system.....</i>	<i>139</i>
Section 6.4 : Discussion .....	144
Section 6.5 : Conclusions .....	148
Section 6.6 : Figures .....	150
<b>Chapter 7 : Overall Conclusions .....</b>	<b>153</b>
Section 7.1 : Summary and Significance .....	153
<i>Introduction.....</i>	<i>153</i>

<i>Results</i> .....	155
<i>Discussion and future directions</i> .....	157
<i>Overall conclusions</i> .....	163
<b>Section 7.2 : List of major limitations and caveats</b> .....	<b>164</b>
<b>Section 7.3 : List of future directions</b> .....	<b>166</b>
<b>Appendices</b> .....	<b>171</b>
<b>Appendix A : Determining GPER protein expression</b> .....	<b>171</b>
<b>Appendix B : FACS sorting strategies</b> .....	<b>174</b>
<i>CD4<sup>+</sup>CD62L<sup>hi</sup> naïve T cells</i> .....	174
<i>CD4<sup>+</sup>Foxp3<sup>+</sup> natural T<sub>REG</sub> cells</i> .....	174
<i>CD4<sup>+</sup>CD45RB<sup>hi</sup> naïve T cells for T cell-mediated colitis</i> .....	175
<i>CD4<sup>+</sup>CD44<sup>lo</sup>CD45RB<sup>hi</sup>GFP(Foxp3)<sup>+/-</sup> naïve T cells</i> .....	175
<i>CD4<sup>+</sup>CD44<sup>hi</sup> memory T cells</i> .....	176
<b>Appendix C : Other data from in vivo treatments</b> .....	<b>178</b>
<b>Appendix D : Other data from ex vivo T cell stimulation assays</b> .....	<b>181</b>
<b>Appendix E : The T cell-mediated colitis model</b> .....	<b>184</b>
<b>References</b> .....	<b>186</b>

## List of Figures

FIGURE 1 : THE "FATHER OF MEDICINE".....	xv
FIGURE 2 : INTRODUCTION: THE MAJOR CLASSES OF T CELL. ....	16
FIGURE 3 : INTRODUCTION: LINEAGES OF CD4+ T CELL. ....	17
FIGURE 4 : INTRODUCTION: THE T <sub>REG</sub> -T <sub>H</sub> 17 AXIS. ....	34
FIGURE 5 : INTRODUCTION: IL10 PRODUCTION IN CD4 <sup>+</sup> T CELL LINEAGES. ....	37
FIGURE 6 : INTRODUCTION: CURRENT MODEL OF ESTROGEN SIGNALING. ....	41
FIGURE 7 : INTRODUCTION: THE G COMPOUNDS AND E2.....	44
FIGURE 8 : PRELIMINARY DATA: G-1 DRIVES IL17A AND IL10 SECRETION FROM T CELLS. ....	54
FIGURE 9 : PRELIMINARY DATA: G-1 ALTERS TGFβ-DRIVEN T CELL DIFFERENTIATION.....	55
FIGURE 10 : PRELIMINARY DATA: G-1 ALTERS IL17A/IL10 EXPRESSION IN NAIVE T CELLS. ....	55
FIGURE 11 : DIAGRAM OF HYPOTHESIS. ....	57
FIGURE 12 : GPER EXPRESSION IN T CELLS, AND G-1 INDUCTION OF FOXP3 mRNA. ....	85
FIGURE 13 : <i>IN VIVO</i> TREATMENT WITH G-1 INCREASES FOXP3 EXPRESSION, BUT NOT PD-1. ....	86
FIGURE 14 : G-1 TREATMENT OF NAÏVE T CELLS INCREASES FOXP3 EXPRESSION. ....	87
FIGURE 15 : PD-1/CTLA-4 FOLLOWING G-1 TREATMENT OF NAÏVE T CELLS. ....	88
FIGURE 16 : PD-1/CTLA-4 ON FOXP3 <sup>+</sup> CELLS FOLLOWING G-1 TREATMENT OF NAÏVE T CELLS.....	89
FIGURE 17 : G-1 TREATMENT OF NAÏVE T CELLS EXPANDS THE HYBRID POPULATION. ....	90
FIGURE 18 : G-1 TREATED T CELLS EXHIBIT ENHANCED SUPPRESSIVE ACTIVITY <i>IN VITRO</i> . ....	91
FIGURE 19 : THE GPER AGONIST G-1 INDUCES IL10 PRODUCTION FROM CD4 <sup>+</sup> T CELLS.....	118
FIGURE 20 : IFNγ STAINING FOLLOWING <i>EX VIVO</i> TREATMENT WITH G-1.....	119
FIGURE 21 : CYTOKINE SECRETION FOLLOWING <i>EX VIVO</i> TREATMENT WITH G-1.....	120
FIGURE 22 : THE GPER-DIRECTED AGONIST G-1 INDUCES IL10 FROM IN IL17A <sup>+</sup> CELLS.....	121
FIGURE 23 : G-1-MEDIATED IL10 PRODUCTION IS BLOCKED BY GPER ANTAGONISTS.....	122
FIGURE 24 : G-1-INDUCED IL10 PRODUCTION IS ERK DEPENDENT. ....	123
FIGURE 25 : G-1 EFFECTS ARE NOT DEPENDENT ON PROLIFERATION.....	124
FIGURE 26 : G-1 INDUCES IL10 PRODUCTION WITHIN THE HYBRID T CELL POPULATION. ....	125
FIGURE 27 : CYTOKINE SECRETION FROM GPERKO SPLENOCYTES. ....	126
FIGURE 28 : CYTOKINE SECRETION FOLLOWING <i>IN VIVO</i> TREATMENT WITH G-1. ....	127
FIGURE 29 : G-1 INDUCES IL17A UNDER CERTAIN CONDITIONS. ....	129
FIGURE 30 : EVALUATION OF IL10 IN GPERKO AND GPERHET MICE. ....	130
FIGURE 31 : T CELL-MEDIATED COLITIS: <i>EX VIVO</i> TREATMENT OF T CELLS WITH G-1. ....	150
FIGURE 32 : T CELL-MEDIATED COLITIS: G-1 PELLET EXPERIMENTS.....	150
FIGURE 33 : T CELL-MEDIATED COLITIS: G-1 INJECTIONS STARTING AT DAY 1. ....	151
FIGURE 34 : T CELL-MEDIATED COLITIS: G-1 TREATMENT DURING FULMINATE COLITIS.....	151
FIGURE 35 : T CELL-MEDIATED COLITIS: G-1 EFFECTS ON CYTOKINE PRODUCTION. ....	152
FIGURE 36 : PROTEIN EXPRESSION OF GPER. ....	173
FIGURE 37 : FACS SORTING STRATEGIES .....	177
FIGURE 38 : RATIO OF CD4 <sup>+</sup> CELLS IN THE SPLEEN FOLLOWING <i>IN VIVO</i> TREATMENTS.....	178
FIGURE 39 : <i>IN VIVO</i> TREATMENT WITH G-1 DRIVES FOXP3 EXPRESSION IN GPERKO MICE. ....	179
FIGURE 40 : SPLEEN WEIGHTS FOLLOWING <i>IN VIVO</i> TREATMENTS. ....	180
FIGURE 41 : G-1-MEDIATED FOXP3 EXPRESSION IS INDEPENDENT OF MAPK SIGNALING. ....	181
FIGURE 42 : G-1-TREATED CULTURES EXHIBIT ELEVATED APOPTOSIS.....	182
FIGURE 43 : OTHER DATA: CYTOKINE SECRETION FROM GPERKO SPLENOCYTES.....	183
FIGURE 44 : THE T CELL-MEDIATED COLITIS MODEL .....	184
FIGURE 45 : SAMPLE SECTIONS FROM COLON FROM TCMC MICE .....	185

## Glossary & Abbreviations

Ab	Antibody
AhR	Aryl hydrocarbon receptor
Antigen	The component of an invading pathogen, foreign moiety, host cell, or other immunogenic material that elicits an adaptive immune response.
Biome	The full complement of all biological species, in total or for a given group.
CD	Crohn's disease
Classical ERs	The two estrogen receptors that fall into the category of ligand-activated nuclear transcription factors, namely ER $\alpha$ and ER $\beta$ .
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
Epitope	The portion of an antigen that forms the 3 dimensional structure recognized by mediators of the adaptive immune system, such as the T cell receptor or antibodies.
<i>Ex vivo</i>	Experimentation on living tissue that has been removed from a living multicellular organism and manipulated outside of it, for example collecting cells from a mouse and culturing them in a dish.
GALT	Gut-associated lymphoid tissue
GPCR	G protein-coupled receptor
IBD	Inflammatory bowel disease
ICS	Intracellular cytokine staining
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
Uterine imbibition	The uptake of water into the uterus, leading an increase in uterine size and wieght
IHC	Immunohistochemistry
<i>In vitro</i>	Experimentation on samples or systems that have been established or developed in a laboratory, and are completely independent of any multicellular organism. For example experiments with tissue culture cell lines.

<i>In vivo</i>	Experimentation through the manipulation of the conditions within an living multicellular organism. For example systemic treatment of a mouse with a drug.
IPEX	Immune dysfunction, Polyendocrinopathy, Enteropathy, X-linked (syndrome)
locus	genetically relevant segment of DNA, often describing a gene of interest.
LPS	Lipopolysaccharide
Lymphopenic	The state of having below normal levels of circulating lymphocytes, or in the case of RAGKO mice a complete lack of lymphocytes.
MHC	Major histocompatibility complex
MS	Multiple Sclerosis
Mucosal immunity	The mucosal immune system is defined as all components on the innate and adaptive immune systems that play a role in immunity within mucosal tissue and its draining lymph nodes.
ORF	Open Reading Frame
Parenchyma	The physiologically functional tissue of an organ. Compare with stroma.
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RBC	Red blood cell
qRT-PCR	quantitative real-time PCR
RT-PCR	reverse transcription PCR
Stroma	The supportive connective tissue that helps provide a structural framework to a cell, tissue, or organ.
Systemic immunity	The systemic immune system is defined as all components on the innate and adaptive immune systems that play a role in immunity within internal tissues and their draining lymph nodes, as well as the spleen, excluding systems that are involved in mucosal immunity.
Taxa	(Plural of taxon) A group of one or more organisms that have been determined to be part of a group, usually based on phylogenetic relationships.
TCMC	T cell-mediated colitis
TCR	T cell receptor

TNBS	2,3,4-trinitrobenzene sulfonic acid
UC	Ulcerative colitis
US	United States of America

## Preface



**Figure 1 : The "Father of Medicine"**

This image of Hippocrates was taken from an article in *Environmental Health News*, a publication of the School of Public Health at the University of Washington in Seattle.

It was the great Greek physician Hippocrates (ca. 460 – ca. 370 BC), known in the western world as the “Father of Medicine”, who laid the foundation upon which western medicine and medical science now rests. His great contribution came with his rejection of divine forces and the supernatural as the basis of human illness. His philosophies in medicine are still an integral part of modern allopathic medicine, embedded in the principles outlined by the Hippocratic Oath. Since its inception, many have contributed to the evolution of biomedical science, including Aristotle (384 – 322 BC) and Claudius Galenus (c. 129 – 199 AD), the Syrian physician Ibn al-Nafis (1213-1288), who was the first to correctly describe pulmonary and coronary circulation, as well as aeration of the blood within the lung, and Andres Vesalius (1514-1564), the author of *de humani corporis fabrica libri septem* (on the fabric of the human body in seven books), perhaps the world's first widespread text on human anatomy. The production of this work was made possible by the advances in artistry and printing that evolved during Europe's Renaissance period. For all of these great scientists and philosophers, their knowledge and intellect were only valuable in that a medium of expressing their ideas was at hand, and the freedom for independent thought was accepted. For them, the development of art and science went hand in hand.

In 1838, Germans Matthias Schleiden and Theodor Schwann postulated the “Cell Theory”, stating that organisms are made up of individual units that they called “cells”. Breakthroughs in the 19<sup>th</sup> century also included the work of Frenchman Claude Bernard, whose concept of the *milieu interieur* (internal environment) would later to be described using the more familiar term “homeostasis” by the American physiologist Walter Cannon. Others, including Koch, Watson, Crick, Franklin, and Pauling set the stage stem cells, tumor vaccines, the human genome project, and the age of molecular medicine, indeed ushering in a modern Renaissance in medical science. As in the days of Hippocrates and Vesalius, we rely on increasing robust methods of expression to convey exponentially complex scientific concepts. As I move forward in my career, I hope never to lose sight of the place art, creativity, and expression have in the pursuit of knowledge for the common good, and the endeavor of scientific investigation.



# Chapter 1 : Introduction

## ***Section 1.1 : Why do we need an immune system?***

The beauty and diversity of Earth's many inhabitants have been forged through eons of evolutionary competition. In my mind it is a marvel that bags of chemicals (cells), seemingly infinitely complex in and of themselves, can come together in numbers into the trillions to form the finely tuned systemic relationships that define animals, plants, and insects. No event was more critical in our evolution than the advent of multicellularity, wherein groups of cells developed specific skill sets that were advantageous to the group rather than the cell itself. In essence, cells began to act collectively rather than individually, eventually to become co-dependent on the existence of each other, growing into a state of permanent symbiosis. However, this critical step came with a competitive cost. Bacteria and other microorganisms largely rely on rapid proliferation and genetic diversity to evade challenges from competitors and propagate survival of their species. This simple and effective strategy is not feasible for larger organisms, such as humans. Our complex structure provided the form and function within which consciousness could arise, allowing us to ponder questions about ourselves and world around us. However, this gift came at a competitive cost. We humans must live for years in order to reach sexual maturity before we can produce progeny and propagate our species. Thus we

must have ways to effectively defend ourselves from invading pathogens and tumorigenic transformations over long periods of time to ensure the survival of our species. The immune system provides a key component of that defense, fighting off invading pathogens from the surrounding biosphere and aberrant neoplastic growth from within. However, to truly appreciate our dependence on this astonishing system, one must understand not only the why of it's existence, but the immense difficulty of it's task.

In essence, we are a bag of cells; to be more precise, a well hydrated bag of cells rich in nutrients and with a tightly regulated thermostat. These characteristics make us the ideal incubator for countless bacteria, yeast, helminthes, fungi, and other microscopic species whose existence is defined by the endless search for opportunities to replicate and divide. We must be able to detect, respond to, contain, and destroy all these potential pathogens. Given the biodiversity of these groups, this is not a trivial matter. Let's take bacteria as the example. A study from 2002 estimated that in a single gram of soil contains between 6400 and 38,000 distinct bacterial taxa, and their estimates show a ton of soil could contain up to 6 million (Curtis et al., 2002). While the actual number of distinct bacteria on Earth is unknown, and is currently impractical to measure or even estimate in any objective way (Ward, 2002), the overarching point remains the same; there is an immense amount of biodiversity within the microscopic biome, and our survival depends on the ability to chemically detect and respond to the endless array of molecules expressed on the surface of these invaders, or secreted by them. Additionally, many of these microorganisms have

evolved to take advantage of unique niches that our bodies provide, and their high proliferation rate and lower DNA replication fidelity precipitates genetic drift, meaning their pool of potential antigens can change over short periods of time. Addressing these immense challenges is the charge of our immune system. This next section will discuss how this amazing feat is accomplished.

### ***Section 1.2 : Overview of the immune system***

A complete perspective of the immune system requires an appreciation of concepts based in the macroscopic world to those best understood through conceptualization of protein interactions. A brief discussion of basic immunology will be followed by a more detailed discussion of the principles directly pertinent to the work presented.

#### **Barrier functions and commensal flora**

The most prominent feature of the immune system, at least to the naked eye, is the skin. The skin is the largest organ in the human body and forms a protective coating that constitutes our first line of defense against the harmful pathogens of our surrounding environment. It serves as a fundamental barrier between our body and what lies outside of it, while also playing a key role in water retention. These properties make it a critical component of our host defense (immune) system and systemic homeostasis. Without this protective coating, our entire external surface would be a battlefield pitting our internal

defenses against fervent assaults from opportunistic pathogens. Such a contest is not stacked in our favor. To point, consider the case of burn victims in the clinic. Despite rapid debridement of devitalized tissue, constant monitoring, sterile techniques in a controlled environment, and our modern arsenal of antibiotics, infection is responsible for 75% of deaths in patients who have sustained burns over 40<sup>+</sup>% of their body (Church et al., 2006). Without skin, we would be constantly exposed to such infectious challenges across the surface of our body.

Of course, not all interactions with the external world are protected by skin. There are numerous tissues whose physiological function is subject to highly specialized cell populations in contact with the environmental interface, making a thick layer of skin unequivocally incompatible with their purpose. Take the example of the lungs, where gas exchange brings fresh oxygen into our system and noxious gases like carbon dioxide are excreted. This process depends on the exquisitely thin layer separating the capillary lumen from that of the lung alveoli, placing RBCs in close proximity to the inspired air and its bounty of oxygen. Other examples include the genitourinary tract, the eyes, and the gastrointestinal tract from the oral cavity and nostrils to the anus. These sites have three common ties; (a) they are all found within an internal body cavity, (b) they are all involved in the process of absorption and/or secretion, and (c) they are lined with epithelial cells. Collectively, these sites are referred to as the mucosa.

Mucosal immunology is a complex topic. This entire document could be taken up with discussion of its many attributes and context specific properties.

Instead, I will elaborate on one section of this field that closely relates to this work, namely the unique plots that unfold along the lining of our gastrointestinal tract. The details of specific immune cell populations are addressed later.

The setting of the mucosa presents a unique problem as direct contact to the environment means constant challenge with foreign antigen. Thus the mucosal immune system must approach its task with a discerning eye, avoiding deleterious inflammatory reactions against harmless agents while managing to respond to and control infectious ones. Within the intestinal tract, the gut-associated lymphoid tissue (GALT), intraepithelial lymphocytes (IELs), cells of the lamina propria, and other players in intestinal immunity must dampen responses to nutrients bound for absorption while preventing colonization of pathogens. Failure of this system can have devastating consequences, as is the case of Celiac disease where immune responses to gluten - a constituent of grains such as wheat and barley - leads to; (a) inflammation within the walls of the small intestine, (b) damaged villi (small finger-like projections that line the intestines), (c) malabsorption, (d) seizures, (e) infertility, and/or (f) cancer (Sollid, 2000). This condition afflicts 1 in 133 people here in the US, and is commonly found in Europe as well (Fasano et al., 2003). Conversely, improper control of intestinal flora can assist in the development of inflammatory bowel disease (IBD) (Marteau et al., 2004). Thus a delicate balance must be maintained between action and inaction against foreign antigens.

Luckily, we don't go it alone: efforts to prevent colonization of pathogenic microbes is augmented by a complex ecosystem of resident symbiotic bacteria

known as the commensal flora (Hooper et al., 2002). While much less is known about the colonization by other microbes such as archaea and fungi, it is estimated that the human colon contains on the order of  $10^{14}$  bacteria, 10 times the number of human cells in the body (Hooper et al., 2002). These commensal microbiota are extremely diverse, with an estimated 500-1000 distinct bacterial species present (Eckburg et al., 2005). They play an active role in host defense through the displacement of pathogens, augmented barrier function through fortification of the gut lining, and competition for nutrients (O'Hara and Shanahan, 2006; Salzman et al., 2007). They also serve metabolic functions through the production of key cofactors like vitamin K and folate (O'Hara and Shanahan, 2006), and are thought to be important in host development as animals raised in germ-free environments fail to thrive (Wostmann, 1981). Indeed, O'Hara and Shanahan have gone so far as to refer to our commensal microbiota as the "forgotten organ" (O'Hara and Shanahan, 2006). It is increasingly clear that the commensal flora play a key role in mucosal immunity within the intestinal tract.

There are, of course, host-derived mechanisms in place to mediate gut immune homeostasis as well. The small and large intestine are lined by intestinal epithelial cells (IECs). IECs are critical to establishing a barrier between the colonized gut lumen and the underlying parenchyma. Like all epithelial cells they are bound together by tight junctions, a fluid impermeable seal of tightly-bound proteins and lipids that circumvent the lateral walls of the cell and fuse its membrane with that of neighboring cells. They form the decisive component of the epithelial barrier. For example, disruption of tight junctions using a modified

bacterial product dextran sulfate sodium (DSS) permits gut flora access to the underlying lamina propria and its rich stable of immune mediators. The result is an inflammatory condition in the gut wall that closely mimics IBD (Sollid and Johansen, 2008). IECs also play an active role in host defense. Recruitment of neutrophils and clearance of *Clostridium rodentium* infection in mice is dependent on lymphotoxin beta receptor signaling in IECs, likely attributable to IEC production of the chemotactic factors CXCL1 and CXCL2 (Wang et al., 2010). In addition, IECs can activate the adaptive immune system through presentation of antigen on MHC class II molecules (Bland, 1988), a function usually reserved for professional antigen presenting cells (APCs) such as macrophages and dendritic cells (see below). Paneth cells, which secrete bactericidal products like lysozyme and  $\alpha$ -defensins, and Goblet cells, which secrete a thick mucous layer that lines the gut, also play a role in mucosal immunity. Collectively, this mucinous lining, its stock of bactericidal peptides, the commensal flora, and the tight network of IECs generate a formidable barrier to invading pathogens within the gut.

While our barrier systems serve us well, even a simple everyday activity like brushing our teeth can compromise their integrity, granting dangerous microbiota access to our underlying tissue. I use the term “systemic immune system” to refer to the collection of cell and protein based systems that monitor our internal environment with the sole purpose of detecting and eliminating harmful challenges from foreign invaders and neoplastic growth within our own tissue.

## **Leukocytes**

The cohort of cells responsible for the amalgamated defensive posture of both mucosal and systemic immunity are collectively referred to as white blood cells or leukocytes (leukocyte comes from the Greek words “leuko” or white and “kytappo” or cell). Derived from hematopoietic stem cells within the bone marrow, they utilize the circulatory system to populate tissues throughout the body. Leukocytes exhibit dramatic plasticity in localization and function, being heavily influenced by diverse sets of gene products within the leukocytes themselves and from the surrounding environment. Thus each tissue has a unique complement of immune mediators tailored to the pathogens likely to be encountered.

Some leukocytes fall into the category of granulocyte, characterized by the presence of large granules within their cytoplasm which carry an abundance of immune mediators and/or bactericidal chemicals. Upon stimulation, granulocytes release their contents into the local milieu leading to inflammation and destruction of pathogens. Leukocytes also play an important role in the clearance of debris through the process of phagocytosis. The class of leukocyte most directly pertinent to this work are the lymphocytes. These cells are unique in that they undergo somatic cell gene rearrangement collectively referred to as V(D)J recombination. V(D)J recombination is dependent on the recombination-activating genes 1 and 2 (RAG1/2), and loss of either allele completely disrupts lymphocyte development. This recombination event generates a vast complement of unique binding pockets on lymphocyte antigen recognition receptors from a single set of genes, which include the B cell receptor (BCR) and T cell receptor (TCR). This



allows lymphocytes to detect and react to millions of different epitopes and mold their response to unique pathogens. The different types of lymphocyte include:

**T lymphocytes**, which will be discussed below. These cells complete their development in the thymus, where they undergo V(D)J recombination to generate a unique TCR. Each T cell expresses a single TCR, and is referred to as a clone. Note that there can be multiple copies of a single clone as the result of “clonal expansion”.

**B lymphocytes**, which complete their develop in the bone marrow. Like T cells, they undergo RAG-dependent somatic gene rearrangement, in this case to develop the vast array of BCRs (which are in essence membrane-bound antibodies) and antibodies. Antigen recognition by BCR stimulates B cells to mature and produce secreted antibodies (BCR without the membrane anchoring region). Antibodies can neutralize surface receptors to prevent infection and opsonize invading pathogens so they can be recognized by phagocytic cells or destroyed by the complement cascade. In addition to V(D)J recombination, B cells also undergo the process of somatic hypermutation to generate further diversity. Like T cells, each B cell expresses a single type of BCR/antibody, and is referred to as a clone. B cells are reviewed here (Kurosaki et al., 2009)

**NKT cells**, which utilize a restricted repertoire of TCRs to recognize glycolipids bound to the CD1d molecule presented on APCs. CD1d is

structurally similar to MHC Class I (see **Figure 2**). NKT cells are reviewed here (Godfrey et al., 2004).

Other cells types include the **monocytes and macrophages**, **dendritic cells**, **neutrophils**, **eosinophils**, **basophils**, and **mast cells**, some of which are discussed below. A detailed discussion of these cell types are beyond the scope of this work and will not be included. A general description of these populations can be found on wikipedia ([www.wikipedia.com](http://www.wikipedia.com)).

### **Innate and adaptive immunity**

The systemic immune system is a complex network of specialized cells and soluble mediators which act in a coordinated fashion to fend off pathogenic insults and clear foreign antigen. Incredibly, this scheme has evolved to tailor its response to the type of pathogen it is challenged with and its route of entry. Unlike most tissues which have a defined structure and organization, the immune system is dynamic and constantly evolving. It is divided into two main branches that have distinct properties and kinetics; the innate immune system and the adaptive immune system. Many of the topics discussed above – namely barrier function, mucous production, bactericidal peptides, and neutrophil recruitment – fall into the category of innate immunity, while lymphocyte-mediated antigen-specific immune responses, immunological memory, and the principles underlying vaccination are all dependent on the adaptive immune system. While

typically discussed as two distinct systems, it is important to realize that these systems are not insulated for each other; quite the contrary in fact. Some of the many ways that they communicate and affect each other are highlighted below.

A hallmark of innate immunity is the ability to rapidly respond to an infection. Tissue-resident leukocytes like macrophages and dendritic cells (DCs) constitutively express different combinations of pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs) commonly expressed by invading microbes. Numerous different PRR families exist (Takeuchi and Akira, 2010). PAMPs include bacterial carbohydrates like lipopolysaccharide (LPS), nucleic acid structures like viral double stranded RNA, bacterial peptides such as flagellin, and many other microbial-specific products. The constitutive expression of the toll-like receptors (TLRs) and other PRRs allows cells of the innate immune system to rapidly respond to pathogens by destruction of infected cells, phagocytosis of infectious particles, and the release of antimicrobial peptides. Activation of innate immunity also drives tissue inflammation which leads to the hematological recruitment of other phagocytic populations like neutrophils and monocytes (which turn into macrophages upon entering the tissue). Other soluble mediators, including the complement cascade, also play a role in detecting, neutralizing, and destroying invading pathogens and eliciting inflammation. The critical distinction from the adaptive immune system described below is that the PRRs and other proteins of the innate immune system are encoded in the germline and do not change over time

In contrast to the innate immune system, the development of an adaptive immune response takes days to weeks to mature. As described above, phagocytic cells ingest foreign material and debris from dying cells, or are infected themselves, leading to the destruction of infectious particles. However, some of these phagocytic leukocytes then migrate to secondary lymphoid organs (such as lymph nodes) where they play a role in adaptive immunity. These professional antigen presenting cells (APCs – ex: dendritic cells or macrophages) are programmed to process protein-based antigens and load them onto major histocompatibility type II (MHC class II) molecules for presentation of helper T cell populations. Helper T cells are important in all branches of the adaptive immune system. Similarly, virtually any infected tissue or cell can process and present antigen on the ubiquitously expressed MHC class I molecule, leading to the activation of cytotoxic T cells. Cytotoxic T cells are critical mediators of cellular (adaptive) immunity while antibody-mediated immunity is collectively referred to as humoral (adaptive) immunity. TCR recognition of an MHC-antigen complex initiates clonal expansion of that cell. Note that antigen recognition by the BCR is independent of MHC, and B cells can in fact act as APCs for T cells. The end goal of these actions is the expansion of lymphocytes tailored to the specific challenge at hand. In addition, the adaptive immune system retains clones generated from previous exposures in the form of memory T cells and B cells. This “immunological memory” allows for a more immediate and robust response to re-infection with the same agent, and forms the basis of vaccination.

Thus the hallmark of adaptive immunity is that its complement of molecules and cells changes over time, reflective of its experience with past infections.

## **T lymphocytes**

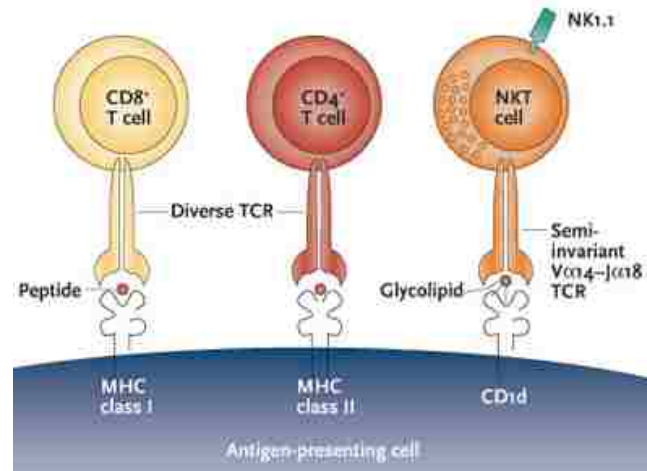
The majority of the work in this document centers on T cell biology, thus a more detailed discussion of their activity, function, and significance in human disease is warranted. T cells can be broken into three main classes (**Figure 2**). The first are the CD8<sup>+</sup> cells, or *cytotoxic T cell* population. These cells recognize antigens presented on the ubiquitously expressed MHC class I molecules, responding by killing the presenting cell as part of the cell-mediated immune system. They are activated by soluble mediators like IFN $\gamma$ , and play a role in the clearance of intracellular infections and tumor rejection (Schepers et al., 2005). The second class of T cell are the *NKT cells*, which were briefly described above. While not discussed in detail, NKT cells have been linked to numerous disease processes including diabetes and cancer (Godfrey et al., 2004).

Finally, the third class of T cell comprise the family of CD4<sup>+</sup> T lymphocytes (or helper T cells), which respond to antigen-MHC class II complexes. The CD4 molecule recognizes and binds to MHC class II, and is critical for stable interaction of the TCR-MHC-antigen complex. It is important to note that other cell types have also been shown to express CD4, for example the NKT cells described above and even macrophages, but these cells lack the TCR co-receptor CD3 and are not part of the helper T cell family. CD4<sup>+</sup> T lymphocytes

are central to the coordination of immune responses. Upon antigen recognition they express potent surface markers and secreting an array of chemokines and cytokines. Their actions lead to recruitment and activation of numerous cells types from both the innate and adaptive immune system, including neutrophils, macrophages, B cells, and cytotoxic T cells. CD4<sup>+</sup> T cells are divided two main groups; *effector T cells*, which play a role in enhancing immune reactions, and *regulatory T cells* (T<sub>REGS</sub>), which suppress immune reactions. They are briefly outlined in **Figure 3**. CD4<sup>+</sup> T cells develop in the thymus and emerge as a population of *naïve T cells*, meaning they have yet to be exposed to antigen and take on one of the characteristic phenotypes described below. Antigen recognition initiates a cascade of signaling events that drive differentiation into one of several distinct T helper populations, each of which is designed to initiate a unique type of immune reaction (see **Figure 3**). Helper T cell differentiation is determined by integration of a complex array of signals provided by the local environment, mostly in the form of surface proteins on neighboring cells and secreted signaling molecules like cytokines. This will be discussed in more detail below. For the sake of simplicity, several poorly described lineages are not included in Figure 3. Follicular T cells express that transcription factor ROR $\gamma$ t, and may develop in a similar fashion to T<sub>H</sub>17 cells. They are found in the B cell zones of lymph nodes, where they assist in affinity maturation and plasma cell development (Fazilleau et al., 2009). Helper T cells expressing IL9 and IL22 have also been described and given the classification T<sub>H</sub>9 and T<sub>H</sub>22 cells, respectively. Whether these represent truly unique lineages of are merely subsets of more

established CD4<sup>+</sup> lineages remains unclear (Murphy and Stockinger, 2010). There is one other population of CD4<sup>+</sup> T cell which bear mentioning, namely  $\gamma\delta$  T cells. This population of cells is found largely within the mucosal immune system of the gut as part of the intraepithelial lymphocyte population. The TCR in vast majority of T cell populations (including all helper/cytotoxic T cells) is a heterodimer containing one copy each of the  $\alpha$  and  $\beta$  TCR subunits or chains. Conversely,  $\gamma\delta$  T cells express a TCR comprised of one  $\gamma$  and one  $\delta$  subunit. Similarly to the  $\alpha$  and  $\beta$  chains, the  $\gamma/\delta$  loci undergo V(D)J recombination and in many respects these cells behave like other members of the adaptive immune system. However, unlike their  $\alpha\beta$  TCR counterparts, the  $\gamma\delta$  T cells play a role in the recognition of lipid antigens, recognize antigen independently of the MHC complexes, and have a restricted repertoires of TCRs, thus they exhibit innate properties as well. While not discussed in detail, they have been reviewed in (Xiong and Raulet, 2007).

The topic of cytokine function in relation to T cell development, differentiation, and function is too vast to discuss further in this proposal, but has been reviewed (Sanchez-Munoz et al., 2008). The pertinent details regarding CD4<sup>+</sup> T cells will be discussed in detail below and throughout this thesis.



**Figure 2 : Introduction: The major classes of T cell.**

Adopted from: Adams JU (2005) *The Scientist* 19(14): 15

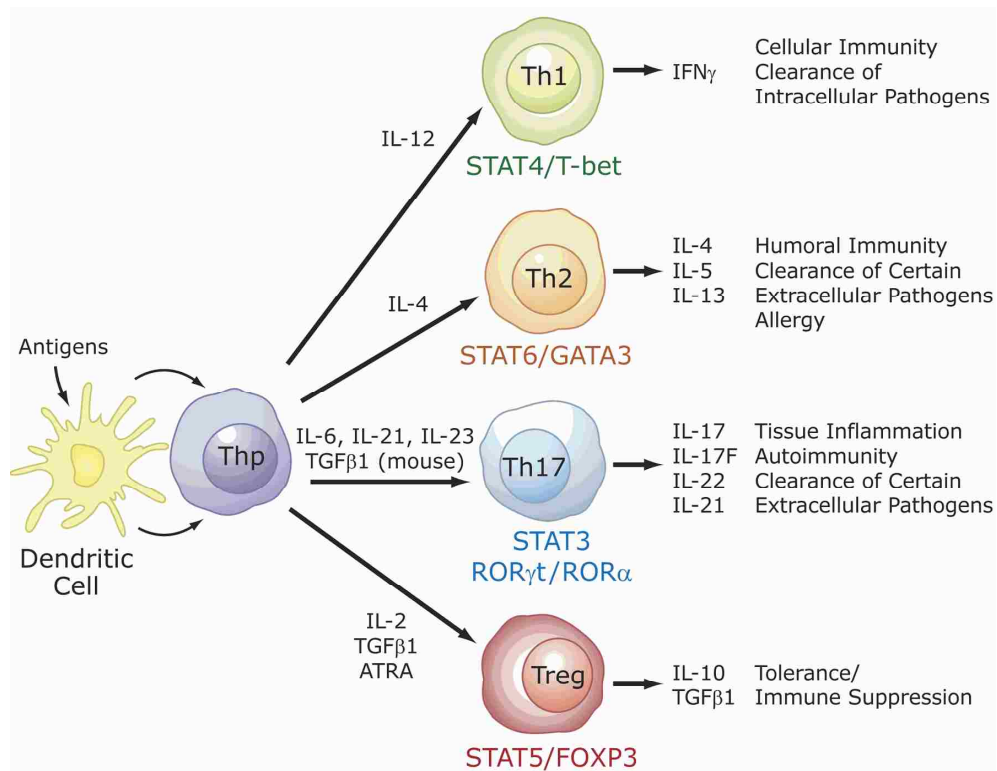
### ***Section 1.3 : Development of CD4<sup>+</sup> T lymphocytes***

#### **Helper T cells and the Mossmann/Coffman paradigm (T<sub>H</sub>1 & T<sub>H</sub>2 cells)**

CD4<sup>+</sup> helper T lymphocytes orchestrate adaptive immune responses to invading pathogens, and are critical to the pathogenesis of numerous disease processes, including autoimmunity and cancer. They are an attractive drug target due to their central role in immunity, and their implication in a wide variety of diseases. The original paradigm described by Coffman and Mossmann divided CD4<sup>+</sup> helper T lymphocytes into the T-helper-1 (T<sub>H</sub>1) and T<sub>H</sub>2 populations (Mosmann and Coffman, 1989), delineated by their secretion of distinct cytokines and expression of characteristic transcription factors. T<sub>H</sub>1 cells express the transcription factor T-bet, secrete IFN<sub>γ</sub>, and are implicated in cell-mediated immunity (Szabo et al., 2003), for example by enhancing the activity of CD8<sup>+</sup> cells and the recruitment of macrophages. The induction of T<sub>H</sub>1 cells requires



interleukin-12 (IL12), and signaling through Signal Transducer and Activator of Transcription (STAT)-1 and STAT4. In contrast,  $T_H2$  cells express the transcription factor GATA3, which is induced by IL4-mediated activation of STAT6 (Zhu et al., 2006). They secrete the cytokines IL4, IL5, and IL13, which play an important role in humoral immunity by driving B cell maturation and antibody class switching. There are also heavily implicated in atopic immune responses (Paul and Zhu, 2010). See **Figure 3**. These two pathways are generally thought to be mutually exclusive, as  $IFN\gamma$  blocks  $T_H2$  differentiation while IL4 blocks  $T_H1$  differentiation and promotes the generation of  $T_H2$  populations (Zhu et al., 2006).



**Figure 3 : Introduction: Lineages of CD4+ T cell.**

Adopted from: *Jetten AM (2009) Nuclear Receptor Signaling 7: e003*

## **T<sub>H</sub>17 cells**

Up until about 2000, the T<sub>H</sub>1-T<sub>H</sub>2 paradigm seemed largely sufficient to account for most of the activities attributed to CD4<sup>+</sup> T cell populations, and much of their proinflammatory capacity was thought to reflect the activity of the T<sub>H</sub>1 arm of the pair (McGeachy and Cua, 2008). It was shown that genetic disruption of the IL12 p40 subunit prevented disease in experimental autoimmune encephalomyelitis (EAE), an animal model of Multiple Sclerosis (MS), leading to the theory that T<sub>H</sub>1 cells were important in the development of autoimmune diseases (Brok et al., 2002). However, evidence from other animal studies slowly built the case that another population must underlie the chronic inflammation associated with these diseases. As early as 1990 it was known that injection of the T<sub>H</sub>1-associated cytokines IL12 and IFN $\gamma$  actually blocked rodent models of acute neurological inflammation (Gran et al., 2004; Voorthuis et al., 1990). Moreover, genetic deletion of IL12 receptor  $\beta$  (IL12R $\beta$ ) resulted in increased disease severity in several models of autoimmune disease (Zhang et al., 2003), further suggesting that the T<sub>H</sub>1 populations played a role in controlling inflammation rather than precipitating it. Other interesting results, for example the finding that loss of T-bet, but not STAT1, prevented the development of EAE (Bettelli et al., 2004), further confounded the story.

In 2000, it was shown the IL12 p40 subunit was in fact a common subunit of both the T<sub>H</sub>1-polarizing cytokine IL12 and the newly identified cytokine IL23

(Oppmann et al., 2000). Studies in mice genetically deficient for the IL23-specific subunit p19 (which have intact IL12) demonstrated that loss of IL23 was associated with protection from autoimmune inflammation, not IL12 (Cua et al., 2003). Moreover, p19-deficient mice were protected despite the presence of neuropeptide-specific T cells expressing IFN $\gamma$ . Conversely, mice lacking the IL12-specific subunit p35 exhibited a rapidly-progressive form of EAE, despite low numbers of IFN $\gamma$ <sup>+</sup> cells (Cua et al., 2003; Gran et al., 2002). Thus it was clear that the T<sub>H</sub>1-polarizing cytokine IL12 and the major T<sub>H</sub>1-associated cytokine product IFN $\gamma$  were not critical to T cell-mediated chronic inflammation, and may in fact suppress the disease. The search was on for another T cell mediator of chronic inflammation.

T<sub>H</sub>17 effector cells were first identified as a distinct helper T cell lineage in 2005 (Langrish et al., 2005) when it was shown that IL17A producing T cell exhibit a distinct transcriptional profile from T<sub>H</sub>1 cells (Langrish et al., 2005), and that IL17A producing effector T cells express a characteristic transcription factor, namely ROR $\gamma$ t (Harrington et al., 2005; Ivanov et al., 2006). This classification was shown to fit well into the Coffman-Mossmann paradigm of mutually exclusive lineage development. Moreover, work from two independent groups demonstrated that both the T<sub>H</sub>1-specific and T<sub>H</sub>2-specific (Harrington et al., 2005; Park et al., 2005) transcriptional regulators inhibit T<sub>H</sub>17 differentiation, further solidifying their status as a unique and independent effector T cell population. T<sub>H</sub>17 cells produce pro-inflammatory cytokines including IL17A/F. IL17A mediates numerous inflammatory responses, including secretion of

proinflammatory cytokines and chemokines from epithelial and endothelial cells (Stockinger et al., 2007) neutrophil mobilization (Aujla et al., 2007), and many of the pathological sequelae that result from chronic inflammation. IL17 and its many functions have been reviewed elsewhere (Ouyang et al., 2008).

Most work to date has centered on the peripheral induction of T<sub>H</sub>17 cells from naïve precursors, but there is evidence that they can develop in the thymus as well (Marks et al., 2009). In mice, peripheral differentiation of T<sub>H</sub>17 cells from antigen-activated naïve T cells depends on the presence of TGFβ and IL6 (Veldhoen et al., 2006; Veldhoen and Stockinger, 2006), while in humans peripheral induction of T<sub>H</sub>17 cells depends on TGFβ paired with either IL-21 or IL-23. Murine T<sub>H</sub>17 express CCR6, while human cells are CCR4<sup>+</sup>CCR6<sup>+</sup>. Other proteins such as IRF1 (Kano et al., 2008), IRF4 (Brustle et al., 2007; Chen et al., 2008), RORα (Yang et al., 2008), and IL23 (Volpe et al., 2008) are also involved in the development of T<sub>H</sub>17 cells (Iwakura and Ishigame, 2006), while the Aryl hydrocarbon receptor (AhR) seems critical to their production of the cytokine IL22 (Ramirez et al., 2010). The differentiation of T<sub>H</sub>17 cells has been reviewed (McGeachy and Cua, 2008). IL23 in particular is important to function and stability of T<sub>H</sub>17 cells. In 2003, IL23 was shown to drive production of IL17A from activated T cells (Aggarwal et al., 2003), while *il23a*<sup>-/-</sup> mice have few IL17A producing cells (Murphy et al., 2003). IL23 was later shown to induce an IL17A<sup>+</sup> T cell populations capable of driving autoimmune inflammation (Langrish et al., 2005). These studies have led to the theory that IL23 plays a role in stabilizing the T<sub>H</sub>17 phenotype (McGeachy and Cua, 2007). In 2007, Daniel Cua's group

showed that T<sub>H</sub>17 cells differentiated in culture with TGF $\beta$  and IL6 were actually protective in an animal model of experimental encephalomyelitis, while those treated with IL23 induced robust disease (McGeachy et al., 2007). Interestingly, the cells differentiated with TGF $\beta$  and IL6 still expressed high levels of IL17A/F, ROR $\gamma$ t, ROR $\alpha$ , IL21, and CCR6, suggesting that none of these factors were important in IL23-mediated neuroinflammation. What the authors did show was that IL10 was a critical factor in the bystander suppressive effects of the TGF $\beta$  and IL6 differentiated cells. Thus the critical mediators of T<sub>H</sub>17 cells proinflammatory phenotype are not clearly defined, at least in certain settings.

A concrete definition of what a T<sub>H</sub>17 cell is has been complicated by the fact that many cell types produce the cytokine IL17A. Indeed  $\gamma\delta$  T cells (which express the  $\gamma\delta$ TCR rather than the conventional  $\alpha\beta$  TCR) are the main producers of IL17A within the CD4<sup>+</sup> compartment, at least in naïve animals (Stark et al., 2005). Other populations which have been shown to express CD4 (but not CD3) like NKT cells (Lee et al., 2008; Michel et al., 2007; Rachitskaya et al., 2008) and lymphoid tissue inducer (LTi)-like cells also produce IL17A (Takatori et al., 2009). Additionally, CD8<sup>+</sup> T cells (He et al., 2006; Shin et al., 1998), alveolar macrophages (Song et al., 2008), mast cells (Hueber et al., 2010; Mrabet-Dahbi et al., 2009) and neutrophils (Li et al., 2010) have also been shown to produce IL17A under certain conditions. These findings, and some of the other findings discussed above led O'Connor et al (O'Connor et al., 2010) to characterize T<sub>H</sub>17 cells as, "CD4<sup>+</sup> effector T cells positive for the  $\alpha\beta$  (TCR), which have high expression of the transcription factors ROR $\alpha$  and ROR $\gamma$ t, low expression of the

transcription factors T-bet and GATA-3, and high surface expression of the chemokine receptor CCR6; produce IL-17A, IL-17F, and IL-22; have surface expression of the IL-23 receptor; and can produce the chemokine CCL20.” While this is without question a clear and exhaustive definition, one wonders what a cell expressing CD4,  $\alpha\beta$ TCR, ROR $\gamma$ t, the TCR co-receptor CD3, and IL17A would then be called. For this work, we chose to go with a simpler definition; all cells expressing CD3, CD4, ROR $\gamma$ t, and IL17A will be referred to as T<sub>H</sub>17 cells.

As discussed above, T<sub>H</sub>17 cells are critical to the pathogenesis of numerous autoimmune diseases (McGeachy and Cua, 2007; Pernis, 2009; Stockinger, 2007), and numerous lines of evidence support their classification as a proinflammatory population. However, like the data discussed above for T<sub>H</sub>1 cells, T<sub>H</sub>17 cells also appears to exhibit both pro- and anti- inflammatory effects. This has led the hypothesis that some of the T<sub>H</sub>17-associated mediators may provide protection against inflammation-induced tissue destruction in a “microenvironment-specific” fashion (O'Connor et al., 2010). For example, studies with the TNBS-induced colitis model has clearly demonstrated that IL17RKO mice are protected from disease (Zhang et al., 2006), while several studies have shown that inflammatory bowel disease (IBD) is associated with increased levels of IL17A expression (Iwakura and Ishigame, 2006). In contrast, in studies with the DSS-based model of acute colitis, *in vivo* neutralization of IL17A was shown to enhance disease severity, while addition of recombinant IL17A reversed this effect (Ogawa et al., 2004). This study was followed by data out of Richard Flavell's lab who used the T cell-mediated colitis model to

demonstrate transfer of IL17KO T cells in precipitates to a more severe disease course than transfer of wild-type T cells, suggesting T cell-derived IL17A acts to dampen disease (O'Connor et al., 2009). Other suppressive effects in the setting of atherosclerosis have also been described (Taleb et al., 2010). Clearly, IL17A can exhibit anti-inflammatory activity.

In addition to the microenvironment hypothesis mentioned above, one possible explanation for these findings may be that IL17A and IL17F have distinct roles in regulating inflammation, and as both IL17A and IL17F rely on the IL17RA/C heterodimer for their signaling, observations made following disruption of the IL17R may reflect the activity of IL17F rather than IL17A. IL17R signaling is reviewed here (Gaffen, 2009). Thus studies in which IL17RA was deleted, or where soluble receptor are used to neutralize IL17, have the caveat that both IL17A and IL17F signaling are being disrupted, and contextual differences observed in IL17 immunobiology may reflect differences in the relative importance of either IL17A or IL17F in that setting. Moreover, IL17A and IL17F can coexist as heterodimers. Therefore systemic neutralization of IL17A may also affect IL17F as well. However, there is also evidence that both IL17A and IL17F have redundant effects in the setting of colitis (Leppkes et al., 2009). Additionally, studies with EAE have shown opposing effects for both IL17A and IL17F, with one group showing that IL17RA signaling is required for disease induction (Hu et al., 2010), while studies from another group demonstrated neutralization of IL17A in IL17FKO mice had only a minimal effect on disease induction and severity (Haak et al., 2009). T<sub>H</sub>17 cells have also been linked to numerous other

diseases, including cancer, where it appears that T<sub>H</sub>17 cells enhance tumor rejection (Kryczek et al., 2007; Martin-Orozco et al., 2009; Muranski et al., 2008; Zou and Restifo, 2010). Thus despite the preponderance of evidence that T<sub>H</sub>17 cells are proinflammatory, there appears to be a great deal of variability in the properties of these cells, and much more work needs to be done to clarify their role in chronic inflammation.

### **Regulatory T cells (T<sub>REG</sub> cells)**

Description of the distinct effector T cell lineages neatly reflected their unique contributions to a developing immune response. However, this paradigm did not appear to offer a clear mechanism for controlling and regulating the inflammation that resulted from their actions. This led to the hypothesis that some form of regulatory T cell must also exist. Early experiments by Fiona Powrie and colleagues with murine colitis models clearly demonstrated that select populations of CD4<sup>+</sup> T cell exhibited potent immunosuppressive properties, including the ability to limit chronic inflammatory responses driven by more pathogenic T cell populations (Powrie et al., 1993a). Yet it was unclear whether these suppressive T cells were a subset of the one of the T<sub>H</sub> lineages, or a unique population not yet described. Indeed, T<sub>H</sub>1 and T<sub>H</sub>2 cells were known to produce IL10 (Del Prete et al., 1993), a potent immunosuppressive cytokine (Moore et al., 2001). In addition, several experiments had shown that two key T<sub>H</sub>2 cytokines, IL4 and IL10, can act together to inhibit cell-mediated immunity



(Powrie and Coffman, 1993; Powrie et al., 1993b, c). The first marker for regulatory T cells was the high affinity IL2 receptor subunit CD25 (Sakaguchi et al., 1995). However, while effective at identifying T<sub>REG</sub> cells in the naïve setting, this molecule is upregulated upon activation of many T cell populations, and was clearly not specific to regulatory T cells (Fontenot et al., 2005b).

A major breakthrough in delineating the characteristics of regulatory T (T<sub>REG</sub>) cells emerged when it was shown that a forkhead box transcription factor, Foxp3, was critical to their function (Hori et al., 2003). The identification of a T<sub>REG</sub>-specific transcription factor established these cells as a unique T cell lineage. Further studies in mice have shown that Foxp3 can be induced during thymic development in response to strong self-antigen recognition (Fontenot et al., 2005b), leading to so called “natural” Foxp3<sup>+</sup> T<sub>REGS</sub> (nT<sub>REG</sub>). Foxp3 expression can also be driven peripherally by TGFβ signaling within naïve T cell populations (Chen et al., 2003; Marie et al., 2005), generating “inducible” T<sub>REGS</sub> (iT<sub>REG</sub>). Several lines of evidence demonstrate that Foxp3 is the critical factor in the development of a regulatory phenotype. Foxp3<sup>+</sup> T<sub>REG</sub> cells suppress numerous animal models of autoimmune disease (Yuan et al., 2007), and a mutation in Foxp3 was found to be the genetic basis for the fatal lymphoproliferative disorder observed in *scurfy* mice, which is characterized by fulminate T<sub>H</sub>1-type inflammation within numerous tissues (Clark et al., 1999). In addition, ectopic expression of Foxp3 in CD4<sup>+</sup> T cells confers a T<sub>REG</sub> phenotype (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003), while sustained expression of Foxp3 was found to be critical to the suppressive function in

nT<sub>REGS</sub> (Williams and Rudensky, 2007). These findings translated to humans as mutations in Foxp3 were identified in over 50% of immune dysfunction, polyendocrinopathy, enteropathy (IPEX) syndrome patients (van der Vliet and Nieuwenhuis, 2007). However, the role of Foxp3 in humans appears to differ from observations made in mice. Several groups have identified Foxp3<sup>+</sup> T cells populations that lack regulatory activity (Allan et al., 2007; Gavin et al., 2006; Morgan et al., 2005; Wang et al., 2007). And while treatment of murine CD4<sup>+</sup> T cells with TGFβ precipitates a regulatory phenotype, the same has not been observed in humans (Tran et al., 2007), despite the induction of Foxp3. Interestingly, ectopic expression of Foxp3 using a lentiviral vector system does confer a regulatory phenotype on human naïve T cells and memory T cells, suggesting that stable expression of Foxp3 may be the critical determinant of its function (Allan et al., 2008). None-the-less, there is evidence that the regulatory T cell transcriptional profile has elements regulated by- and independent of- Foxp3 (Hill et al., 2007). A 2010 report has shown that the transcription factor Helios is specifically upregulated in human nT<sub>REGS</sub>, and functions in the stabilization of Foxp3 expression (Getnet et al., 2010), while suppression of Helios abrogated their regulatory function (Getnet, 2010). More work is needed to clarify whether Foxp3 functions differently in humans and mice, and the implications of Helios in this paradigm.

T<sub>REGS</sub> suppress immune reactions by numerous mechanisms. The simplest method is the secretion of inhibitory cytokines, including TGFβ, IL10, and the recently identified cytokine IL35 (Collison et al., 2007). This is reviewed

here (Vignali et al., 2008). These soluble mediators can act to suppress proinflammatory populations in the local environment and drive the generation of other anti-inflammatory T<sub>REG</sub> cells. For example, IL10 reduces the proinflammatory activity of numerous immune populations (Maynard and Weaver, 2008), can suppress autoimmune disease (Lavasani et al., 2010), and is important to immune homeostasis within the mucosa (Rubtsov et al., 2008), in addition to acting in a feed-forward mechanism by driving its own expression in other T<sub>REG</sub> cells (Maynard and Weaver, 2008).

T<sub>REG</sub> cells also utilize cytotoxicity in regulating inflammatory reactions. Human iT<sub>REG</sub>s have been shown to exhibit target cell killing in a granzyme A and perforin dependent mechanism (Grossman et al., 2004). Similar findings were observed in mice as T<sub>REG</sub> cells from granzyme B-deficient mice had reduced regulatory activity *ex vivo* (Gondek et al., 2005) and *in vivo* (Cao et al., 2007). This correlated with data showing up-regulation of granzyme B in murine T<sub>REG</sub> cells (Herman et al., 2004; McHugh et al., 2002).

A third mechanism employed by regulatory T cells involves suppression by metabolic disruption. This categorical description is best exemplified by the production of adenosine nucleosides. T<sub>REG</sub>s have been shown to express the 5' ribonucleotide phosphohydrolase CD73, which can convert adenosine monophosphate to adenosine. Co-expression of CD73 with CD39 leads to locally produced adenosine, which binds to the adenosine receptor 2A (A<sub>2A</sub>R) and inhibits effector T cell function (Borsellino et al., 2007; Deaglio et al., 2007; Kobie et al., 2006). Interestingly, A<sub>2A</sub>R activation on CD4<sup>+</sup> populations also enhances

the generation of iT<sub>REG</sub> cells at the expense of T<sub>H</sub>17 cells via down-regulation of IL6 and up-regulation of TGF $\beta$  expression (Zarek et al., 2008). Another study has demonstrated T<sub>REG</sub>-mediated mitigation of inflammation by direct transfer of cyclic adenosine monophosphate (cAMP) into effector T cells via gap junctions (Bopp et al., 2007). Furthermore, inhibition of cAMP degradation has been shown to augment T cell regulatory function (Bopp et al., 2009). A more controversial mechanism of T<sub>REG</sub>-induced metabolic disruption is IL2-depletion-induced apoptosis within effector populations (Vignali et al., 2008), which is postulated to depend on T<sub>REG</sub> expression of the high affinity IL2 receptor CD25 (discussed above).

Finally, regulatory T cells mediate suppression by directly targeting the maturation and function of dendritic cell populations. T<sub>REGS</sub> express lymphocyte activation gene 3 (LAG3) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) which bind to MHC class II (Workman and Vignali, 2003) and CD80/86 (Cederbom et al., 2000; Oderup et al., 2006), respectively, on DCs. This leads to a reduction in their ability to activate effector populations. T<sub>REGS</sub> can also drive DCs to produce indoleamine 2,3-dioxygenase (IDO), a potent pro-apoptotic factor in T cells. This appears to occur in a CTLA-4-dependent fashion (Fallarino et al., 2003). Moreover, intravital studies have demonstrated stable interaction between DCs and Foxp3<sup>+</sup> T<sub>REGS</sub> *in vivo* (Tadokoro et al., 2006; Tang et al., 2006), while CTLA-4 is critical to regulatory T cell control of colitis (Read et al., 2000). IL10 and cAMP also appear to play a role in nT<sub>REG</sub>-induced suppression of DC function

(Fassbender et al., 2010). Thus regulatory T cells regulate T cell-mediated immunity through a number of direct and indirect mechanisms.

There has been some debate as to whether nT<sub>REGS</sub> rely on secreted factors for their suppressive function. Several studies have attempted to assess the importance of IL10 and/or TGF $\beta$  signaling in T<sub>REG</sub>-mediated regulatory activity using culture-based T cell suppression assays (Piccirillo et al., 2002), and found that loss of either pathway did not affect the suppressive function of the cells in question. However, more recent evidence suggests that this may be due to the dominant nature of contact-mediated inhibitory mechanisms in this setting. For example, neutralization of IL10 has been shown to affect T<sub>REG</sub> function in culture when T<sub>REGS</sub> and effectors are separated using a transwell assay system (Collison et al., 2009). This line of thinking fits well with *in vivo* data that demonstrate T<sub>REG</sub>-produced IL10 is important in both immune homeostasis (Rubtsov et al., 2008) and regulatory activity in the setting of T cell-mediated colitis (Asseman et al., 1999; Asseman et al., 2003; Uhlig et al., 2006) and EAE (Mann et al., 2007). The role of TGF $\beta$  appears to be a bit more convoluted. Inhibition of TGF $\beta$  by neutralizing antibody or soluble receptor had little effect on T<sub>REG</sub> function *ex vivo*, while T cells that cannot respond to TGF $\beta$  were as susceptible to suppression as those from wild type animals (Piccirillo et al., 2002). These data suggest that TGF $\beta$  is not important in the regulatory activity of T<sub>REGS</sub>. However, *ex vivo* data from the Strober group showed that surface-bound TGF $\beta$  was required for optimal regulatory activity of nT<sub>REGS</sub> (Nakamura et al., 2001). A similar finding was reported *in vivo*, in the setting of the type-1 diabetes using the NOD mouse

model (Green et al., 2003). Additionally, effector T cells that lack a functional TGF $\beta$  receptor (TGF $\beta$ R) could not be controlled by co-transfer of T<sub>REG</sub> cells in the T cell-mediated colitis model (Fahlen et al., 2005), suggesting that the action of TGF $\beta$  is critical to T<sub>REG</sub>-mediated suppression of effector populations in this setting. Therefore, it appears that T<sub>REG</sub>-produced TGF $\beta$  is important in the *in vivo* context, perhaps in a surface bound form that mediates direct interaction with cells expressing the TGF $\beta$ R.

Clearly T<sub>REGS</sub> are important in many pathological settings, including autoimmunity, cancer, and other diseases characterized by chronic inflammation. In the case of autoimmunity and chronic inflammation T<sub>REG</sub> cells act unambiguously to prevent pathological inflammation and tissue destruction that would otherwise harm the host. This is reviewed here (Vignali et al., 2008), and much of the relevant data was discussed above. Conversely, several studies have shown that T<sub>REG</sub> cells are recruited in the setting of cancer and certain infections to prevent protective immune responses, thus contributing to the development of human disease. For example, blockade of the IL10 receptor led to viral clearance in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) (Brooks et al., 2006; Ejrnaes et al., 2006). However, other data from murine models of *Toxoplasma* and *Plasmodium* infection clearly show that loss of IL10 is associated with a lethal inflammatory response (Gazzinelli et al., 1996) or enhancement of infection (Li et al., 1999), without major changes in pathogen load. Thus IL10 can undermine sterilizing immune responses in the case of some infections, while being protective to the host in others. The data in

cancer are far more clear. Experiments with the B16 melanoma model have shown that Foxp3<sup>+</sup> cells help tumors escape from immune surveillance by lysing antigen-carrying DCs in tumor draining lymph nodes (TDLNs). This effect occurred in a perforin-dependent manner (Boissonnas et al., 2010). Furthermore, T<sub>REGS</sub> have also been shown to secrete granzyme B and perforin within the tumor microenvironment, leading to the lysis of tumor-responsive NKT and CD8<sup>+</sup> T cells (Cao et al., 2007). Moreover, tumors have been shown to drive the differentiation of Foxp3<sup>+</sup> T<sub>REG</sub> cells. Further analysis demonstrated that these tumor-associated T<sub>REGS</sub> prevented tumor rejection in an IL10-dependent manner (Bergmann et al., 2007). In another study, tumor-produced TGFβ converted CD4<sup>+</sup>CD25<sup>-</sup> cells into suppressive CD4<sup>+</sup>CD25<sup>+</sup> populations (Liu et al., 2007). Thus, part of the mechanism that tumors use to evade immune surveillance is the induction and recruitment of regulatory T cell populations, making them target of interest in cancer therapy.

### **The T<sub>REG</sub> - T<sub>H</sub>17 axis and T cell-targeted therapy**

There is an intriguing link between iT<sub>REG</sub> cells and T<sub>H</sub>17 cells, both of which depend on TGFβ signaling for their differentiation (Bettelli et al., 2006; Lee et al., 2009a). Foxp3 can inhibit RORγt function and the T<sub>H</sub>17 phenotype (Zhou et al., 2008), likely through a coordinated interaction with the transcription factor Runx1 (Zhang et al., 2008). Foxp3 has also been shown to block the transcription activity of another T<sub>H</sub>17 associated transcription factor, RORα (Du et al., 2008).

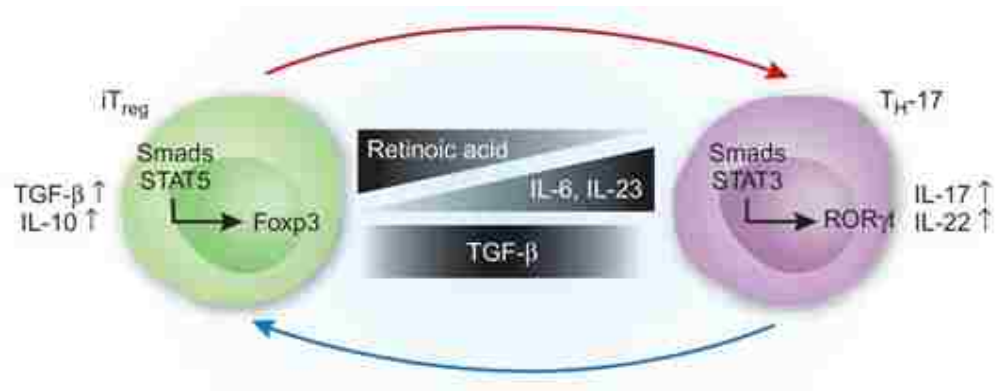
Conversely, IL6 signaling blocks the chromatin binding activity of Foxp3 and promotes T<sub>H</sub>17 differentiation (Samanta et al., 2008; Sauer et al., 2008; Zhang et al., 2008; Zhou et al., 2007). In addition, T<sub>H</sub>17 cells secrete the cytokine IL21, which like IL6 and IL23, activates STAT3 and promotes T<sub>H</sub>17 differentiation at the expense of iT<sub>REG</sub> induction (Wei et al., 2007). Autocrine activity of IL21 may account for the surprising result that re-stimulation of differentiated T<sub>H</sub>17 cells with exogenous TGF $\beta$  alone maintains expression of both IL17A and IL17F (Lee et al., 2009b).

The choice between iT<sub>REG</sub> cells and T<sub>H</sub>17 cells can have profound pathological implications. For example, as discussed above, T<sub>REGS</sub> actively participate in tumor escape from immune surveillance (Boissonnas et al., 2010; Kryczek et al., 2007; Sharma et al., 2009), while T<sub>H</sub>17 cells enhance tumor rejection (Kryczek et al., 2007; Martin-Orozco et al., 2009; Muranski et al., 2008; Zou and Restifo, 2010). One can imagine dampening the function of regulatory T cell populations within the tumor bed or in the TDLNs to enhance anti-tumor immunity. Conversely, work out of Nicholas Restifo's group at the NIH has demonstrated that T<sub>H</sub>17-polarized cells exhibit enhanced tumoricidal activity after adoptive transfer in B16 melanoma mice (Muranski et al., 2008; Zou and Restifo, 2010), at least in part through the induction of cytotoxic T cells (Martin-Orozco et al., 2009). Thus a drug designed to shift tumor-associated regulatory T cells toward the T<sub>H</sub>17 end of the spectrum might serve to diminish the pro-tumor activity of resident T<sub>REG</sub> cells while at the same time driving T<sub>H</sub>17-mediated anti-tumor activity within the same population.



This intimate interplay between critical factors in  $T_{REG}$  and  $T_H17$  development, along with the dual reliance on  $TGF\beta$  signaling for their differentiation, has led to the conceptualization of the  $T_{REG}$ - $T_H17$  axis. From a therapeutics perspective, the identification of drugs that promote pro- or anti-inflammatory responses by influencing differentiation along this axis has gained momentum as examples of T cell plasticity continue to be characterized (Bluestone et al., 2009). Interestingly, the  $T_{REG}$  and  $T_H17$  populations appear to be particularly prone to late developmental plasticity (Lee et al., 2009a). These results suggest it may be possible to treat disease by shifting the balance along the  $T_{REG}$ - $T_H17$  axis *in situ* during ongoing immune responses, either to attenuate inflammation in the setting of autoimmune disease, or to enhance immunity to aid in pathogen clearance or tumor rejection. The developmental plasticity between the  $T_H17$  and  $T_{REG}$  populations is reviewed here (Lee et al., 2009a).

To that end, targeting non-cytokine signaling pathways may be a viable option. There are several factors that appear to preferentially polarize cells towards either the  $iT_{REG}$  or  $T_H17$  phenotype. For example, ATP (Atarashi et al., 2008), shinogosine-1-phosphate (Liao et al., 2007) and vitamin D (Colin et al., 2010) can modulate  $T_H17$  development, while APC-derived indolamine 2,3-dioxygenase (Sharma et al., 2009) and retinoic acid (Mucida et al., 2007) can promote  $T_{REG}$  populations. Targeting such pathways has the potential advantage of avoiding the severe side-effect profiles that have plagued cytokine-targeted therapies.



**Figure 4 : Introduction: The T<sub>REG</sub>-T<sub>H</sub>17 axis.**

Adopted from: *Tato CM & Cua DJ (2008) Nat Immunol. 9:1323-25*

### **Other regulatory T cells (Hybrid T cells and Foxp3<sup>-</sup>IL10<sup>+</sup> cells)**

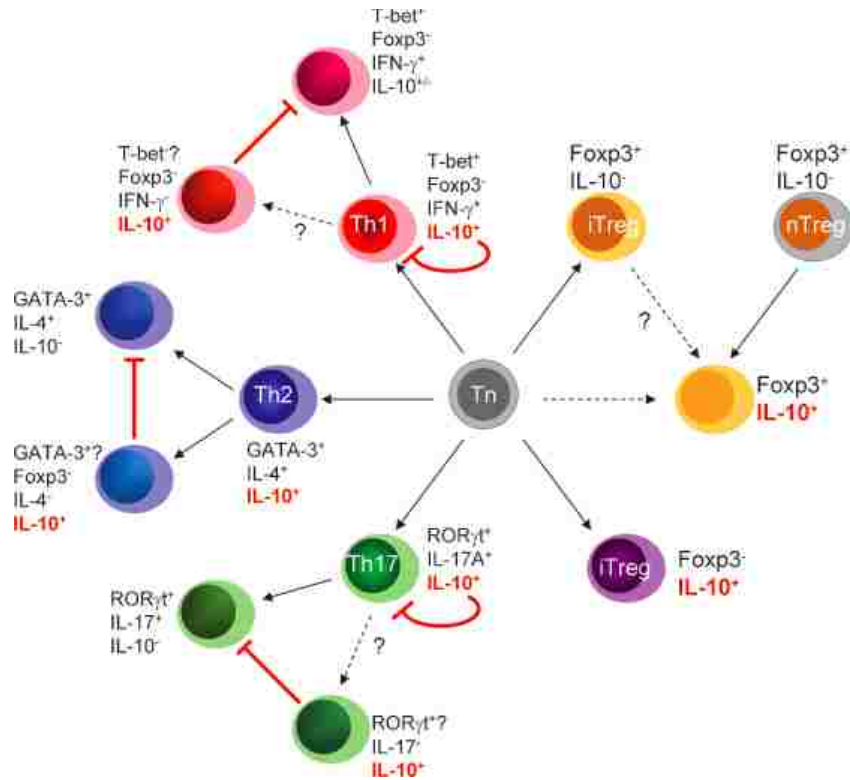
It is now known that all helper T cell lineages can express IL10 (**Figure 5**), and there is clear evidence that both Foxp3<sup>+</sup> and Foxp3<sup>-</sup> T cell populations can exhibit regulatory activity through the production of IL10 (Maynard et al., 2007). There is also evidence that IL10 can actually stabilize Foxp3 expression *in vivo* (Murai et al., 2009). While the types and classifications of the various T<sub>REG</sub> populations is a dynamic process, and often varies by investigator, it is clear that at least some regulatory T cell populations do not express Foxp3. One such population are the type-1 regulatory T (Tr1) cells, which were originally described as be IL10-induced T<sub>REG</sub> cells that express IL10 (Roncarolo et al., 2006). However, it is now clear that Foxp3<sup>-</sup>IL10<sup>+</sup> regulatory T cells can be generated without exogenous IL10 (Maynard et al., 2007). While clear categorical

descriptions of IL10 producing iT<sub>REGS</sub> are still lacking, the importance CD4<sup>+</sup> T cell produced IL10 in regulating immune responses is not in question (Maynard and Weaver, 2008), including in the setting of human inflammatory bowel disease (Amre et al., 2009; Glocker et al., 2009).

The induction of IL10 within each of the distinct effector lineages is thought to be important in limiting ongoing inflammatory reactions locally. The implicit function of this negative feedback loop has led to the characterization of IL10<sup>+</sup> effector populations as “autoregulatory T cells”. Work with cultured T cells clearly shows that ERK signaling is necessary for the induction of IL10 in T<sub>H1</sub> and T<sub>H2</sub> cells, and contributes to IL10 expression in T<sub>H17</sub> populations, with no detectable difference when p38 signaling is blocked (Saraiva et al., 2009). Furthermore, IL10 production in T<sub>H1</sub> cells requires IL12-signaling through STAT4, whereas T<sub>H2</sub> cells are dependent on IL4-signaling through STAT6, and IL10 production in T<sub>H17</sub> cells requires signaling through STAT3 (Stumhofer et al., 2007). IL27 is also capable of inducing IL10 in all three lineages (Fitzgerald et al., 2007; Stumhofer et al., 2007). Moreover, TGFβ blocks IL10 expression in CD4<sup>+</sup> T cells differentiating in culture (Saraiva et al., 2009), while efficient induction of IL10 secretion from T<sub>H17</sub>-polarized cells requires both TGFβ and an activator of STAT3 such as IL6 (McGeachy et al., 2007), IL21 (Spolski et al., 2009), or IL27 (Stumhofer et al., 2007). The ability to incite localized IL10 production suggests that one mechanism to therapeutically treat the chronic inflammatory reactions associated with autoimmune disease would be to induce IL10 expression within the effector populations (ex: T<sub>H17</sub> cells) contributing to the pathological

inflammation. Indeed, IL10 has been explored as a therapeutic option in many disease settings (O'Garra et al., 2008).

Finally, several reports have characterized “hybrid” T cell populations where Foxp3 is expressed in various effector T cell populations (Barnes and Powrie, 2009). The thought is that induction of one of the canonical effector T cell transcription factors (T-bet, GATA-3, or ROR $\gamma$ t) in conjunction with Foxp3 allows lineage-specific trafficking factors to be expressed in cells that are programmed to function in a regulatory fashion. This induces trafficking of T<sub>REGS</sub> in the circulation to sites of ongoing inflammation. Such a response is not unprecedented as T-bet-induced CXCR3 expression in Foxp3<sup>+</sup> cells has been shown to be important in targeting T<sub>REGS</sub> to sites of T<sub>H</sub>1-type inflammation and limiting tissue damage (Koch et al., 2009).



**Figure 5 : Introduction: IL10 production in CD4<sup>+</sup> T cell lineages.**

Adopted from: *Maynard CL & CT Weaver (2008) Immunol Rev 226: 229-33*

## Section 1.4 : Estrogen

The other major topic relevant to this work centers on estrogen signaling and the role of the G protein coupled receptor GPER, thus a brief discussion of estrogen biology is presented.

### Estrogens physiological effects

Estrogens belong to the family of lipid soluble steroid hormones. There are numerous forms of estrogen, including estrone (E1), estradiol (E2), estriol (E3),

and the estrogen sulfates (Pasqualini et al., 1989). In mammals, the form associated with the highest activity is 17 $\beta$ -estradiol (hereafter referred to as E2) (Prossnitz et al., 2008). E2 is shown in **Figure 7** below. Estrogen is most prominently associated with women of reproductive age, who generally have much higher circulating levels than prepubescent females, postmenopausal women, and males. However, it is important to note that estrogens play a critical role in numerous physiological processes ranging from reproductive biology and bone metabolism (Dupont et al., 2000) to cardiovascular and lipid homeostasis (Baker et al., 2003). Moreover, although the major site of female estrogen production is in the ovary; adipose tissue, the testis, and the central nervous system (among others) are also capable of producing estrogen (Simpson et al., 1993), and estrogen receptor deficiency is associated with female and male infertility (Akingbemi, 2005). Indeed many cell types express the enzyme aromatase which converts testosterone to estrogen (Simpson et al., 1993), and extraovarian synthesis has been described (Baquedano et al., 2007). Thus, estrogens are important in many settings, and in both sexes.

Estrogens have also been linked to numerous human diseases. There is a long history linking estrogen to breast cancer in women (Beatson, 1898; Moore et al., 1967). There are also environmental contaminants that can mimic estrogen and activate estrogen receptors. These include synthetic compounds (xenoestrogens), plant products, (phytoestrogens), and fungal products (mycoestrogens) (Prossnitz and Maggiolini, 2009). Thus understanding estrogen signaling is critical in many areas of human exposure and disease.

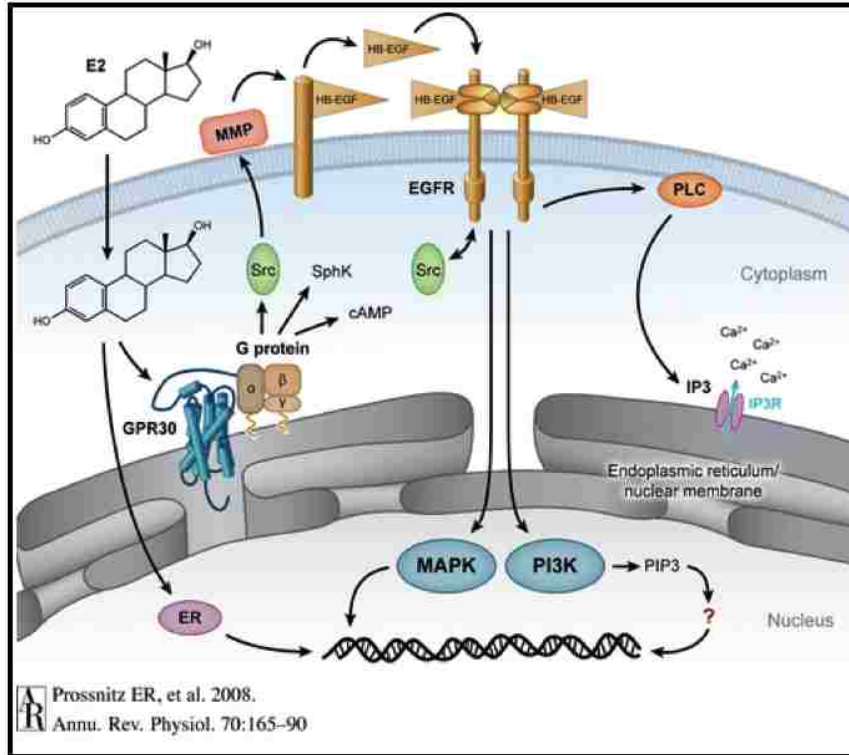
## **ER $\alpha$ and ER $\beta$**

The classic estrogen receptors ER $\alpha$  (Jensen and DeSombre, 1973) and ER $\beta$  (Kuiper et al., 1996) contain DNA binding domains that recognize EREs (estrogen response elements) on target genes where they act as ligand-activated nuclear transcription factors. Upon estrogen binding, they undergo conformational changes that allow coordination of a complex transcriptional profile through a multitude of protein-protein interactions with numerous enhancer and regulatory elements (Marino et al., 2006). In addition to the transcriptional events that result from activation of ER $\alpha$  and ER $\beta$ , estrogen responses are known to include several rapid signaling events more commonly associated with surface receptors such as receptor tyrosine kinases and G protein coupled receptors (GPCRs). Examples include calcium mobilization and transactivation of mitogen-activated protein (MAP) kinase cascades. While the majority of the work on the classical estrogen receptors has focused on their ability to regulate transcription, there is evidence that they can elicit some of the rapid signaling responses associated with estrogen binding (Edwards, 2005). In reality, the designation as rapid or “non-genomic” signaling versus “genomic” transcription based signaling is largely arbitrary as each plays a substantial role in the outcomes associated with the other. In general, these are collectively referred to as estrogen signaling in this manuscript.

## **GPER (GPR30)**

At the start of the decade, dogma in the estrogen field stated that the classical ER's (ER $\alpha$  and ER $\beta$ ) accounted for all estrogen signaling events. However, evidence in the literature demonstrating estrogenic effects in ER $\alpha/\beta$  double knockout mice suggested that other estrogen receptors may exist (Das et al., 2000; Shughrue et al., 2002). In 2000, it was demonstrated that the orphan G protein-coupled receptor GPR30 could mediate estrogen-dependent ERK activation in SKBr3 cells (which lack ER $\alpha$  and ER $\beta$ ) (Filardo et al., 2000). These findings were extended in 2005, when our group along with the team led by Filardo and Thomas published work demonstrating estrogen binding to GPR30 in cells lacking both ER $\alpha$  and ER $\beta$  (Revankar et al., 2005; Thomas et al., 2005). In these studies, it was shown that E2 activation of GPER led to calcium mobilization and activation of PI3 kinase. Since these initial reports, GPR30 (renamed GPER) has been linked to several estrogen-mediated events. A current model of estrogen signaling is included below as **Figure 6**.





**Figure 6 : Introduction: Current model of estrogen signaling.**

Adopted from: Prossnitz et al (2008) Annu Rev Physiol 70:165-90

The physiological functions of GPER remain largely unknown (Prossnitz and Barton, 2009b), partly due to the fact that many reports prior to 2005 utilized drugs that have yet uncharacterized functions in GPER signaling, and because there is evidence of synergistic effects between ER $\alpha$  and GPER (Albanito et al., 2007). However, some links have been identified. It appears that GPER plays a role in estrogen-mediated secretion from pancreatic beta cells (Nadal et al., 2011), while genetic disruption of GPER in female mice leads to altered bone growth and blood pressure, with older mice exhibiting hyperglycemia and impaired glucose tolerance (Martensson et al., 2008). Moreover male GPERKO mice exhibit increased bone mass, bone mineralization, and overall weight (Ford

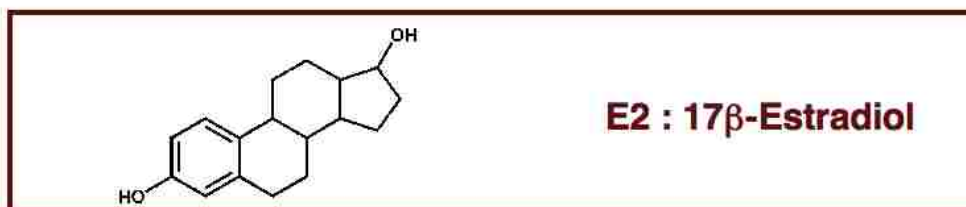
et al., 2010). Links to several other human pathologies have also been established, including pain disorders (Dun et al., 2009; Hazell et al., 2009; Liverman et al., 2009; Lu et al., 2009) and cardiovascular disease (Bopassa et al., 2010; Filice et al., 2009; Jessup et al., 2010; Meyer and Barton, 2009; Weil et al., 2010; Yang and Reckelhoff, 2010). In terms of cancer, a multitude of reports suggest that GPER can serve as a prognostic marker in gynecological cancers (Filardo et al., 2006; Filardo et al., 2008; Giess et al., 2010; Liu et al., 2009; Prossnitz and Barton, 2009a; Smith et al., 2007; Tu et al., 2009) and alter cancer cell proliferation (Arias-Pulido et al., 2010; Ariazi et al., 2010; Chan et al., 2010; Dong et al., 2010; Maggiolini et al., 2004). Links to Tamoxifen resistance in breast cancer have also been established (Ignatov et al., 2010).

Several fundamental questions regarding GPERs function remain. Indeed, the status of GPER as an estrogen receptor is not without controversy (Langer et al., 2010; Otto et al., 2009). Some have speculated that GPER drives expression of a little known ER $\alpha$  splice variant, termed ER36, which lacks much of the 5' transactivation domain and contains a unique 3' exon. These authors have hypothesized that it is this receptor which binds estrogen when GPER is expressed (Kang et al., 2010). However, bioinformatics analysis of protein and DNA sequences from the published C57BL/6 mouse genome (NCBI) shows there is no homologous 3' exon within 1Mb of the murine GPER locus (data not shown) that would match the reported ER36 exon. In addition, we and others have been unable to replicate the findings with ER36 described above (personal

communication). Thus it appears that ER36 is unlikely to account for the observed effects of GPER. The subcellular localization is also a point of question, with some group (ours included) seeing it localized to the endoplasmic reticulum (Revankar et al., 2007), and others localizing it to the plasma membrane (Filardo et al., 2007; Sanden et al., 2010). Work is ongoing to resolve the exact mechanism and setting for GPER-mediated estrogen signaling.

Given the importance of estrogen in breast cancer and other human health conditions, and given the success in targeting G protein-coupled receptors pharmacologically, our lab has developed a series of small molecules that specifically target GPER. This class of compounds currently includes the GPER-directed agonist, G-1 (Bologa et al., 2006), and the antagonists G15 (Dennis et al., 2009) and G36 (unpublished). These membrane-permeable small molecules are highly specific for GPER. G-1 in particular shows minimal binding to classical estrogen receptors (Arterburn et al., 2009) and has been tested against 25 other GPCRs (Blasko et al., 2009). Like the estrogens (Muller et al., 1979), the G compounds are uncharged, hydrophilic compounds that freely migrate across cell membranes via passive diffusion, and are therefore attractive candidates for rapid incorporation into the pharmaceutical pipeline. In addition, the growing evidence that GPER plays a more subtle role in the majority of estrogens most prominent physiological effects suggest that the G compounds may be associated with a more attractive side-effect profile. See structures in **Figure 7**. These compounds were used throughout this study.

## Drug Compounds



**Not pictured: G-36 : Antagonist**

Figure 7 : Introduction: The G compounds and E2

### ***Section 1.5 : Estrogen and the immune system***

It has long been recognized that women exhibit a much higher prevalence of numerous autoimmune diseases (Whitacre et al., 1999), with several lines of evidence linking this observation to estrogen signaling. For example, the PRIMS study showed that patients with multiple sclerosis (MS) experience a decrease in relapses over the course of their pregnancy, most notably in the third trimester, with a subset of women exhibiting a period of increased symptoms immediately post-partum (Vukusic et al., 2004). These studies translate to animal models as

estrogen has been shown to protect animals from experimental autoimmune encephalomyelitis (EAE), a widely accepted animal model of multiple sclerosis (Ito et al., 2001; McClain et al., 2007; Offner and Polanczyk, 2006; Polanczyk et al., 2004a; Polanczyk et al., 2004b; Polanczyk et al., 2005). Analysis of cells from the draining lymph nodes of EAE mice showed that estrogen can increase Foxp3 expression (Polanczyk et al., 2005). Other important immunoregulatory molecules have been linked to estrogen as well. Increased expression of the surface receptor programmed death (PD) -1 was observed on Foxp3<sup>+</sup> cells in estrogen treated EAE mice, and PD-1 has been linked to estrogen-mediated suppression of this model (Polanczyk et al., 2007; Wang et al., 2009a). Yet a recent study showed that estrogen-mediated protection from EAE was not dependent on Foxp3 expression (Subramanian et al., 2010). Thus it appears that multiple mechanisms are responsible for estrogens protective effects in EAE. Moreover, estrogens effects do not appear to be limited to the setting of neurological inflammation as studies of patients with systemic lupus erythematosus (SLE) (Clowse, 2007) and Rheumatoid arthritis (RA) (Da Silva and Spector, 1992) have also linked these diseases to estrogen.

The evolutionary logic behind estrogen-mediated immune regulation may stem from the need to suppress responses to paternal epitopes. Experiments using allogeneic rejection models of pregnancy have demonstrated that T<sub>REG</sub> populations are important in tolerance to foreign epitopes *in utero* (Schumacher et al., 2007). Another reason that estrogen may impart an effect on the immune system is the role of leukocytes in the remodeling processed during and post

delivery (Read et al., 2007). Studies with cervical biopsies from post term women undergoing prostaglandin-induced parturition showed that women who didn't respond to therapy showed reduced leukocyte influx, including reduced numbers of IL8<sup>+</sup> cells, as compared to women who did respond to therapy or who gave birth at term. However, no differences in estrogen receptor expression were observed (Dubicke et al., 2008). Interestingly, IL8 can be produced by T<sub>H</sub>17 cells (Stockinger et al., 2007). Moreover, work with animal models has shown that delivery of oral ethinyl estradiol protects animals from the development of EAE while inducing IL10 secretion (Yates et al., 2010).

While it is clear that estrogen imparts a direct effect on immune physiology, many questions remain. The majority of work to date has focused on pathways within the T<sub>H</sub>1 and T<sub>H</sub>2 subpopulations, yielding variable results (Pernis, 2007). However, efforts to delineate the role for specific estrogen receptors have uncovered a few interesting insights. Estrogen-mediated induction of Foxp3 within the CD4<sup>+</sup> T cell compartment was lost in ER $\alpha$ KO mice (Polanczyk et al., 2003), and both ER $\alpha$  and ER $\beta$  have been linked to estrogens protective effects in this model (Polanczyk et al., 2004a). Further insights emerged when it was shown that G-1 can suppress EAE induced by direct immunization with peptide from either myelin oligodendrocyte glycoprotein (MOG) (Wang et al., 2009a) or myelin proteolipid protein (PLP) (Blasko et al., 2009). This latter study also used an adoptive transfer protocol where pathogenic cells from the draining LN (DLN) of PLP peptide-treated mice were harvested and expanded *ex vivo* prior to injection into naïve hosts, who subsequently developed

severe disease (Blasko et al., 2009). They were able to show that co-culture of DLN cells with G-1 during *ex vivo* conditioning reduced disease severity without the need for direct treatment to recipient mice. This effect was associated with reduced production of IL17A and IFN $\gamma$  from CNS lymphocytes, as well as reduced levels of IL23 and CCL2 (a critical factor for macrophage recruitment to the CNS in EAE) within the tissue itself. Despite the similarities in disease outcome, these two studies focused on two distinct immune populations. In one study, G-1 induced suppression of EAE was associated with increased PD-1 expression on Foxp3<sup>+</sup> T cells with no change in the number of Foxp3<sup>+</sup> cells overall, while the protective effects of G-1 were largely absent in PD-1KO mice. In addition, the protective effects of estrogen were significantly (but not completely) attenuated in GPER<sup>-/-</sup> mice. This suggested that estrogen-mediated suppression of EAE may be partially due to GPER-mediated induction of PD-1 expression on Foxp3<sup>+</sup> T<sub>REGS</sub> (Wang et al., 2009a). Conversely, in the second study, G-1 treatment inhibited polyI:C-induced TNF $\alpha$  and IL6 production as well as LPS-induced IL12(p40) and CCL5 production from primary human macrophages. They went on to show that ectopic expression of GPER in the human promyelocytic cell line HL60 allowed for G-1 induced calcium mobilization, and that G-1 treatment reduced macrophage recruitment to the CNS of EAE mice. Such observations suggest that G-1 may affect the macrophage population to suppress EAE. These two conclusions are not mutually exclusive (Blasko et al., 2009), but they bring up a common theme within the literature exploring

estrogens effects within the immune system; these interactions are complex and multifaceted.



## Chapter 2 : The hypothesis

### ***Section 2.1 : Preliminary findings and figures***

The overarching theories that guided our preliminary work are summarized in the following list;

1. Estrogen is a known modulator of the immune system and T cells.
2. GPER is a novel estrogen receptor.
3. GPER is a G protein-coupled receptor.
4. G protein-coupled receptors have been successfully targeted with small molecules for therapeutic benefit.
5. We have developed a small molecule agonist for GPER, G-1, and two small molecule antagonists, G15 and G36.
6. G-1 has already been shown to attenuate an animal model of multiple sclerosis.
7. CD4<sup>+</sup> T cells are central mediators of many immune diseases.
8. Small molecules that could modulate CD4<sup>+</sup> T cell populations would be of immense therapeutic interest, both for systemic treatment and for alteration of T cells *ex vivo* for tumor vaccines and other adoptive therapies.

Given these starting concepts, we set out to investigate whether our GPER-directed agonist G-1 could alter CD4<sup>+</sup> T cell function by acting directly on the T cells. Specific action within the T cell populations was delineated by using cultures of purified, primary murine T cells stimulated *ex vivo*.

### **GPER affects cytokine secretion from CD4<sup>+</sup> T cells**

To begin, CD4<sup>+</sup> splenocytes were collected using positive selection on a magnetic bead-based cell purification system (AutoMACS) from the spleens and lymph nodes of 7-11 week old C57BL/6 male mice. Purified cells were cultured in 96-well plates and stimulated with antibodies directed to CD3 $\epsilon$  and CD28, which mimics APC-driven T cell activation, along with various combinations of the GPER-directed compounds G-1 and G36. Samples of medium were collected at day 3 or day 5 post stimulation and screened for IL2, IL4, IL5, IL10, IL12, IL17A, TNF $\alpha$ , and IFN $\gamma$  production by Luminex Multiplex assay (Invitrogen). We observed no trends for IL2, IL4, IL5, IL12, IFN $\gamma$  and TNF $\alpha$  (data not shown) in the G-1 treated cultures, although this was possibly due to autocrine/paracrine uptake of these cytokines. Interestingly, G-1 treatment did induce the production of the cytokines IL17A on day 3, and IL10 on day 5. These effects could be inhibited by G36. (**Figure 8**). Combined, these findings suggest that GPER-signaling could enhance production of IL17A and IL10. As 10% of these cultures consisted of cells other than CD4<sup>+</sup> T cells (data not shown), it was possible that our observations were due to effect on other non-T cell populations. Additionally,

the CD4<sup>+</sup> population contained both memory and naïve lineages, thus even if our findings were the result of direct activity within the CD4<sup>+</sup> T cell population, these data do not distinguish whether the effect was due to G-1 altering T cell differentiation or rather a reflection of alterations in memory T cell cytokine secretion. Thus our next question was whether G-1 could modulate T cell differentiation.

### **G-1 modulates TGFβ-induced differentiation of naive CD4<sup>+</sup> T cells**

To determine if GPER has an effect on the differentiation of naive T cell populations, CD4<sup>+</sup>CD62L<sup>hi</sup> naive T cells were purified by fluorescence-activated cells sorting (FACS) from the spleens and lymph nodes of 7-11 week old C57BL/6 male mice. Purified cells were stimulated in culture for 5 days with anti-CD3ε and anti-CD28 antibody (Ab) supplemented with TGFβ (10ng/mL) in the presence and absence of G-1. No additional treatments were added after day 0. The decision to add TGFβ was based on the fact that;

(A) it is a common factor in the differentiation of both T<sub>H</sub>17 and iT<sub>REG</sub> cells, forming a critical cog in the T<sub>REG</sub>-T<sub>H</sub>17 axis (**Figure 3**). This is interesting given our data show that G-1 can drive secretion of IL17A (the canonical T<sub>H</sub>17 cytokine) and IL10 (a well known suppressive cytokine), and;

(B) published work in human cancer cell lines had established that GPER could inhibit TGF $\beta$ -induced cancer cell migration (Kleuser et al., 2008), suggesting that GPER-mediated signaling could integrate with signals from the TGF $\beta$  receptor.

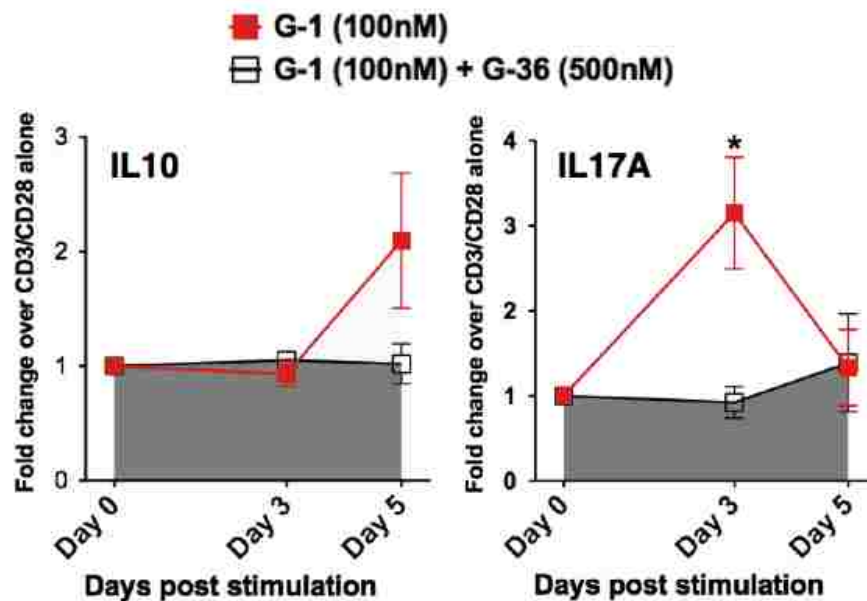
The addition of IL2 to the culture medium served as the negative control, as this cytokine blocks T<sub>H</sub>17 differentiation (Kryczek et al., 2007). Consistent with our findings from Figure 8, the addition of G-1 lead to an increase in the number of IL17<sup>+</sup> cells, as assessed by intracellular cytokine staining (ICS) (**Figure 9A**). Interestingly, when the cells were re-stimulated with anti-CD3 Ab following 5 days of culture, we observed drastically reduced IL17 and IFN $\gamma$  secretion from the cultures treated with G-1 (**Figure 9B,C**). These results implicated a regulatory T cell population in the G-1 treated cultures. Given that our results with enriched CD4<sup>+</sup> demonstrated increased secretion of IL10 at day 5, we wanted to determine if G-1 could drive IL10 production as well. However, we were unable to detect IL10<sup>+</sup> cells in these cultures (data not shown), which is consistent with a previous study from Anne O'Garra's group which showed that continuous treatment with TGF $\beta$  alone can block IL10 expression in cultured CD4<sup>+</sup> T cells (Saraiva et al., 2009). It is important to note that removal of TGF $\beta$  or addition of other factors after TGF $\beta$  treatment may lead to IL10 expression in these cells, meaning this is not a stable inhibition of IL10 expression. Thus we next sought to investigate IL10 and IL17A expression during CD4<sup>+</sup> T cell differentiation in conditions that were amenable to the production of IL10.

## **G-1 enhances differentiation of a CD4<sup>+</sup>IL10<sup>+</sup>IL17<sup>+</sup> T cell population**

As stated above, when G-1 was added to cultures of CD4<sup>+</sup> splenocytes, there was a spike in IL17 production observed on day 3, which was subsequently lost by day 5. The loss of IL17 production correlated with an increase IL10 production, although this trend never reached statistical significance (P = 0.11 by student's t-test, **Figure 8**). To determine whether G-1 can drive IL10 expression during T cell differentiation, naïve CD4<sup>+</sup> T cells were collected, stimulated in culture as described above, and stained for intracellular IL10 and IL17. In this case, IL6 (20ng/mL) was added to the cultures along with TGFβ (5ng/mL). This was based on the fact that a report out of Daniel Cua's lab had shown that an IL10<sup>+</sup>IL17<sup>+</sup> population of cells developed under these conditions, and that these cells exhibited immunosuppressive activity *in vivo* (McGeachy et al., 2007). Notably, these cells were shown to attenuate EAE, the same disease model used in studies demonstrating G-1's *in vivo* immunosuppressive properties (Blasko et al., 2009; Wang et al., 2009a). It is also known that IL10 production within T<sub>H</sub>17 populations requires transactivation of STAT3 (Saraiva and O'Garra, 2010), which can be elicited by treatment with several cytokines, including IL6 (Stumhofer et al., 2007). Thus these conditions presented the ideal opportunity to investigate both IL10 and IL17A expression. Exposure to G-1 increased the number of IL10<sup>+</sup>IL17<sup>+</sup> cells and IL10<sup>+</sup>IL17<sup>-</sup> cells (**Figure 10**), consistent with our findings in Figures 8 and 9.

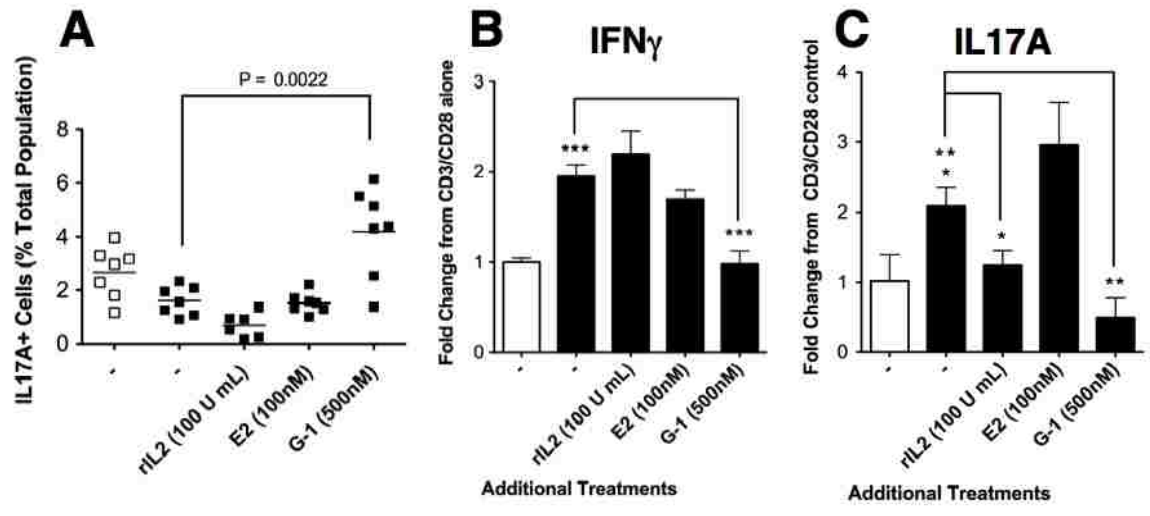
## Conclusions from preliminary studies

In summary, preliminary work suggested that G-1 drives expression of IL10 and IL17A within differentiating CD4<sup>+</sup> T cells. We also noted an expansion of the CD4<sup>+</sup>IL17<sup>+</sup>IL10<sup>+</sup> T cell population under T<sub>H</sub>17-polarizing conditions. These cells have been previously shown to exhibit *in vivo* T<sub>REG</sub> activity. It is important to again note that IL17A has been shown to exhibit immunosuppressive properties, in addition to its role in proinflammatory processes (O'Connor et al., 2010). Thus the induction of both IL10 and IL17A does not necessarily preclude G-1 from therapeutic development.



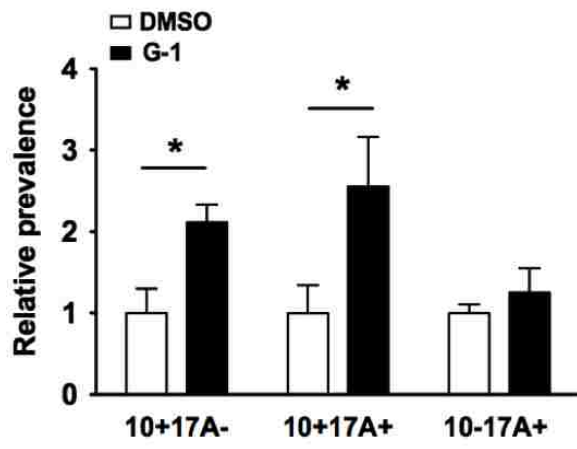
**Figure 8 : Preliminary data: G-1 drives IL17A and IL10 secretion from T cells.**

CD4<sup>+</sup> splenocytes were collected by AutoMACS (Miltenyi) stimulated in culture with G-1 (Red) or G-1 + G-36 (open boxes, gray area). Medium was collected 3 and 5 days later and analyzed for IL10 and IL17 by Luminex Multiplex Assay. Summary of data from 3 independent experiments. Statistical analysis done by student's t-test. \* = P < 0.05. Error bars = S.E.M.



**Figure 9 : Preliminary data: G-1 alters TGF $\beta$ -driven T cell differentiation.**

CD4<sup>+</sup>CD62L<sup>hi</sup> naïve T cells were stimulated with for 5 days in presence (black) or absence (white) of TGF $\beta$  along with treatments indicated. Cultures were then stained for intracellular IL17A (A) or were re-stimulated with antiCD3 Ab for evaluation of IFN $\gamma$  (B) and IL17A (C) secretion by ELISA assay. Summary of data from 3 experiments. Statistical analysis done by student's t-test. \*\*\* = P < 0.0005, \*\* = P = P < 0.005, \* = P < 0.05. Error bars = S.E.M.



**Figure 10 : Preliminary data: G-1 alters IL17A/IL10 expression in naive T cells.**

CD4<sup>+</sup>CD62L<sup>hi</sup> naïve T cells from Foxp3<sup>egfp</sup> mice were collected by FACS and cultured for 4 days with TGF $\beta$  + IL6, supplemented with either 100nM G-1 or DMSO, as indicated. Cells were then stained for intracellular IL10 and IL17A, then analyzed by flow cytometry. Data from one of five independent experiments shown. P values determined by student's t-test. \* = P < 0.05. Error bars = S.D.

## ***Section 2.2 : Development of a hypothesis***

Numerous studies have implicated the hormone estrogen in T cell function and autoimmune pathogenesis (See Section 1.5). Yet to date, few studies have definitively clarified the relationship between estrogen receptor signaling and T cell physiology. The emerging evidence of GPER involvement in immune regulation opens the door to new therapeutic targets for immune-related diseases. Preliminary results with cultured CD4<sup>+</sup> T cells demonstrated that selectively stimulating GPER leads to production of the suppressive cytokine IL10 and the T<sub>H</sub>17-associated cytokine IL17A, including induction of an IL10<sup>+</sup>IL17<sup>+</sup> T cell population. The ability to drive IL10 production from T<sub>H</sub>17 cells is of therapeutic interest for two main reasons; this cytokine exhibits enormous immunosuppressive potential, and numerous autoimmune diseases are associated with a large number of T<sub>H</sub>17 cells (see Section 1.3). Interestingly, previous reports have linked GPER to TGFβ signaling, a critical regulator of the T<sub>REG</sub>-T<sub>H</sub>17 axis, while E2 is known to drive expression of the canonical T<sub>REG</sub> transcription factor Foxp3. These results led to the following **HYPOTHESIS**:

**The GPER-directed agonist G-1 drives differentiation of traditional and hybrid regulatory T cells by direct action on CD4+ T cell populations, including in the context of T<sub>H</sub>17 differentiation. (See Figure 11)**



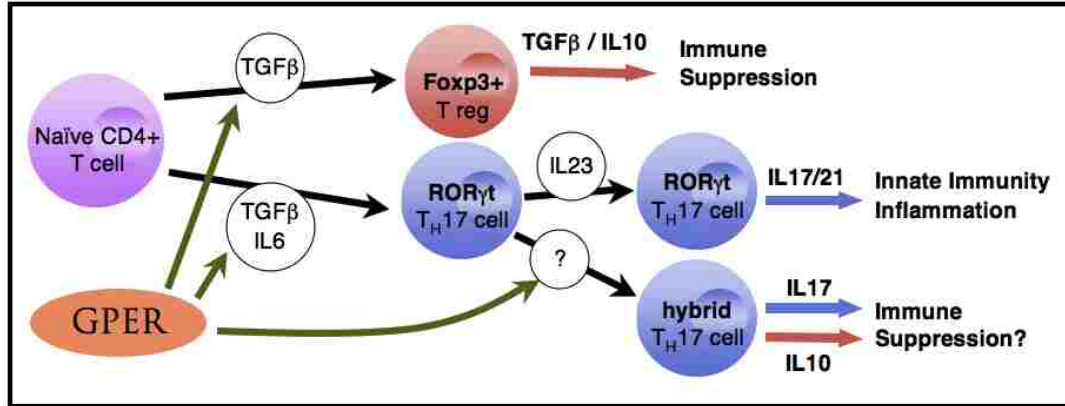


Figure 11 : Diagram of hypothesis.

### Section 2.3 : Aims of the study

In order to test the veracity of the hypothesis, the following Aims were outlined, and the associated experiments were carried out:

**Specific Aim 1 – To determine the effect of G-1 on the expression of key regulatory T cell markers.**

The ability of G-1 to induce development of regulatory T cell populations was investigated *in vivo* and *ex vivo*. G-1 and physiological estrogen (E2) were injected subcutaneously into various Foxp3<sup>egfp</sup> transgenic mice (Haribhai et al., 2007) (Methods: Chapter 3) for *in vivo* studies of Foxp3, PD-1 and CTLA-4 expression. Similarly, CD4<sup>+</sup> naïve T cells were purified by fluorescence-activated cell sorting (FACS) and stimulated in culture under various conditions to identify G-1-mediated effects on Foxp3, RORγt, PD-1, and CTLA-4 expression by direct action, or where assessed for suppressive function by T cell suppression assay.

**Specific Aim 2 – To delineate changes in T cell cytokine profiles following treatment with G-1.**

The ability of G-1 to modulate the expression of key cytokines within CD4<sup>+</sup> T cell populations was investigated following *in vivo* and *ex vivo* treatment. G-1 or E2 was injected subcutaneously into either wildtype or GPERKO mice, after which splenocytes were collected and stimulated *ex vivo* to analyze cytokine production. In addition, CD4<sup>+</sup> naïve T cells were purified by FACS and stimulated in the presence of G-1 under various conditions, including T<sub>H</sub>17-polarizing conditions. Following culture, cells were either stained for intracellular cytokines, transcription factors, or other moieties, or were re-stimulated and analyzed for cytokine secretion. Signaling pathways were investigated by employing chemical inhibitors, and proliferation was studied using fluorescent dyes.

**Specific Aim 3 – To determine if G-1 treated T cells demonstrate suppressive function *in vivo* in the setting of chronic inflammation.**

The *in vivo* suppressive function of G-1 treated T cells was delineated using a T cell-mediated model of colitis (TCMC; see Methods Chapter 3 and Appendix E). We also investigated whether systemic treatment with G-1 could inhibit disease induction. This paradigm was selected on the basis that Foxp3 (Murai et al., 2009; Uhlig et al., 2006), IL10 (Asseman et al., 2003; Coquerelle et al., 2009; Uhlig et al., 2006), and IL17A (O'Connor et al., 2009) have been shown to attenuate wasting disease and colonic inflammation in this model.

## **Chapter 3 : Materials and Methods**

### ***Section 3.1 : Cell sorting with MoFlo and AutoMACS***

T cells were obtained from single cell suspensions following homogenization of spleens and lymph nodes by mechanical disruption and passage through a 70 $\mu$ m nylon filter. Suspensions were stained with anti-CD4, antiCD62L, and anti-CD44 antibodies (Biolegend). Enriched populations of CD4<sup>+</sup>CD62L<sup>hi</sup> and CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> naïve T cells (See Appendix B) were collected by flow cytometric cell sorting on MoFlo cell sorter (Cytomation) using the Sort Single setting. Purity was regularly >96%. In most cases, experiments were repeated with both types of sorted naïve T cell, and no differences were noted. Other populations were collected in a similar fashion. See Appendix B for more details on sorting logic and criteria.

### ***Section 3.2 : Culture of CD4<sup>+</sup> T cells***

All experiments and cell purification were carried out in RPMI 1640 medium supplemented with fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, HEPES, sodium pyruvate, and 2-mercaptoethanol. Phenol red-free buffers and charcoal-stripped FBS were used to minimize exposure to estrogens or phyto/xenoestrogens that could have confounded our results (Berthois et al., 1986). This medium was used for all T cell culture experiments (**T cell media**).

Cells were stimulated in culture with soluble anti-CD3 $\epsilon$  (1.0  $\mu$ g/mL) and anti-CD28 (2.5  $\mu$ g/mL) antibodies (Biolegend), and supplemented with various combinations of TGF $\beta$  (0.5-10.0ng/mL), IL6 (20ng/mL), and IL23 (20ng/mL) as described (Biolegend and eBiosciences). Non-polarizing conditions (T<sub>H</sub>0) contained no exogenous cytokines. T<sub>H</sub>17 conditions contained TGF $\beta$  + IL6  $\pm$  IL23. Experiments were carried out using 96 well plates with 2x10<sup>5</sup> cells per well (10<sup>6</sup> cells/mL). For experiments using GPER and MAPK inhibitors, freshly sorted cells were incubated at 37°C (+5% CO<sub>2</sub>) for 60-90 minutes with 25 $\mu$ M PD98059 (MEK inhibitor), 250nM JNK II inhibitor, 100nM SB203580 (p38 inhibitor), or 500nM G15 or G36 (GPER antagonist (Dennis et al., 2009), provided by Dr. Jeffrey Arterburn at New Mexico State University) where indicated, prior to addition of stimulatory antibodies or cytokines. All compounds used in the study were dissolved in DMSO. All cultures were incubated at 37°C (+5% CO<sub>2</sub>).

### ***Section 3.3 : Staining cells for flow cytometry***

**Intracellular cytokine staining:** Following 4 days in culture, cells were washed with medium and “rested” for 60-90 min at 37°C (+5% CO<sub>2</sub>). Cultures were then treated with PMA (50ng/mL) and ionomycin (500ng/mL) for 4-5 hours in the presence of Brefeldin A (Biolegend) followed by fixation in Fixation Buffer (Biolegend). Samples were then washed and stained for intracellular proteins in Permeabilization Wash buffer (PWB – Biolegend) for 2 hours at room temperature, and washed with excess PWB for 15 minutes at room temperature

prior to centrifugation and analysis. Immediately after staining, data were collected on a FACScalibur (Becton Dickinson). Data analysis was performed using FlowJo software (TreeStar). Antibodies for staining included anti-IL10-allophycocyanin (APC), anti-IL10-phycoerythrin (PE), anti-IL17A-PE, anti-IL17A-PerCP, and anti-IFN $\gamma$ -APC all from Biolegend, as well as anti-ROR $\gamma$ t-PE from eBiosciences.

**Staining of surface markers:** Cells were collected and spun down, either from single cell suspensions of homogenized tissue or from purified cultures of T cells as indicated. Cells were resuspended in 100 $\mu$ l 50% PBS + 50% T cell media (See Section 3.2 above) with appropriate antibodies diluted 1:100. Cells were stained for 15-30 minutes at room temperature, after which 500 $\mu$ l of 50%PBS/T cell media was added to dilute out the antibody, and incubated for an additional 2-5 minutes before being spun down. Cells were then either resuspended in PBS or fixed with Fixation Buffer (Biolegend) and stored at 4°C for up to 2 weeks prior to analysis by flow cytometry. For Annexin V and 7-AAD staining, cells were spun down and resuspended in 100 $\mu$ l 1X Annexin V staining buffer (BD Pharmingen), to which 5 $\mu$ l of Annexin-V-PE and 7-AAD were added. Staining was followed as described above for other surface markers, only Annexin staining buffer was used to wash off excess antibody/stain.

**Proliferation studies (eFluor670):** For analysis of proliferation, freshly sorted T cells were stained with 2.5 $\mu$ M eFluor670 according to the manufacturer's

protocols (eBiosciences). Cells were then cultured, stained, and analyzed as indicated above. Geometric mean fluorescence intensity (GMFI) of eFluor670 was determined using FlowJo software (TreeStar), and un-stimulated controls were used to differentiate between proliferating and non-proliferating cells.

### ***Section 3.4 : Flow cytometry***

The data were collected on a FACScalibur (Becton Dickinson). Data analysis was performed using FlowJo software (TreeStar). All equipment was provided by the Shared Flow Cytometry Resource at UNM.

### ***Section 3.5 : ELISA and Luminex multiplex assays***

Following 4 days in culture, cells were washed with cold medium to remove any cytokines in solution, resuspended in fresh medium, and counted. Cell were then plated in a 96 well plate with  $2 \times 10^5$  cells per well ( $10^6$  cells/mL), allowed to incubate for 60-90 min at 37°C (+5% CO<sub>2</sub>), and re-stimulated with soluble anti-CD3 $\epsilon$  (2.5  $\mu$ g/mL) antibody. Following the indicated incubation times hours, culture medium was collected and spun down to remove any residual cells. The concentration of IL6, IL10, IL17A, IFN $\gamma$ , and TNF $\alpha$  in the cell-free culture medium was analyzed using custom bead arrays from Millipore, and quantified on a Luminex 100 system with the Luminex XY plate handling platform. Assays were performed according to the manufacturers protocols. Duplicate wells were

assayed for each sample, and data are representative of the average median value for each sample. Analysis was performed using IS 2.3 software (Luminex).

### ***Section 3.6 : Immunofluorescence and cryosectioning***

Samples were fixed in PBS + 4% paraformaldehyde + 10% sucrose, then embedded in OCT for cryosectioning on a cryostat. 7-10 $\mu$ m sections were used. Sections were then mounted on slides and immersed in VectaShield with DAPI, and analyzed on a Zeiss Meta Confocal microscope provided by the UNM Microscopy Facility.

### ***Section 3.7 : Western blots***

Protein samples for western blot were collected in RIPA buffer and incubated on ice for 30 minutes. Samples were stored at -20°C prior to use. 15  $\mu$ g of protein was loaded onto an acrylamide gel, and run using standard western blot protocols.

### ***Section 3.8 : (q)RT-PCR***

Cells were homogenized with QIAshredder tubes (Qiagen) and RNA was extracted using the RNeasy mini kit (Qiagen) following manufacturer instructions. RNA was then quantitated using a Nanodrop spectrophotometer (Thermo Scientific). Reverse transcription was performed in a 20 $\mu$ l reaction volume using

100ng of RNA and Applied Biosystems High Capacity cDNA Reverse Transcription kit with RNase inhibitor (Applied Biosystems). Samples were then prepared for quantitative PCR using Applied Biosystems SYBR Green Master Mix. Reactions were carried out in 20ul reaction volume containing 10ul 2X SYBR Green master mix, 0.5uM forward and reverse primer, and 2ul (10ng) cDNA template. Quantitative PCR was performed on Applied Biosystems 7500 Fast Real-time PCR system and standard conditions consisting of 50°C for 2 min followed by 40 cycles of 95° for 15 sec, 60° for 1 min. GAPDH was used as a loading control for all samples. 7500 Fast software was used for data collection. Data was analyzed using the standard  $\Delta\Delta\text{CT}$  method.

### ***Section 3.9 : T cell-mediated colitis model***

A  $T_H1$ -type model of IBD can be elicited by intraperitoneal injection of  $4 \times 10^5$   $\text{CD4}^+\text{CD45RB}^{\text{hi}}$  naïve T cells into Rag1KO mice (Ostanin et al., 2009). For these experiments,  $\text{CD45RB}^{\text{hi}}$  were defined as the top 40% of  $\text{CD4}^+$  cells, and  $\text{CD45RB}^{\text{lo}}$  included the bottom 15% of  $\text{CD4}^+$  cells. Disease progression was followed by monitoring the weight of the mice. Histological sections and intracellular cytokine staining were used to evaluate disease severity. See Appendix E for examples of data from one of these experiments.



### **Section 3.10 : Mice**

Male (7-11 weeks old) wildtype and  $Foxp3^{egfp}$  mice were used for this study for collection of purified T cell populations by AutoMACS or FACS. Rag1KO mice were used as the recipient mice for T cell-mediated colitis experiments. All mice were on the C57BL/6 genetic background and were purchased from Jackson Laboratory. Animals were subsequently housed, bred, and cared for according to the institutional guidelines in the Animal Resource Facility at the University of New Mexico, and studies were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) under approved protocols.  $Foxp3$ -IRES-GFP ( $Foxp3^{egfp}$ ) transgenic mice, which contain *egfp* under the control of an IRES inserted downstream of the *foxp3* coding region, have been previously described (Haribhai et al., 2007).

### **Section 3.11 : G compounds and estrogen**

Our lab has developed a series of small molecules that specifically target GPER, including an agonist, G-1 (Bologa et al., 2006), and an antagonist G-15 (Dennis et al., 2009). Extensive studies will need to be conducted to determine the potential side effects and off-target activity of the G compounds if they are to move into pharmaceutical production. However, some studies have begun to address these concerns. In 2009, a study demonstrating that G-1 could protect mice against disease in the multiple sclerosis (MS) model experimental autoimmune encephalomyelitis (EAE) reported G-1 had a greater than 1000-fold

selectivity for GPER relative to 27 other endocrine receptors, with minimal binding observed at a concentration of 10 $\mu$ M (Blasko et al., 2009). In another study from that same year, G-1 was able to protect mice from EAE without exhibiting many of the physiological side effects associated with estrogen treatment, including increased uterine weight, decreased femur length, and induction of progesterone (Wang et al., 2009a). They observed that G-1 decreased serum corticosteroid levels to a similar degree as estrogen treatment. However, GPER plays a smaller role in the majority of classical estrogen-mediated physiological responses as compared to its more well known counterpart ER $\alpha$  (Prossnitz and Barton, 2009b).

### ***Section 3.12 : Administration of compounds in vivo.***

**Injections:** A vehicle consisting of 90% emulsion solution (PBS + 0.9% Tween-20 + 0.9% BSA) and 10% ethanol was used. For delivery of compounds, E2 or G-1 was dissolved in ethanol and added at appropriate concentrations such that 100 $\mu$ l per animal per injection was used. The compound was added to each injection as part of the 10% ethanol found in the vehicle, thus it was diluted such that less than 10 $\mu$ l per animal per injection was required. Injections were done in the afternoon, and to limit stress from the long series of injections inherent to this study, animals were sedated using isofluorane (provided by ARF) prior to injection. Compound was delivered subcutaneously on the dorsum adjacent to the hind limb, and the side of injection was alternated every two days.

**Pellets:** For the delivery of compounds over extended periods of time, compound was packaged into cholesterol-based pellets (Innovative Research of America) and implanted sub-dermally in the posterior flank via incision immediately cranial to the hind limb. The pellets were implanted one week (7 days) prior to the animals being used in any experiment. The pellets are designed by the manufacturer to release compound over 60 days, and for control animals pellets devoid of compound were used.

### ***Section 3.13 : Statistical analysis***

**Exclusion of data points:** Any point that was determined to be greater than or equal to 2.5 standard deviations away from the mean of a set was excluded from the set. Standard deviation was calculated prior to removal of the data point in question.

**Calculations:** Statistics were calculated using Prism 5 for Mac OS X software (GraphPad), and some values were verified using the online student's t-test tool found at the following website: [http://www.physics.csbsju.edu/stats/t-test\\_bulk\\_form.html](http://www.physics.csbsju.edu/stats/t-test_bulk_form.html). This site is provided Saint Benedict & Saint John's University. Observations were considered statistically significant if they were associated with a P value of less than or equal to 0.05.

## **Chapter 4 : Foxp3 induction**

### ***Section 4.1 : Preface***

In this first data chapter, we investigate the ability of G-1 to affect naïve T cell differentiation, and establish it as a T cell modulating drug compound. Our preliminary data suggest that G-1 may modulate expression and secretion of the critical suppressive cytokine IL10, which is known to be produced by a multitude of regulatory and effector T cell populations. Additionally, we saw that G-1 drove IL17A expression, suggesting that it may modulate T<sub>H</sub>17 differentiation. This is interesting considering G-1's ability to suppress disease induction in an animal model of experimental encephalomyelitis, which is heavily linked to the T<sub>H</sub>17 population. A brief review of pertinent background is presented first.

### ***Section 4.2 : Introduction***

The immune system is faced with the complex task of responding to nature's endless array of pathogenic microorganisms, many of which exhibit high mutations rates. To meet this challenge, the adaptive immune system uses somatic cell gene rearrangement during lymphocyte development to generate vast repertoires of unique antigen recognition receptors, including the T cell receptor (TCR). In the case of CD4<sup>+</sup> T cells, TCR activation leads to clonal expansion and differentiation into one of three main lineages; T<sub>H</sub>1, T<sub>H</sub>2, or T<sub>H</sub>17

cells. These divergent populations coordinate distinct immune responses through the expression of unique mediators and signaling molecules. While effective at its purpose, this approach is marked with an inherent risk of autoimmunity through the generation of self-reactive clones, or cells responsive to common environmental contaminants that pose no risk to the host. In order to mitigate this danger, the immune system has evolved a series of approaches aimed at eliminating and/or limiting self-reactive antigen receptors and the cells bearing them, as well as mechanisms to limit the extent of inflammation *in situ*. One such mechanism is the induction of regulatory T cell populations.

CD4<sup>+</sup> regulatory T (T<sub>REG</sub>) cells are an eclectic population which play a critical role in this system. The most well defined class of T<sub>REG</sub> cells express the transcription factor Foxp3 (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). This protein is critical to immune homeostasis as loss of Foxp3 function in both humans and mice precipitates a fatal multi-organ autoimmune condition marked by the inability to control T cell responses (Clark et al., 1999; Patey-Mariaud de Serre et al., 2008). Experimentally, Foxp3<sup>+</sup> T<sub>REG</sub> cells suppress numerous animal models of autoimmune disease (Yuan et al., 2007). Conversely, excessive T<sub>REG</sub> activity has been shown to inhibit protective anti-tumor immune responses (Boissonnas et al., 2010; Zou, 2006) and immunity against infection (Belkaid and Tarbell, 2009). Thus Foxp3<sup>+</sup> cells are a critical point of control in many disease settings. Foxp3 expression can be elicited during thymic development in T cell receptor (TCR) dependent fashion (Nunes-Cabaco et al., 2010), leading to “natural” or nT<sub>REG</sub> cells. Additionally, conventional T cells

in the periphery can be driven towards a suppressive phenotype during the course of an antigenic immune response when stimulated in the presence of TGF $\beta$  (Chen et al., 2003), IL2 (Fontenot et al., 2005a), IL10 (Murai et al., 2009), IL35(Kochetkova et al., 2010), and/or retinoic acid (Elias et al., 2008), or when antigen recognition occurs in the absence of appropriate costimulatory ligands like CD80 and CD86 (Gottschalk et al., 2010). This leads to numerous types of inducible regulatory T (iT<sub>REG</sub>) cell (Vignali et al., 2008), many of which express Foxp3.

It has long been recognized that women exhibit a much higher prevalence of numerous autoimmune diseases (Whitacre et al., 1999), with several lines of evidence linking this observation to estrogen signaling. Recent work has identified the G protein coupled receptor GPER as an estrogen binding receptor, making it a third estrogen receptor identified, along with the two ligand-activated nuclear transcription factors ER $\alpha$  and ER $\beta$ . This shift in paradigm is of great interest to those seeking to target or exploit estrogens many functions for therapeutic benefit. In light of this our group has developed a series GPER-targeted small molecules, including an agonist, G-1 (Bologa et al., 2006). In binding assays, G-1 shows >1000-fold selectivity for GPER relative to 27 other known endocrine receptors (including the classical estrogen receptors) (Blasko et al., 2009), with minimal off-target binding detected at 10 $\mu$ M. Two reports have shown that G-1 can suppress EAE induced with either MOG (Wang et al., 2009a) or PLP (Blasko et al., 2009) peptide. One group found that G-1-mediated protection was dependent on up-regulation of PD-1 within the Foxp3 population

(Wang et al., 2009a), while the second reported that the effect was associated with changes in the cytokine profile of macrophages (Blasko et al., 2009). Neither group did comprehensive studies to investigate potential direct effects of G-1 on T cell populations. These facts made G-1 an interesting compound to investigate for immunomodulatory properties in the context of CD4<sup>+</sup> T cell biology.

In our preliminary work we showed that G-1 could drive production of the T<sub>H</sub>17 associated cytokine IL17A, as well as the potent suppressive cytokine IL10 from CD4<sup>+</sup> T cells stimulated polyclonally in culture. Thus we wanted to determine if G-1 might affect the expression of any of the CD4<sup>+</sup> lineage specific transcription factors, namely T-bet (T<sub>H</sub>1), GATA-3 (T<sub>H</sub>2), ROR $\gamma$ t (T<sub>H</sub>17), and Foxp3 (T<sub>REG</sub>). Here we show that G-1 can induce Foxp3 expression in cultured CD4<sup>+</sup> T cells, and enhances the suppressive function of T<sub>H</sub>17-polarized T cells. Given G-1 has several properties that make it attractive for rapid incorporation into the pharmaceutical pipeline, these initial findings demonstrate that G-1 and the other G compounds warrant further investigation for their T cell regulatory properties, and the utility of targeting GPER in immunopathologies should be further delineated.

### ***Section 4.3 : Results***

#### **GPER expression in T cells**

It has been reported that human regulatory T cells (Blasko et al., 2009) and murine splenocytes (Isensee et al., 2009) express GPER. However, no

reports investigating GPER expression in murine CD4<sup>+</sup> T cells have been published to date. To begin our studies, we sought to determine if GPER is expressed within various CD4<sup>+</sup> T cell populations from C57BL/6 mice. Hence CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>REGS</sub> and CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup>Foxp3<sup>-</sup> naïve T cells were sorted by FACS from Foxp3-IRES-EGFP knockin transgenic mice (Haribhai et al., 2007). Expression of GPER mRNA was determined by endpoint RT-PCR (**Figure 12A**), with GPERKO and ER $\alpha$ KO splenocytes serving as controls. We detected GPER expression in both cell types. To verify that the message was being expressed into protein, we attempted western blots with two distinct rabbit antisera raised against short peptide sequences of GPER, one from mouse and one from human GPER (Details are found in Appendix A). Unfortunately we were unable to clearly corroborate protein expression of GPER as the antibodies appeared to crossreact with a roughly 40-45 kDa protein in splenocytes from GPERKO mice (see **Figure 36E** in Appendix A). As GPER has a molecular weight of approximately 42kDa, this non-specific binding made it impossible to adequately resolve GPER expression by western blot. Experiments using cryosectioning of tissue and cytopinning of purified T cells were also unclear as GPERKO cells and tissues also exhibited robust staining. Thus protein expression of GPER could not be verified.



## G-1 increases Foxp3 expression in CD4<sup>+</sup> T cells

We next turned our attention to assessing the impact of G-1 on expression of the lineage-specific transcription factors responsible for programming the various helper T cell sub-sets. Our preliminary data demonstrated that G-1 treatment could increase production of both IL10 and IL17A from T cells, suggesting it may have an impact on the number of T<sub>H</sub>17 cells, and hence the expression of ROR $\gamma$ t (**Figure 8**). It is also known that estrogen can elicit IFN $\gamma$  expression (Karpuzoglu et al., 2006), and can decrease IRF1 expression, which in theory would alleviate IFN $\gamma$ -mediated suppression of IL4 (Lengi et al., 2006). As GPER appears to act in concert with the classical estrogen receptors in some settings (Albanito et al., 2007), it is possible that G-1 may alter the T<sub>H</sub>1-T<sub>H</sub>2 axis that is largely regulated by IFN $\gamma$  and IL4, and thus the expression of T-bet and GATA3 (See Section 1.4). To determine if G-1 could affect the expression of the canonical transcription factors by direct action on CD4<sup>+</sup> T cells, naïve T cells were collected by FACS and stimulated *ex vivo* with antiCD3 and antiCD28 antibodies under T<sub>H</sub>0 conditions (meaning without the addition of exogenous cytokines or neutralizing antibodies), either with 10nM E2, 100nM G-1, or equivalent concentrations of DMSO. Samples were collected after 4 days in culture and analyzed for mRNA expression of T-bet, GATA3, ROR $\gamma$ t, and Foxp3 by qRT-PCR. As can be seen in **Figure 12B**, no major effects with E2 were observed. Conversely, G-1 led to an increase in the expression of Foxp3, with no change in the expression of the three effector T cell transcription factors noted. The difference between treatment with estrogen and the GPER-specific agonist

G-1 likely reflects the impact of activating the classical estrogen receptors, which may counterbalance activation of GPER when cells are stimulated with E2. These data suggested that G-1 could act on T cell populations to drive Foxp3 mRNA expression.

This result was interesting given the previous findings from the Offner group, who showed that estrogen could expand the Foxp3 population *in vivo* (Polanczyk et al., 2005), but G-1-mediated suppression of EAE was associated with an increase in the expression of PD-1 on Foxp3<sup>+</sup> cells, rather than an increase in the number of Foxp3<sup>+</sup> cells themselves (Wang et al., 2009a). To determine if we could recapitulate our finding *in vivo*, male naïve Foxp3<sup>egfp</sup> mice were treated for 7 seven consecutive days with either G-1, E2, or vehicle via subcutaneous injection. Following treatment, mice were sacrificed and the spleens were analyzed for the expression of CD4<sup>+</sup> and GFP (Foxp3) (**Figure 13A,B**). We noted a small but significant increase in the number of Foxp3<sup>+</sup> cells within the CD4<sup>+</sup> compartment. The increase was similar to the increase observed for E2-treated mice. Given the importance of PD-1 in G-1-mediated EAE suppression, we also sought to describe any changes in the expression of PD-1. To our surprise we were unable to detect any change in the surface expression of PD-1 (**Figure 13C,D**) on either the total CD4<sup>+</sup> T cell population or the Foxp3<sup>+</sup> sub-set following treatment with G-1. It is important to note that these two results, namely the data presented here and the data from the Offner lab, are not necessarily incongruous. Our data were collected in naïve male mice, and we looked specifically at the populations within the spleen, while the Offner group

collected data from the draining lymph nodes as well as the spleen of female EAE mice. These two settings may result in differential effects of G-1 signaling, leading to related but distinct outcomes. Future work will be needed to address this hypothesis.

### **G-1-mediated induction of Foxp3 in T<sub>H</sub>17 polarizing conditions**

We hypothesized that the difference in Foxp3 and PD-1 expression in splenocytes of naïve mice versus cells in the setting of EAE following systemic G-1 treatment reflected the presence of T<sub>H</sub>17-polarizing conditions in the EAE mice. This would not be unexpected as it is known that IL6, one of the cytokines implicated in T<sub>H</sub>17 development, can inhibit Foxp3 expression (Samanta et al., 2008). If there is a high concentration of IL6 during the preclinical stages of EAE development it is possible that this would mask the effects of G-1 in terms of Foxp3 induction, even without the development of overt disease. It is also possible that the immunoprivileged environment of the CNS lacks certain key signals that are required for G-1-mediated induction of Foxp3, although the fact that we observed that G-1 could elicit Foxp3 expression *ex vivo* without the addition of any exogenous mediators indicates this explanation is less likely. To determine if any of the local mediators important in T<sub>H</sub>17-like inflammation were necessary for G-1-mediated increases in Foxp3, naïve T cells were collected by FACS from Foxp3<sup>egfp</sup> mice and stimulated in culture as before, in the presence of either G-1 or DMSO. In this case, cultures were supplemented with various combinations of the key T<sub>H</sub>17-polarizing cytokines TGFβ, IL6, and IL23.

Following 4 days in culture, cells were analyzed for the expression of GFP (Foxp3), PD-1 and CTLA-4 by flow cytometry. Consistent with our findings in **Figure 12**, we observed that G-1 treatment precipitated an increase in the number of Foxp3<sup>+</sup> cells in cultures which lacked exogenous cytokines (**Figure 14**). We also observed that G-1 led to an increase in the number of cells expressing Foxp3 in cultures supplemented with IL6 and IL6 + TGFβ. Interestingly no effect was observed in cultures supplemented with TGFβ alone. However, given the large number of cells expressing Foxp3 in these cultures (>50%), it is possible that there are few cells remaining capable of expressing Foxp3. Thus the loss of G-1-mediated Foxp3 induction in cultures treated with TGFβ alone may reflect the lack of uncommitted cells capable of switching on Foxp3 rather than any relevant alterations in signal integration related to G-1's activity. Finally, we also observed a trend towards increased numbers of Foxp3<sup>+</sup> cells in G-1-treated cultures supplemented with IL23, although this trend never reached statistical significance (**Figure 14**).

We next sought to determine if we could observe any changes in the expression of the inhibitory molecules PD-1 and CTLA-4 following *ex vivo* G-1 treatment of purified T cells cultures. Like PD-1, CTLA-4 inhibits T cell activation and is upregulated on T<sub>REG</sub> populations. It has also been implicated in the pathogenesis of animal models of colitis (Coquerelle et al., 2009). As before, we utilized naïve T cell cultures purified by FACS and cultured with various combinations of T<sub>H</sub>17-polarizing cytokines TGFβ, IL6, and IL23, in addition to adding either G-1 or DMSO. Analysis of the entire culture showed that under T<sub>H</sub>0

conditions, G-1 treatment led to a modest increase in the expression of PD-1 and CTLA-4 (**Figure 15**). A similar pattern was noted in cultures supplemented with IL6, while cultures treated with TGF $\beta$  alone also demonstrated increased CTLA-4 (**Figure 15**). However, all observable differences were extremely small, equating to a roughly 25% change in the expression, or less. Similarly, when we looked specifically at the Foxp3<sup>+</sup> population, changes were seen exclusively in the non-polarizing conditions (**Figure 16**). Again, increases in both PD-1 (**Figure 16A,B**) and CTLA-4 (**Figure 16C,D**) were minimal, on the order of 20%.

### **G-1 induced Foxp3 expression occurs within hybrid T cells**

The observation that G-1 can drive Foxp3 expression under T<sub>H</sub>17-polarizing conditions raises the question of whether G-1 affects the expression of the transcription factor ROR $\gamma$ t. We didn't note any changes in ROR $\gamma$ t expression in Figure 12 under T<sub>H</sub>0 conditions, but it is possible that G-1 cannot elicit ROR $\gamma$ t without the addition of IL6 and TGF $\beta$ , especially given the importance of these cytokines to activation of *rorc* locus. Additionally, the developing paradigm of hybrid T cell populations, which appear to function by targeting to sites of lineage specific inflammation, raise the question of whether G-1-mediated Foxp3 expression occurs within the ROR $\gamma$ t<sup>+</sup>. Therefore, naïve T cells were collected from Foxp3<sup>egfp</sup> mice and cultured under T<sub>H</sub>17-polarizing conditions. After 4 days in culture, cells were collected and stained for the intracellular moieties IL10, IL17A, and ROR $\gamma$ t, while GFP expression was again used as a surrogate for Foxp3. Samples were then analyzed by flow cytometry. We did not observe any

differences in ROR $\gamma$ t expression between G-1 and DMSO treated cultures (**Figure 17**). However, virtually all of the Foxp3 expressing cells fell into the hybrid T cell category (Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup>), thus G-1 treatment increased the number of hybrid T cells in differentiating cultures of naïve T cells.

### **G-1 treated cells exhibit increased suppressive function *ex vivo***

Finally, we wanted to know if G-1 treated cells would still exhibit enhanced suppressive function. Given that we were not observing any changes to the expression of PD-1 or CTLA-4 when T cells were differentiated under T<sub>H</sub>17-polarizing conditions (**Figure 15,16**), or following *in vivo* treatment with G-1 (**Figure 13**), but were able to observe increased Foxp3 expression (**Figure 13,14**), G-1-induced suppression would likely be through a distinct mechanism from the up-regulation of PD-1 on Foxp3<sup>+</sup> cells reported in the suppression of the T<sub>H</sub>17-mediated disease EAE. Thus naïve T cells were collected by FACS and cultured with TGF $\beta$  + IL6 in the presence of either 100nM G-1 or DMSO. Following 4 days of differentiation, cells were collected, washed, and used in a T cell suppression assay with eFluor670 stained splenocytes as the responder cells (see Chapter 3). These cultures were then stimulated with antiCD3 $\epsilon$  antibody for three days, and dilution of eFluor670 was determined by flow cytometry. As can be seen in **Figure 18**, G-1 treated cells were able to inhibit proliferation of the responder splenocytes more efficiently than the DMSO treated controls, demonstrating that G-1 can enhance the suppressive function of T<sub>H</sub>17-polarized T cells.

## **Section 4.4 : Discussion**

In this chapter, we have begun to delineate the effects of G-1 treatment on the differentiation of CD4<sup>+</sup> T cell populations, and its impact on the polarization towards different helper T cell lineages. We observed that treatment of naïve T cells with G-1 under non-polarizing conditions (T<sub>H</sub>0 conditions) drives expression of the canonical regulatory T cell transcription factor Foxp3, while not affecting the expression of any of the established effector transcription factors T-bet, GATA3, or ROR $\gamma$ t, as determined by qPCR. Interestingly, E2 treatment did not have the same affect, despite the evidence in the literature that it can expand the Foxp3<sup>+</sup> population *in vivo*. Whether this is a product of the dose of E2 that we chose to employ (10nM), or reflective of the fact that induction of Foxp3 is a secondary effect of estrogens action within another immune population remains unclear.

We now know that GPER is implicated in estrogen-induced immune regulation, building on evidence for the role of ER $\alpha$ . One study by the Offner group investigated the role of ER $\alpha$  in estrogen-induced Foxp3 expression in CD4<sup>+</sup> T cells from the draining lymph nodes of EAE mice. They found that estrogen did not increase Foxp3 expression in ER $\alpha$ <sup>-/-</sup> mice, which corroborated previous findings that disruption of ER $\alpha$  was associated with a loss in E2-mediated protection from EAE (Polanczyk et al., 2004a). However, these studies only looked at Foxp3 expression by RT-PCR and western blot, which does not

actually address that number of Foxp3<sup>+</sup> cells. Additionally, as mentioned in the introduction there is compelling evidence that GPER and ER $\alpha$  can act in a coordinated fashion such that loss of one moiety interferes with signaling from the other. To further delineate the conditions in which G-1 could elicit Foxp3 expression, and ascertain as to whether increased Foxp3 mRNA reflected an increase in the number of Foxp3<sup>+</sup> cells, we cultured naïve T cells from Foxp3<sup>egpf</sup> knockin mice, which express GFP anytime the Foxp3 transcript is made. Flow cytometric analysis of FACS purified naïve T cells stimulated in culture under T<sub>H</sub>0 conditions demonstrated that G-1 treatment led to an expansion of the number Foxp3<sup>+</sup> cells, consistent with the qPCR data. These findings translated to the *in vivo* setting as systemic delivery of G-1 in Foxp3<sup>egpf</sup> knockin mice drove expansion of the CD4<sup>+</sup>Foxp3<sup>+</sup> population, without significant changes in the relative percentages of CD4<sup>+</sup> cells (**Figure 39**, Appendix C).

In our preliminary data we noted an increase in IL17A secretion upon treatment of CD4<sup>+</sup> cells with G-1. Hence, we also investigated the impact of G-1 on Foxp3 expression under T<sub>H</sub>17-polarizing conditions. Our data demonstrate that G-1 can elicit Foxp3 expression in cultures treated with IL6 and IL6 + TGF $\beta$ . Collectively, these data suggest; (a) that G-1-mediated Foxp3 expression resulting from direct action on the T cell populations can occur in a variety of inflammatory milieu, and (b) that differences in the observed *in vivo* effects of G-1 are not the result of variable levels of the T<sub>H</sub>17-polarizing cytokines TGF $\beta$  or IL6. However, as we did see some instability in the induction of Foxp3 in IL23 treated cultures (**Figure 14**), it is possible that stabilization of the T<sub>H</sub>17 lineage following



prolonged exposure to IL23 (McGeachy et al., 2007; McGeachy et al., 2009; McGeachy and Cua, 2007; Yen et al., 2006) may block G-1-mediated Foxp3 expression. This would indicate that G-1 is acting on polarized, but uncommitted, T<sub>H</sub>17 cells to drive Foxp3 expression. This concept warrants further study.

Two previous reports demonstrated that G-1 can suppress disease in the MS-like animal model EAE (Blasko et al., 2009; Wang et al., 2009a). In one study, the authors found that G-1's protective effects correlated with increased PD-1 expression on Foxp3<sup>+</sup> T<sub>REG</sub> cells, and were dependent on intact PD-1 expression in the host animal as PD-1KO mice were not protected from disease by G-1 (Wang et al., 2009a). However, these experiments were based on *in vivo* administration of G-1, and analysis was based on experiments with cells from the draining lymph nodes of diseased animals. Thus, it is not clear whether these observation reflect a direct effect of G-1 driving PD-1 expression within the Foxp3<sup>+</sup> population itself, or is the result of G-1 effects on another cell type, perhaps leading to the induction of other mediators. We were unable to detect any changes in PD-1 or CTLA-4 expression following *in vivo* treatment with G-1. We were able to detect increased expression of both molecules in a few conditions tested *ex vivo*, but these effects were much smaller than those reported in the literature following *in vivo* G-1 treatment of EAE mice in which the percent of Foxp3<sup>+</sup>PD-1<sup>+</sup> cells nearly doubled (Wang et al., 2009a). These effects were also smaller than the 50% increase in the number of Foxp3<sup>+</sup> cells observed in **Figure 15**. Additionally, no changes were detected under T<sub>H</sub>17-polarizing conditions (TGFβ + IL6 ± IL23), suggesting that if this effect is valid it is likely the

result of a distinct mechanism from that responsible for G-1-mediated Foxp3 expression.

We do not feel that these data represent a counterpoint to the findings of Halina Offner and her colleagues. Rather, these results suggest that the effects of G-1 are dependent on context, which makes sense given the variable effects that many signaling pathways within the immune system can have, depending on the setting in which they occur. As mentioned in the introduction, estrogens are produced in reproductive organs, adipose tissue, and the central nervous system (among others), and estrogen receptor deficiency is associated with both female and male infertility (Akingbemi, 2005). We chose to use male mice in our studies to eliminate the confounding effects of surgery (ovariectomy) and/or high levels of endogenous estrogens found in female mice. However, an important caveat to this approach is that the findings herein may not be recapitulated in female mice. Thus some of the differences that we noted from other published work, namely the induction of Foxp3 expression with only small changes to PD-1 expression, may in fact represent a sexual dimorphism of GPER signaling. Moreover, previous studies have focused on Foxp3 expression within the setting of EAE. Thus it remains possible that a GPER signaling could also play a role in estrogen-induced Foxp3 expression, in particular in settings outside of EAE. Studies with other animals models of disease will be needed to address this hypothesis.

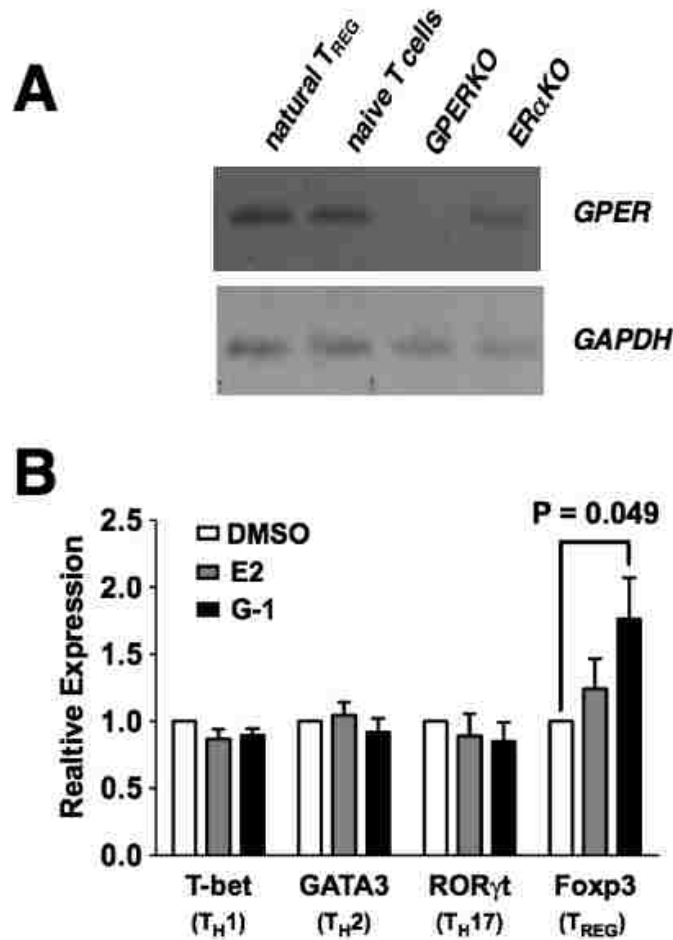
The data from the T cell suppression assay showed that G-1 induced Foxp3 expression correlated with increased suppressive function *ex vivo*. However, it should be noted that the results from this assay were highly variable,

and further studies will be needed to determine the veracity of these results (**Figure 18** is only one example from four independent experiments, and exhibited the most robust response we saw). Moving forward, it would be instructive to test the effects of neutralizing PD-1 to compare results, so as to verify that G-1-induced suppression is indeed independent of PD-1 signaling, as suggested by our data showing the absence of changes to PD-1 expression in G-1 treated cultures. Also, it was recently noted that GPER is required for suppression of EAE by oral ethinyl estradiol (Yates et al., 2010). It is possible that G-1 induced changes in T cell cytokine secretion are responsible for the *ex vivo* suppressive activity, especially given our preliminary data which shows that G-1 can elicit IL10 production from CD4<sup>+</sup> cells (Chapter 2). Interestingly, we also noted an increase in IL17A production from G-1 treated cultures (See preliminary data **Figure 8-10**). While classically thought of as a proinflammatory cytokine, several reports have demonstrated that IL17A can serve in an anti-inflammatory capacity in several setting, including in the induction of atherosclerosis (Ait-Oufella et al., 2010; Taleb et al., 2009) and colitis (O'Connor et al., 2009). Moreover, it appears that some of the *in vivo* suppressive activity associated with IL17A production can be attributed to autocrine activity directly on the CD4<sup>+</sup> T cell populations themselves, as the adoptive transfer of IL17A receptor knockout T cells was associated with more severe disease in an animal model of colitis (O'Connor et al., 2009). Thus increased IL10 or IL17A secretion could play a part in G-1's enhancement of T cell suppressive function. The work in Chapter 5 and Chapter 6 attempt to build upon this idea.

## **Section 4.5 : Conclusions**

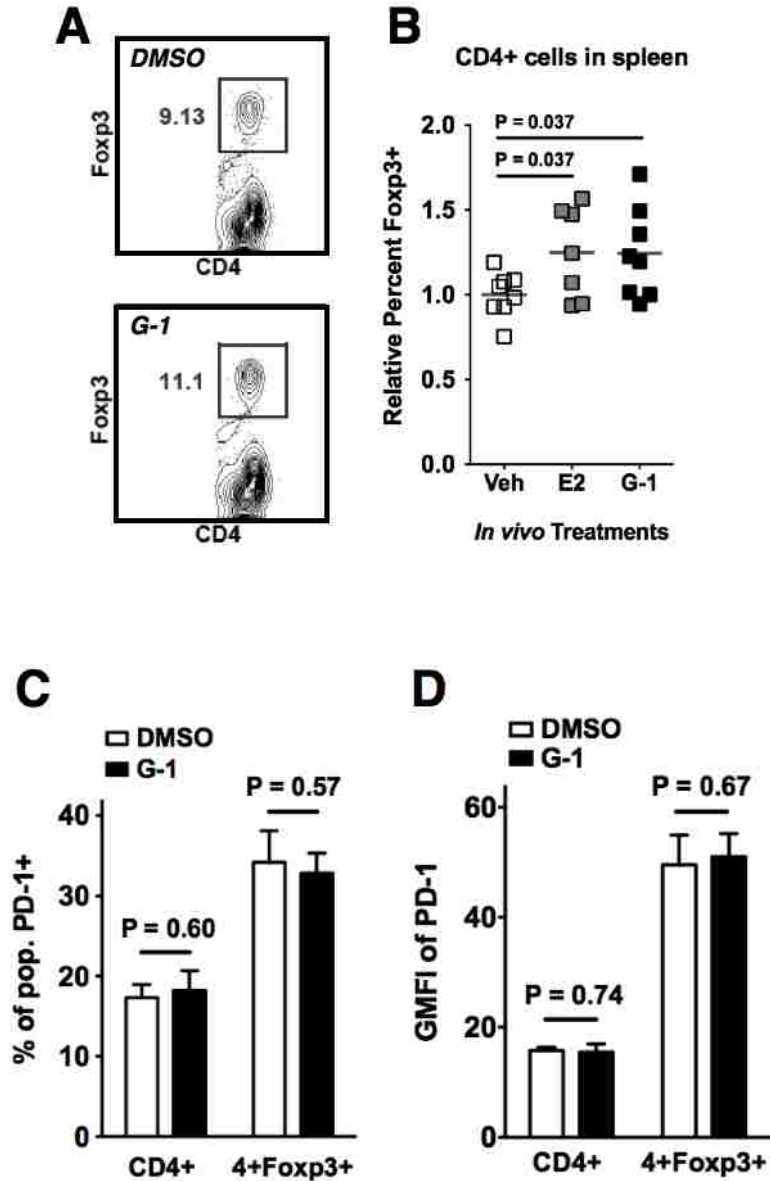
We provide evidence that the GPER-directed small molecule G-1 can elicit Foxp3 expression within CD4<sup>+</sup> T cells, and expand the percentage of cells expressing Foxp3 *in vivo*. We also noted no major changes in the expression levels of the well-characterized inhibitory receptor PD-1 following *in vivo* or *ex vivo* treatments of G-1 on the total CD4<sup>+</sup> populations or on CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>REGS</sub>. Our results vary from previously published report that demonstrated G-1 can increase the surface expression of PD-1 in Foxp3<sup>+</sup> regulatory T cells in the setting of experimental encephalomyelitis, suggesting the G-1 exhibits context-specific effects on immune populations. These data demonstrate that further study of G-1s immunomodulatory properties are necessary to fully elucidate the full scope of its therapeutic potential, including a detailed study of its effects of T cell populations.

**Section 4.6 : Figures**



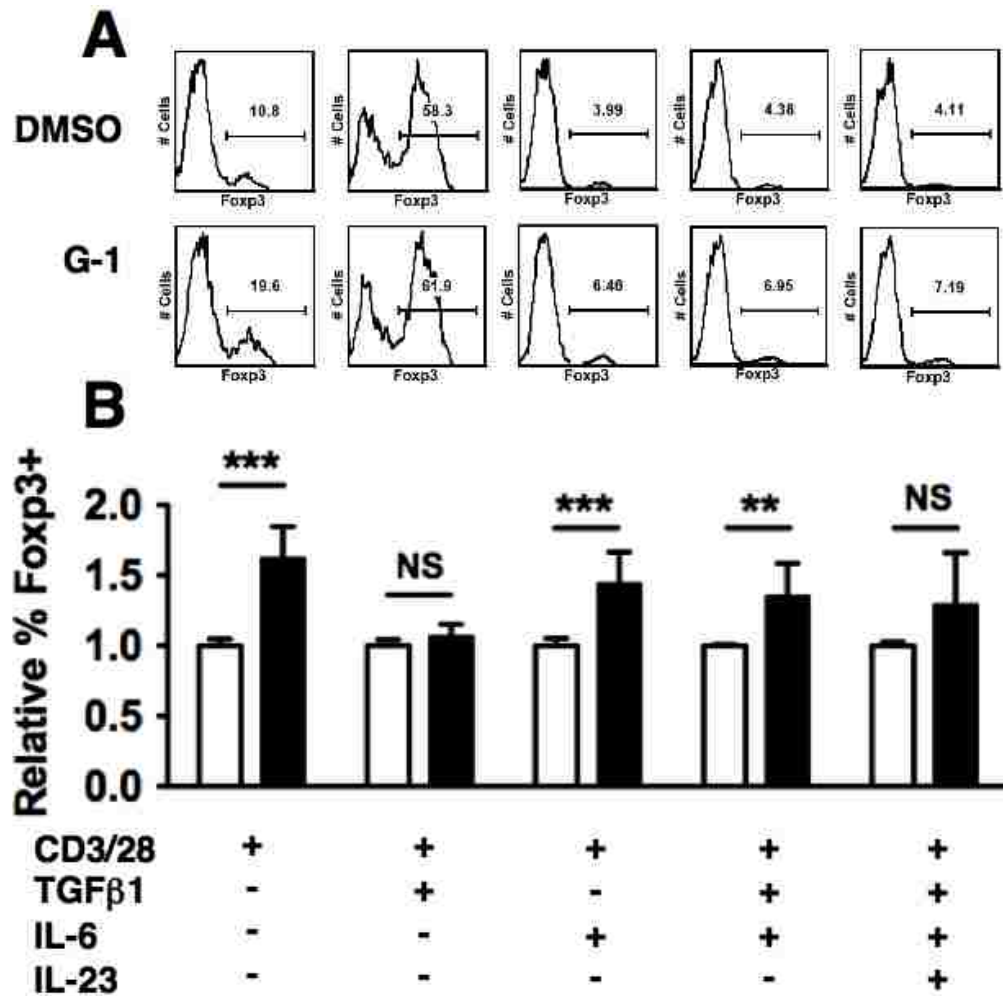
**Figure 12 : GPER expression in T cells, and G-1 induction of Foxp3 mRNA.**

CD4<sup>+</sup>GFP<sup>+</sup> natural regulatory T cells and CD4<sup>+</sup>CD62L<sup>hi</sup>CD44<sup>lo</sup>GFP<sup>-</sup> naive T cells were collected by FACS from male Foxp3<sup>egfp</sup> mice and were; (A) analyzed for GPER and GAPDH expression by RT-PCR, or (B) where cultured with antiCD3 $\epsilon$  and antiCD28 antibody for 4 days then analyzed for expression of the canonical CD4<sup>+</sup> T cell transcription factors T-bet, GATA3, ROR $\gamma$ t, and Foxp3 by qRT-PCR. (B) Summary of the means from 4 independent experiments. P values determined by student's t-test. Error bars = S.E.M. These data were collected by Kristin Owens.



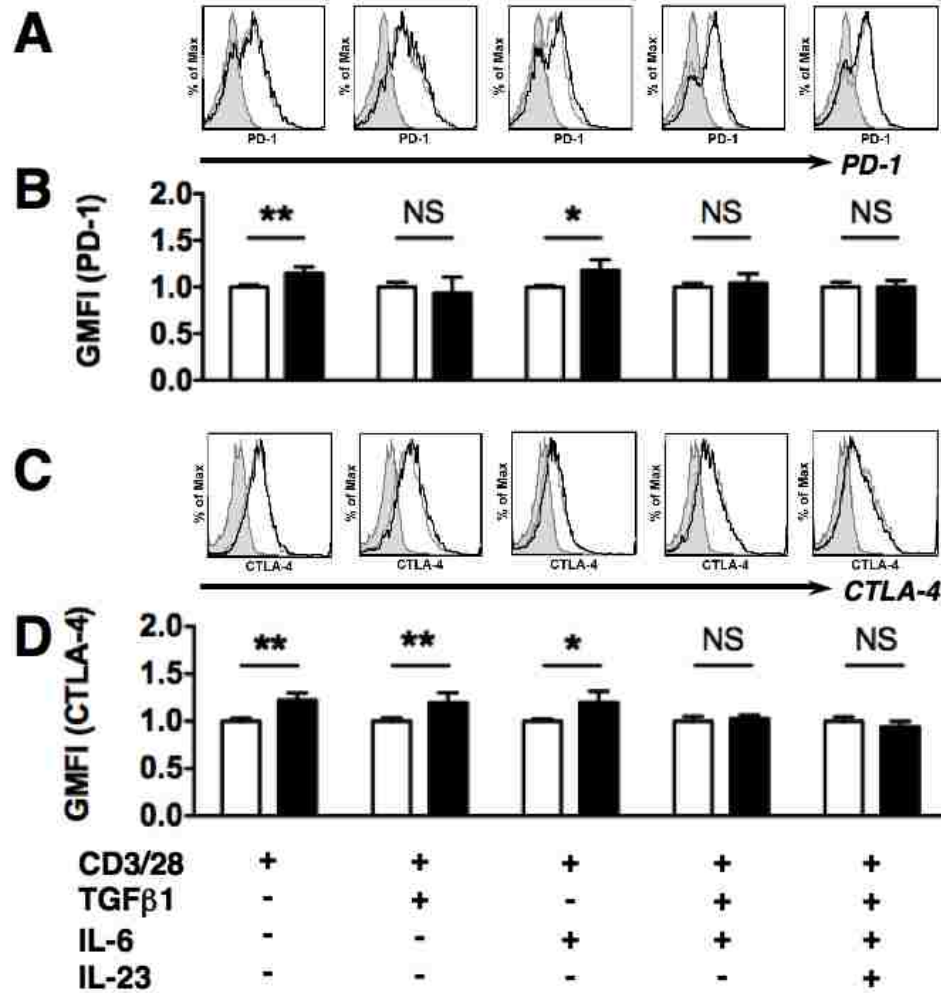
**Figure 13 : *In vivo* treatment with G-1 increases Foxp3 expression, but not PD-1.**

Seven to eleven week old male Foxp3<sup>egfp</sup> mice were injected with 17 $\beta$ -estradiol (E2 - 0.5 $\mu$ g/day) , G-1 (5 $\mu$ g/day) or vehicle for 7 consecutive days. One day following the last injection, single cell suspensions were made from spleen. Cell were then stained for CD4, PD-1, and CTLA-4 and analyzed by flow cytometry. (A) Individual dot plots showing GFP (Foxp3) expression in CD4<sup>+</sup> T cells from the spleen of vehicle or G-1 treated mice, with each square representing one mouse. Summary of all data collected from the spleen showing; (B) the percent of CD4<sup>+</sup> cells expressing Foxp3, (C) the percent of either CD4<sup>+</sup> or CD4<sup>+</sup>Foxp3<sup>+</sup> cells expressing PD-1, or (D) the GMFI of PD-1 on CD4<sup>+</sup> Cells or CD4<sup>+</sup>Foxp3<sup>+</sup> cells. P values determined by student's t-test. Error bars = S.D.



**Figure 14 : G-1 treatment of naïve T cells increases Foxp3 expression.**

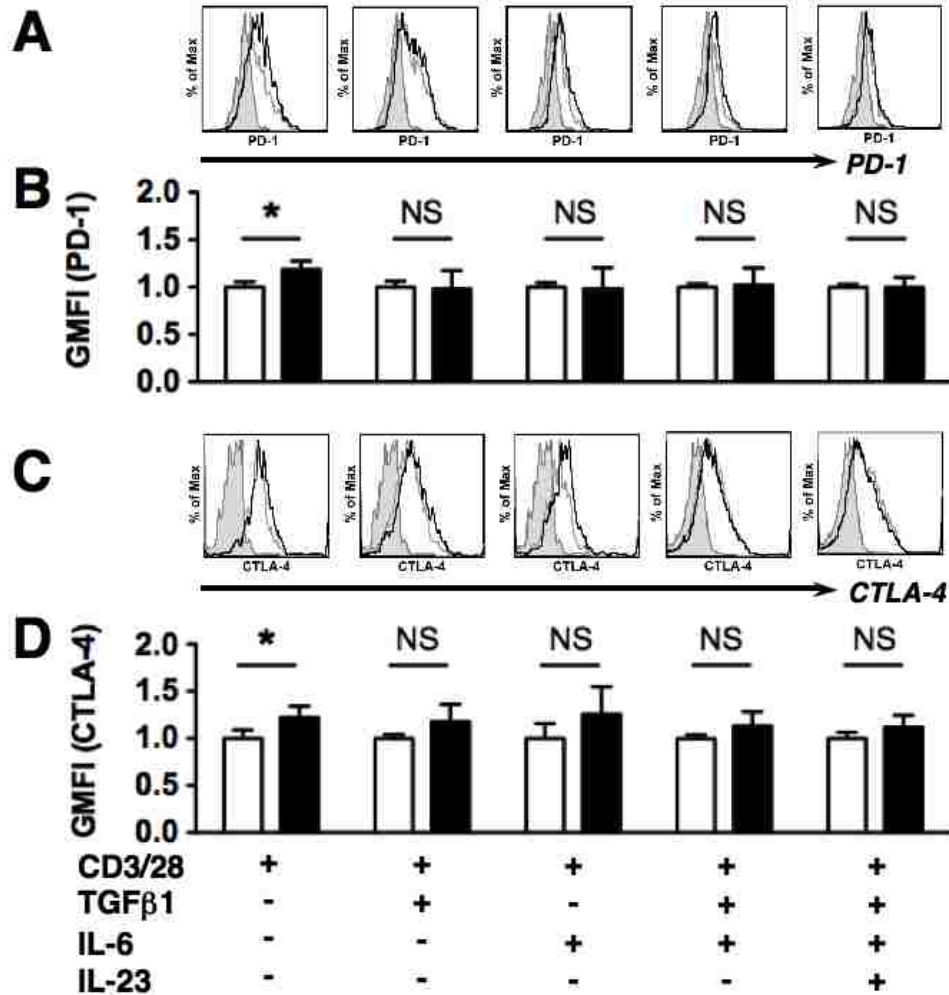
CD4<sup>+</sup>CD62L<sup>hi</sup>CD44<sup>lo</sup> naïve T cells from Foxp3<sup>egfp</sup> mice were collected by FACS and cultured for 4 days in the conditions indicated. Individual wells were supplemented with either 100nM G-1 (Black bars) or DMSO (White bars). GFP (Foxp3) expression was assessed by flow cytometry. (A) Representative plots and (B) summarized data from three to four independent experiments is shown, with conditions for both panels indicated at the bottom of the figure. P values determined by student's t-test. \*\*\* = P < 0.0005, \*\* = P < 0.005, \* = P < 0.05, N.S. = not significant. Error bars = S.D.



**Figure 15 : G-1 treatment has minimal effect on PD-1/CTLA-4 expression.**

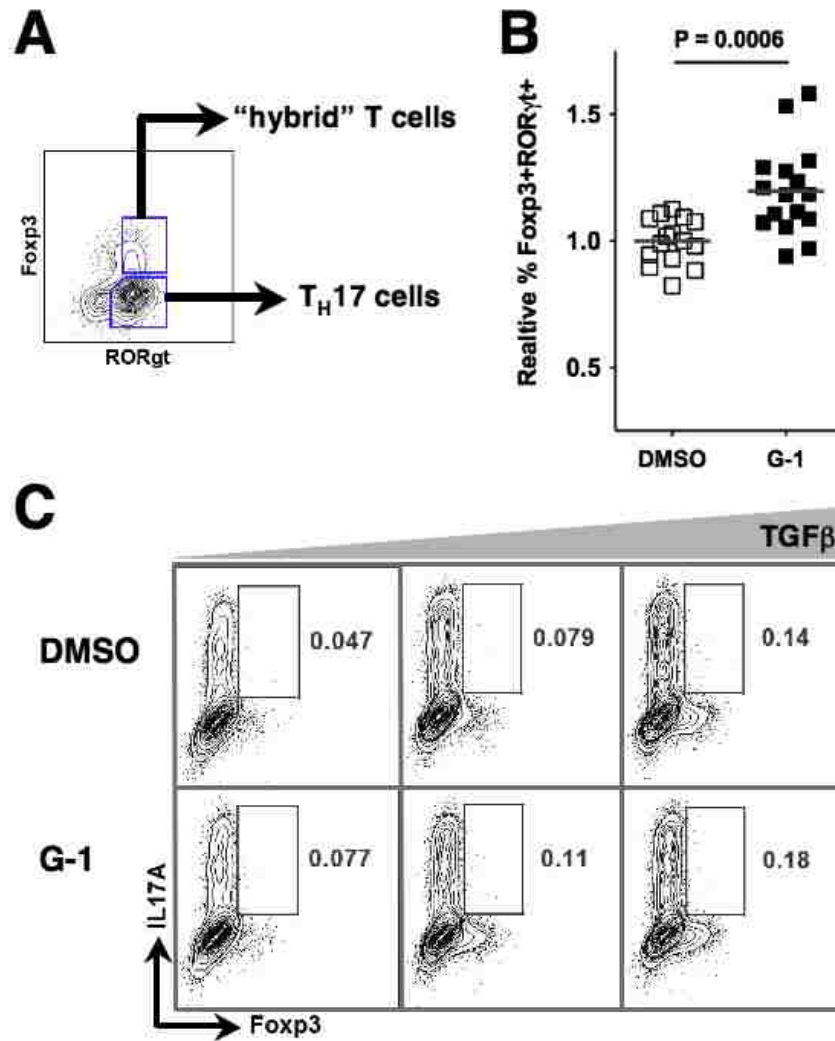
CD4<sup>+</sup>CD62L<sup>hi</sup>CD44<sup>lo</sup> naive T cells from Foxp3<sup>egfp</sup> mice were collected by FACS and cultured for 4 days in the conditions indicated. Individual wells were supplemented with either 100nM G-1 (black bars) or DMSO (white bars). Cells were then stained for PD-1 and CTLA-4 then analyzed by flow cytometry. Representative histograms from the total population are included for PD-1 (A) and CTLA-4 (C), with the isotype control (shaded region in gray), DMSO treated cells (Gray line without shading) and G-1 treated cells (Black line) shown. Data for geometric mean fluorescence intensity (GMFI) for PD-1 expression (B) and CTLA-4 expression (D) are summarized from three independent experiments. P values determined by student's t-test. \* = P < 0.05. N.S. = not significant. Error bars = S.D.





**Figure 16 : G-1 has minimal effect on PD-1/CTLA-4 expression on T<sub>REG</sub> cells.**

CD4<sup>+</sup>CD62L<sup>hi</sup>CD44<sup>lo</sup> naive T cells from Foxp3<sup>egfp</sup> mice were collected by FACS and cultured for 4 days in the conditions indicated. Individual wells were supplemented with either 100nM G-1 (black bars) or DMSO (white bars). Cells were then stained for PD-1 and CTLA-4 then analyzed by flow cytometry. Representative histograms from the Foxp3<sup>+</sup> population are included for PD-1 (A) and CTLA-4 (C), with the isotype control (shaded region in gray), DMSO treated cells (Gray line without shading) and G-1 treated cells (Black line) shown. Data for geometric mean fluorescence intensity (GMFI) for PD-1 expression (B) and CTLA-4 expression (D) are summarized from three independent experiments. P values determined by student's t-test. \* = P < 0.05. N.S. = not significant. Error bars = S.D.



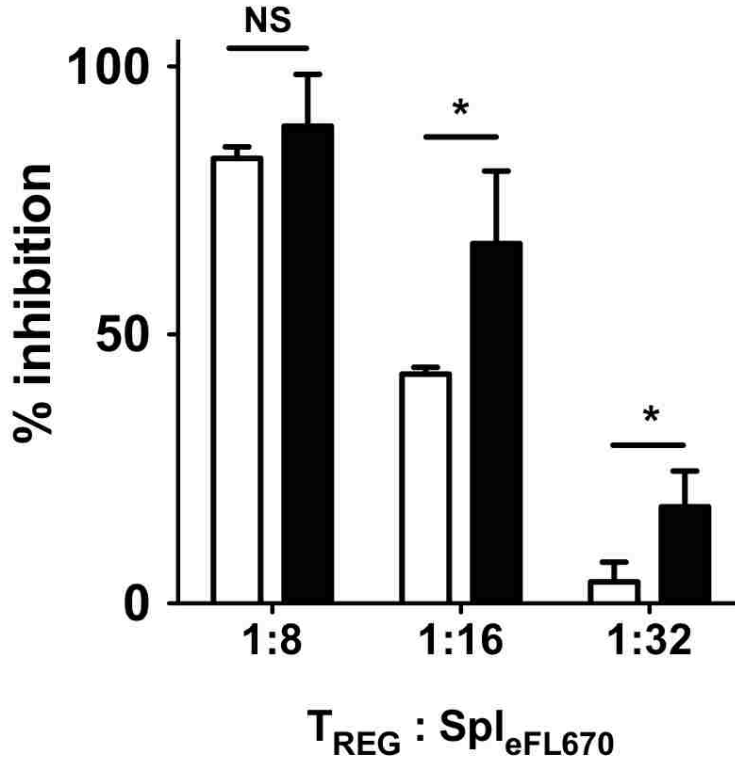
**Figure 17 : G-1 treatment of naïve T cells expands the hybrid population.**

CD4<sup>+</sup>CD62L<sup>hi</sup> naïve T cells from Foxp3<sup>egfp</sup> mice were collected by FACS and cultured for 4 days with TGF $\beta$  + IL6, supplemented with either 100nM G-1 or DMSO, as indicated. Cells were then stained for ROR $\gamma$ t and IL17A. (A) A representative histogram showing the gating for determining hybrid T cells is. (B) Summary of data from five experiments showing the relative percent of hybrid T cells in DMSO versus G-1 treated cultures. (C) Representative plots from one of two independent experiments done in triplicate showing IL17A and Foxp3 staining. P values determined by student's t-test. Error bars = S.D.

2 way ANOVA : P = 0.0116

□ DMSO treated cells as Tregs

■ G-1 (100nM) treated cells as Tregs



**Figure 18 : G-1 treated T cells exhibit enhanced suppressive activity *in vitro*.**

CD4<sup>+</sup>CD62L<sup>hi</sup> naive T cells from Foxp3<sup>egfp</sup> mice were collected by FACS and cultured for 4 days with TGFβ + IL6, supplemented with either 100nM G-1 or DMSO, as indicated. Cells were then collected, washed, and used as regulatory T cell in an *in vitro* T cell suppression assay. For responder cells, splenocytes from male wild-type C57BL/6 mice were collected and stained with the proliferation dye eFluor670 (eBiosciences). Cells were mixed in the ratios indicated and stimulated with antiCD3ε Ab. After 3 days in culture, samples were analyzed for dilution of eFluor670 by flow cytometry. Example from one of four independent experiments. Statistics determined by student's t-test and 2-way ANOVA test. \* = P < 0.05 (student's t-test). N.S. = not significant. Error bars = S.D.

## **Chapter 5 : ERK-dependent IL10 induction**

### ***Section 5.1 : Preface***

In this chapter, we investigate the role of GPER in altering cytokine production from T cell populations (Aim 2). This builds on the data presented in Chapter 4, wherein G-1 led to an increase in Foxp3 expression within CD4<sup>+</sup> T cells. While Foxp3 expression imparts a suppressive phenotype on cells in which it is expressed, other secreted factors, including cytokines and chemokines, are also important in regulating immune reactions. Thus in order to build a more comprehensive picture of G-1's activity in T cell populations, we decided to investigate the ability of G-1 to modulate the production of several key cytokines, including IL10, IL17A, and IFN $\gamma$ , under conditions that drive differentiation of the T<sub>H</sub>17 lineage. The introduction starts with a short review of the various helper T cell lineages, and the contextual framework from which they are characterized. Some review of IL10 function, and estrogen and G-1 in immunity, follows.

### ***Section 5.2 : Introduction***

CD4<sup>+</sup> helper T lymphocytes orchestrate adaptive immune responses to invading pathogens, and are critical to the pathogenesis of numerous disease processes, including autoimmunity and cancer. They are an attractive drug target due to their central role in immunity, and their implication in a wide variety of

diseases. There are several distinct lineages of CD4<sup>+</sup> helper T cell, each specialized in enhancing specific branches of the immune system. The original paradigm described by Coffman and Mossmann divided CD4<sup>+</sup> helper T lymphocytes into the T-helper-1 (T<sub>H</sub>1) and T<sub>H</sub>2 populations (Mosmann and Coffman, 1989), with T<sub>H</sub>1 producing IFN $\gamma$  and coordinating cellular immunity responses and T<sub>H</sub>2 secreting humoral immunity mediators such as IL4, IL5, and IL13. In 2005, the T<sub>H</sub>1-T<sub>H</sub>2 paradigm was expanded as the T<sub>H</sub>17 population emerged as a third class of helper/effector T cell. T<sub>H</sub>17 cells are characterized by expression of the transcription factor ROR $\gamma$ t (Harrington et al., 2005; Ivanov et al., 2006), and secrete pro-inflammatory cytokines including IL21 (Wei et al., 2007) and IL17A/F. These cells are important to controlling infections by extracellular pathogens, but also play a deleterious role in human health by contributing to the pathogenesis of numerous autoimmune diseases (Torchinsky and Blander, 2010). In mice, T<sub>H</sub>17 differentiation depends on TGF $\beta$  and IL6 or IL21 within the local milieu (Torchinsky and Blander, 2010), while IL23 signaling plays a critical role in stabilizing the T<sub>H</sub>17 phenotype (McGeachy et al., 2009). Although T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 effector T cells coordinate a robust and diverse arsenal of adaptive immune responses necessary for the maintenance of human health, mechanisms of restraint must limit effector responses to protect the host from immune-mediated damage.

A major breakthrough in elucidating the mechanisms of adaptive immune regulation emerged with the identification of an array of regulatory T cell (T<sub>REG</sub>) populations. The best defined class of T<sub>REG</sub> cells express the forkhead

transcription factor Foxp3 and suppress numerous animal models of autoimmune disease (Vignali et al., 2008), whereas loss of Foxp3 function in humans and mice precipitates a fatal multi-organ autoimmune condition marked by the inability to control T cell responses (Clark et al., 1999; Patey-Mariaud de Serre et al., 2008). T<sub>REG</sub>S function to dampen immune responses through a variety of approaches, including contact-mediated inhibition, secretion of perforin and granzyme A/B, sequestration of key growth factors such as IL2, and secretion of suppressive cytokines including TGF $\beta$ , IL10, and IL35 (Vignali et al., 2008). IL10 in particular plays an important role in immune homeostasis, both in mice (Kuhn et al., 1993) and humans (Glocker et al., 2009), suggesting it has several non-redundant roles in regulating inflammatory responses. Many cell types in addition to Foxp3<sup>+</sup> cells (Saraiva and O'Garra, 2010) can produce IL10, most notably several lineages of CD4<sup>+</sup> T cells (Saraiva et al., 2009), including T<sub>H</sub>1 (Del Prete et al., 1993; Jankovic et al., 2007; Meyaard et al., 1996), T<sub>H</sub>2 (Del Prete et al., 1993; Fiorentino et al., 1989), and T<sub>H</sub>17 (Fitzgerald et al., 2007; McGeachy et al., 2007; Stumhofer et al., 2007) cells, as well as various types of regulatory T cells (Maynard and Weaver, 2008). In a feed forward mechanism, IL10 can drive its own expression through the induction of an IL10-producing T<sub>REG</sub> population termed Tr1 cells (Battaglia et al., 2006; Roncarolo et al., 2006). Conversely, IL10 can also be induced independently of IL10 signaling in both Foxp3<sup>+</sup> and Foxp3<sup>-</sup> T<sub>REG</sub> populations (Maynard et al., 2007). Studies using conditional knockout mice have begun to identify specific roles for IL10 produced by distinct T cell populations. For example, loss of IL10 production in Foxp3<sup>+</sup> T<sub>REG</sub>S leads to

inflammation within the wall of the gut (Rubtsov et al., 2008). IL10 acts on antigen presenting cells to downregulate expression of costimulatory molecules and decrease their production of proinflammatory cytokines, in addition to acting on T cell themselves (Moore et al., 2001). Given its potent anti-inflammatory effects, various strategies are being explored to target IL10 for therapeutic intervention (O'Garra et al., 2008).

Estrogen is a well-documented modulator of immune function in humans and mice, capable of increasing the expression of Foxp3 (Polanczyk et al., 2005) and IL10 (Yates et al., 2010). These effects translate to human disease wherein multiple sclerosis (MS) patients experience a decrease in symptoms during pregnancy (Confavreux et al., 1998), and to murine models of autoimmune disease where estrogen inhibits development of experimental autoimmune encephalomyelitis (EAE) (Wang et al., 2009a), an animal model of MS. Although the effects of estrogen are presumed to be mediated by the classical estrogen receptors, ER $\alpha$  and ER $\beta$ , recent studies have pointed to the newly described G protein-coupled estrogen receptor GPR30/GPER as contributing to many of these responses. We and others have recently shown that, like E2, the GPER-selective agonist G-1 can attenuate EAE (Blasko et al., 2009; Wang et al., 2009a). In the current work we show that G-1 can evoke IL10 expression and secretion from CD4<sup>+</sup> T cells differentiated under T<sub>H</sub>17-polarizing conditions. G-1-mediated IL10 expression was blocked by the GPER-directed antagonist G15 (Dennis et al., 2009), and was dependent on ERK signaling, consistent with known mechanisms of IL10 production within effector T cell populations (Saraiva

and O'Garra, 2010). Analysis of IL17A, Foxp3 and ROR $\gamma$ t expression demonstrated that these responses occurred in cells expressing both IL17A and ROR $\gamma$ t, as well as in a population of Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> hybrid T cells. G-1-mediated IL10 expression was blocked by the GPER-directed two antagonists, G15 (Dennis et al., 2009) and the unpublished compound G36. However, the use of T cells from GPER<sup>+/-</sup> and GPER<sup>-/-</sup> mice yielded unexpected results as G-1 appeared to have no effect on cells derived from heterozygous mice, while cells from knockout mice appeared to exhibit a similar trend as was observed in wild types. Taken together, our results demonstrate a novel immunomodulatory property for G-1, and suggest that this small molecule may serve as a model compound for a new class of T cell-targeted pharmaceuticals. In addition, these data suggest that the family of GPER-directed small molecules may serve as model compounds for a new class of T cell-targeted pharmaceuticals in the treatment of autoimmune disease and cancer. However, more elegant studies are going to be required to delineate the role of GPER and/or other cellular targets in our observed G-1-mediated effects, as well as any effects of other G compounds.

### ***Section 5.3 : Results***

#### **G-1 elicits IL10 in CD4<sup>+</sup> cells under T<sub>H</sub>17 polarizing conditions**

As in chapter 4, in order to investigate the direct effects of G-1 on CD4<sup>+</sup> T cells, we chose to utilize purified cultures of naïve T cells activated by polyclonal



stimulation with antiCD3 $\epsilon$  and antiCD28 antibody. This eliminated secondary effects due to the activity of G-1 on APCs within the culture. Furthermore, primary cells from male mice were used throughout the study to avoid potential confounding effects of either; (a) varying estrogen levels in female mice, or (b) the inflammatory effects of ovariectomy. We have also shown that CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> naïve T cell and CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>REG</sub> populations express the G-1 target GPER (Section 4.3).

Given our preliminary findings wherein G-1 drove expression of IL10 and IL17A, and given that G-1 can protect mice from EAE (Blasko et al., 2009; Wang et al., 2009a) and the importance of the T<sub>H</sub>17 lineage to this model (Ivanov et al., 2006), we began by determining the effects of G-1 on naïve T cell differentiation under T<sub>H</sub>17-polarizing conditions (TGF $\beta$ /IL6  $\pm$  IL23). Thus, naïve T cells from 7-11 week old male C57BL/6 mice were collected by FACS and stimulated for 4 days *ex vivo*, supplemented with various combinations of TGF $\beta$ , IL6, and IL23. Following 4 days of stimulation, cells were analyzed for expression of IFN $\gamma$ , IL17A, and IL10 by intracellular cytokine staining (ICS). Expression of IL10 was present exclusively in cultures treated with IL6 (**Figure 19A**), consistent with previous findings using *ex vivo* culture systems where treatment with TGF $\beta$  alone blocks IL10 expression in differentiating CD4<sup>+</sup> T cells (Saraiva et al., 2009), and efficient induction of IL10 secretion from T<sub>H</sub>17-polarized cells requires both TGF $\beta$  and IL6 (McGeachy et al., 2007). As expected, IL17A expression was dependent on T<sub>H</sub>17-polarizing conditions [i.e. treatment with both TGF $\beta$  and IL6 (**Figure 19A-C**)], and was enhanced by the addition of IL23. G-1 treatment resulted in an

increase in the number of IL10<sup>+</sup> cells within T<sub>H</sub>17-polarized cultures (**Figure 19B**), as well as in the presence of IL23 (**Figure 19C**), which is known to be important in stabilizing the phenotype of T<sub>H</sub>17 populations. G-1-mediated IL10 expression was not reflective of a general effect on cytokine production as no increase in the number IL17A<sup>+</sup> cells was observed in either condition (**Figure 19B,C**). In addition, G-1 had no effect on IFN $\gamma$  expression in cultures stimulated with CD3/28 alone (**Figure 19D**); however, few IFN $\gamma$ <sup>+</sup> cells were detected in the other culture conditions tested (**Figure 19D, 20**).

To determine whether the increased numbers of IL10<sup>+</sup> cells translated into a specific increase in the secretion of IL10 from G-1 treated cultures, naïve T cells were collected and stimulated as above, in the presence of TGF $\beta$  and IL6. After 4 days of differentiation, DMSO and G-1 treated cells were collected, washed with medium to remove any cytokines released over the course of differentiation, and re-plated. Cells were then re-stimulated with anti-CD3 $\epsilon$  Ab for 24 hours, after which culture medium was analyzed for the presence of newly secreted IL6, IL10, IL17A, TNF $\alpha$ , and IFN $\gamma$  by Luminex multiplex assay. Cells differentiated in the presence of G-1 produced approximately three-fold more IL10 than control cultures (**Figure 21A**), consistent with our observation that G-1 increased the number of IL10-producing cells. No difference in the secretion of IL6, IL17A, TNF $\alpha$ , or IFN $\gamma$  was detected (**Figure 21B-E**), again suggesting that G-1 was specifically driving the production of the anti-inflammatory cytokine IL10, and not proinflammatory mediators such as IL17A and IFN $\gamma$ . Taken together,

these data show that G-1 can specifically drive IL10 expression within and secretion from CD4<sup>+</sup> T cell populations.

### **Induction of an IL10<sup>+</sup>IL17A<sup>+</sup> and IL10<sup>+</sup>IL17A<sup>-</sup> population by G-1**

As G-1-induced IL10 expression was dependent on T<sub>H</sub>17-polarized conditions, we sought to determine the relationship of G-1-induced IL10<sup>+</sup> cells to those expressing the characteristic T<sub>H</sub>17 cytokine IL17A. Thus, naïve T cells were again collected by FACS and polyclonally stimulated in the presence of TGFβ and IL6. Cells were cultured with increasing doses of G-1 and analyzed for IL17A and IL10 by ICS (**Figure 22**). Our data reveal a dose-dependent increase in the number of IL10<sup>+</sup>IL17A<sup>-</sup> (**Figure 22A, B**) and IL10<sup>+</sup>IL17A<sup>+</sup> cells (**Figure 22A, C**) within G-1-treated cultures. A similar trend was observed under IL23 polarizing conditions (**Figure 19A** and data not shown). In addition, G-1-mediated IL10 expression was blocked by the recently described GPER antagonist G15 (Dennis et al., 2009) or G36 (manuscript in preparation) (**Figure 23**). Furthermore, the induction of a population of IL10<sup>+</sup>IL17A<sup>+</sup> cells suggests that G-1 can elicit IL10 expression within cells that have differentiated to the T<sub>H</sub>17 lineage. Taken together, these data show that G-1 can elicit IL10 production within the T<sub>H</sub>17 compartment, a response that is blocked by preincubation with the GPER antagonists G15 and G36.

## **ERK signaling is critical for G-1-mediated IL10 expression**

IL10 production within T<sub>H</sub> populations has been shown to be dependent on signaling through extracellular signal-regulated kinases (ERK1/2) (Saraiva et al., 2009; Saraiva and O'Garra, 2010), one of three MAP kinase cascades, the others comprising JUN N-terminal kinases (JNK1/2) and p38. GPER has been shown to activate the ERK pathway, although predominantly in cancer cells (Filardo et al., 2000). To test whether G-1-mediated induction of IL10 was dependent on MAP kinase signaling, naïve T cells were treated with either PD98059, an inhibitor of the ERK pathway, SB203580, an inhibitor of the p38 pathway, or the JNK II inhibitor, and stimulated under T<sub>H</sub>17-polarizing conditions as before. Consistent with other published reports (Saraiva et al., 2009), we found that inhibition of p38 had no effect on IL10 expression in T<sub>H</sub>17-polarized cells. Similarly, JNK signaling appeared not to be required for G-1-mediated induction of IL10 (**Figure 24A**). In contrast, there was no difference in the number of IL10<sup>+</sup> cells observed between control and G-1-treated cultures when cells were cultured with the ERK inhibitor PD98059 (**Figure 24A-C**), consistent with a role for ERK signaling specifically in G-1-mediated IL10 induction. Of note, PD98059 prevented expansion of both the IL10<sup>+</sup>IL17A<sup>+</sup> and the IL10<sup>+</sup>IL17A<sup>-</sup> populations (**Figure 24C**). These data demonstrate that G-1 mediates IL10 expression by increasing ERK signaling in CD4<sup>+</sup> T cells.

The ERK pathway is known to be a potent activator of cell proliferation. To determine if G-1-mediated increases in IL10 were the result of increased proliferation of cells expressing IL10 rather than induction of IL10 *de novo*, naïve

T cell were stained with the proliferation dye eFluor670 prior to stimulation in culture. We were unable to detect any significant difference in the proportion of dividing cells following G-1 treatment. The observation that G-1 treated cultures demonstrated attenuated dilution of the eFluor dye as compared to the DMSO-treated cultures (**Figure 25**) indicates that the increased number of IL10<sup>+</sup> cells following G-1 treatment is not due an increase in cell proliferation, and in fact show that proliferating cells are going through fewer divisions when treated with G-1, perhaps due to the action of IL10. In addition, the dramatic increase in the number of non-dividing cells expressing IL10 in some G-1 treated cultures (as indicated in the upper right quadrant in **Figure 25B**) suggests that G-1 can specifically drive expression of IL10 independent of cell division. Taken together, these data show that G-1 stimulates *de novo* IL10 expression within differentiating T<sub>H</sub>17 through direct action on T cells via an ERK-dependent mechanism.

### **IL10-induction occurs within a hybrid T cell population**

An emerging paradigm in T cell biology is the induction of “hybrid” T cell populations that express one of the canonical effector T cell transcription factors (for example T-bet from the T<sub>H</sub>1 lineage) as well as Foxp3 (Barnes and Powrie, 2009). These cells appear to play a role in the regulation of specific types of inflammatory responses, where the expression of Foxp3 imparts a suppressive phenotype, and the expression of the lineage-specific factor such as T-bet leads to a repertoire of gene products (e.g. chemokine receptors) that allow for

targeting to sites of inflammation. Presumably, this provides a mechanism for the recruitment of regulatory T cells to sites of ongoing inflammatory responses. To investigate the expression of Foxp3 together with ROR $\gamma$ t, naïve T cells were collected from Foxp3<sup>egfp</sup> transgenic mice (Haribhai et al., 2007). Cells were stimulated for 4 days in the presence of TGF $\beta$  and IL6 with or without G-1 added to the culture. Following differentiation, IL10, IL17A, ROR $\gamma$ t, and Foxp3 were analyzed by ICS or detection of endogenous GFP expression by flow cytometry. G-1 was equally effective at inducing IL10 production within Foxp3<sup>-</sup>ROR $\gamma$ t<sup>+</sup> T<sub>H</sub>17 cells as in Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> hybrid T cells (**Figure 26**). The T<sub>H</sub>17 subset saw an increase in both IL10<sup>+</sup>IL17A<sup>+</sup> and IL10<sup>+</sup>IL17A<sup>-</sup> cells, while only IL10<sup>+</sup>IL17A<sup>-</sup> cells were detected in the hybrid T cell population. In fact no IL17A<sup>+</sup> cells were present in the Foxp3<sup>+</sup> population (**Figure 26**). These data demonstrate the ability of G-1 to induce IL10 within the recently described hybrid T<sub>H</sub>17 population in addition to conventional (Foxp3<sup>-</sup>ROR $\gamma$ t<sup>+</sup>) T<sub>H</sub>17 cells.

### **Splenocytes from GPER<sup>-/-</sup> mice produce less IL17A and IL10**

To begin to determine if our results in discussed so far translated to the setting of intact animals, GPER<sup>-/-</sup> mice were treated with E2 *in vivo*, and splenocytes were collected *for ex vivo* analysis. As we were interested in looking at cytokine production specifically from T cell populations, the splenocytes were stimulated with antiCD3 $\epsilon$  and antiCD28 antibodies, which, as mentioned previously, activates T cells by mimicking antigen presentation. Cells were

stimulated for 48 hours after which cell free culture medium was analyzed for a series of cytokines by Luminex Multiplex assay (**Figure 27** and Appendix D). Several trends were observed, including drastic reductions in the production of IL4, IL10, and IL17A from GPER<sup>-/-</sup> splenocytes. The reduction in IL4 is interesting because of previous work demonstrating the E2 can elicit IL4 production, possibly by alleviating IFN $\gamma$ -induced suppression of the *il4* locus (Lengi et al., 2006). However, the fact the IL13 production from these same cells is not affected by a lack of GPER suggests that GPER was not involved in T<sub>H2</sub> differentiation. Of note, another T<sub>H2</sub> cytokine, IL5, also appeared to be affected by the loss of GPER (Appendix D). The loss of IL17A and IL10 production in GPER<sup>-/-</sup> cells was also interesting as both cytokines are elicited by the combination of IL6 and TGF $\beta$  signaling during T<sub>H17</sub> differentiation (McGeachy et al., 2007), and G-1 can attenuate the T<sub>H17</sub>-dependent disease model EAE (Blasko et al., 2009; Wang et al., 2009a).

The fact that a loss of GPER expression can lead to decreased IL10 and IL17A expression suggests that systemic treatment with the GPER-directed agonist G-1 should be able to stimulate production of these cytokines following T cell activation. Moreover, our results leading up to these *in vivo* studies show that treatment of naïve T cells with G-1 in culture can lead to increased IL10 expression and secretion. To determine if these findings translated to *in vivo* G-1 treatment, wild-type mice were injected subcutaneously with G-1 for 7 consecutive days, after which they were sacrificed and splenocytes were stimulated in culture with antiCD3 $\epsilon$  and antiCD28 antibodies. Samples of

supernatant were collected 24, 48, and 72 hours after stimulation and analyzed for secreted IL6, IL10, IL17A, IFN $\gamma$ , and TNF $\alpha$  by Luminex multiplex assay. No trends were observed for any of the analytes following 24 hours of stimulation (**Figure 28**). In agreement with our results with cultured naïve T cells, cells from G-1 treated mice secreted increased levels of IL10 (**Figure 28A**). Notably, we also observed that splenocytes from G-1-treated mice secreted increased levels of IL17A (**Figure 28B**). This varied from our findings in **Figure 19**, wherein no increase in the number of IL17A<sup>+</sup> cells was observed, and **Figure 21** were naïve T cells cultured with G-1 produced similar levels of IL17A as compared cells treated with DMSO. However, these data reflect our observations following E2 treatment in GPER<sup>-/-</sup> mice (**Figure 27**). Moreover, we were able to detect G-1-mediated IL17A induction within naïve T cells under specific experimental conditions. For example, stimulation of naïve T cells in culture with TGF $\beta$  alone showed that G-1 could elicit a small increase in the number of IL17A<sup>+</sup> cells (**Figure 29A**). These results are also in agreement with data described within our preliminary data, where G-1 treatment led to a sequential increase in IL17A and IL10, respectively, in cultures of enriched CD4<sup>+</sup> T cells (see Chapter 2). A portion of this same data is showing the trend of IL17A production from G-1 treated cells versus untreated cells has been re-graphed in **Figure 29B**. We also noted that only IL17A secretion from enriched CD4<sup>+</sup> T cells is altered, not IL4 or IFN $\gamma$  (**Figure 29C**). The difference in the temporal dynamics of those results and the data reported here following *in vivo* G-1 treatment are likely due to the presence of numerous other immune populations within the unsorted splenocyte



cultures, and may also reflect differences in systemic treatment with G-1 relative to co-culture *ex vivo*. Therefore our observations that systemic G-1 enhances TCR-mediated IL17A secretion likely reflects the variable nature of the *in vivo* environment, or perhaps secondary effects resulting from activity on other immune populations. In addition, IL17A has been shown to exhibit immunosuppressive properties in several settings, including in the development of atherosclerosis (Ait-Oufella et al., 2010; Taleb et al., 2009; Taleb et al., 2010) and the induction of T cell mediated colitis (O'Connor et al., 2009). Moreover, cells treated with TGF $\beta$  + IL6 have been shown to exhibit bystander suppressive effects in EAE, despite producing higher levels of IL17A (McGeachy et al., 2007). As there appeared to be no induction of IL17A when exogenous IL6 was added to the culture, it is also possible that the setting of the CNS does not provide the appropriate conditions required for G-1-mediated IL17A induction. Thus the induction of IL17A is reconcilable to its ability to attenuate EAE, despite the established importance of T<sub>H</sub>17 cells to EAE induction (Cua et al., 2003; Ivanov et al., 2006; Langrish et al., 2005), and the fact that systemic neutralization of IL17A/F attenuates clinical symptoms in this model (Hofstetter et al., 2005). Conversely, splenocytes from G-1 treated mice produced decreased levels of IFN $\gamma$  relative to those that were treated with vehicle alone (**Figure 28C**), suggesting that in addition to driving production of IL10 and IL17A, G-1 may act systemically to reduce the levels of IFN $\gamma$ . No changes in the secretion of TNF $\alpha$  (**Figure 28D**) or IL6 (**Figure 28E**) were noted, in agreement with our findings from **Figure 21**. Collectively, these data suggest that pharmacological stimulation

of GPER *in vivo* leads to an increase in the production of the cytokines IL10 and IL17A, and decreased production of the proinflammatory cytokine IFN $\gamma$  following T cell activation.

### **G-1 exhibits unexpected activity in GPER<sup>-/-</sup> cells**

G-1 was originally described as an agonist directed towards GPER, and we saw in previous findings from this chapter that the GPER-directed antagonists G15 and G36 could block G-1-mediated IL10 expression (**Figure 23**). To determine if our observations were in fact due to signaling through GPER, naïve T cells were collected simultaneously from GPER<sup>+/-</sup> and GPER<sup>-/-</sup> mice, then treated in culture as before. To our surprise, we observed no G-1-mediated induction of IL10 expression in cells derived from GPER<sup>+/-</sup> mice. Conversely, G-1 treatment led to increased number of IL10<sup>+</sup> and IL10<sup>+</sup>IL17A<sup>+</sup> cells within cultures of GPER<sup>-/-</sup> T cells. This result repeated to varying degrees in three independent experiments (**Figure 30**). As in Chapter 4, the reasons for this observation are not clear, but it is possible that the role of GPER in thymic T cell development contributes (Wang et al., 2008). Irrespective of the basis for this observation, these data cast doubt over the actual target of G-1 in the data presented, despite the correlation between the findings outline above with GPER<sup>-/-</sup> mice and systemic G-1 treatment. Unfortunately, the question of G-1 treatment in GPER<sup>-/-</sup> mice was not addressed in these studies, but should be a priority for future work.

## **Section 5.4 : Discussion**

CD4<sup>+</sup> T cells play a critical role in the pathogenesis of many of the most prominent diseases of the Western world, including cancer, autoimmunity, and infectious disease. The cytokine IL10 is a potent suppressor of immune responses, capable of acting on a multitude of cells types to dampen inflammatory responses and limit host damage to infection and autoimmune disease. In this chapter, we demonstrated that *in vivo* treatment with the GPER-directed agonist G-1 can drive IL10 production from splenocytes following T cell activation. Furthermore, our findings with purified cultures of naïve T cells suggests that these observations are due to the direct action of G-1 on CD4<sup>+</sup> T cell populations themselves, as treatment with G-1 drove IL10 production from T<sub>H</sub>17-polarized naïve T cell populations. We observed an increase in the number of cells expressing IL10 within the G-1-treated cultures, as measured by ICS. This response was not due to global changes in cytokine production as G-1 had no effect on the expression of IL17A under T<sub>H</sub>17-polarizing conditions, or in the induction of IFN $\gamma$  in non-polarizing (T<sub>H</sub>0) conditions. Results from ICS translated into increased cytokine secretion. G-1-treated cultures of purified T cells produced three-fold more IL10 in response to re-stimulation compared to controls, with no significant change in the secretion of proinflammatory cytokines IL6, IL17A, TNF $\alpha$ , or IFN $\gamma$ , demonstrating high selectivity for the mechanism of immune regulation by G-1. Similarly, systemic administration of G-1 had no effect of IL6 or TNF $\alpha$  secretion. Interestingly, we did note increased secretion of IL17A following *in vivo* treatment with G-1, while also observing a decrease in the

production of IFN $\gamma$ . The difference in IL17A and IFN $\gamma$  regulation following systemic G-1 treatment as compared to our the results from purified T cell cultures may reflect effects of G-1 on other immune populations following *in vivo* treatment. Another possibility would be that G-1-mediated IL10 production is elicited during the week long injections of G-1, leading to inactivation splenic APCs and a decrease in the secretion of T<sub>H</sub>1-polarizing cytokines like IL12, and thus lower IFN $\gamma$  production. It is worth noting that in our preliminary data we observed increases in both IL10 and IL17A production with G-1 treatment in enriched cultures of CD4<sup>+</sup> T cells, and in some experiments with the purified cultures of naïve T cells G-1 increased the number of IL17A<sup>+</sup> cells. Thus it seems as though G-1 can drive IL17A expression by acting directly on CD4<sup>+</sup> T cells in some settings. Future studies will be required to clearly elucidate the contextual framework required. Such studies may prove valuable in determining G-1's potential as a therapeutic, as IL17A can act in both a pro- and anti-inflammatory capacity (O'Connor et al., 2010).

These results build upon previous studies that demonstrate G-1 can influence immune responses under autoimmune conditions (Blasko et al., 2009; Wang et al., 2009a; Wang et al., 2008). T<sub>H</sub>17 cells are implicated in the pathogenesis of numerous autoimmune diseases and are localized in high numbers to sites of autoimmune inflammation. Our data suggest that it may be possible to induce IL10 *in situ* where large numbers of T<sub>H</sub>17 cells persist, through systemic treatment with G-1. That this may be a feasible therapeutic approach is suggested by experiments in which co-injection of IL10-producing cells

differentiated in the presence of TGF $\beta$  and IL6, as was done here, inhibits the development of EAE following adoptive transfer of neuropeptide-reactive T<sub>H</sub>17 cells (McGeachy et al., 2007). This effect was dependent on IL10 production (McGeachy et al., 2007) and suggests that such cells can inhibit fully differentiated pathogenic T cell populations through the secretion of IL10 *in situ*, as would likely be required in the case of a viable therapeutic intervention based on the findings of our study.

Our findings also suggest that GPER-mediated induction of IL10 may play a role in estrogen's ability to suppress autoimmune diseases. Two previous reports demonstrated that G-1 can suppress disease in the MS-like animal model EAE (Blasko et al., 2009; Wang et al., 2009a). In one study, the authors also observed increased IL10 production from G-1-treated splenocytes collected from diseased animals as compared to placebo controls (Wang et al., 2009a). This correlated well with our results in Figure 28, where we observed increased IL10 production following *ex vivo* stimulation of splenocytes derived from G-1 treated mice. Interestingly, in our work we also noted increased IL17A and decreased IFN $\gamma$  secretion from G-1-treated splenocytes, which stands in contrast to the findings in the report discussed above, where the opposite trend was detected in the draining lymph nodes of EAE mice treated with G-1. They also noted an increase in IL6, while no observable difference was detected in our experiments.

As was the case in Chapter 4, whether these differences are the result of effects on other cell types or differential expression of other immune mediators requires further investigation. The fact that our work was done in male mice and

the previous report was done in female mice may also have contributed to differences between our results and previous observations. It is also possible that G-1 has divergent effects on T cells depending on the context in which it is acting, and perhaps the inflammatory environment of the CNS provides the appropriate context to precipitate G-1-mediated induction of PD-1. This would explain the differences between our results following systemic G-1 treatment in naïve mice, and the results reported by Wang et al. In contrast to their results described above, we have not observed any changes in PD-1 expression on Foxp3<sup>+</sup> T cells following *in vivo* administration of G-1 in naive animals or after *ex vivo* treatment of cultured CD4<sup>+</sup> T cells (Section 4.3). It has also been recently shown that estrogen can protect mice from EAE in a Foxp3-independent manner (Subramanian et al., 2010). The authors noted an increase in IL10 production, though it is not known what cells were responsible for this effect. Interestingly, IL10 production in CD4<sup>+</sup> T cells can inhibit the development of EAE (Fitzgerald et al., 2007), a disease whose pathogenesis is dependent on ROR $\gamma$ t expression (Ivanov et al., 2006). The fact that we demonstrated G-1 leads to an increase in IL10 within Foxp3<sup>-</sup>ROR $\gamma$ t<sup>+</sup> cells, and that IL10 induction occurs even in the presence of IL23, suggest that one explanation for the results observed with the EAE model above is the induction of IL10 through E2-mediated activation of GPER in, and subsequent IL10 production from, ROR $\gamma$ t<sup>+</sup> cells specifically within the CNS. This would be consistent with results discussed above, and other studies that have shown; (a) E2 can increase IL10 production *in vivo* in a GPER-dependent manner (Yates et al., 2010), and (b) the *in vitro* suppressive activity of

T<sub>REG</sub> cells from PD-1KO mice was enhanced following *in vivo* treatment with E2, without changing the expression levels of Foxp3 (Polanczyk et al., 2007). Further studies using conditional knockouts of IL10 within the CD4<sup>+</sup> compartment will be needed to definitively address these questions.

G-1 has been characterized as a selective agonist for the G protein-coupled estrogen receptor GPER (Bologa et al., 2006), a recently identified non-classical member of the estrogen receptor family (Prossnitz et al., 2008). Consistent with this mechanism of action, G-1-mediated IL10 expression was inhibited by the addition of the GPER-directed antagonist G15 (Dennis et al., 2009). Our results are also supported by observations that G-1-mediated inhibition of EAE is dependent on GPER expression (Wang et al., 2009a). Although small molecules can be subject to off-target activity, it is unlikely that both G-1 and G15 would exhibit off-target profiles that mimic their established activities towards GPER. Nevertheless, further investigation into the G-1 target(s) in T cells is warranted. To begin to address this question, we employed GPER<sup>-/-</sup> mice to investigate whether G-1 was acting through GPER. Our finding that G-1-mediated IL10 expression is lost in GPER<sup>+/-</sup>, only to return in GPER<sup>-/-</sup> cells, is difficult to reconcile with an assertion that G-1 is acting exclusively through GPER. Interestingly, investigation of some of our findings from Chapter 4 also showed a similar trend (see **Figure 39**, Appendix C), in that systemic treatment with G-1 led to an increase in the number of Foxp3<sup>+</sup> cells in GPER<sup>-/-</sup> mice, while no trend was observed in GPER<sup>+/-</sup> mice. It is worth noting again that these mice exhibit higher levels of apoptosis in double negative thymocytes at baseline, and

lack the E2-mediated increase in this population following systemic estrogen administration (Wang et al., 2008). However, even if one postulates that aberrant thymic development or insufficient expression of GPER in the GPER<sup>+/-</sup> T cells constitutes the basis for the loss of G-1 induced IL10 production in this population, the observation that G-1 can drive IL10 expression in GPER<sup>-/-</sup> T cells indicate that it is capable to eliciting a response in the complete absence of GPER; suggesting that in this context G-1 is acting via off target activity. It would be interesting to determine if G-1-mediated IL10 expression within GPER<sup>-/-</sup> cells can be blocked by the GPER-directed antagonists. If G15 and G36 prove incapable of blocking the G-1 effect in GPER<sup>-/-</sup> cells, this would further support the hypothesis that two distinct targets are indeed responsible for these effects in wildtype versus GPER<sup>-/-</sup> cells. That finding may not be as statistically unlikely as random chance would predict as other sex steroids such as androgens and progestins also effect the immune system, receptors whose ligands are chemically similar to G-1. This notion is further supported by the fact that GPER gene expression is clearly involved in G-1-induced IL10 expression, based on the observation that the effect is consistently lost in GPER<sup>+/-</sup> cells. It may be that in the complete absence to GPER, a compensatory mechanism within the developing thymocytes is induced which drives expression of another G-1 binding receptor that carries redundant function within the cell; for example one might postulate the up-regulation of another sex steroid receptor. Why lower expression of GPER would only impart an effect on the G-1-mediated induction of IL10 and not the expression of the proposed secondary G-1-binding receptor



may reflect differences the activities of G-1 and its natural ligand, E2, which would presumably be responsible for the second effect. Therefore, it will be worth looking at the effect of E2 in IL10 induction within T cell populations. Moving forward, future studies aimed at verifying GPER as the target of G-1 within the T cell population should ideally employ inducible knockout strategies or retroviral RNAi targeting of GPER to avoid the confounding effects of aberrant thymic T cell development observed in GPER<sup>-/-</sup> mice.

Our results have begun to elucidate the mechanisms by which G-1 induces IL10 expression and production. Addition of the MEK1 inhibitor PD98059 blocked G-1-mediated IL10 induction, whereas addition of inhibitors of the p38 and JNK pathways had no effect. These findings are consistent with data reported in the literature (Saraiva et al., 2009). Given that ERK signaling is implicated in IL10 expression within T<sub>H</sub>1 and T<sub>H</sub>2 cells, it will be interesting to determine whether G-1 can drive IL10 production under T<sub>H</sub>1 or T<sub>H</sub>2-polarizing conditions. The lack of IL10 expression in unpolarized (T<sub>H</sub>0) cells is not unexpected. IL10 production in T<sub>H</sub>1 cells requires IL12-signaling through STAT4, whereas T<sub>H</sub>2 cells are dependent on IL4-signaling through STAT6, and IL10 production in T<sub>H</sub>17 cells requires signaling through STAT3 (Stumhofer et al., 2007). Furthermore, IL27 is also capable of inducing IL10 in all three lineages (Fitzgerald et al., 2007; Stumhofer et al., 2007). However, IL4, IL6, IL12, IL21, and IL27 are produced by APCs and/or differentiated T cell populations; therefore the cytokines necessary for IL10 production are likely limited in pure cultures of naïve T cells that we employed. Additionally, our findings with TGFβ alone are

consistent with previous findings using *ex vivo* culture systems that suggest treatment with TGF $\beta$  blocks IL10 expression in differentiating CD4<sup>+</sup> T cells (Saraiva et al., 2009), and efficient induction of IL10 secretion from T<sub>H</sub>17-polarized cells requires both TGF $\beta$  and an activator of STAT3 such as IL6 (McGeachy et al., 2007), IL21 (Spolski et al., 2009), or IL27 (Stumhofer et al., 2007). We observed that G-1 was unable to induce IL10 production in differentiating naïve T cell without the addition of both TGF $\beta$  and IL6 to the culture medium, suggesting the G-1 cannot replace any of the critical signals necessary to induce IL10 in T<sub>H</sub>17 cells. A study using T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> double knockout mice suggests that the sole function of TGF $\beta$  in T<sub>H</sub>17 development is blocking the differentiation of T<sub>H</sub>1 and T<sub>H</sub>2 cells (Das et al., 2009). Thus our observation that G-1 treatment with IL6 alone does not consistently elicit IL10 production despite detectable levels of IL10<sup>+</sup> cells may reflect a dependence on T<sub>H</sub>17 differentiation. Future studies will need to address this question. Given our findings in Chapter 4, we also investigated whether G-1-mediated Foxp3 expression was dependent MAPK signaling. However, our findings indicate that G-1 drives Foxp3 expression via a mechanisms independent of ERK, p38, or JNK II signaling (**Figure 41**, Appendix D). This suggests that IL10 induction and Foxp3 induction occur via distinct mechanisms.

Another interesting observation from this study was that G-1 was capable of eliciting IL17A production under certain conditions. There is a longstanding debate about how the apparent immunosuppressive activities of E2 can be reconciled with the higher prevalence of autoimmune disease in women. It is

possible that E2-mediated activation of GPER may drive increased IL17A production under specific circumstances, and that this contributes to augmented autoimmune pathogenesis in women. Future studies aimed at investigating this possibility should be directed at delineating the specific conditions in which GPER activation leads to IL17A, and perhaps IL17F, production. It would be interesting to correlate these findings with studies investigating the expression of ER $\alpha$ , ER $\beta$ , and GPER, which can vary over time. An explanation for the sexual dimorphism in the prevalence of autoimmune disease may reside in identifying a setting where GPER plays a predominant role in estrogen signaling, perhaps due to downregulation of ER $\alpha$  and/or ER $\beta$  within specific cell populations, under conditions where GPER activation leads to production of IL17A or even IL17F. If these properties can be definitively described, there is also the possibility that G-1 may serve a role in T-cell based tumor vaccine strategies. Evidence suggests that polarization of tumor-specific T cells towards a T<sub>H</sub>17 phenotype prior to adoptive transfer can enhance tumor eradication (Muranski et al., 2008). It is possible that G-1 or a related compound may serve as a cost effective and safe alternative to recombinant cytokines during T cell culture, or even as a systemic adjuvant treatment to help stabilize the cells post adoptive transfer, especially given the fact we saw increased IL17A production following *in vivo* G-1 treatments in the data presented here. Moreover, further delineating the role of GPER in polarization along the T<sub>REG</sub>-T<sub>H</sub>17 axis may uncover other pharmacological mechanisms, for example the use of G15, that can elicit antitumor responses by driving conversion of T<sub>REG</sub> cells into T<sub>H</sub>17 populations, a

strategy which was validated in principle with the use of IDO-inhibitors in the B16 melanoma model (Sharma et al., 2009).

Finally, the IL10<sup>+</sup>IL17A<sup>+</sup> cells we identified appear to be part of the autoregulatory pathway (Maynard and Weaver, 2008), as they express ROR $\gamma$ t but not Foxp3. In fact we detected virtually no IL17A<sup>+</sup> cells within the Foxp3<sup>+</sup> population (Figure 6 and data not shown). While not completely unexpected since Foxp3 can inhibit some of the transcriptional activity of ROR $\gamma$ t (Zhou et al., 2008), Foxp3<sup>+</sup>IL17A<sup>+</sup> cells have previously been reported (Beriou et al., 2009). Our observation that G-1 induces IL10 expression in Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> hybrid T cells suggests that in addition to generating IL10 production in populations already localized at the site of inflammation, G-1 may also enhance the suppressive function of T<sub>REG</sub> populations drawn in from the circulation. If IL10 can be stably induced in hybrid T cell populations following *in vivo* G-1 treatment, their suppressive activity may be enhanced as they are recruited to sites of ongoing inflammation.

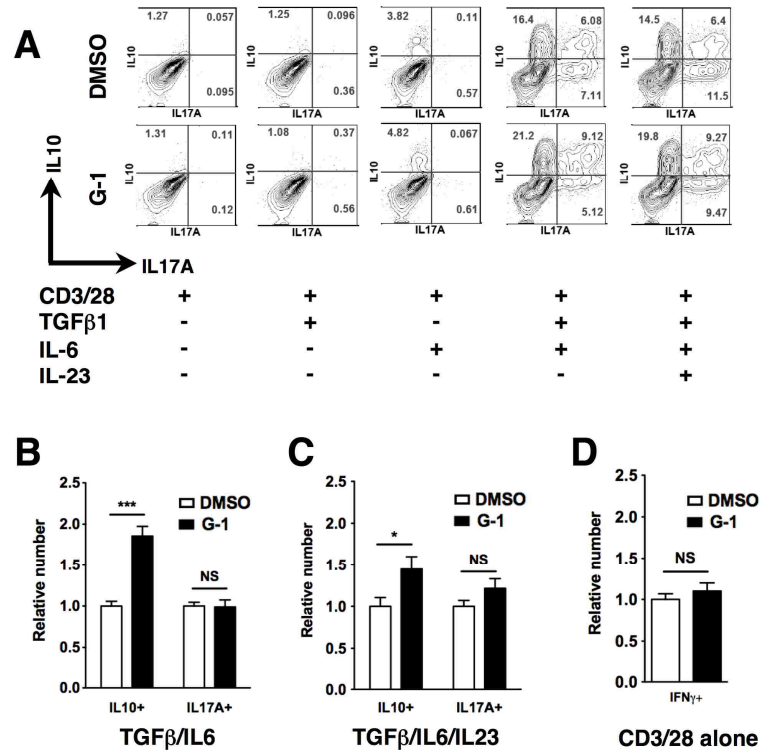
Numerous attempts have been made to harness the immunosuppressive properties of IL10 for therapeutic benefit, many of which have been based on the use of biologics (O'Garra et al., 2008). To our knowledge, this is the first evidence that a synthetic small molecule can shift the balance along the T<sub>REG</sub>-T<sub>H</sub>17 axis in favor of IL10 production, in this case by acting directly on T cell populations. These data build on prior results demonstrating that dexamethasone and retinoic acid can elicit IL10 from polyclonally stimulated naïve T cells when IL4, IL12 and IFN $\gamma$  are neutralized (Spolski et al., 2009). Also worth noting is the fact that it is

becoming increasingly clear that GPER likely plays a smaller role in the majority of classical estrogen responses, such as uterine imbibition, as compared to its more well known counterpart ER $\alpha$  (Dennis et al., 2009). Thus G-1 may be associated with a more tolerable side effect profile.

### **Section 5.5 : Conclusions**

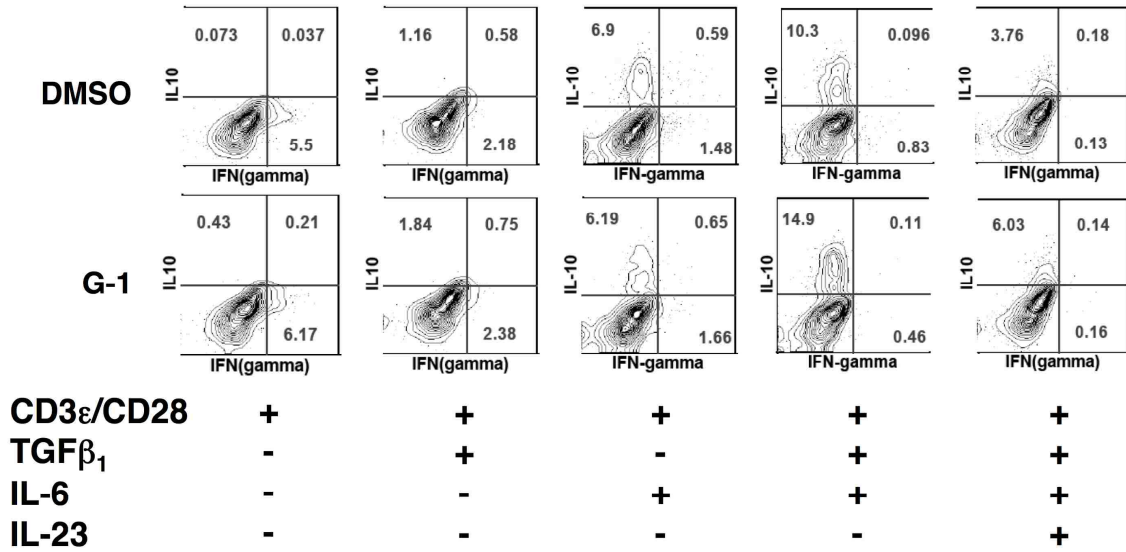
Our findings suggest that the membrane-permeable small molecule G-1 may serve as a novel T cell-targeted immunosuppressive agent through the induction of IL10 in settings where large populations of T<sub>H</sub>17 cells exist, for example in rheumatoid arthritis, inflammatory bowel disease, or psoriasis. G-1 may also prove useful for *in vitro* generation of IL10-producing cells for adoptive immunotherapy. Future studies delineating the specific signaling mechanisms and molecular targets of G-1 and other related compounds will be seminal to the continued development of this new class of immunoregulatory estrogenic small molecules. The selectivity of G-1 (Blasko et al., 2009; Bologna et al., 2006) and its attractive pharmacological properties (Wang et al., 2009a) make this compound a strong candidate for pharmaceutical development, paving the way for the development of novel T-cell targeted immunotherapeutics.

## Section 5.6 : Figures



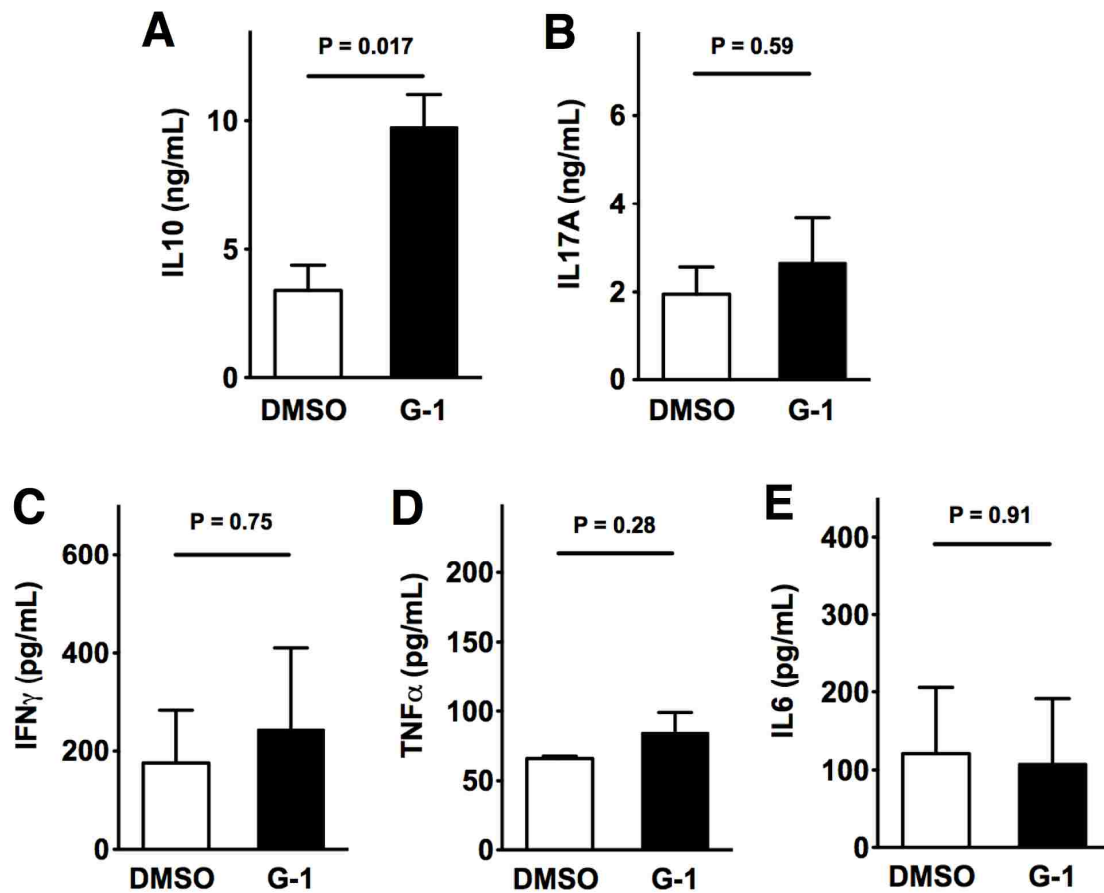
**Figure 19 : The GPER agonist G-1 induces IL10 production from CD4<sup>+</sup> T cells.**

CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and cultured for 4 days *ex vivo* with various combinations of TGFβ, IL6, and IL23, and supplemented with 100nM G-1 or vehicle (DMSO, control). Cells were subsequently stained for intracellular IFN $\gamma$ , IL17A, and IL10, then analyzed by flow cytometry. (A) Representative plots from the various conditions showing intracellular IL17A and IL10. (B) Quantification of data from five to seven independent experiments showing relative number of total IL10<sup>+</sup> cells and total IL17A<sup>+</sup> cells cultures treated with TGFβ + IL6. (C) Quantification of data from four to seven independent experiments showing relative number of total IL10<sup>+</sup> cells and total IL17A<sup>+</sup> cells cultures treated with TGFβ + IL6 + IL23. (D) Quantification of the number of IFN $\gamma$ <sup>+</sup> cells in cultures stimulated with CD3/28 in non-polarizing conditions (i.e. without the addition of any cytokines). P values determined by student's t-test; \* p<0.05; \*\*\* p<0.0005. Error bars = S.E.M; NS: not significant.



**Figure 20 : G-1 does not alter the number of IFN $\gamma$ <sup>+</sup> cells.**

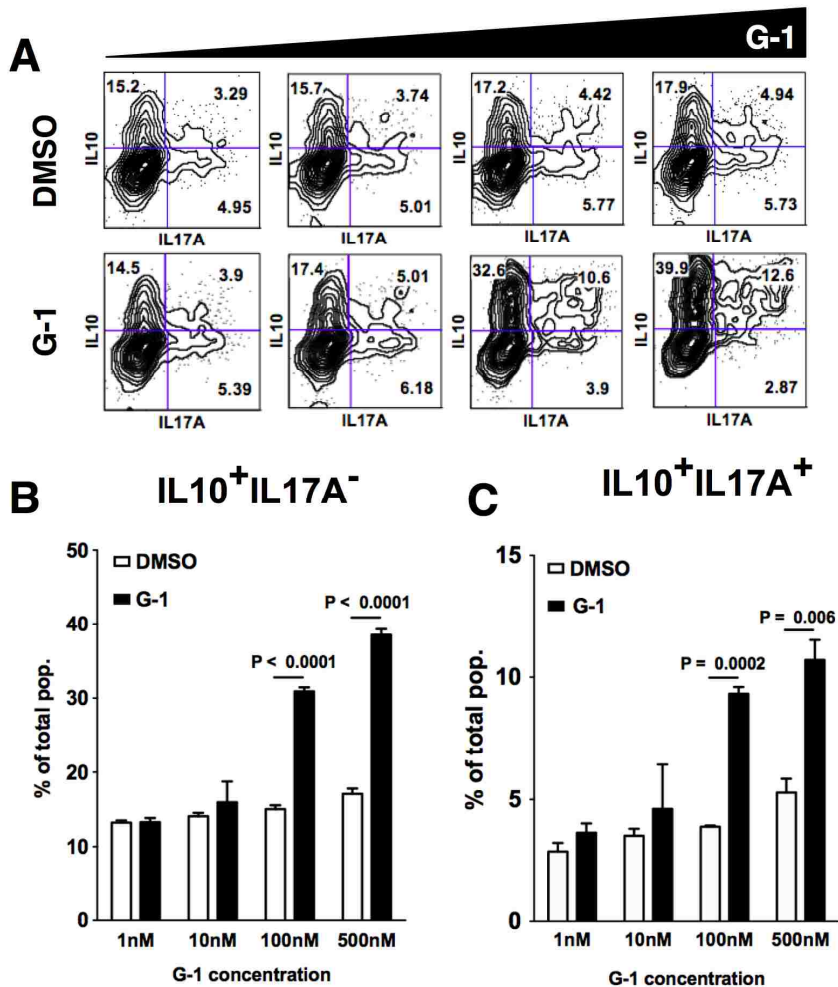
CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and cultured for 4 days *ex vivo* with various combinations of TGF $\beta$ , IL6, and IL23, and supplemented with 100nM G-1 or vehicle (DMSO, control). Cells were subsequently stained for intracellular IFN $\gamma$ , IL17A, and IL10, then analyzed by flow cytometry. (A) Representative plots from the various conditions showing intracellular IFN $\gamma$  and IL10.



**Figure 21 : G-1 increases IL10 secretion from T cells, but not proinflammatory cytokines.**

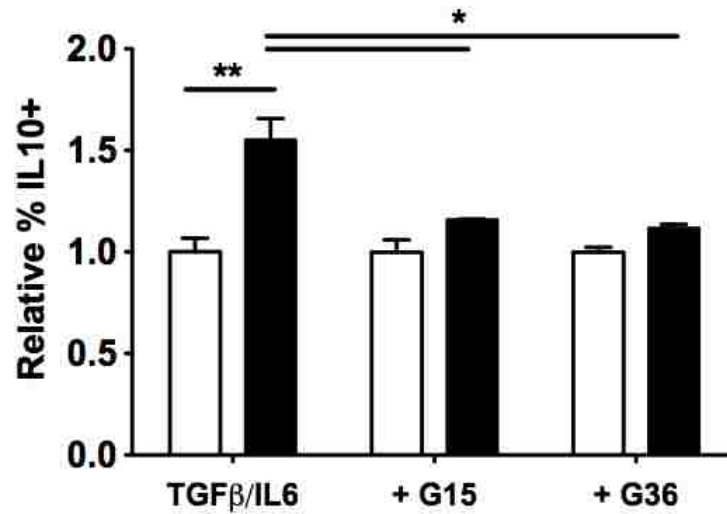
CD4<sup>+</sup>CD62L<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and cultured for 4 days *ex vivo* with anti-CD3/28 + IL6 + TGF $\beta$  in the presence of 100nM G-1 (black bars) or DMSO (white bars). Cells were washed on day 4 and re-stimulated with antiCD3/28 alone. Culture medium was collected after 24 hours and analyzed for the presence of secreted (A) IL10, (B) IL17A, (C) IFN $\gamma$ , (D) TNF $\alpha$ , (E) and IL6 by Luminex multiplex assay. Data are the means from three independent experiments done in triplicate. P values determined by student's t-test. Errors bars = S.E.M.





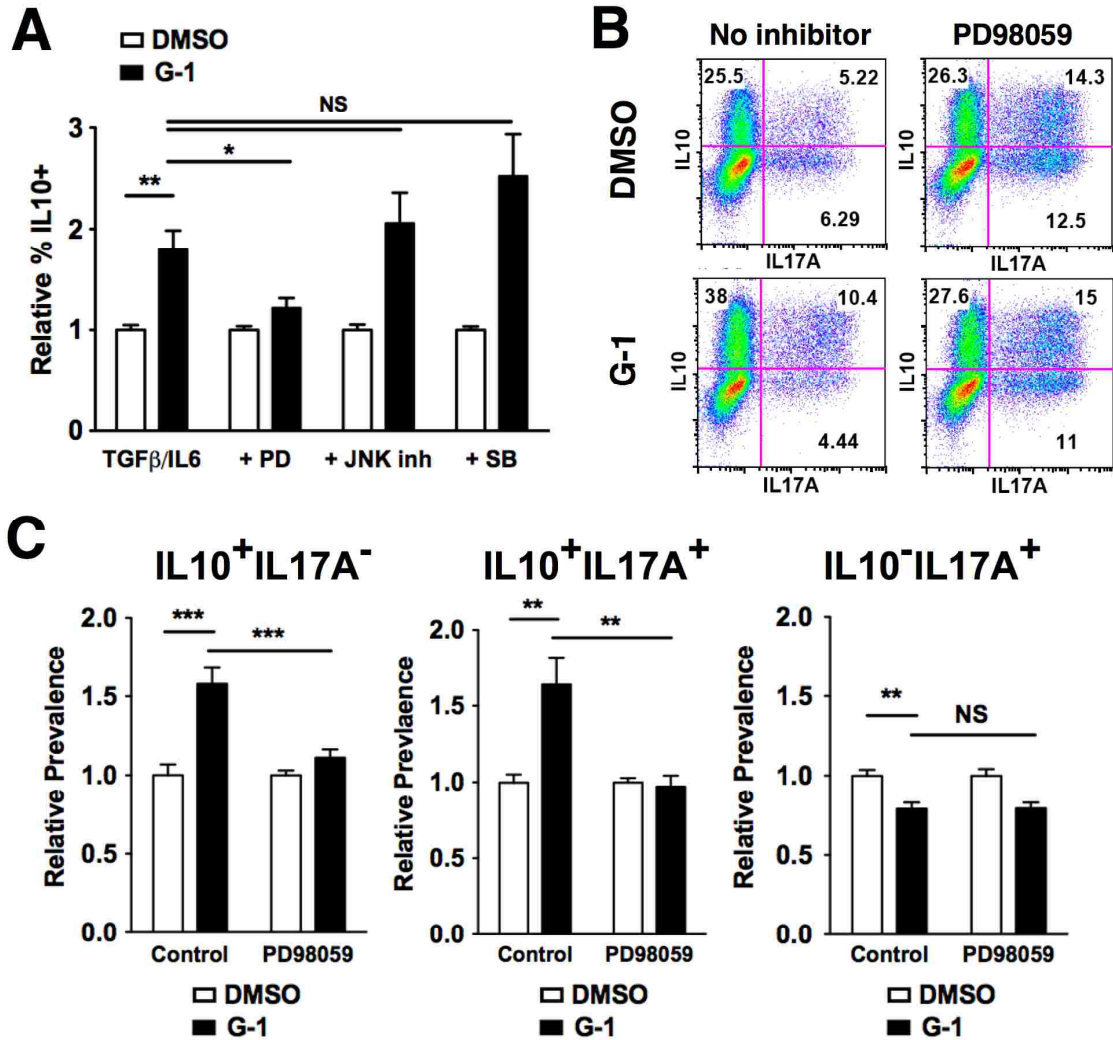
**Figure 22 : G-1 induces IL10 expression in IL17A<sup>+</sup> cells.**

CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> or CD4<sup>+</sup>CD62L<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and cultured for 4 days *ex vivo* with anti-CD3/28 + IL6 + TGFβ. Increasing doses of G-1 (1 – 500nM, black bars) or equivalent amounts of vehicle (DMSO, white bars) were added. Cells were subsequently stained for intracellular IL17A and IL10, and analyzed by flow cytometry. (A) Representative plots from the various conditions showing intracellular IL17A and IL10. (B-C) Quantitation of data from one of two independent experiments showing the percent of cells that are (B) IL10+IL17A<sup>-</sup> and (C) IL10+IL17A<sup>+</sup> for the given conditions. P values determined by student's t-test. Errors bars = S.D.



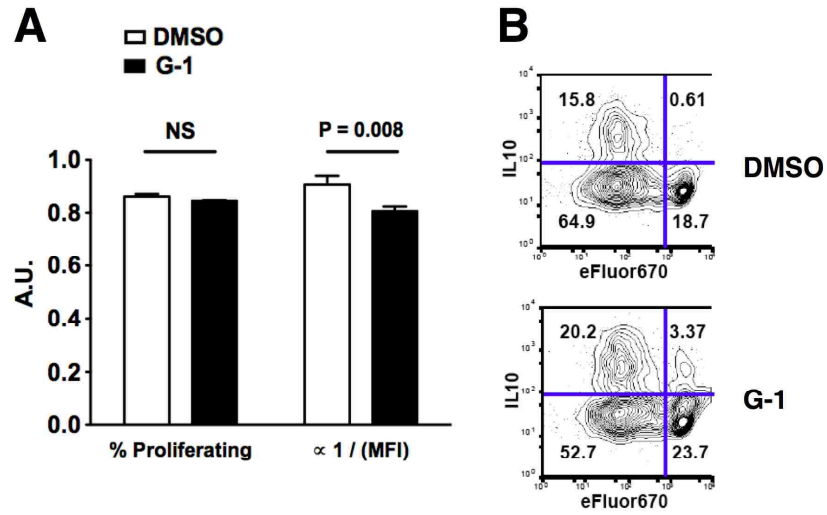
**Figure 23 : G-1-mediated IL10 production is blocked by GPER antagonists.**

CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> or CD4<sup>+</sup>CD62L<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and cultured for 4 days *ex vivo* with anti-CD3/28 + IL6 + TGFβ, and cultures were pretreated with the GPER antagonist G15 or G36. Summary of data from two independent experiments showing that the GPER-directed antagonists G15 and G36 can block G-1-mediated IL10 induction within CD4<sup>+</sup> T cells. P values determined by student's t-test; \* p<0.05; \*\* p<0.005. Error bars = S.E.M.



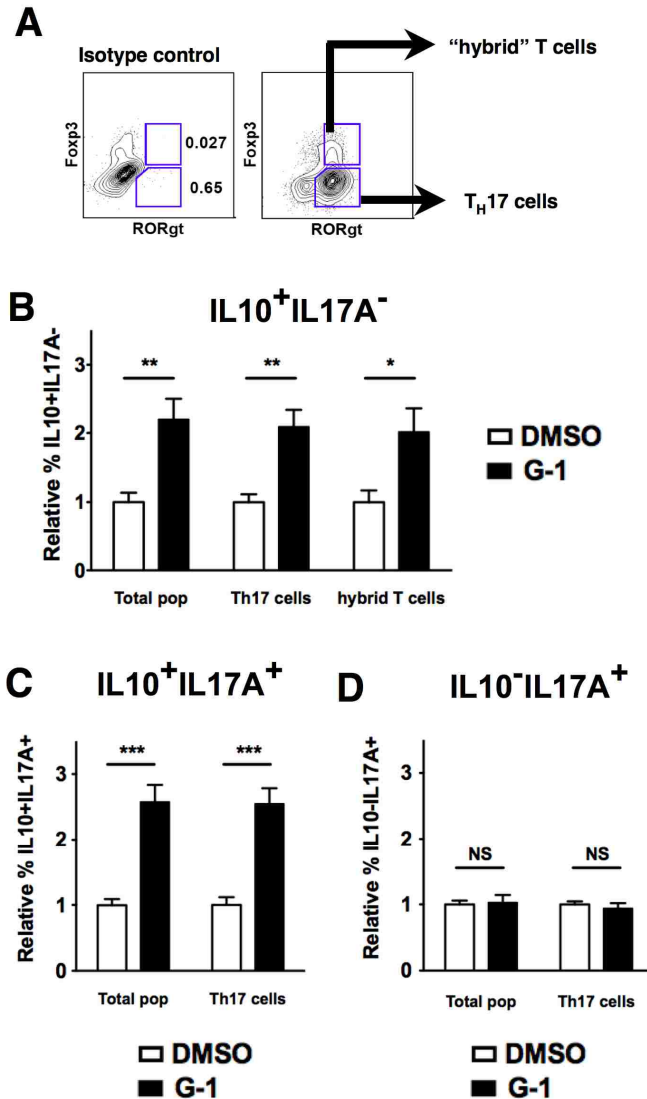
**Figure 24 : G-1-induced IL10 production is ERK dependent.**

CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> naive CD4<sup>+</sup> T cells were collected by FACS and cultured for 4 days *ex vivo* with anti-CD3/28 + IL6 + TGFβ in the presence of 100nM G-1 (black bars) or DMSO (white bars). Cultures were supplemented with inhibitors of the ERK (PD98059), JNK (JNK II inhibitor), or p38 (SB203580) signaling cascades. Following culture, cells were collected and stained for intracellular IL10 and IL17A, and analyzed by flow cytometry. (A) Graphs represent summary of data from three independent experiments. (B) Representative plots from one of four independent experiments with PD98059. (C) Summary of data from four PD98059 experiments analyzing induction within populations expressing combinations of IL10 and IL17A. P values determined by student's t-test. \* p<0.05; \*\* p<0.005; \*\*\* p<0.0005. Errors bars = S.E.M.



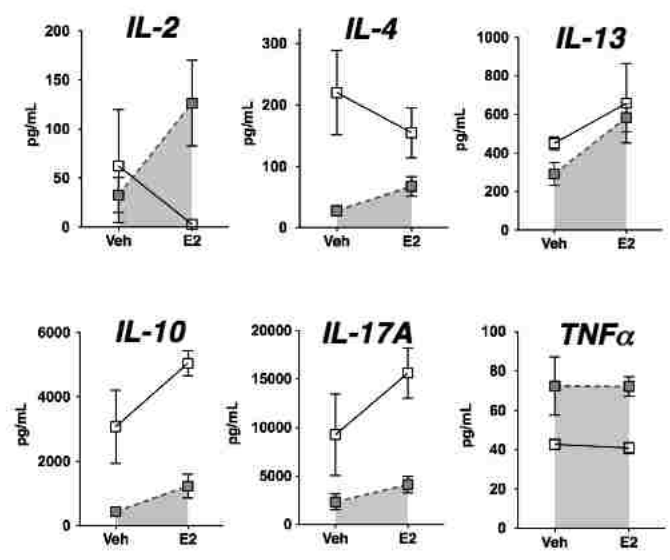
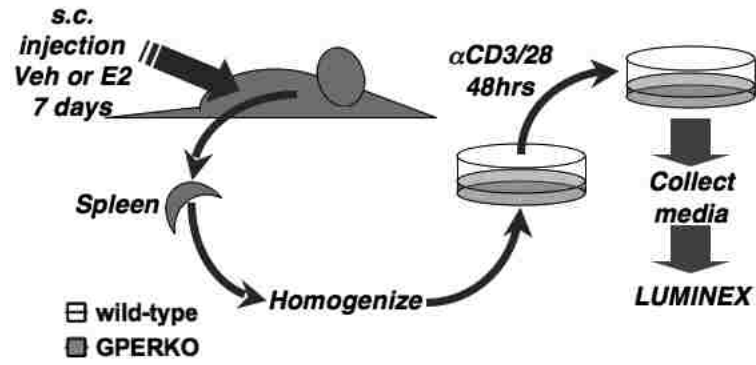
**Figure 25 : G-1 effects are not dependent on proliferation.**

CD4<sup>+</sup>CD62L<sup>hi</sup> naive CD4<sup>+</sup> T cells were collected by FACS and stained with the proliferation dye eFluor670 (eBiosciences) prior to culture. Following differentiation for 4 days *ex vivo* in culture with anti-CD3/28 + IL6 + TGF $\beta$  in the presence of 100nM G-1 (black bars) or DMSO (white bars), cells were stained for intracellular IL10. (A) Percent of cells proliferating and the inverse of GMFI, a measure of total proliferation. (B) Sample plots showing IL10 expression and eFluor670 staining. The upper right quadrant shows cells expressing IL10 without evidence of proliferation. Data show one of two independent experiments. Error bars = S.D. NS = not significant.



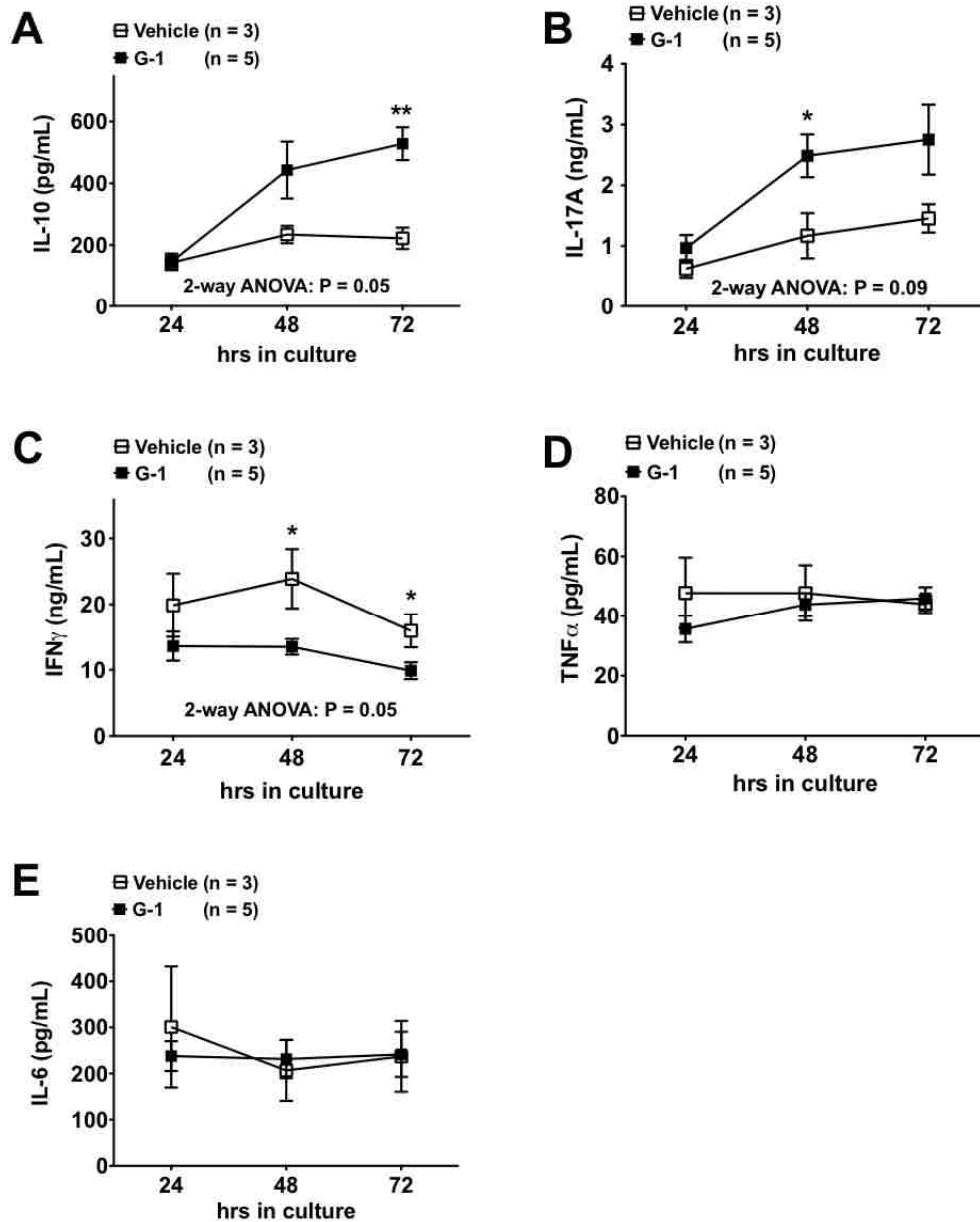
**Figure 26 : G-1 induces IL10 production within the hybrid T cell population.**

CD4<sup>+</sup>CD62L<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS from Foxp3<sup>egfp</sup> mice and cultured for 4 days *in vitro* with anti-CD3/28 + IL6 + TGFβ1 in the presence of 100nM G-1 (black bars) or DMSO (white bars). Cells were collected and stained for intracellular IL10, IL17A, and RORγt, and analyzed by flow cytometry. Cells that were Foxp3<sup>+</sup> RORγt<sup>+</sup> were designated as hybrid T cells, while those that were Foxp3<sup>-</sup> RORγt<sup>+</sup> were designated as T<sub>H</sub>17 cells. (A) Gating logic to determine hybrid T cell and T<sub>H</sub>17 populations. (B-D) Graphs represent summary of data from three independent experiments showing the relative number of (B) IL10<sup>+</sup>IL17A<sup>-</sup>, (C) IL10<sup>+</sup>IL17A<sup>+</sup>, and (D) IL10<sup>-</sup>IL17A<sup>+</sup> populations. Error bars = S.E.M. \* p<0.05; \*\* p<0.005; \*\*\* p<0.0005. NS, not significant.



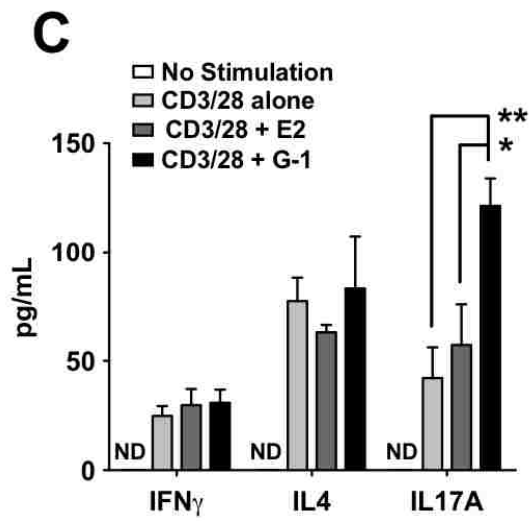
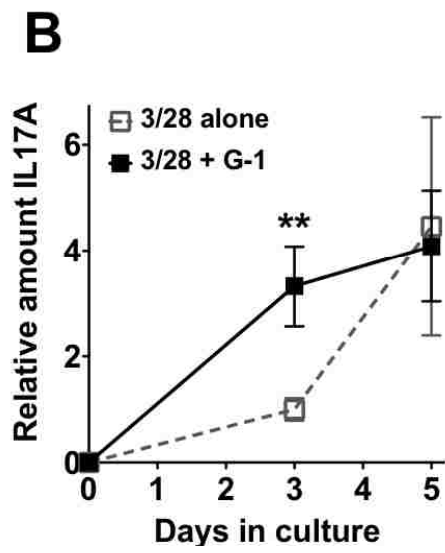
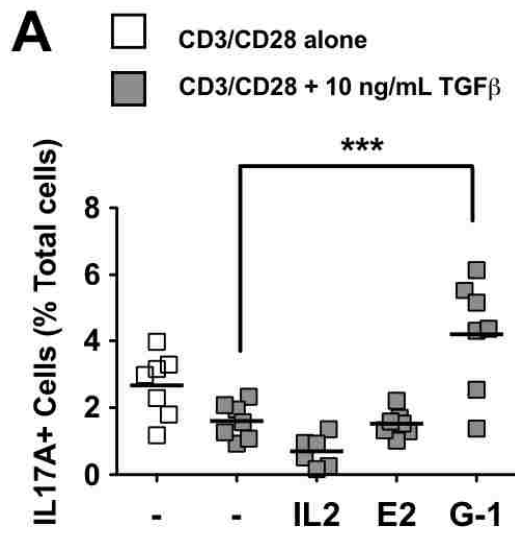
**Figure 27 : IL10 and IL17A secretion is reduced from GPERKO splenocytes.**

Seven to eleven week old male wild-type or GPERKO C57BL/6 mice were injected with estrogen (E2 – 500ng/day) or vehicle for 7 consecutive days. One day following the last injection, splenocytes were collected and cultured in the presence of antiCD3 $\epsilon$  (1.0  $\mu$ g/mL) and antiCD28 (2.5  $\mu$ g/mL) Ab. Culture medium was collected after 48 hours and analyzed for the presence of secreted IL2, IL4, IL13, IL10, IL17A, and TNF $\alpha$  by Luminex multiplex assay.. Graphs are mean data with three mice per group. Errors bars = S.E.M.



**Figure 28 : Cytokine secretion following *in vivo* treatment with G-1.**

Seven to eleven week old male wild-type C57BL/6 mice were injected with G-1 (5 $\mu$ g/day) or vehicle for 7 consecutive days. One day following the last injection, splenocytes were collected and cultured in the presence of antiCD3 $\epsilon$  (1.0  $\mu$ g/mL) and antiCD28 (2.5  $\mu$ g/mL) Ab. Culture medium was collected after 24, 48, and 72 hours and analyzed for the presence of secreted IL6, IL10, IL17A, IFN $\gamma$ , and TNF $\alpha$  by Luminex multiplex assay. Graphs are mean data with three to five mice per group. Errors bars = S.E.M. P values determined by student's t-test, except where 2-way ANOVA is indicated. \* = P  $\leq$  0.05, \*\* = P  $\leq$  0.01.

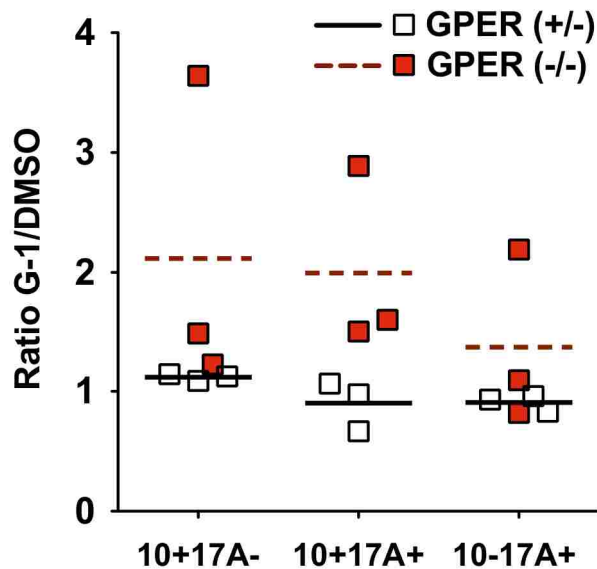




**Figure 29 : G-1 transiently induces IL17A from total CD4<sup>+</sup> cells in culture.**

(A) CD4<sup>+</sup>CD62L<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS from C57BL/6 mice and cultured for 4 days *ex vivo* with anti-CD3/28 antibody, both with (grey squares) and without (white squares) TGFβ (10 ng/mL). Cultures were supplemented with rIL2 (100 U/mL), E2 (10nM), or G-1 (100nM) as indicated. Cells were collected and stained for intracellular IL17A then analyzed by flow cytometry. Summary of data from three independent experiments. (B) CD4<sup>+</sup> cells were enriched by magnetic bead sorting (AutoMACS) and stimulated in culture with anti-CD3/28 supplemented with G-1 (100nM: black boxes with solid black line) or without (white boxes with dashed grey line). Culture medium was collected following 3 days and 5 days of stimulation, then analyzed for secreted IL17A by Luminex multiplex assay. Summary of data from 2 independent experiments. (C) CD4<sup>+</sup> cells were enriched by magnetic bead sorting (AutoMACS) and cultures; with media only (white bars), with anti-CD3/28 only (light grey bars), with anti-CD3/28 + 10nM E2 (dark grey bars), or with anti-CD3/28 + 100nM G-1 (Black bars). Culture medium was collected after 3 days of stimulation and analyzed for IL4, IL17A, and IFN<sub>γ</sub> secretion by Luminex multiplex assay. Example from one of two independent experiments. Errors bars = S.E.M. P values comparing two points were determined by student's t-test, P values comparing G-1 and vehicle curves were determined by 2-way ANOVA as indicated. \* = P ≤ 0.05, \*\* = P ≤ 0.01, \*\*\* = P ≤ 0.005.

NOTE: Figure 29A is the same as Figure 9A in preliminary data.



**Figure 30 : G-1 induces IL10 expression in GPERKO cells, but not GPERhet cells.**

CD4<sup>+</sup>CD62L<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS from GPERhet x Foxp3<sup>egfp</sup> or GPERKO x Foxp3<sup>egfp</sup> mice and cultured for 4 days *in vitro* with anti-CD3/28 + IL6 + TGFβ in the presence of 100nM G-1 or DMSO. Cells were collected and stained for intracellular IL10 and IL17A by flow cytometry. The graph shows the ratio of a given population (indicated along the X-axis) observed in G-1 treated cultures relative to cultures treated with DMSO alone. Naïve T cells from GPERhet (clear, white boxes) and GPERKO mice (dark, red boxes) were collected and stimulated the same day for a given experiment. Each box indicates the mean from one individual experiment done in duplicate or triplicate, with three independent experiments shown.

## **Chapter 6 : Data from the T cell-mediated colitis model**

### ***Section 6.1 : Preface***

Perhaps the most attractive model to investigate the therapeutic potential of G-1 is the T cell mediated colitis model. While the T<sub>H</sub>17 population has been widely discussed for its proinflammatory attributes and its role in autoimmunity, there is an emerging story which suggests these cells exhibit regulatory properties in certain settings through the activity of IL17A, perhaps best exemplified by the pleiotropic effects of IL17A in the setting of autoimmune colitis, as discussed in the introduction (Section 1.3). Additionally, as has been discussed previously, IL10 has been shown to play a role in immune homeostasis at environmental interfaces, such as the gut, and is effective in suppressing animal models of colitis. Therefore, in this chapter we investigated G-1's ability to function prophylactically and therapeutically in an animal model of T cell-mediated colitis.

### ***Section 6.2 : Introduction***

Inflammatory bowel disease (IBD) is characterized by inflammation and ulceration of the gastrointestinal mucosa (Podolsky, 2002), and numerous lines of evidence have linked T cell to disease pathogenesis and prevention. The importance of Foxp3<sup>+</sup> cells in mucosal immunity is highlighted by patients with

immunodeficiency, polyendocrinopathy, and enteropathy X-linked (IPEX) syndrome, at least 50% of whom harbor a mutation in the *Foxp3* gene (van der Vliet and Nieuwenhuis, 2007). These patients present clinically with watery diarrhea due to villous atrophy and severe intestinal inflammation (Patey-Mariaud de Serre et al., 2008). Experimentally, T<sub>REGS</sub> suppress the colitis associated with adoptive transfer of pathogenic T cells into lymphopenic hosts (Yuan et al., 2007). Some of their suppressive function is mediated by the cytokine IL10, which reduces the proinflammatory activity of numerous immune populations (Maynard and Weaver, 2008). Furthermore, IL10 is essential to gut immune homeostasis, because IL10KO mice exhibit spontaneous colitis (Etling et al., 2007), and loss of IL10 production by Foxp3<sup>+</sup> T<sub>REGS</sub> leads to mucosal inflammation (Rubtsov et al., 2008).

T<sub>H</sub>17 cells have also been linked to IBD (Harrington et al., 2005; Ivanov et al., 2006). Given the established pro-inflammatory activity of IL17A/F, it is attractive to hypothesize that T cell-mediated mucosal inflammation is determined by the balance between Tregs and T<sub>H</sub>17 cells. In reality, the role of T<sub>H</sub>17 cells in colitis has proven controversial. Patients with UC express high levels of IL17 in the intestinal mucosa (Kobayashi et al., 2008), large numbers of activated T<sub>H</sub>17 cells have been found in lesions from CD patients (Kleinschek et al., 2009), IL23KO mice are protected from colitis (Yen et al., 2006), and two studies have identified IL23R gene (*Il23r*) variants that protect against IBD (Dubinsky et al., 2007; Duerr et al., 2006). However, there is evidence IL17A is actually protective in both a T cell independent (Ogawa et al., 2004) and T cell

dependent (O'Connor et al., 2009) model of IBD. Moreover, one of the major contributing factors to IBD pathogenesis is compromised epithelial barrier function along the GI tract. It is thought that loss of tight junction integrity between the epithelial cells lining the gut lumen allows commensal flora access to immune mediators within the mucosal wall where they elicit a robust inflammatory response (Yu et al., 2004). Whether this is a precipitating event or simply contributes to the chronic stages of IBD is still debated, though numerous animal models provide clear evidence that transient disruption of barrier function can induce colitis (Sollid and Johansen, 2008).  $T_H17$  cells can impact the gut wall integrity through production of the cytokine IL22 (Ouyang and Valdez, 2008), which acts on IECs to promote mucosal wound healing in a STAT3-dependent manner (Pickert et al., 2009). Interestingly, recent work demonstrated that IL23 enhances colitis by blocking  $Foxp3^+$   $T_{REG}$  activity (Izcue et al., 2008). IL23 also drives production of the  $T_H1$ -specific cytokine  $IFN\gamma$  in the GI mucosa (Hue et al., 2006). This is important as there is clear evidence that  $T_H1$  responses can drive colonic inflammation. Adoptive transfer of pathogenic T cells from  $IFN\gamma$ KO mice does not induce colitis (Ito and Fathman, 1997), and  $IFN\gamma$  neutralization abrogates disease in this model (Powrie et al., 1994). These results appear to translate to human disease as IL23-mediated  $IFN\gamma$  production has been correlated with the chronic inflammation associated with CD (Kamada et al., 2008), and elevated levels of  $IFN\gamma$  have been detected in the intestinal mucosa of IBD patients (Breese et al., 1993). Thus, it may be that IL23-mediated  $T_H1$  are responsible for the T cell component of IBD.

As mentioned in Section 1.3, there is an intriguing link between the developmental pathways for Foxp3<sup>+</sup> T<sub>REGS</sub> and T<sub>H</sub>17 cells. Like other settings within the body, the decision between cell types depends on the other signals present (Samanta et al., 2008; Sauer et al., 2008; Zhang et al., 2008). For example, ATP from gut flora (Atarashi et al., 2008) or LPS in the presence of apoptotic cells (Torchinsky et al., 2009) appears to aid T<sub>H</sub>17 development in the colonic lamina propria.

In the previous chapters we showed that the GPER-directed agonist G-1 can drive Foxp3 and IL10 expression in polyclonally stimulated naïve T cells. We chose to investigate whether these findings translated to the *in vivo* setting using the T cell-mediated colitis model of IBD. The reason for selecting this model was based on the fact that the adoptive transfer step allowed us to treat cells in culture prior to injection into recipient mice and, if needed, we could control the genotype of the donor T cells specifically without affecting the genotype of the recipient animal. Moreover, IL17A and IL10 have both been shown to contribute in T<sub>REG</sub>-mediated suppression of colonic inflammation in this model. To our surprise, G-1 was unable to prevent the development of colitis following *ex vivo* treatment of donor cells, or by sustained systemic treatment prior disease induction. However, treatment of animals with clinically apparent wasting disease with G-1 led to a rapid increase in the rate of disease progression, which correlated with a decrease in the number of IL17A<sup>+</sup> cells within the mucosal immune system of the gut. These findings suggest that G-1-mediated regulation

of T cell cytokine production may not be a viable treatment in the setting of colitis, and other models will need to be explored.

### **Section 6.3 : Results**

Prior to initiating these studies, we wanted to determine if we could detect IL10 induction within cells used to induce disease. Thus, CD4<sup>+</sup>CD45RB<sup>hi</sup> cells were collected by FACS and stimulated with antiCD3/28 Ab in the presence of TGFβ and IL6, supplemented with DMSO or 100nM G-1. We detected an increase in the number of IL10<sup>+</sup> cells within the G-1 treated cultures, which correlated with our findings in Chapter 5 for CD4<sup>+</sup>CD62L<sup>hi</sup> naïve T cells (data not shown). Thus, we moved forward with our *in vivo* experiments.

#### **Treatment of T cells with G-1 in culture does not alter colitis**

To investigate the *in vivo* suppressive properties of G-1 treated naïve T cells, CD4<sup>+</sup>CD45RB<sup>hi</sup> naïve T cells were collected and stimulated as before, namely plus or minus G-1 in the presence of TGFβ and IL6. Following 4 days in culture, DMSO or G-1 treated cells were injected into Rag1KO recipient mice to test for any variability in the induction of colitis. Given the fact that T cells cultured in the presence of TGFβ and IL6 exhibit reduced pathogenic potential in the animal model EAE due to attenuated recruitment of proinflammatory leukocytes and can confer bystander protection through the secretion of IL10 (McGeachy et al., 2007), it was possible that the mice from both groups would fail to develop

colitis. However, we observed a typical clinical course in mice treated *ex vivo* with TGF $\beta$  and IL6 (**Figure 31**). We predicted that if G-1-mediated IL10 induction is stable following transfer and expansion *in vivo*, then mice injected with G-1 treated cells should demonstrate a more indolent disease course and/or a reduction in disease severity relative to the DMSO treated control cells. To our surprise, G-1 treated cells were capable of driving disease onset and progression with identical characteristics as the DMSO treated cells (**Figure 31**), despite the known protective effects of IL10 in this model. There are two possible interpretations of this data; either (a) G-1-mediated IL10 induction is not stable following transfer into lymphopenic mice, or (b) the amount of IL10 secreted is insufficient to confer bystander protective effects in this model.

### **Systemic treatment with G-1 does not inhibit colitis**

To determine if the failure of G-1-treated cells was the result of changes in effector phenotype following homeostatic proliferation, we chose to treat recipient mice with systemic G-1 immediately after adoptive transfer of freshly sorted CD4<sup>+</sup>CD45RB<sup>hi</sup> pathogenic naïve T cells. This should expose the cells to G-1 up to and following their *in vivo* expansion, and during their differentiation into effector lineages in the gut lamina propria, where many cells undergo T<sub>H</sub>17 differentiation, likely due to an abundance TGF $\beta$  and IL6. Previous work has demonstrated the ability of *in vivo* derived peptide-specific effector T cells from the draining lymph nodes of EAE mice to respond to TGF $\beta$  and IL6 by the stable production of IL10 (McGeachy et al., 2007), hence it was reasonable to



hypothesize that systemic G-1 would expand IL10 production *in vivo* in the setting of colitis, especially given G-1 is known to suppress EAE (Blasko et al., 2009; Wang et al., 2009a) and induce IL10 expression *in vivo* (Yates et al., 2010) and (**Figure 28**). In the first series of experiments G-1 was delivered using custom-made 60 day-release cholesterol pellets (either 1.125 mg or 0.225 mg), which have been previously used to effectively deliver both E2 and G-1 in the treatment of EAE (Wang et al., 2009a). The pellets were implanted 7 days prior to the adoptive transfer of T cells to ensure that wound had healed properly and to give time for the drug to begin being released. While this time frame was chosen based on previous experiences, it is important to note that we have no tool for directly analyzing the presence of drug circulating in the animals, and thus verify the effectiveness of this strategy. Animals implanted with G-1 pellets demonstrated slightly reduced weights relative to the vehicle treated animals (**Figure 32**), but a similar pattern was also noted in the non-diseased mice, suggesting a small effect of G-1 treatment on weight. This is consistent with previous data which showed increased body mass in male GPERKO mice (Ford et al., 2010). However, these data suggest that *in vivo* G-1 treatment with implanted pellets was not effective in preventing T cell-mediated colitis.

As we have no mechanism for tracking the release of G-1 from the pellets it was possible that there was not sufficient G-1 circulating at a critical stage following the adoptive transfer of the pathogenic T cell populations. One line of evidence supporting this hypothesis was our observations that the G-1 pellets never broke down, as compared to the sham control pellets which did on

occasion (data not shown). To verify our findings from the pellet experiments, mice were treated with 5  $\mu$ g of G-1 via daily subcutaneous injection for the first 21 days following transfer of freshly sorted T cells. This would ensure daily delivery of G-1 up until the typical onset of clinical symptoms, which usually present 3-4 weeks after ignition of disease. In agreement with our previous results, daily delivery of G-1 had no effect on disease course (**Figure 33**), even when the mice were followed for 9 weeks. This finding further demonstrates that G-1 cannot act to prevent disease in the T cell-mediated colitis model.

### **G-1 accelerates wasting disease when given therapeutically**

These data show that that G-1 treatment cannot prevent the onset of wasting disease in an adoptive transfer-based T cell-mediated colitis model of IBD. However, one final possibility is that effective treatment with G-1 requires ongoing T cell differentiation, and in particular the presence of T<sub>H</sub>17 polarizing conditions, as we observed for the induction of IL10 in Chapter 5 (**Figure 19**). If indeed there is an issue with the use of cholesterol pellets, perhaps specific to the Rag1KO mice, and treatment with G-1 prior to the onset of clinical symptoms was ineffective due to a lack of T<sub>H</sub>17 polarizing conditions, then it was possible that treatment of animals with fulminate colitis with G-1 would finally uncover a therapeutically viable activity of G-1 in this model. Along those lines, a cohort of Rag1KO mice were injected with colitogenic T cells and allowed to progress to the onset of clinical symptoms (**Figure 34**). Starting on day 35 post T cell injection, mice were treated with 5  $\mu$ g of G-1 via daily subcutaneous injection for

7 consecutive days, matching the paradigm used in Chapter 5 to demonstrate G-1-enhanced IL10 production from TCR activated splenocytes following systemic G-1 treatment (**Figure 28**). To our surprise, administration of G-1 in this context led to an immediate and significant increase in the development of wasting disease (**Figure 34**), as compared to the vehicle controls.

### **G-1 has distinct effects on the mucosal & systemic immune system**

To investigate what the underlying cause of this unexpected result might be, animals were sacrificed upon completion of the experiment and spleens, mesenteric lymph nodes (MLN), inguinal lymph nodes (ILN), and colonic lamina propria cells were collected for analysis of surface markers and intracellular cytokine expression (**Figure 35**). We were unable to detect any intracellular IL10 in any of the tissue samples (data not shown). As mentioned, IL17A is also protective in this model and has been observed to be modulated by G-1 *in vivo* (**Figure 28**), and *in vitro* (**Figure 8, 9 10, 19, & 22**). Thus, intracellular IL17A was analyzed (**Figure 35A**). We saw that mice treated with G-1 had an increase in the percentages of CD4<sup>+</sup>IL17A<sup>+</sup> cells in their ILNs, in agreement with our previous findings (**Figure 9, 10, and 22**), although it is important to note that unlike the majority of our *ex vivo* experiments these cells did not express IL10 (data not shown). Interestingly, when cells from the MLN were analyzed for IL17 expression, the opposite trend was observed, wherein G-1 treated mice had a lower percentage IL17A<sup>+</sup> cells within the CD4<sup>+</sup> compartment (**Figure 35A**). This result reached statistical significance. A similar pattern was noted in the colonic

lamina propria cells, although it never reached statistical significance. No difference in CD4<sup>-</sup>IL17A<sup>+</sup> cells was noted in any setting (**Figure 35A**). Our observation that augmentation of wasting disease in response to G-1 treatment correlated with reduced percentages of IL17A<sup>+</sup> cells within the mucosal immune system is consistent with a previous report using this same model which showed IL17AKO and IL17A receptor KO T cells impart a more severe disease course following adoptive transfer.

To determine if alterations in the expression of other proinflammatory cytokines may also be implicated in G-1's effects, cells from the various tissues were stained for IFN $\gamma$  (**Figure 35B**) and TNF $\alpha$  (**Figure 35C**). Staining for IFN $\gamma$  demonstrated that G-1 treated mice had lower percentages of CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells. As there is strong evidence that IFN $\gamma$  is important in promoting the development of colitis (Ito and Fathman, 1997), these data suggest that alterations in IFN $\gamma$  expression are not responsible for G-1 effects. However, the decrease in CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells correlates well with our findings in Chapter 5 where we observed a decrease in the secretion of IFN $\gamma$  in response to *ex vivo* TCR crosslinking from splenocytes exposed to G-1 *in vivo* (**Figure 28**). Staining for TNF $\alpha$  yielded results that were a bit more varied. While we observed no effects on TNF $\alpha$  secretion following systemic G-1 treatment in Chapter 5 (**Figure 28**), here we noted that there were fewer TNF $\alpha$ <sup>+</sup> cells in the CD4<sup>+</sup> T cell population (CD3<sup>+</sup>CD4<sup>+</sup> cells) from the colonic lamina propria of G-1 treated mice. Conversely, there were more TNF $\alpha$ <sup>+</sup> cells in the CD3<sup>-</sup>CD4<sup>-</sup> population from the MLN of G-1 treated mice (**Figure 35C**). Neither pattern reached statistical

significance ( $P > 0.05$ ). Collectively, these data show that modulation of  $\text{IFN}\gamma$  and  $\text{TNF}\alpha$  expression do not contribute to the G-1 effect observed in Figure 33.

Perhaps the most interesting observation from the entirety of this work was our identification of distinct and opposite compartment-specific effects following systemic administration of G-1 in the colitis model; specifically, increased percentages of  $\text{CD4}^+\text{IL17A}^+$  cells in the systemic immune system (ILN) of G-1 treated mice correlating with a decreased in the percentage of  $\text{IL17A}^+$  cells among the  $\text{CD4}^+$  population in the mucosal immune system (MLN) of the same animals. One possible explanation for the altered response to G-1 treatment in the mucosa is that naive T cells within the mucosa and its draining lymph nodes exist in a unique resting state due to the constant challenge of the commensal flora and the distinct milieu of immune mediators within the gut wall (see Introduction). To determine if the apparent reversal of G-1's effects within the mucosal immune system was reflective of a unique property intrinsic to T cells from this setting, naive T cells from the MLN were collected by FACS, stimulated in culture with antiCD3/28 antibody in the presence of  $\text{TGF}\beta + \text{IL6}$ , and supplemented with either 100nM G-1 or DMSO. MLN-derived naïve T cells responded to G-1 treatment with an increase in the number of  $\text{IL10}^+$  and  $\text{IL10}^+\text{IL17A}^+$  cells (data not shown), similar to what was observed when cells were collected from the spleen and other LN populations (**Figure 19 and 22**). However, the effect was much weaker than what was observed in our previous studies, and trend did not reach statistical significance. These data suggest that compartment-specific differences in G-1's effect on  $\text{IL17}^+$  T cell number may

reflect inherent differences in naïve T cell signaling and the response to G-1 within the local environment of the mucosal immune system.

Another possible explanation for the observed discrepancy in G-1 modulation of IL17A expression is that more than one CD4<sup>+</sup> population is responsible for our observation.  $\gamma\delta$  T cells express CD4, can produce IL17A, and are found in abundance in the lining of the gut (Xiong and Raulet, 2007). In fact, in naïve mice it appears that the majority of IL17A producing cells are of the  $\gamma\delta$  T cell variety. However, staining for the  $\gamma\delta$ TCR demonstrated that less than 2% of the cells within the MLN were in fact  $\gamma\delta$ TCR<sup>+</sup> (**Figure 35E**), and no  $\gamma\delta$ TCR<sup>+</sup>IL17A<sup>+</sup> cells were detected in either the colonic lamina propria or the MLN (**Figure 35E**). This is likely due to the fact that the majority of gut associated  $\gamma\delta$  T cells are part of the intraepithelial lymphocyte population which reside within and immediately underneath the ICE population lining the gut lumen (see Section 1.2). These cells are lost during the process of purifying lamina propria cells (Ostanin et al., 2009). NKT T cells are also CD4<sup>+</sup> and are capable of producing IL17A (Rachitskaya et al., 2008). However, similar to our results for  $\gamma\delta$  T cells, staining for NK1.1 and IL17A demonstrated that this population did not vary between treatment groups, and was likely too small to account for our observations (**Figure 35D/E**). Another CD4<sup>+</sup> population known to produce an abundance of IL17A are the lymphoid tissue inducer-like (LTi) cells (Takatori et al., 2009). However, these cells are distinguished from T cell populations based on their lack of the TCR co-receptor CD3. However, we were unable to detect any CD3<sup>-</sup>CD4<sup>+</sup> cells in any tissue tested (**Figure 35E** and data not shown). It is

also worth noting that we did not observe any changes in the relative number of any of the major immune populations (**Figure 35E**). Collectively, these data show that the  $CD4^+IL17A^+$  population shown in Figure 33 are  $T_H17$  cells and not another IL17A producing cell line.

One final possibility we investigated was the hypothesis that the differential effects of G-1 in Figure 33 were in fact secondary to effects on other critical immune populations within the gut, most notably dendritic cells (DCs) and macrophages. Work from Bali Pulendran's lab has shown that by using surface expression of CD11b and CD11c as markers, one can identify populations of APCs within the gut lamina propria that promote either  $T_H17$  or  $T_{REG}$  differentiation (Denning et al., 2007). If G-1 was reducing the number of  $T_H17$ -promoting DCs ( $CD11b^+CD11c^+$ ), it may account for the reduction in  $T_H17$  cells observed in diseased mice following treatment with G-1. Cells from the MLN and lamina propria from G-1 and control treated mice were stained for CD11b and CD11c, and their relative numbers analyzed. We noted a statistically significant decrease in the number of  $T_{REG}$  promoting  $CD11b^-CD11c^+$  DCs, which correlated with an increase in the number of  $T_H17$ -promoting  $CD11b^+CD11c^+$  DCs (**Figure 35F**). The increased proportion of  $T_H17$ -promoting DCs suggested that the reduction in  $T_H17$  was not due to changes in this population. However, we did not evaluate the actual function of any APC population, and thus drawing firm conclusions must be done with caution. In addition, the observation that G-1 may reduce the number of  $T_{REG}$ -promoting DCs suggests that a loss of  $T_{REG}$ s may underlie the inability of G-1 to protect against colitis. While we could not detect

any IL10 expressing cells in the tissues tested (data not shown), we did not analyze Foxp3 expression. Thus it remains a possibility that alteration of T<sub>REG</sub> number or function play a critical role in our observations.

### ***Section 6.4 : Discussion***

In this chapter we attempted to build on our findings from Chapter 4 and Chapter 5 by investigating the utility of G-1 in the treatment of T cell-mediated colitis, an established animal model of IBD. It was interesting that cells cultured *ex vivo* with TGF $\beta$  and IL6 were capable of eliciting a similar disease pattern as compared to cells that were injected immediately after collection. In Chapter 5 we demonstrated that cells stimulated in this fashion expressed IL10 and produced IL10 upon re-stimulation (**Figure 19 and Figure 21**), in agreement with previous studies using peptide-specific T cells from EAE mice (McGeachy et al., 2007). We also confirmed the ability of the specific naïve T cell population used to induce TCMC also showed the ability to up-regulate IL10 (data not shown). The unabridged ability of these cells to drive colitis following transfer may be due to changes in their effector properties as they undergo homeostatic proliferation. The studies by McGeachy et al, which initially identified the IL10-producing capacity of TGF $\beta$  and IL6 treated cells and their IL10-dependent bystander suppressive activity, were conducted using the EAE model in which the recipient mice have an intact immune system, and thus transferred cells were not subject to



homeostatic proliferation following transfer. Thus this could account for the differences between our data and their findings.

Given that the G-1 treated cells induced the same disease pattern as the DMSO treated cells, another possibility was that we were no longer observing IL10 induction due to subtle changes in our culture protocol, for example the new operator of the MoFlo sorter used to collect the naïve T cell population or a different lot of one of the key cytokines. This was partly based on the fact that a few of the *ex vivo* culture experiments with CD4<sup>+</sup>CD62L<sup>hi</sup> cells that we ran around that same time had also shown no induction of IL10. Subsequent analysis of IL10 induction in CD4<sup>+</sup>CD45RB<sup>hi</sup> cells demonstrated a loss of G-1-mediated effects in this population as well (data not shown). The issue with our CD4<sup>+</sup>CD62L<sup>hi</sup> cells was resolved by changing the batch of G-1 being used. However, even after restoring G-1-driven IL10 induction within the CD4<sup>+</sup>CD62L<sup>hi</sup> population, we continued to get variable results with the CD4<sup>+</sup>CD45RB<sup>hi</sup> cells. As of yet, it is unclear why. Thus the possibility remains that this particular population of T cells simply do not produce sufficient IL10 to resolve any G-1-mediated effects.

Another possible scenario is that G-1 treatment effects the function of IECs in the setting of colonic inflammation, affecting barrier integrity or the production of other immunomodulatory factors from this population. Additionally, while we observed a reduction in the ratio of T<sub>REG</sub> to T<sub>H17</sub> promoting DCs in Figure 34F, we didn't actually check the function of any of the APC populations within the gut. Future experiments comparing the ability of CD11c+ populations

from the MLN and lamina propria of diseased mice treated with either vehicle or G-1. The reduction in T<sub>REG</sub>-promoting DCs also warrants further investigation. It may be that in addition to reducing the number of T<sub>H</sub>17 cells, G-1 treatment of diseased mice leads to a reduction in the number of T<sub>REG</sub> cells within the MLN or the gut wall, further exacerbating the chronic inflammation associated with the disease. Experiments looking at Foxp3 expression in G-1 treated mice will help answer this question.

Finally, the pharmacodynamics and pharmacokinetics of G-1 are at this point unknown, so it is unclear how G-1 is metabolized *in vivo*, and where it tends to accumulate. It is possible that rapid metabolization of G-1 yields products that lack the functions that we delineated *ex vivo* in tissue culture dishes. There is also the possibility that G-1 itself does not efficiently accumulate in the mucosa. Along those lines, it would be interesting to test whether oral G-1 or perhaps oral ethinyl estradiol (EE), which has been used to effectively attenuate the development of EAE, would be prophylactically or therapeutically effective in the T cell-mediated colitis paradigm.

There were some insightful results that came out of this study as well. The fact that we observed a reversal of G-1 effects in terms of IL17A induction in the MLN of diseased mice (relative to ILN within the same mice and the bulk of our *ex vivo* data) does suggest that G-1 can behave differently in different contexts. We attempted to determine if there was a detectable difference in naïve T cells from the MLN relative to cells collected from other secondary lymphoid tissue, utilizing the *ex vivo* T cell differentiation paradigm employed widely in Chapter 5,

however there were no apparent differences in G-1-mediated induction of IL10<sup>+</sup> and IL10<sup>+</sup>IL17A<sup>+</sup> cells from this population. Moving forward, it would be instructive to collect T cells from the draining lymph nodes and lamina propria of mice with fulminate colitis and see if the same pattern of IL10 and IL17A induction occurs following *ex vivo* stimulation. In this case, both naïve T cells and activated/memory T cells would need to be evaluated. While we did note that cultured CD4<sup>+</sup>CD44<sup>hi</sup> memory cells also respond to G-1 treatment with an increase in the number of IL10<sup>+</sup> cells (data not shown), it is unclear that such cells would behave similarly when extracted from the setting of ongoing colonic inflammation.

There is also the possibility that rather than affecting the differentiation of IL17A producing cells, systemic G-1 treatment affects the expression of different chemokine receptors on a subset of T cell populations, altering their trafficking. Blasko et al noted decreased production of the chemokines CCL4 and CCL5 following antigen-specific stimulation of cells from the draining lymph nodes of EAE mice (Blasko et al., 2009). This provides evidence that G-1 can alter chemokine secretion. It is possible that the reason we saw increased CD4<sup>+</sup>IL17A<sup>+</sup> cells systemically while at the same time noting fewer in the mucosa was due to altered trafficking of this population, perhaps via down regulation of CCR6 or CD103 (see Section 1.3). Further studies looking at G-1-mediated effects on trafficking molecules *in vivo* will be instructive to addressing these questions.

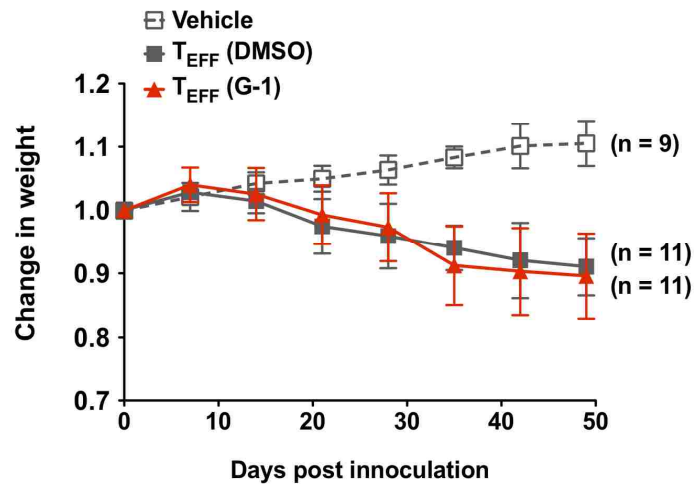
In summary, these data show that systemic treatment with G-1 cannot treat fulminate colitis, prevent the onset of disease, or imprint a stable suppressive phenotype on colitogenic T cell populations following *ex vivo* treatment.

### ***Section 6.5 : Conclusions***

Despite the compelling evidence that G-1 can drive IL10 production from CD4<sup>+</sup> T cell populations and expand the number of Foxp3<sup>+</sup> cells, we were unable to delineate any conditions in which this drug could effectively suppress CD4<sup>+</sup> T cell-mediated colitis. Cells treated with G-1 *ex vivo* failed to demonstrate restrained disease development, let alone be protective in this model, as was predicted. It is possible that the variable induction of IL10 within the colitogenic CD4<sup>+</sup>CD45RB<sup>hi</sup> cells was partly to blame for the failure (data not shown), or that G-1-mediated IL10 expression is not stable during the homeostatic proliferation that follows adoptive transfer of the cells. In addition, systemic treatment with G-1 immediately following adoptive transfer of colitogenic T cells conferred no appreciable protection from disease, even when 60 day release pellets were utilized. There is a distinct possibility that G-1 or its metabolites do not accumulate within the colonic lamina propria, rendering blood-borne delivery useless. It would be interesting to see if oral delivery would yield different results. Collectively, these data suggest that G-1 is ineffective in this model, and another

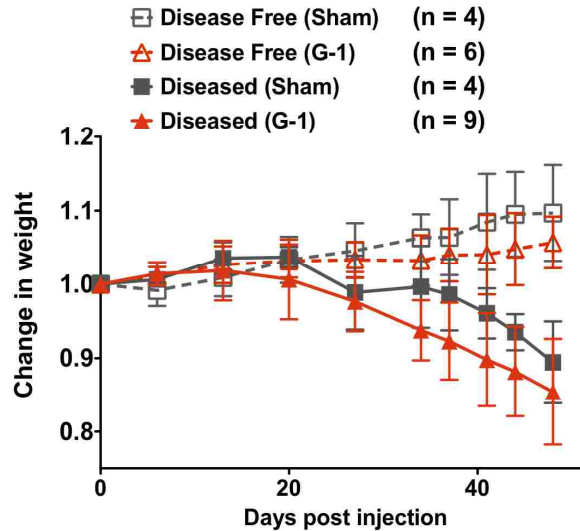
approach is warranted to investigate the ability of G-1-treated T cells to function therapeutically *in vivo*.

## Section 6.6 : Figures



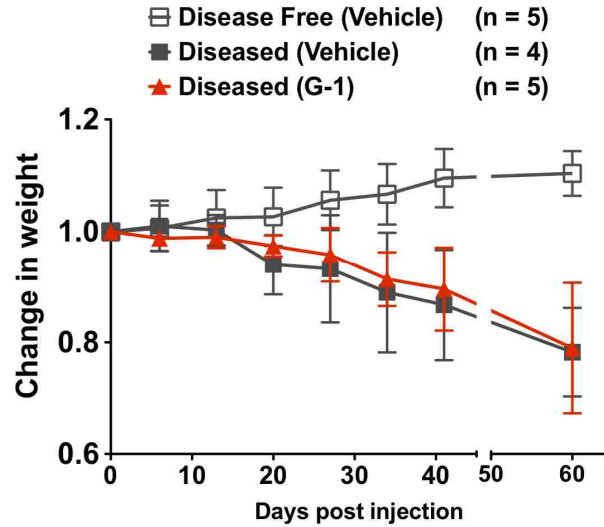
**Figure 31 : T cell-mediated colitis: *Ex vivo* treatment of T cells with G-1.**

CD4<sup>+</sup>CD45RB<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and stimulated in culture for 4 days in the presence of TGFβ + IL6 and either DMSO (grey filled boxes) or G-1 (red filled triangles). Cells were then injected into Rag1KO mice, with animals receiving vehicle alone (open boxes) serving as the control. N = number of animals. Error bars = 95% C.I.



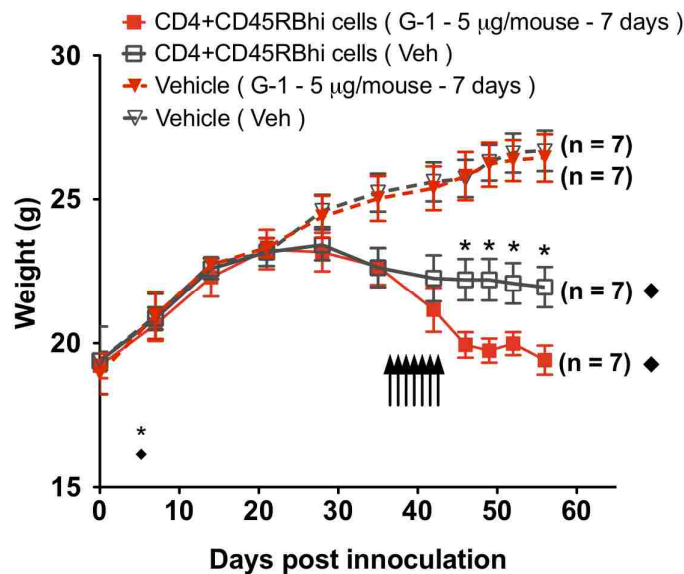
**Figure 32 : T cell-mediated colitis: G-1 pellet experiments.**

CD4<sup>+</sup>CD45RB<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and injected into Rag1KO mice that were implanted with either a sham pellet (grey filled boxes) or G-1 pellet (red filled triangles). Rag1KO mice implanted with either a sham pellet (grey open boxes) or G-1 pellet (red open triangles) which received saline injections served as the control. Pellets were implanted 7 days prior to adoptive transfer. N = number of animals. Error bars = S.D.



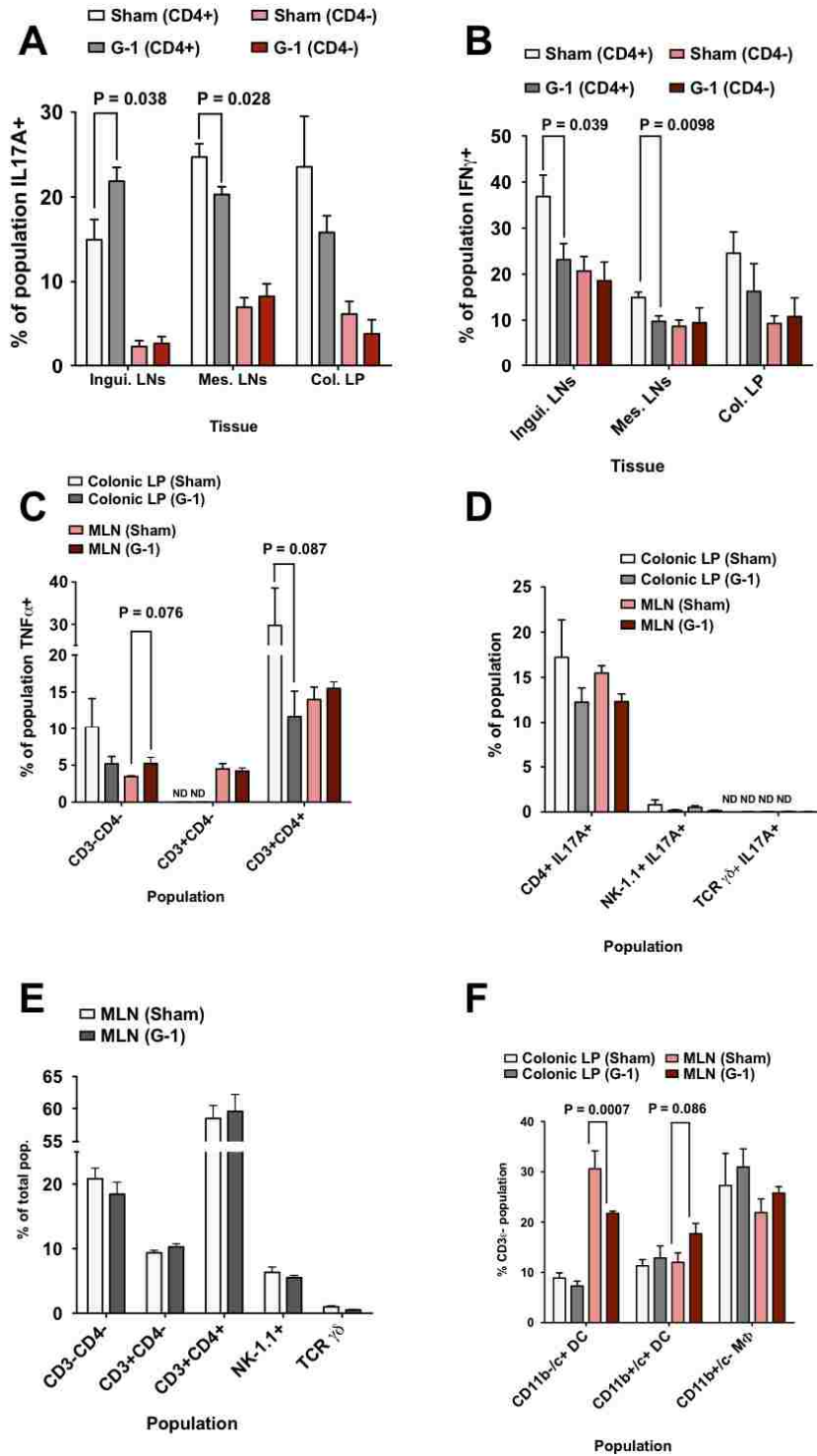
**Figure 33 : T cell-mediated colitis: G-1 injections starting at day 1.**

CD4<sup>+</sup>CD45RB<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and injected into Rag1KO mice. Mice received either vehicle (black filled boxes) or 5 $\mu$ g G-1 (red filled triangles) subcutaneously for 21 consecutive days starting the day after adoptive transfer. Non-diseased mice receiving vehicle injections served as controls (black open boxes). N = number of animals. Error bars = S.D.



**Figure 34 : T cell-mediated colitis: G-1 treatment during fulminate colitis.**

CD4<sup>+</sup>CD45RB<sup>hi</sup> naïve CD4<sup>+</sup> T cells were collected by FACS and injected into Rag1KO mice. Mice received either vehicle (grey open boxes) or 5 $\mu$ g G-1 (red filled boxes) subcutaneously for 7 consecutive days starting 35 days after adoptive transfer. Non-diseased mice receiving vehicle injections (grey open triangles) or G-1 (red filled triangles) as controls (black open boxes). N = number of animals. Error bars = S.E.M.



**Figure 35** : T cell-mediated colitis: G-1 effects on cytokine production.

Tissue samples were collected from the mice in Figure 34 and stained as described in the methods found in Chapter 3 . P values determined by students t-test. Error bars = S.E.M.



## Chapter 7 : Overall Conclusions

### *Section 7.1 : Summary and Significance*

#### **Introduction**

Several concepts about estrogen-mediated immune regulation have been established. They first emerged when epidemiological data demonstrated that the prevalence of autoimmune diseases was heavily skewed toward females (Whitacre et al., 1999). However, while it is clear that estrogen imparts a direct effect on immune physiology, many questions remain. Studies of patients with systemic lupus erythematosus (SLE) (Clowse, 2007) and rheumatoid arthritis (RA) (Da Silva and Spector, 1992) demonstrated estrogen's role in autoimmune pathogenesis. Interestingly,  $T_H17$  cells have been implicated in both diseases as well (Pernis, 2009). Conversely, work with animal models have shown that estrogen can illicit IL10 expression in  $CD4^+$  T cells and protect animals from Experimental Autoimmune Encephalomyelitis (EAE), a widely accepted animal model of multiple sclerosis (Ito et al., 2001; McClain et al., 2007; Offner and Polanczyk, 2006; Polanczyk et al., 2004a; Polanczyk et al., 2004b; Polanczyk et al., 2005). In addition, estrogen has been linked to several important mediators within  $T_H17$  and  $T_{REG}$  populations, including decreasing IRF1 expression in murine splenocytes (Lengi et al., 2006), increasing IRF4 expression in dendritic

cells (Carreras et al., 2010), inducing Foxp3 expression in CD4<sup>+</sup> T cells (Polanczyk et al., 2004b), and enhancing T<sub>REG</sub> function (Polanczyk et al., 2006, 2007). Thus, evidence for both pro-inflammatory and immunosuppressive properties of estrogen exists. Despite a few isolated studies looking at Foxp3 expression (Polanczyk et al., 2004b) and EAE suppression (Polanczyk et al., 2004a), little evidence exists for specific functions of distinct estrogen receptors in CD4<sup>+</sup> T cell differentiation. Recent findings demonstrating a regulatory role in thymic atrophy (Wang et al., 2008), TGFβ signaling (Kleuser et al., 2008), and suppression of EAE (Blasko et al., 2009; Wang et al., 2009a) suggest that GPER is important in estrogen-induced immune regulation. Consistent with this notion, our preliminary results showed that the treatment with the GPER-directed agonist G-1 enhanced IL10 and IL17A production from CD4<sup>+</sup> T cells in several settings, including the induction of a CD4<sup>+</sup>IL10<sup>+</sup>IL17A<sup>+</sup> population. These data suggested that GPER signaling may modulate balance along the T<sub>REG</sub>-T<sub>H</sub>17 axis.

Given the attractive pharmacological properties of the GPER-directed compounds, and the historical success in targeting GPCR's therapeutically, the importance of the T<sub>REG</sub>-T<sub>H</sub>17 axis to immunopathology, the functional plasticity of T<sub>REG</sub> and T<sub>H</sub>17 cells, and previous successes using G-1 to treat EAE, we decided to ask the following questions: (i) What is the role of GPER signaling in regulating CD4<sup>+</sup> T cell differentiation, in particular the T<sub>REG</sub> and T<sub>H</sub>17 populations? (ii) Does G-1 exhibit any T cell-modulatory properties that can be developed for clinical use?

## Results

In Chapter 4 we showed that naïve T cells and nT<sub>REG</sub> cells express GPER mRNA, while G-1 treatment of unpolarized, polyclonally stimulated naïve T cells selectively increased expression of the T<sub>REG</sub>-associated transcription factor Foxp3 without eliciting changes in the other lineage specific transcription factors T-bet, GATA-3, or ROR $\gamma$ t. Flow cytometry based studies demonstrated that the increased Foxp3 expression reflected an increase in the number of Foxp3<sup>+</sup> cells *in vivo* and in culture. G-1 was also able to drive expansion of a Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> T cell population in cells cultured under T<sub>H</sub>17-polarizing conditions. T<sub>H</sub>17-polarized cells treated with G-1 showed increased suppressive function in a T cell suppression assay. Interestingly, we did not note any changes to PD-1 expression following *in vivo* treatment with G-1, which stands in contrast to previous studies demonstrating G-1-mediated inhibition of EAE is dependent on PD-1, which correlated with increased PD-1 expression on Foxp3<sup>+</sup> T cells. However, this may reflect differences in experimental conditions (see Section 4.5, 5.5, 7.2 and 7.3). Similar trends were observed for CTLA-4.

In Chapter 5, we built upon our preliminary findings by exploring alterations in cytokine secretion, focusing on IL10, IL17A, and IFN $\gamma$ . We demonstrated that G-1 was able to drive IL10 expression and secretion under T<sub>H</sub>17-polarizing conditions. This effect was specific as secretion of IL6, IL17A, TNF $\alpha$ , and IFN $\gamma$  were unaffected by G-1 treatment. We also began the work of delineating the signaling mechanisms responsible for the G-1-mediated effects. We noted that G-1-mediated induction of IL10 was ERK-dependent, and did not

appear to result from proliferation of IL10<sup>+</sup> cells as G-1 was able to drive IL10 expression in non-dividing cells. This finding fits well with the known mechanisms of IL10 induction in helper T cell populations, which are dependent on the STAT and ERK signaling pathways. One important note is that we did observe increased IL17A expression in a few conditions, including some of our preliminary findings. These findings translated *in vivo* as subcutaneous injection of G-1 resulted in increased IL10/IL17A secretion and decreased IFN $\gamma$  secretion from harvested splenocytes stimulated in culture.

Our data demonstrating *in vivo* and *ex vivo* treatment with G-1 drives Foxp3 expression and IL10 secretion in CD4<sup>+</sup> T cells gave us confidence moving forward testing G-1 in the setting of T cell-based chronic inflammation. Therefore in Chapter 6 we evaluated G-1 as a prophylactic and therapeutic treatment in the setting of T cell-mediated colitis. To our surprise, G-1 treatment of donor cells prior to adoptive transfer was ineffective in altering the course of disease, while systemic treatment of G-1 also elicited no detectable protection. Interestingly, treatment of diseased mice with G-1 led to an acute exacerbation of the wasting disease that correlated with a decrease in the percentage of IL17A<sup>+</sup> cells specifically within gut mucosal tissues and mesenteric lymph nodes. Consistent with preliminary data and a subset of experiments from Chapter 5, G-1 treatment led to an increase in the percentage of IL17A<sup>+</sup> cells within the systemic immune system, as measured in the inguinal lymph nodes.

## **Discussion and future directions**

These findings will prove critical to the development of T cell-targeted therapies aimed at exploiting GPER, or in the pharmacological development of the G compounds. Despite the great promise of therapies tailored to direct the immune system toward a specific response, few such regimens have made it to the clinic. Cytokine targeted biologics such as Ustekinumab (human mAb directed against IL12/IL23) and Etanercept (anti-TNF $\alpha$  therapy) can elicit robust reductions in disease severity, but they are associated with a number of disadvantages, including severe side-effects and immense cost (Uhlenhake and Feldman, 2010). These issues are compounded by the need for repeated dosing. Other drugs such as Methotrexate and Cytosin are associated with even more severe side-effect profiles. Dosing can be a challenge, often leading to significant detriment to quality of life despite limited efficacy in resolving symptoms and controlling disease. Orally available treatments that elicit refined changes to T cell populations will profoundly change how we treat immunological and neoplastic disease and revolutionize medicine. Such immunomodulatory therapies will require identifying signaling targets amenable to disruption and highly specific in their function. This work demonstrates that G-1 can serve in this capacity, and that GPER may be one such target.

This study provides several lines of evidence that the context of G-1 treatment is important to its ability to modulate T cell activity. First, we present

clear evidence that systemic treatment of G-1 does not alter the expression of PD-1 on the Foxp3<sup>+</sup> T cell population in naïve (non-diseased) male mice. We also have data which shows an increase in the number of Foxp3<sup>+</sup> T cells. This finding is clearly distinct from data showing systemic G-1 treatment in female EAE mice greatly enhances PD-1 expression on Foxp3<sup>+</sup> T cells, without altering cell number. Whether these reflect gender-based differences or result from environmental differences a naïve mouse and the setting of encephalomyelitis are not clear. Second, G-1 increased the number of CD3<sup>+</sup>CD4<sup>+</sup>IL17A<sup>+</sup> cells within the systemic immune system when delivered via subcutaneous injection in diseased TCMC mice (**Figure 35**), yet led to a reduction in the CD3<sup>+</sup>CD4<sup>+</sup>IL17A<sup>+</sup> population within the mucosa of the same mice. This suggests that G-1 can elicit distinct responses in a context-specific manner within the same animals, although other possibilities are discussed in Section 6.4 and 6.5. Our data demonstrating that G-1 is ineffective in the setting of colitis should not undermine future attempts to investigate this compound in the context of disease. Furthermore, our study does not include an analysis of G-1 in the setting of T<sub>H</sub>1 or T<sub>H</sub>2 polarizing conditions. The fact that G-1-mediated IL10 induction was mediated through the ERK signaling pathway suggests that similar findings will be observed in T<sub>H</sub>1 and T<sub>H</sub>2 polarized cells as ERK signaling is a common factor in the induction of IL10 for all three effector lineages (Saraiva et al., 2009). This hypothesis is supported by our observation that CD4<sup>+</sup>CD44<sup>hi</sup> memory T cells can also be driven to express IL10 following G-1 treatment in culture (data not

shown). Thus, the effects of G-1 in various *in vivo* settings, disease models, and in T<sub>H</sub>1 and T<sub>H</sub>2 polarizing conditions warrant further study.

If G-1 is to be developed for therapeutic use with the purpose of addressing immunological conditions, several paths of investigation need to be pursued. One factor that has recently received more attention is the mechanisms by which T cell populations (and other immune populations) are targeted to specific tissue sites or inflammatory settings. Undoubtedly, the site where manipulated populations accumulate within the body will have far-reaching implications in determining therapeutic success. Our observation in Chapter 4 that G-1 can elicit what appear to be Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> hybrid T cells suggests that G-1 treatment can alter the trafficking of T<sub>REG</sub> populations. For example, one of the pivotal studies discussed previously that substantiated the concept of hybrid T cell populations looked at the importance of T<sub>REG</sub> trafficking. They showed that T-bet induced CXCR3 expression in Foxp3<sup>+</sup> cells was critical for targeting T<sub>REG</sub>s to sites of T<sub>H</sub>1-type inflammation during chronic infection with *M. tuberculosis*, and adoptive transfer of Foxp3<sup>+</sup> cells from T-betKO mice were unable to rescue Foxp3KO (*scurfy*) mice from their fatal T<sub>H</sub>1-mediated lymphoproliferative disorder, while Foxp3<sup>+</sup> from wildtype mice completely abrogated disease (Stock et al., 2004). Along these same lines, future work aimed at delineating G-1-mediated effects in inducing hybrid T cell populations, and any resultant changes in pertinent trafficking molecules, will be vital to any therapeutic development of this compound.

Other therapeutic paradigms are also worth considering. If there is a sufficient pool of target cells available at the site where an intervention is needed, G-1 may serve to act on the local populations *in situ* to modulate disease. For example, if the large number of T<sub>H</sub>17 cells within the inflamed mucosal wall of Crohn's disease (CD) patients (Kleinschek et al., 2009) can be converted to produce IL10, there may be a sufficiently large target population available at the site of inflammation to elicit a therapeutic effect without the need to recruit peripherally induced populations. Indeed there is promising evidence that conversion of T cell populations *in situ* can reverse the course of autoimmunity. In an elegant study from the Santamaria group, nanoparticles coated with peptide-loaded MHC complexes were able to drive endogenous low-avidity CD8<sup>+</sup> T cells into an autoregulatory population that blunted disease progression and reversed hyperglycemia in a non-obese diabetic (NOD) mice (Anderson et al., 1999). There are certain to be instances where site-specific recruitment determines treatment success and side effect profiles, and conditions where local alteration of T cell function play a predominant role in therapeutic viability. Thus both topics will be of great interest as therapies aimed at T cell populations increase in sophistication.

The fact that we observed such a large amount of variability in the induction of IL10 within the CD4<sup>+</sup>CD45RB<sup>hi</sup> population made it difficult to draw any firm conclusions from this data. Attempts to determine the difference between the CD4<sup>+</sup>CD45RB<sup>hi</sup> population and the CD4<sup>+</sup>CD62L<sup>hi</sup> population will be needed to better characterize the nature of the problem, and help guide future



studies. One of the major differences between these two populations is the number of Foxp3<sup>+</sup> cells (See Appendix B), thus it may be that the presence of Foxp3<sup>+</sup> T<sub>REGS</sub> is necessary for G-1 induced IL10 production. Future work delineating the precise cellular populations wherein G-1 elicits its effects are needed. This will be important in guiding development in that appropriate pathologies can be targeted. This will also prove seminal to attempts to utilize this compound for adoptive therapies in that purification of appropriate target populations will be critical to therapeutic success.

What of the other G compounds? The striking effects of G-1 on T cell activity and cytokine secretion beg the question; can G15 or G36 drive a proinflammatory phenotype in T cell populations? These complex questions may best be delineated in models from the field of tumor immunology. Work by the Restifo group at the NIH has shown that polarization of naïve T cell populations toward a T<sub>H</sub>17 phenotype *ex vivo* enhances tumor killing in a mouse xenograft model of melanoma. Strangely, this effect was lost in mice housed in a sterile environment which lack commensal flora in the gut. Reintroduction of the flora restored the effect. The plasticity of T<sub>H</sub>17 cells arises from these studies as well, as the benefit of T<sub>H</sub>17-polarization *ex vivo* effect is dependent on *in vivo* production of IFN $\gamma$  (Muranski et al., 2008). When molded into a single picture, it appears that the benefit of T<sub>H</sub>17 polarization is to induce surface receptors that target these cells to the gut, where they acquire enhanced tumor killing by a mechanism dependent on the commensal gut flora. Conversely, in a xenograft model of melanoma, Foxp3<sup>+</sup> cells suppress immunity by destroying DC within

tumor draining LNs in an IDO and perforin dependent manner (Boissonnas et al., 2010). Thus animal models of tumorigenesis should prove to be an appropriate venue to investigate the G compounds.

Finally, this work offers some interesting estrogen-related questions that warrant further investigation. A major point that needs to be addressed by future studies is gender-based differences in estrogen-driven effects within the CD4<sup>+</sup> T cell compartment. A 2007 study showed that regulatory T cell-derived IL10 participates in the development of tolerance to paternal antigens during pregnancy (Schumacher et al., 2007; Thuere et al., 2007). It has also been shown that GPER is responsible for the protective effects of orally delivered ethinyl estradiol in EAE (Yates et al., 2010) in female mice. These data along with our findings, wherein G-1 induced IL10 in cells collected from male mice, appear to indicate that there are some similarities between systemic G-1 treatment in female and male mice. Furthermore, our findings may be helpful reconciling the predominantly anti-inflammatory properties of estrogen that have been described empirically with the irrefutable evidence that women suffer from a high prevalence of autoimmunity. Estrogen protects animals from EAE, yet women suffer from a four-fold higher prevalence of MS than men. Why is that? One can imagine that during high-estrogen periods of estrous cycle there is an expansion of these T<sub>H</sub>17-like T<sub>REG</sub> populations via GPER signaling, likely under conditions where some low-level T<sub>H</sub>17-like inflammation is already present. Upon shifting to a low estrogen state, these cells lose their IL10 and/or Foxp3 expression, reverting to a more proinflammatory phenotype in response to

another unknown factor or simply in response to the local milieu. This could lead to sequential amplification of an ongoing inflammatory process. A hint that this might indeed be the case comes from the clinic, where MS patients experience a decrease in flare-ups during pregnancy but, conversely, a dramatic increase in symptomatology postpartum (Vukusic et al., 2004). Might this increase in disease severity postpartum reflect reversion of E2-induced hybrid T cells to a more pathogenic phenotype? If so, why then does this effect disappear over time (patients will generally revert to their prepartum disease status over time)? As more and more examples of T cell plasticity are described along the T<sub>REG</sub>-T<sub>H</sub>17 axis, more explicit hypotheses are likely to be developed. Further studies will be required to explore these possibilities. As these questions relate to the stability of these induced populations, answers to these questions will also be instructive in guiding how drugs aimed at targeting these properties are designed and utilized, and in what context they are most appropriately used.

## **Overall conclusions**

In conclusion, this work may prove seminal in understanding the complex interactions of estrogen signaling and the T cell differentiation by linking G-1/GPER with two critical T cell populations; T<sub>REG</sub> and T<sub>H</sub>17 cells. Thus these findings will help to provide further understanding in the complex story of estrogen-immune interactions. Moreover, these data lay the groundwork for analyzing the G compounds, and G-1 in particular, as T cell-targeted therapies.

The surprising findings that G-1 cannot protect in the animal model of colitis should not dissuade from future studies aimed at addressing its therapeutic potential in other disease settings. Furthermore, the unexpected results in GPERKO mice must be addressed, and the molecular target of G-1 responsible for our findings clearly delineated, be it GPER or another unknown protein. Collectively, our findings highlight the need to further understand the intricacies of T cell biology and estrogen physiology if we are to intelligently manipulate their function for medical purposes.

### ***Section 7.2 : List of major limitations and caveats***

Detailed discussions about the various limitations and caveats has been presented throughout the manuscript, and are intertwined with various discussions, conclusions, the preceding summary section of this chapter (Section 7.1). This next section offers a neat summary of the major points that have been discussed, and offers some guidance as to where they are discussed.

#### **Molecular target of G-1 is not clear**

The true target of G-1 that is responsible for these observations remains unclear. While testing against ER $\alpha$ /ER $\beta$  and small panel of receptors suggest that it does indeed specifically target GPER, our findings that the G-1-mediated effects were lost in GPER(+/-) mice and returned in GPER(-/-) mice strongly

suggest that G-1 can exhibit off-target activity that is relevant to the work presented here. Given the role of GPER in estrogen-induced thymic atrophy and apoptosis of maturing thymocytes, future experiments utilizing conditional knockout strategies will be needed to adequately address the role of GPER in E2 and G-1-mediated T cell effects. (See Section 5.4).

### **Limited data in the *in vivo* disease setting**

In this work our goal was to analyze a multitude of cytokines, chemokines, and surface markers in the setting of colitis. We were able to achieve a small part of that goal with the data presented in **Figures 34 and 35**. However, future work will be needed to address whether our findings of G-1-mediated Foxp3, IL10, or IL17A expression are of therapeutic interest. (See Section 6.4, 6.5, and 7.1)

### **Male versus female mice**

The entirety of this work was done using male mice and cells from male mice. The reasoning behind this was discussed previously, but centered on the desire to avoiding the confounding effects of the cyclical nature of endogenous estrogen in female, and the potentially inflammatory effects of surgical intervention that would be required to mitigate the problem via ovariectomy. While this gave us clean results, the veracity of these findings in sexually mature females is unclear, and nothing that we presented addresses this concern. There are differences (PD-1 expression on Foxp3<sup>+</sup> T<sub>REGS</sub>) and similarities (induction of IL10) to previous work carried out in female animals. How these findings translate

to female mice (and people) should be a focus of future work. (See Section 4.4, 5.4, and 7.1)

### **G-1-mediated Foxp3 induction may be the result of apoptosis/proliferation**

The majority of the effects that we observed throughout Chapter 4 were modest, generally in the neighborhood of 50% increases or less. While in general the results did achieve statistical significance, there is the possibility that G-1 was exhibiting selective apoptosis in the non-Foxp3<sup>+</sup> populations. In fact we did detect significantly higher levels of apoptosis following *ex vivo* treatment with G-1 (**Figure 42**, Appendix D). While we did not detect any significant changes in the number of CD4<sup>+</sup> T cell following systemic treatment of G-1, given the modest increases that we observed, more work needs to be done to corroborate our findings. Additionally, it is possible that the increased apoptosis observed in G-1 treated cultures was the result of increased T<sub>REG</sub> activity, as one of the mechanisms employed by regulatory T cells in suppressing immunity is cytotoxic lysis of other immune populations (see Section 1.3).

### **Section 7.3 : List of future directions**

Like the limitations and caveats, discussions about the various future directions were presented throughout the manuscript, and are intertwined with various discussions, conclusions, and the preceding summary section of this chapter (Section 7.1). Like Section 7.2, this next section offers a neat summary of

the most pertinent future directions, and offers some guidance as to where they are discussed, though not all topics have been discussed in depth.

### **What is the effect of G-1 on T cell under T<sub>H</sub>1 or T<sub>H</sub>2 polarizing conditions?**

As discussed in Section 7.1, the finding that G-1-mediated IL10 induction requires ERK signaling strongly suggests that a similar effect would be observed in the other effector lineages. Therefore, repeating our findings from Chapter 5 under T<sub>H</sub>1 and T<sub>H</sub>2-polarizing conditions is an obvious next step to take.

### **What is the mechanism of ERK activation?**

We show that G-1 drives IL10 secretion by activation of ERK, however, the mechanism of the activation remains unclear. Studies of GPER signaling have led to a model wherein GPER activation drives activation of epidermal growth factor receptor (EGFR) activation and subsequent transactivation of MAP kinase cascades (such as ERK) and the PI3 kinase pathway (See **Figure 6**). In order to further address the signaling mechanisms responsible for G-1-mediated IL10 secretion, we attempted to treat cultured T cells with the EGFR inhibitor AG1478. However, this led to near complete blockade of T cell proliferation and widespread cell death. This may be due to the unique culture conditions that we use, which lack phenol red and utilize charcoal stripped FBS (See Chapter 3), but that remains a hypothesis at this point. Thus future studies determining EGFR expression on T cell populations, and more sophisticated attempts at disrupting the activity of EGFR are warranted. One example might be to try retroviral

expression of a kinase dead EGFR. Experiments looking at EGFR activation, either by western blot or using Luminex arrays, are needed as well. Experiments designed to investigate downstream transcriptional profiles are described below.

### **What is the effect of G-1 on other key T<sub>REG</sub> and T<sub>H17</sub> molecules?**

Numerous other proteins, including Helios (Getnet, 2010; Getnet et al., 2010), AhR (Ramirez et al., 2010), IRF1 (Kano et al., 2008), IRF4 (Brustle et al., 2007; Chen et al., 2008), ROR $\alpha$  (Yang et al., 2008), and IL23 (Volpe et al., 2008) are also involved in T<sub>H17</sub> development (Iwakura and Ishigame, 2006), and warrant further investigation. Understanding how these proteins are affected by G-1 treatment will have important implications in designing experiments to test G-1 therapeutically. For example, CCR4 has been shown to play a role in T<sub>REG</sub>-mediated suppression of colitis, and other receptors such as CD103 (Leithauser et al., 2006) and CCR6 are also important in targeting cells to the mucosa (Wang et al., 2009b). Thus delineating G-1 effects on trafficking receptor expression profiles would be of immense value, and may help explain why our studies with the T cell-mediated colitis model failed. (See Section 7.1).

Future studies could include simple approaches like qRT-PCR and flow cytometry-based techniques, as well as more technically refined approaches like microarray analysis. Perhaps the best approach would be to conduct microarray analysis following *ex vivo* G-1 treatment of highly enriched (>99%) naïve T cells under non-polarizing and TH17-polarizing conditions, as well as following



systemic G-1 treatment in a relevant animal model. The obvious choice here might be EAE.

### **Why did the colitis model fail, what about other models?**

One obvious approach would be to go back to the model where G-1 is known to exert a protective effect, namely EAE, and see if G-1 effects are lost in IL10KO mice. A second option would be to attempt to bring work using a mouse tumor model. As described in Section 7.1, this would allow investigation of all 3 G compounds in T cell based immunotherapies.

### **Do these findings translate into female mice?**

All of our work was done in male mice and cells from male mice. As discussed in Section 4.5, 5.5, and 7.1, this may underlie differences between our data and other published reports. Thus repeating experiments where disparate results were obtained from those in the literature in ovariectomized female mice will be needed to address whether these reflect gender based differences in the effects of G-1. Moreover, it is important to understand the properties of these compounds in the female setting as they make up over half of all autoimmune disease patients, aside from the obvious ethical implications.

### **Do these findings translate into human samples?**

Since the ultimate goal outlined in the summary is to focus on therapeutic development of these compounds, future studies addressing how these

molecules function within human T cell populations are pivotal to our stated goals. As was discussed in the introductory discussion about regulatory T cells (Section 1.3), these appear to be some differences in the form and function of human versus murine T<sub>REG</sub> populations. Thus studies utilizing T cell populations derived from preparations of human PBMCs are a critical future direction. In addition, given the recent data demonstrating that the transcription factor Helios is important in human T<sub>REG</sub> function and stabilization of Foxp3 expression (Getnet, 2010; Getnet et al., 2010), carrying out the studies described above to investigate Helios expression may prove pivotal in determining the ability of G-1 to stably affect T<sub>REG</sub> activity clinically.

## Appendices

### ***Appendix A : Determining GPER protein expression***

In Chapter 4 it was shown by qRT-PCR that GPER mRNA is expressed in murine naïve T cells and in nT<sub>REG</sub> cells (see **Figure 12**, Chapter 4). This was consistent with previous published reports which demonstrated GPER expression in Foxp3<sup>+</sup> T cells from human PBMC preparations using IHC (Blasko et al., 2009). In this work, they also demonstrated GPER staining in primary human macrophages and the mouse monocyte/macrophage cell line RAW 264.7. To corroborate that the mRNA we detected was being translated into protein, we attempted to detect GPER using western blots and immunofluorescence. Our own custom-made rabbit sera were used for these experiments. The first, serum 8073, was raised against a peptide sequence from the C-terminus of human GPER, although all but one or two residues are identical in mice (there are two published sequences), and both are highly conserved. It is worth noting that the published findings discussed above utilized our 8073 rabbit serum as well. The second, serum 9368, was raised against a peptide derived from extra cellular loop 2 (ECL2) of murine GPER.

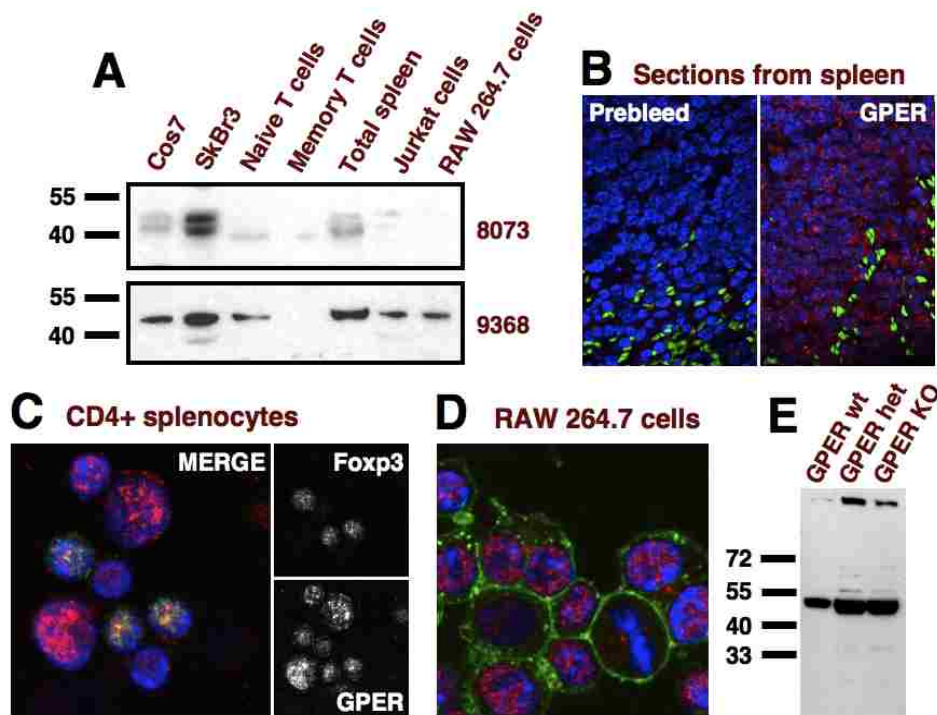
We started our analysis with western blots. Blotting with 8073 yielded expected results for African green monkey derived Cos7 cells (which express very low levels of GPER) and human SkBR3 cells (which express high levels of GPER) (**Figure 36A**). However, despite data suggesting the GPER is expressed

in the murine spleen (Wang et al., 2008) and our qRT-PCR data, we detected very little cross-reaction with murine whole spleen, FACS sorted naïve T cells, or RAW cells (**Figure 36A**). This discrepancy was not due to loading, as indicated by Coomassie staining of the membrane post-transfer (data not shown). It is possible that, despite the substantial similarity between the relevant peptide sequences in human and mouse, the single residue difference abrogates antibody binding to murine GPER. Notably, the authors of the previous report demonstrating 8037 staining in RAW 264.7 cells were able to eliminate antibody binding by the addition of excess target peptide (Blasko et al., 2009). However, we conducted several *in silico* blast searches within the NCBI protein databank using our target peptide as bait. We found no similar sequences within any known human or mouse protein or putative ORF, with the exception of being able to identify the expected target sequence in hGPER and mGPER (data not shown).

We next tested whether we could detect GPER using the 9368 serum. While we were able to detect a signal at the appropriate MW (42/44 kDa, **Figure 36A**) in nearly all lysates tested, we also detected a band in Cos7 cells which largely lack GPER mRNA. These findings were further confounded by the detection of a band of roughly 44kDa in whole spleen lysate from GPERKO mice (**Figure 36E**), which lack detectable GPER mRNA (See **Figure 12**, Chapter 4).

Finally, we analyzed primary CD4<sup>+</sup> T cells, RAW 264.7 cells, and tissue sections from mouse spleen for GPER expression using immunofluorescence. In all three samples, we detected staining with the 8073 serum while staining with

Prebleed control serum yielded no detectable signal (**Figure 36B-D**) and data not shown). Of note, the staining was largely nuclear, suggesting one of the following; (A) the GPCR GPER is expressed in the nucleus, or (B) that these antibodies recognize additional target(s) distinct from GPER. Overall, given the inconsistency of our findings, it was not possible to draw any concrete conclusions from these data.



**Figure 36 : Protein expression of GPER.**

The expression of GPER was analyzed using western blots and immunofluorescence assay (IFA). (A) Western blots of various cell lysates using two distinct rabbit sera, 8073 (raised against human C-terminal peptide) and 9368 (raised against mouse peptide from ECL2). (B) IFA showing GPER (Red, serum 8073) in a paraffin embedded section, demonstrating the border between the red and white pulp of the spleen from a C57BL/6 mouse (C) AutoMACS sorted CD4+ T cell (purity >90%) stained for GPER (Red, serum 8073) and Fcpx3 (Green), with DAPI counterstain to identify nuclei. (D) Murine macrophage cell line RAW 264.7 stained for GPER (Red, serum 8073) and actin (Green). (E) Western blot probing for GPER (9368) on total spleen protein lysate from wild-type (Left lane), GPER heterozygote (Middle lane), and GPERKO (Right lane) mice.

## ***Appendix B : FACS sorting strategies***

### **CD4<sup>+</sup>CD62L<sup>hi</sup> naïve T cells**

The collection of naïve T cells was based on surface expression of the CD4 co-receptor, which distinguishes the CD4<sup>+</sup> helper T cell populations from the CD8<sup>+</sup> cytotoxic T cell population, and the expression of L-selectin (CD62L), which is used by T cell and other immune populations to enter secondary lymphoid organs such as the lymph nodes through high endothelial venules (HEVs) by binding to sialylated carbohydrate groups like those found on GlyCam-1. While other immune populations like macrophages have been known to express CD4, they are usually observed to express lower levels than naïve T cells and can be easily sorted out (see **Figure 37**).

### **CD4<sup>+</sup>Foxp3<sup>+</sup> natural T<sub>REG</sub> cells**

As is discussed in the intro and in Chapter 4, Foxp3 expression within the CD4<sup>+</sup> T cell compartment can be driven during thymic development or induced peripherally. When induced during thymic T cell development, Foxp3 expression tends to be more stable. In the unchallenged (naïve) mice that we are using for our donors, the majority of the Foxp3 populations should fall into the category of natural regulatory T cell. This is an oversimplification as the mice are not raised in a germ-free environment, and hence have developed immunity to commensal flora in the alimentary tract and the lungs. The intestinal tract in particular is a

known site for robust generation of inducible regulatory T cells. None-the-less, cells expressing Foxp3 in naïve mice should predominantly fall into the natural T<sub>REG</sub> category. Thus to collect natural T<sub>REG</sub>S, cells from Foxp3<sup>egfp</sup> mice were stained with CD4, and CD4<sup>+</sup>GFP<sup>+</sup> cells were sorted out. (see **Figure 37**)

### **CD4<sup>+</sup>CD45RB<sup>hi</sup> naïve T cells for T cell-mediated colitis**

The induction of T cell-mediated colitis is exquisitely sensitive to the presence of regulatory T cells, in particular Foxp3<sup>+</sup> T<sub>REG</sub>S. Thus the CD4<sup>+</sup>CD62L<sup>hi</sup> populations sorted above are not colitogenic because of the presence of a small population of Foxp3<sup>+</sup> cells. In order to induce disease, the surface marker CD45RB must be used in place of CD62L. In this case the population is collected by taking the 40% of the total CD4<sup>+</sup> population which express the highest amount of CD45RB, as determined by flow. (see **Figure 37**)

### **CD4<sup>+</sup>CD44<sup>lo</sup>CD45RB<sup>hi</sup>GFP(Foxp3)<sup>+/-</sup> naïve T cells**

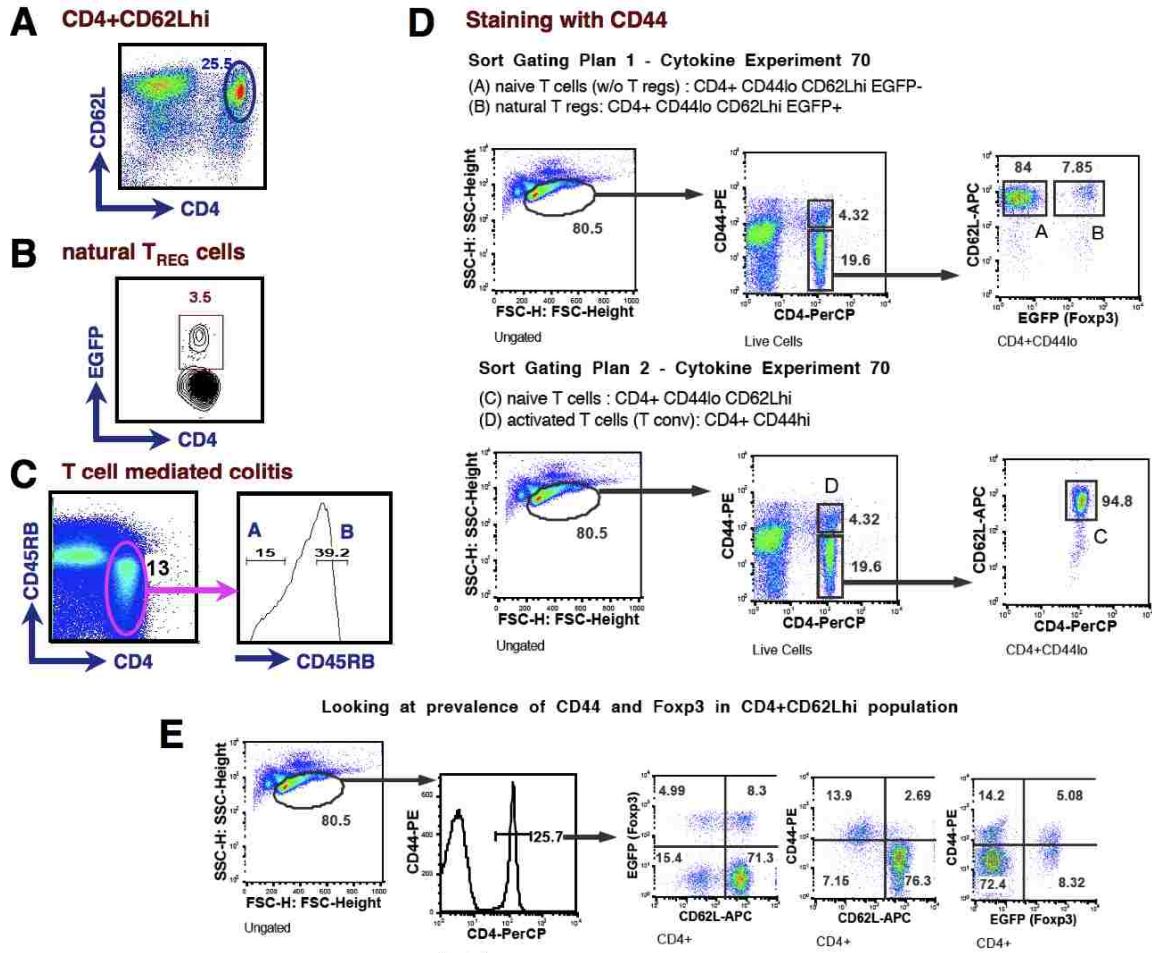
As mentioned above, the naïve mice that we use are not, in fact, truly naïve because they are not housed in a germ-free environment. Thus there are some memory T cells that have developed in these mice. Interestingly, there are two main types of memory T cells, effector memory cells and central memory cells. While generally thought to be a smaller portion of the total memory T cell repertoire, central memory cells can express CD62L, and hence can be found in the same secondary lymphoid organs we use to collect our T cell populations. To

eliminate these cells, co-staining with the memory cell marker CD44 allows us to sort out any CD44<sup>hi</sup> cells from our naïve T cell preps. We can also gate out any GFP<sup>+</sup> cells to eliminate T<sub>REG</sub> cells. As you can see in the figure below, the population of CD44<sup>hi</sup>CD62L<sup>hi</sup> cells is generally very small. In this case 2.69% of all CD4<sup>+</sup> cells fall into that category, as compared to the CD44<sup>lo</sup>CD62L<sup>hi</sup> population which comprises 76.3% of CD4<sup>+</sup> cells. (see **Figure 37**)

### **CD4<sup>+</sup>CD44<sup>hi</sup> memory T cells**

The sorting logic of memory T cell is similar to naïve T cells, only CD4<sup>+</sup>CD44<sup>hi</sup> are collected in this case. CD44 is a surface protein that has a wide range of functions and appears to play a role in T cell activation, aside from being the classical marker for memory T cells. It can bind to several ligands, including many of the canonical proteins of the extracellular matrix, and in some cases even L- and P-selectin. (see **Figure 37**)

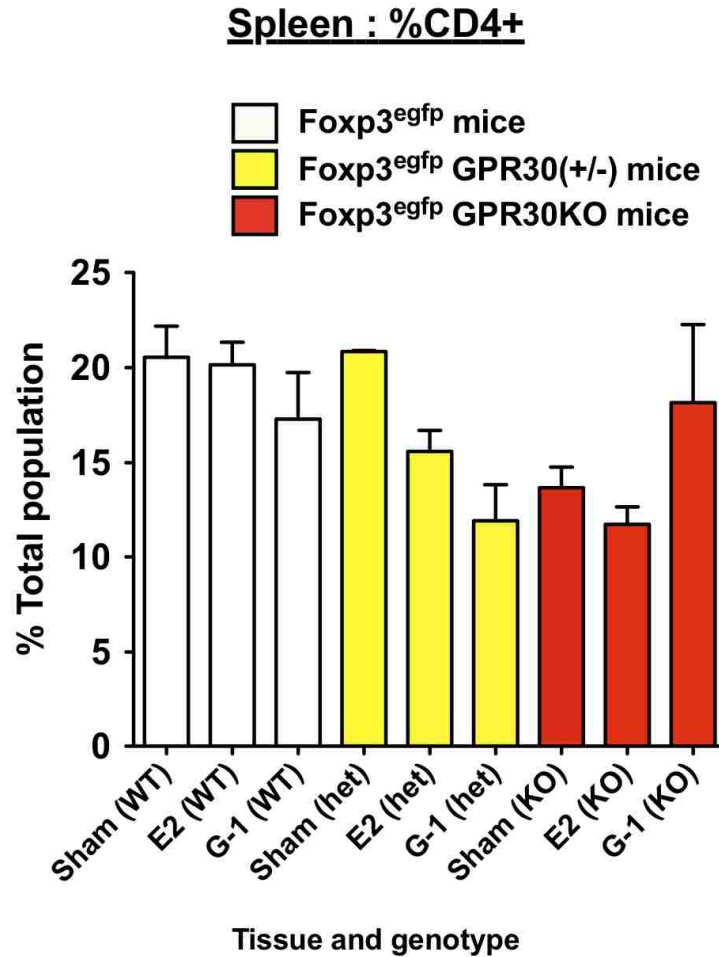




**Figure 37 : FACS sorting strategies**

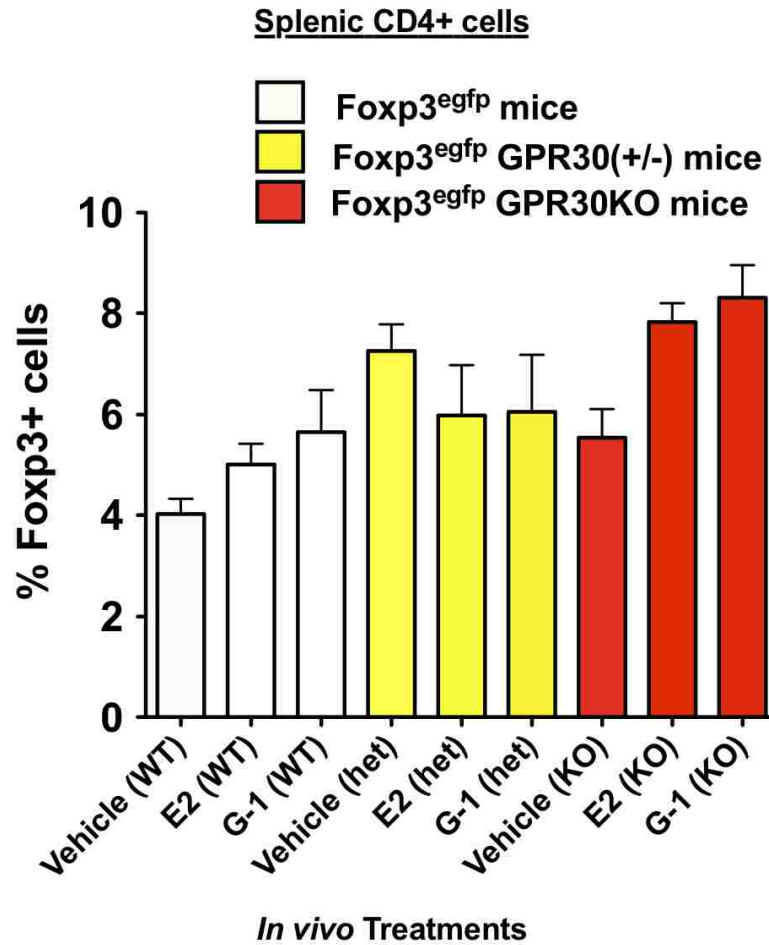
The gating logic for different populations of cells that were collected sorting on the MoFlo system here at the UNM Shared Flow Facility. The various antibodies used for staining and the reasoning for the markers chosen is described in the figure and the text above, respectively. These data were collected on the Beckton Dickinson FACScalibur, also in the UNM Shared Flow Facility. It is important to note that the MoFlo system is not a proficient at detection as the FACScalibur, and therefore one should anticipate some differences in the appearance of various populations when actually sorting, in particular with the PerCP stains.

## Appendix C : Other data from *in vivo* treatments



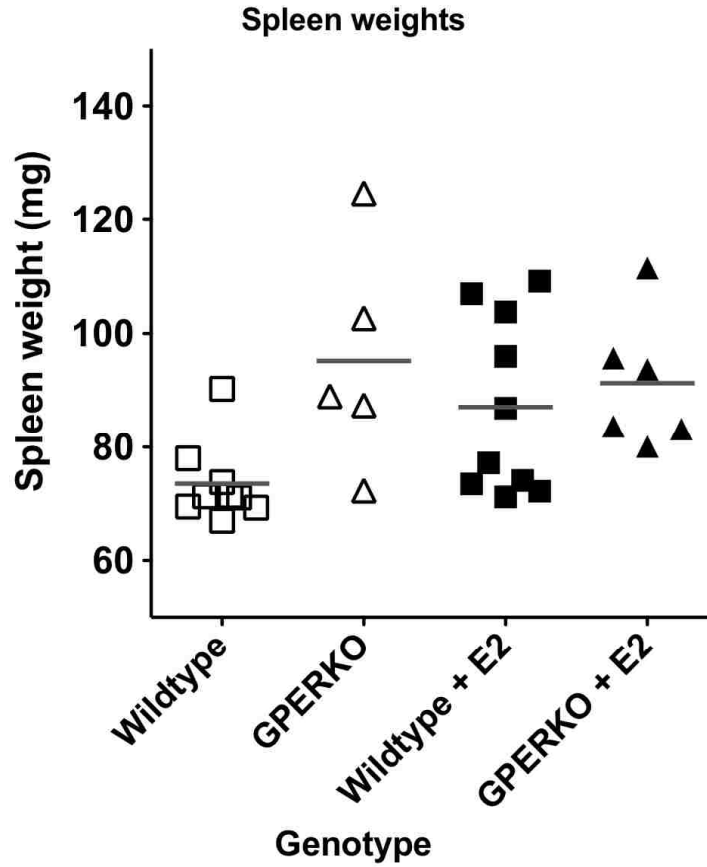
**Figure 38 : Ratio of CD4<sup>+</sup> cells in the spleen following *in vivo* treatments.**

Spleens from the mouse indicated were collected following treatment with 500ng/day of E2 or 5 $\mu$ /day of G-1, as indicated. See Methods chapter for details. Collected tissue was homogenized by mechanical disruption into a single cells suspension, treated with PBC lysis buffer and stained with anti-CD4-PE antibody, and in some cases others as described elsewhere, and analyzed by flow cytometry on a BD FACScalibur. The graphs depicts the percent of the total cell population that was CD4<sup>+</sup>. GPER(+/+), mice have minimum 3 mice per group, GPER (+/-) mice have minimum 2 mice per group, and GPER (-/-) mice have minimum 3 mice per group. These results are considered preliminary.



**Figure 39 : *In vivo* treatment with G-1 drives Foxp3 expression in GPERKO mice.**

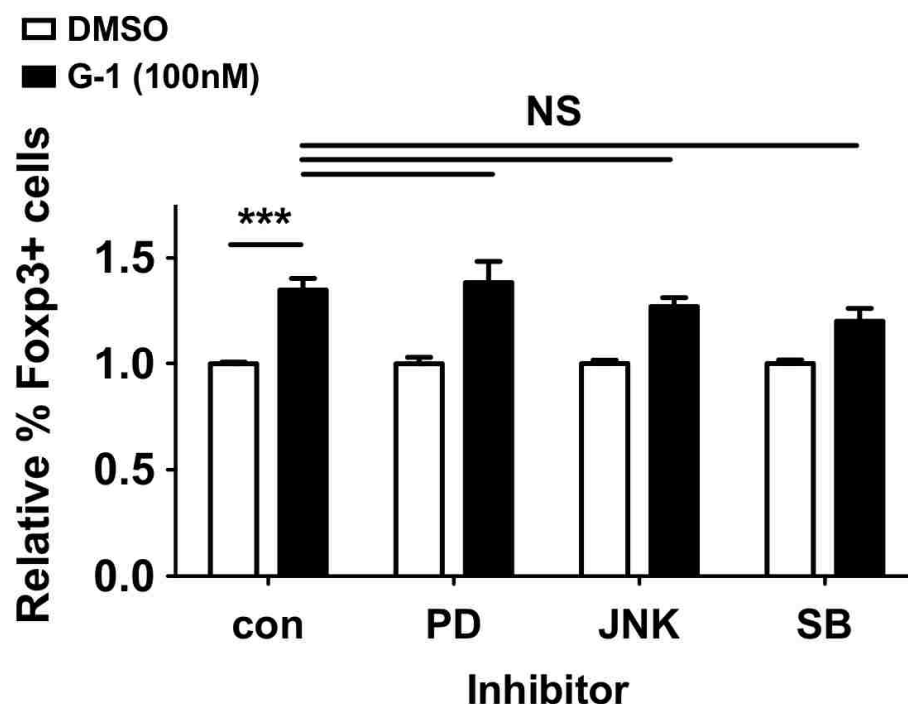
Seven to eleven week old male Foxp3<sup>egfp</sup> Foxp3<sup>egfp</sup>xGPERhet, or Foxp3<sup>egfp</sup>xGPERKO mice were injected with 17 $\beta$ -estradiol (E2 - 0.5 $\mu$ g/day) , G-1 (5 $\mu$ g/day) or vehicle for 7 consecutive days. One day following the last injection, single cell suspensions were made from spleen. Cell were then stained for CD4 and analyzed by flow cytometry. GPER(+/-) mice have minimum 3 mice per group, GPER (+/-) mice have minimum 2 mice per group, and GPER (-/-) mice have minimum 3 mice per group. These results are considered preliminary.



**Figure 40 : Spleen weights following *in vivo* treatments.**

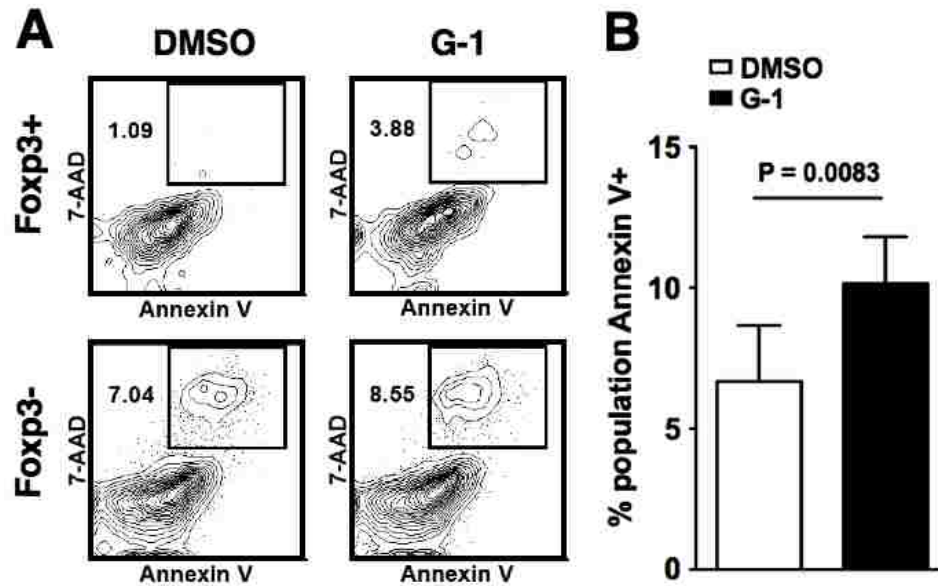
Spleens from the mouse indicated were collected following treatment with 500ng/day of E2 or 5 $\mu$ /day of G-1, as indicated. See Methods chapter for details. Wet weight of collected tissues was determined after dabbing on cellulose paper to remove any residual fluid from the surface.

## Appendix D : Other data from ex vivo T cell stimulation assays



**Figure 41 : G-1-mediated Foxp3 expression is independent of MAPK signaling.**

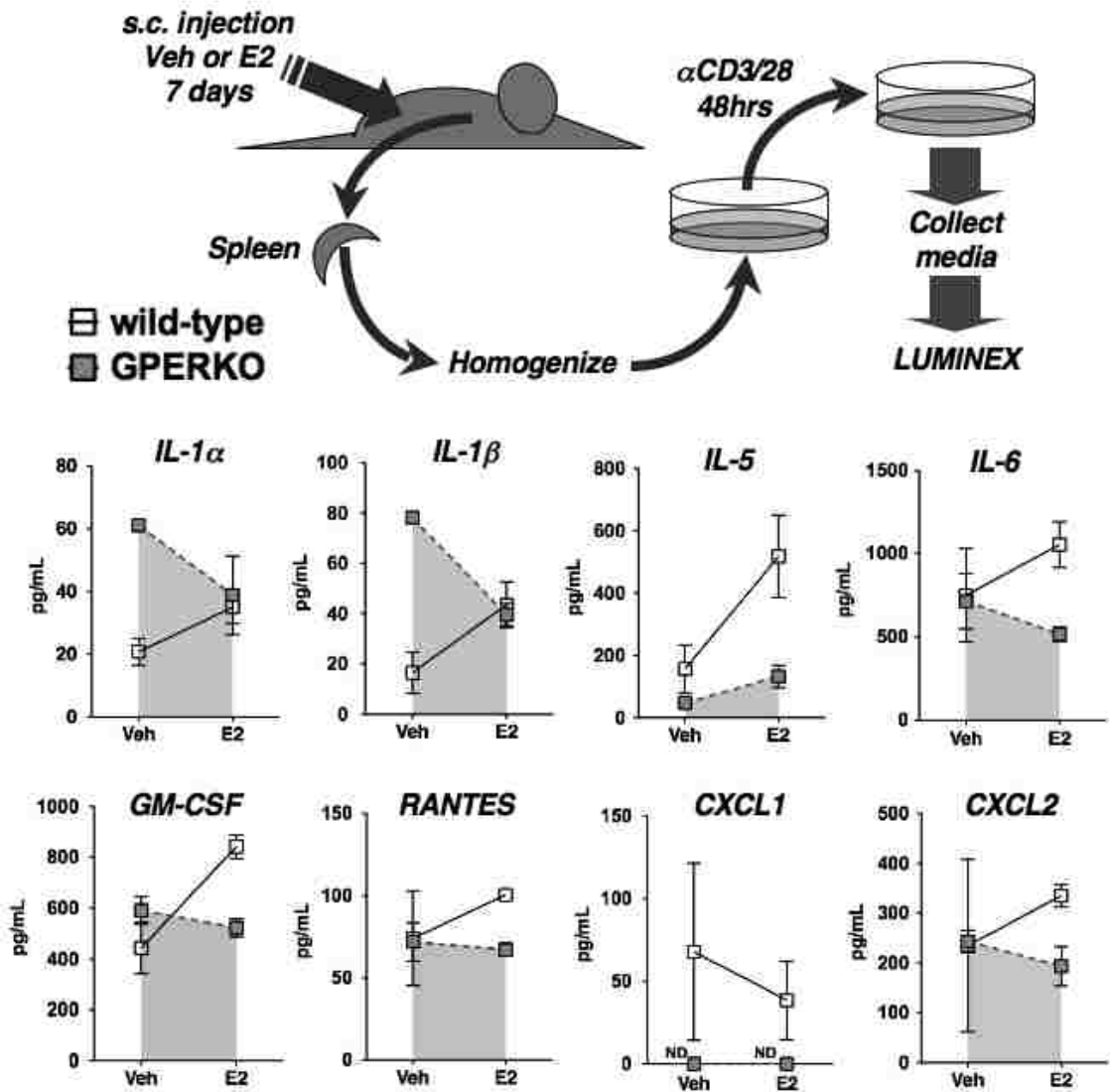
CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> naive CD4<sup>+</sup> T cells were collected by FACS from Foxp3<sup>gfp</sup> mice and cultured for 4 days *ex vivo* with anti-CD3/28 + IL6 + TGFβ in the presence of 100nM G-1 (black bars) or DMSO (white bars). Cultures were supplemented with inhibitors of the ERK (PD98059), JNK (JNK II inhibitor), or p38 (SB203580) signaling cascades. Following culture, cells were collected analyzed by flow cytometry for GFP expression. Summary of data from three independent experiments. Statistical analysis done by student's t-test. \*\*\* = P < 0.0005. Error bars = S.E.M. NS = Not significant.



**Figure 42 : G-1-treated cultures exhibit elevated apoptosis.**

CD4<sup>+</sup>CD62L<sup>hi</sup> or CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> naive T cells from Foxp3<sup>egfp</sup> or wild-type mice were collected by FACS and cultured for 4 days with TGFβ + IL6, supplemented with either 100nM G-1 or DMSO, as indicated. Cells were then stained for Annexin V (+/- 7-AAD). (A) A representative dot plots showing Annexin V and 7-AAD staining for both the Foxp3+ and FOxp3- populations following treatment with DMSO or G-1. (B) Summary of data from three experiments showing the relative percent of apoptotic cells in the total population. (C) P values determined by student's t-test. Error bars = S.D.

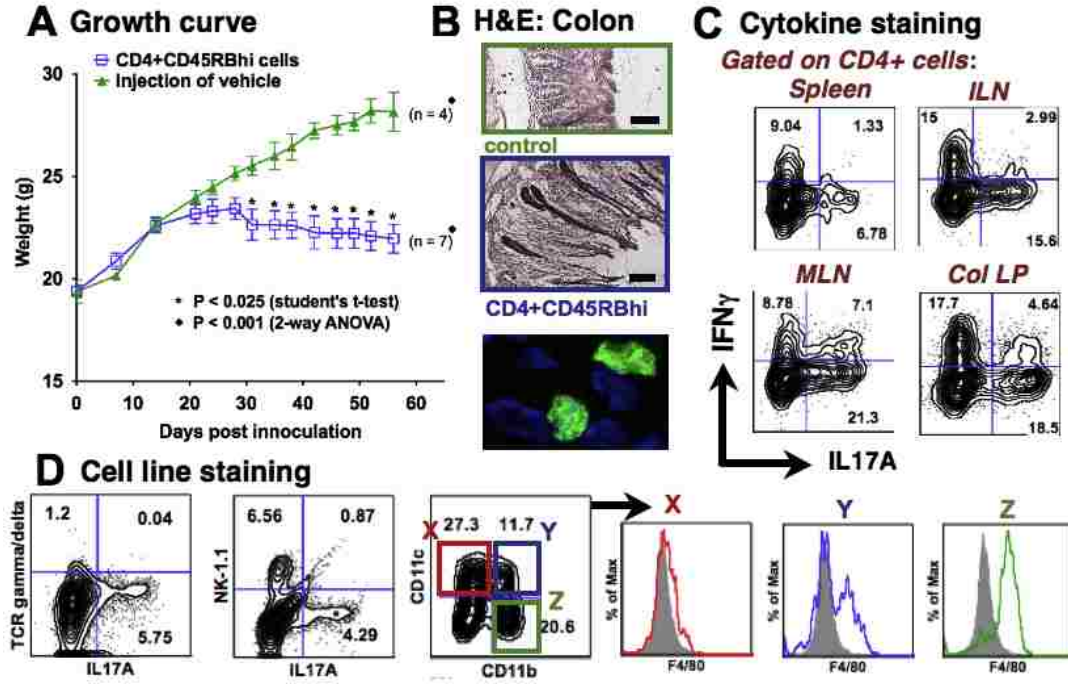
## Appendix E : Other data from Luminex multiplex assays



**Figure 43 : Other data: cytokine secretion from GPERKO splenocytes.**

Seven to eleven week old male wild-type or GPERKO C57BL/6 mice were injected with estrogen (E2 – 500ng/day) or vehicle for 7 consecutive days. One day following the last injection, splenocytes were collected and cultured in the presence of antiCD3 $\epsilon$  (1.0  $\mu$ g/mL) and antiCD28 (2.5  $\mu$ g/mL) Ab. Culture medium was collected after 48 hours and analyzed for the presence of secreted cytokines by Luminex multiplex assay. Graphs are mean data with three mice per group. Errors bars = S.E.M.

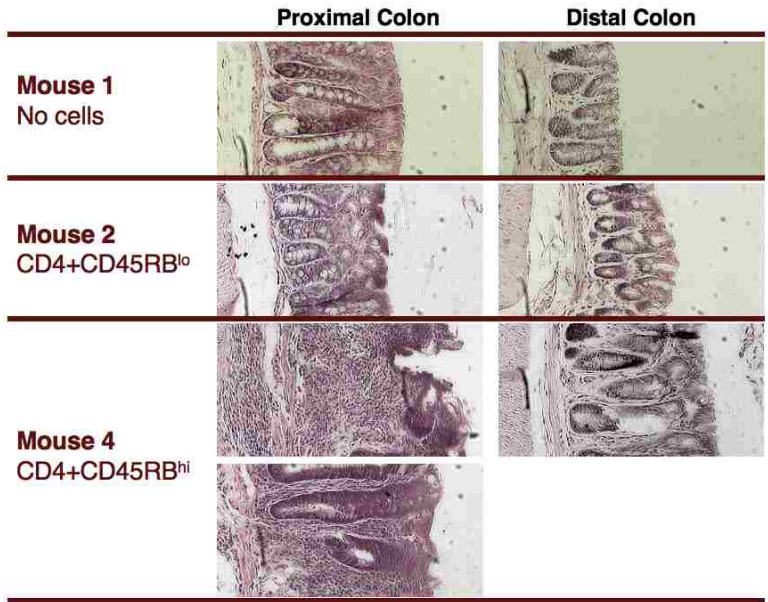
## Appendix E : The T cell-mediated colitis model



**Figure 44 : The T cell-mediated colitis model**

The T cell-mediated colitis model is induced by the injection of CD4+CD45RBhi cells into Rag1KO mice. (A) Growth curve from an experiment carried out in our lab. (B) Cryosections from the distal colon of Foxp3<sup>egfp</sup> mice from experiment in A, stained with H&E (top & middle) or imaged by IF (bottom). (C) Examples of intracellular cytokine staining, in this case for IL17A and IFN $\gamma$ , from single cell suspensions of various tissues from the animals in A. (D) Staining for lineage markers relevant to study, from MLN cells of diseased animals in A.





**Figure 45 : Sample sections from colon from TCMC mice**

## References

Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J., and Gurney, A.L. (2003). Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *The Journal of biological chemistry* 278, 1910-1914.

Ait-Oufella, H., Herbin, O., Bouaziz, J.D., Binder, C.J., Uyttenhove, C., Laurans, L., Taleb, S., Van Vre, E., Esposito, B., Vilar, J., *et al.* (2010). B cell depletion reduces the development of atherosclerosis in mice. *The Journal of experimental medicine* 207, 1579-1587.

Akingbemi, B.T. (2005). Estrogen regulation of testicular function. *Reprod Biol Endocrinol* 3, 51.

Albanito, L., Madeo, A., Lappano, R., Vivacqua, A., Rago, V., Carpino, A., Oprea, T.I., Prossnitz, E.R., Musti, A.M., Ando, S., *et al.* (2007). G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. *Cancer Res* 67, 1859-1866.

Allan, S.E., Alstad, A.N., Merindol, N., Crellin, N.K., Amendola, M., Bacchetta, R., Naldini, L., Roncarolo, M.G., Soudeyns, H., and Levings, M.K. (2008). Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. *Mol Ther* 16, 194-202.

Allan, S.E., Crome, S.Q., Crellin, N.K., Passerini, L., Steiner, T.S., Bacchetta, R., Roncarolo, M.G., and Levings, M.K. (2007). Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *International immunology* 19, 345-354.

Amre, D.K., Mack, D.R., Morgan, K., Israel, D., Lambrette, P., Costea, I., Krupoves, A., Fegury, H., Dong, J., Grimard, G., *et al.* (2009). Interleukin 10 (IL-10) gene variants and susceptibility for paediatric onset Crohn's disease. *Aliment Pharmacol Ther* 29, 1025-1031.

Anderson, B., Park, B.J., Verdaguer, J., Amrani, A., and Santamaria, P. (1999). Prevalent CD8(+) T cell response against one peptide/MHC complex in autoimmune diabetes. *Proc Natl Acad Sci U S A* 96, 9311-9316.

Arias-Pulido, H., Royce, M., Gong, Y., Joste, N., Lomo, L., Lee, S.J., Chaher, N., Verschraegen, C., Lara, J., Prossnitz, E.R., *et al.* (2010). GPR30 and estrogen receptor expression: new insights into hormone dependence of inflammatory breast cancer. *Breast cancer research and treatment* 123, 51-58.

Ariazi, E.A., Brailoiu, E., Yerrum, S., Shupp, H.A., Slifker, M.J., Cunliffe, H.E., Black, M.A., Donato, A.L., Arterburn, J.B., Oprea, T.I., *et al.* (2010). The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res* *70*, 1184-1194.

Arterburn, J.B., Oprea, T.I., Prossnitz, E.R., Edwards, B.S., and Sklar, L.A. (2009). Discovery of selective probes and antagonists for G-protein-coupled receptors FPR/FPRL1 and GPR30. *Current topics in medicinal chemistry* *9*, 1227-1236.

Asseman, C., Mauze, S., Leach, M.W., Coffman, R.L., and Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *The Journal of experimental medicine* *190*, 995-1004.

Asseman, C., Read, S., and Powrie, F. (2003). Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4+ regulatory T cells and IL-10. *J Immunol* *171*, 971-978.

Atarashi, K., Nishimura, J., Shima, T., Umesaki, Y., Yamamoto, M., Onoue, M., Yagita, H., Ishii, N., Evans, R., Honda, K., *et al.* (2008). ATP drives lamina propria T(H)17 cell differentiation. *Nature* *455*, 808-812.

Aujla, S.J., Dubin, P.J., and Kolls, J.K. (2007). Th17 cells and mucosal host defense. *Semin Immunol* *19*, 377-382.

Baker, L., Meldrum, K.K., Wang, M., Sankula, R., Vanam, R., Raiesdana, A., Tsai, B., Hile, K., Brown, J.W., and Meldrum, D.R. (2003). The role of estrogen in cardiovascular disease. *J Surg Res* *115*, 325-344.

Baquedano, M.S., Saraco, N., Berensztein, E., Pepe, C., Bianchini, M., Levy, E., Goni, J., Rivarola, M.A., and Belgorosky, A. (2007). Identification and developmental changes of aromatase and estrogen receptor expression in prepubertal and pubertal human adrenal tissues. *The Journal of clinical endocrinology and metabolism* *92*, 2215-2222.

Barnes, M.J., and Powrie, F. (2009). Hybrid Treg cells: steel frames and plastic exteriors. *Nature immunology* *10*, 563-564.

Battaglia, M., Gregori, S., Bacchetta, R., and Roncarolo, M.G. (2006). Tr1 cells: from discovery to their clinical application. *Semin Immunol* *18*, 120-127.

- Beatson, G.T. (1898). Case of Excision of a Mass of Enlarged Cervical Glands (Tuberculous) with a Portion of the Internal Jugular Vein. *British medical journal* *2*, 1124-1125.
- Belkaid, Y., and Tarbell, K. (2009). Regulatory T cells in the control of host-microorganism interactions (\*). *Annual review of immunology* *27*, 551-589.
- Bergmann, C., Strauss, L., Zeidler, R., Lang, S., and Whiteside, T.L. (2007). Expansion and characteristics of human T regulatory type 1 cells in co-cultures simulating tumor microenvironment. *Cancer Immunol Immunother* *56*, 1429-1442.
- Beriou, G., Costantino, C.M., Ashley, C.W., Yang, L., Kuchroo, V.K., Baecher-Allan, C., and Hafler, D.A. (2009). IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* *113*, 4240-4249.
- Berthois, Y., Katzenellenbogen, J.A., and Katzenellenbogen, B.S. (1986). Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci U S A* *83*, 2496-2500.
- Betelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., and Kuchroo, V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* *441*, 235-238.
- Betelli, E., Sullivan, B., Szabo, S.J., Sobel, R.A., Glimcher, L.H., and Kuchroo, V.K. (2004). Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *The Journal of experimental medicine* *200*, 79-87.
- Bland, P. (1988). MHC class II expression by the gut epithelium. *Immunol Today* *9*, 174-178.
- Blasko, E., Haskell, C.A., Leung, S., Gualtieri, G., Halks-Miller, M., Mahmoudi, M., Dennis, M.K., Prossnitz, E.R., Karpus, W.J., and Horuk, R. (2009). Beneficial role of the GPR30 agonist G-1 in an animal model of multiple sclerosis. *Journal of neuroimmunology* *214*, 67-77.
- Bluestone, J.A., Mackay, C.R., O'Shea, J.J., and Stockinger, B. (2009). The functional plasticity of T cell subsets. *Nature reviews* *9*, 811-816.
- Boissonnas, A., Scholer-Dahirel, A., Simon-Blancal, V., Pace, L., Valet, F., Kissenpfennig, A., Sparwasser, T., Malissen, B., Fetler, L., and Amigorena, S. (2010). Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. *Immunity* *32*, 266-278.

Bologna, C.G., Revankar, C.M., Young, S.M., Edwards, B.S., Arterburn, J.B., Kiselyov, A.S., Parker, M.A., Tkachenko, S.E., Savchuck, N.P., Sklar, L.A., *et al.* (2006). Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* 2, 207-212.

Bopassa, J.C., Eghbali, M., Toro, L., and Stefani, E. (2010). A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 298, H16-23.

Bopp, T., Becker, C., Klein, M., Klein-Hessling, S., Palmethofer, A., Serfling, E., Heib, V., Becker, M., Kubach, J., Schmitt, S., *et al.* (2007). Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *The Journal of experimental medicine* 204, 1303-1310.

Bopp, T., Dehzad, N., Reuter, S., Klein, M., Ullrich, N., Stassen, M., Schild, H., Buhl, R., Schmitt, E., and Taube, C. (2009). Inhibition of cAMP degradation improves regulatory T cell-mediated suppression. *J Immunol* 182, 4017-4024.

Borsellino, G., Kleinewietfeld, M., Di Mitri, D., Sternjak, A., Diamantini, A., Giometto, R., Hopner, S., Centonze, D., Bernardi, G., Dell'Acqua, M.L., *et al.* (2007). Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110, 1225-1232.

Breese, E., Braegger, C.P., Corrigan, C.J., Walker-Smith, J.A., and MacDonald, T.T. (1993). Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. *Immunology* 78, 127-131.

Brok, H.P., van Meurs, M., Blezer, E., Schantz, A., Peritt, D., Treacy, G., Laman, J.D., Bauer, J., and Hart, B.A. (2002). Prevention of experimental autoimmune encephalomyelitis in common marmosets using an anti-IL-12p40 monoclonal antibody. *J Immunol* 169, 6554-6563.

Brooks, D.G., Trifilo, M.J., Edelman, K.H., Teyton, L., McGavern, D.B., and Oldstone, M.B. (2006). Interleukin-10 determines viral clearance or persistence in vivo. *Nature medicine* 12, 1301-1309.

Brustle, A., Heink, S., Huber, M., Rosenplanter, C., Stadelmann, C., Yu, P., Arpaia, E., Mak, T.W., Kamradt, T., and Lohoff, M. (2007). The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nature immunology* 8, 958-966.

Cao, X., Cai, S.F., Fehniger, T.A., Song, J., Collins, L.I., Piwnica-Worms, D.R., and Ley, T.J. (2007). Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27, 635-646.

Carreras, E., Turner, S., Frank, M.B., Knowlton, N., Osban, J., Centola, M., Park, C.G., Simmons, A., Alberola-Ila, J., and Kovats, S. (2010). Estrogen receptor signaling promotes dendritic cell differentiation by increasing expression of the transcription factor IRF4. *Blood* 115, 238-246.

Cederbom, L., Hall, H., and Ivars, F. (2000). CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *European journal of immunology* 30, 1538-1543.

Chan, Q.K., Lam, H.M., Ng, C.F., Lee, A.Y., Chan, E.S., Ng, H.K., Ho, S.M., and Lau, K.M. (2010). Activation of GPR30 inhibits the growth of prostate cancer cells through sustained activation of Erk1/2, c-jun/c-fos-dependent upregulation of p21, and induction of G(2) cell-cycle arrest. *Cell death and differentiation* 17, 1511-1523.

Chen, Q., Yang, W., Gupta, S., Biswas, P., Smith, P., Bhagat, G., and Pernis, A.B. (2008). IRF-4-binding protein inhibits interleukin-17 and interleukin-21 production by controlling the activity of IRF-4 transcription factor. *Immunity* 29, 899-911.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *The Journal of experimental medicine* 198, 1875-1886.

Church, D., Elsayed, S., Reid, O., Winston, B., and Lindsay, R. (2006). Burn wound infections. *Clin Microbiol Rev* 19, 403-434.

Clark, L.B., Appleby, M.W., Brunkow, M.E., Wilkinson, J.E., Ziegler, S.F., and Ramsdell, F. (1999). Cellular and molecular characterization of the scurfy mouse mutant. *J Immunol* 162, 2546-2554.

Clowse, M.E. (2007). Lupus activity in pregnancy. *Rheum Dis Clin North Am* 33, 237-252, v.

Colin, E.M., Asmawidjaja, P.S., van Hamburg, J.P., Mus, A.M., van Driel, M., Hazes, J.M., van Leeuwen, J.P., and Lubberts, E. (2010). 1,25-dihydroxyvitamin D(3) modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. *Arthritis Rheum* 62, 132-142.

Collison, L.W., Pillai, M.R., Chaturvedi, V., and Vignali, D.A. (2009). Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. *J Immunol* *182*, 6121-6128.

Collison, L.W., Workman, C.J., Kuo, T.T., Boyd, K., Wang, Y., Vignali, K.M., Cross, R., Sehy, D., Blumberg, R.S., and Vignali, D.A. (2007). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* *450*, 566-569.

Confavreux, C., Hutchinson, M., Hours, M.M., Cortinovis-Tourniaire, P., and Moreau, T. (1998). Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group. *The New England journal of medicine* *339*, 285-291.

Coquerelle, C., Oldenhove, G., Acolty, V., Denoed, J., Vansanten, G., Verdebout, J.M., Mellor, A., Bluestone, J.A., and Moser, M. (2009). Anti-CTLA-4 treatment induces IL-10-producing ICOS<sup>+</sup> regulatory T cells displaying IDO-dependent anti-inflammatory properties in a mouse model of colitis. *Gut* *58*, 1363-1373.

Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., *et al.* (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* *421*, 744-748.

Curtis, T.P., Sloan, W.T., and Scannell, J.W. (2002). Estimating prokaryotic diversity and its limits. *Proc Natl Acad Sci U S A* *99*, 10494-10499.

Da Silva, J.A., and Spector, T.D. (1992). The role of pregnancy in the course and aetiology of rheumatoid arthritis. *Clin Rheumatol* *11*, 189-194.

Das, J., Ren, G., Zhang, L., Roberts, A.I., Zhao, X., Bothwell, A.L., Van Kaer, L., Shi, Y., and Das, G. (2009). Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. *The Journal of experimental medicine* *206*, 2407-2416.

Das, S.K., Tan, J., Raja, S., Halder, J., Paria, B.C., and Dey, S.K. (2000). Estrogen targets genes involved in protein processing, calcium homeostasis, and Wnt signaling in the mouse uterus independent of estrogen receptor-alpha and -beta. *The Journal of biological chemistry* *275*, 28834-28842.

Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enyoloji, K., Linden, J., Oukka, M., *et al.* (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *The Journal of experimental medicine* *204*, 1257-1265.

Del Prete, G., De Carli, M., Almerigogna, F., Giudizi, M.G., Biagiotti, R., and Romagnani, S. (1993). Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* *150*, 353-360.

Denning, T.L., Wang, Y.C., Patel, S.R., Williams, I.R., and Pulendran, B. (2007). Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nature immunology* *8*, 1086-1094.

Dennis, M.K., Burai, R., Ramesh, C., Petrie, W.K., Alcon, S.N., Nayak, T.K., Bologa, C.G., Leitao, A., Brailoiu, E., Deliu, E., *et al.* (2009). In vivo effects of a GPR30 antagonist. *Nat Chem Biol* *5*, 421-427.

Dong, S., Terasaka, S., and Kiyama, R. (2010). Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. *Environ Pollut* *159*, 212-218.

Du, J., Huang, C., Zhou, B., and Ziegler, S.F. (2008). Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. *J Immunol* *180*, 4785-4792.

Dubicke, A., Akerud, A., Sennstrom, M., Hamad, R.R., Bystrom, B., Malmstrom, A., and Ekman-Ordeberg, G. (2008). Different secretion patterns of matrix metalloproteinases and IL-8 and effect of corticotropin-releasing hormone in preterm and term cervical fibroblasts. *Molecular human reproduction* *14*, 641-647.

Dubinsky, M.C., Wang, D., Picornell, Y., Wrobel, I., Katzir, L., Quiros, A., Dutridge, D., Wahbeh, G., Silber, G., Bahar, R., *et al.* (2007). IL-23 receptor (IL-23R) gene protects against pediatric Crohn's disease. *Inflamm Bowel Dis* *13*, 511-515.

Duerr, R.H., Taylor, K.D., Brant, S.R., Rioux, J.D., Silverberg, M.S., Daly, M.J., Steinhardt, A.H., Abraham, C., Regueiro, M., Griffiths, A., *et al.* (2006). A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* *314*, 1461-1463.

Dun, S.L., Brailoiu, G.C., Gao, X., Brailoiu, E., Arterburn, J.B., Prossnitz, E.R., Oprea, T.I., and Dun, N.J. (2009). Expression of estrogen receptor GPR30 in the rat spinal cord and in autonomic and sensory ganglia. *Journal of neuroscience research* *87*, 1610-1619.



Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., and Mark, M. (2000). Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* *127*, 4277-4291.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., and Relman, D.A. (2005). Diversity of the human intestinal microbial flora. *Science* *308*, 1635-1638.

Edwards, D.P. (2005). Regulation of signal transduction pathways by estrogen and progesterone. *Annual review of physiology* *67*, 335-376.

Ejrnaes, M., Filippi, C.M., Martinic, M.M., Ling, E.M., Togher, L.M., Crotty, S., and von Herrath, M.G. (2006). Resolution of a chronic viral infection after interleukin-10 receptor blockade. *The Journal of experimental medicine* *203*, 2461-2472.

Elias, K.M., Laurence, A., Davidson, T.S., Stephens, G., Kanno, Y., Shevach, E.M., and O'Shea, J.J. (2008). Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* *111*, 1013-1020.

Etling, M.R., Davies, S., Campbell, M., Redline, R.W., Fu, P., and Levine, A.D. (2007). Maturation of the mucosal immune system underlies colitis susceptibility in interleukin-10-deficient (IL-10<sup>-/-</sup>) mice. *J Leukoc Biol* *82*, 311-319.

Fahlen, L., Read, S., Gorelik, L., Hurst, S.D., Coffman, R.L., Flavell, R.A., and Powrie, F. (2005). T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *The Journal of experimental medicine* *201*, 737-746.

Fallarino, F., Grohmann, U., Hwang, K.W., Orabona, C., Vacca, C., Bianchi, R., Belladonna, M.L., Fioretti, M.C., Alegre, M.L., and Puccetti, P. (2003). Modulation of tryptophan catabolism by regulatory T cells. *Nature immunology* *4*, 1206-1212.

Fasano, A., Berti, I., Gerarduzzi, T., Not, T., Colletti, R.B., Drago, S., Elitsur, Y., Green, P.H., Guandalini, S., Hill, I.D., *et al.* (2003). Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Arch Intern Med* *163*, 286-292.

Fassbender, M., Gerlitzki, B., Ullrich, N., Lupp, C., Klein, M., Radsak, M.P., Schmitt, E., Bopp, T., and Schild, H. (2010). Cyclic adenosine monophosphate and IL-10 coordinately contribute to nTreg cell-mediated suppression of dendritic cell activation. *Cellular immunology* *265*, 91-96.

Fazilleau, N., Mark, L., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2009). Follicular helper T cells: lineage and location. *Immunity* *30*, 324-335.

Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., and Thomas, P. (2007). Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. *Endocrinology* *148*, 3236-3245.

Filardo, E.J., Graeber, C.T., Quinn, J.A., Resnick, M.B., Giri, D., DeLellis, R.A., Steinhoff, M.M., and Sabo, E. (2006). Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin Cancer Res* *12*, 6359-6366.

Filardo, E.J., Quinn, J.A., Bland, K.I., and Frackelton, A.R., Jr. (2000). Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* *14*, 1649-1660.

Filardo, E.J., Quinn, J.A., and Sabo, E. (2008). Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor. *Steroids* *73*, 870-873.

Filice, E., Recchia, A.G., Pellegrino, D., Angelone, T., Maggiolini, M., and Cerra, M.C. (2009). A new membrane G protein-coupled receptor (GPR30) is involved in the cardiac effects of 17beta-estradiol in the male rat. *J Physiol Pharmacol* *60*, 3-10.

Fiorentino, D.F., Bond, M.W., and Mosmann, T.R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *The Journal of experimental medicine* *170*, 2081-2095.

Fitzgerald, D.C., Zhang, G.X., El-Behi, M., Fonseca-Kelly, Z., Li, H., Yu, S., Saris, C.J., Gran, B., Ciric, B., and Rostami, A. (2007). Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. *Nature immunology* *8*, 1372-1379.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature immunology* *4*, 330-336.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005a). A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nature immunology* *6*, 1142-1151.

Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G., and Rudensky, A.Y. (2005b). Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22, 329-341.

Ford, J., Hajibeigi, A., Long, M., Hahner, L., Gore, C., Hsieh, J.T., Clegg, D., Zerwekh, J., and Oz, O.K. (2010). GPR30 deficiency causes increased bone mass, mineralization, and growth plate proliferative activity in male mice. *J Bone Miner Res.*

Gaffen, S.L. (2009). Structure and signalling in the IL-17 receptor family. *Nature reviews* 9, 556-567.

Gavin, M.A., Torgerson, T.R., Houston, E., DeRoos, P., Ho, W.Y., Stray-Pedersen, A., Ocheltree, E.L., Greenberg, P.D., Ochs, H.D., and Rudensky, A.Y. (2006). Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* 103, 6659-6664.

Gazzinelli, R.T., Wysocka, M., Hieny, S., Scharon-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G., and Sher, A. (1996). In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J Immunol* 157, 798-805.

Getnet, D. (2010). Differential expression of Helios in regulatory T cells (The Johns Hopkins University), pp. 119.

Getnet, D., Grosso, J.F., Goldberg, M.V., Harris, T.J., Yen, H.R., Bruno, T.C., Durham, N.M., Hipkiss, E.L., Pyle, K.J., Wada, S., *et al.* (2010). A role for the transcription factor Helios in human CD4(+)CD25(+) regulatory T cells. *Molecular immunology* 47, 1595-1600.

Giess, M., Latrich, C., Springwald, A., Goerse, R., Ortmann, O., and Treeck, O. (2010). GPR30 gene polymorphisms are associated with progesterone receptor status and histopathological characteristics of breast cancer patients. *The Journal of steroid biochemistry and molecular biology* 118, 7-12.

Glocker, E.O., Kotlarz, D., Boztug, K., Gertz, E.M., Schaffer, A.A., Noyan, F., Perro, M., Diestelhorst, J., Allroth, A., Murugan, D., *et al.* (2009). Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *The New England journal of medicine* 361, 2033-2045.

Godfrey, D.I., MacDonald, H.R., Kronenberg, M., Smyth, M.J., and Van Kaer, L. (2004). NKT cells: what's in a name? *Nature reviews* 4, 231-237.

Gondek, D.C., Lu, L.F., Quezada, S.A., Sakaguchi, S., and Noelle, R.J. (2005). Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* *174*, 1783-1786.

Gottschalk, R.A., Corse, E., and Allison, J.P. (2010). TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. *The Journal of experimental medicine* *207*, 1701-1711.

Gran, B., Chu, N., Zhang, G.X., Yu, S., Li, Y., Chen, X.H., Kamoun, M., and Rostami, A. (2004). Early administration of IL-12 suppresses EAE through induction of interferon-gamma. *Journal of neuroimmunology* *156*, 123-131.

Gran, B., Zhang, G.X., Yu, S., Li, J., Chen, X.H., Ventura, E.S., Kamoun, M., and Rostami, A. (2002). IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. *J Immunol* *169*, 7104-7110.

Green, E.A., Gorelik, L., McGregor, C.M., Tran, E.H., and Flavell, R.A. (2003). CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. *Proc Natl Acad Sci U S A* *100*, 10878-10883.

Grossman, W.J., Verbsky, J.W., Tollefsen, B.L., Kemper, C., Atkinson, J.P., and Ley, T.J. (2004). Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* *104*, 2840-2848.

Haak, S., Croxford, A.L., Kreymborg, K., Heppner, F.L., Pouly, S., Becher, B., and Waisman, A. (2009). IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. *The Journal of clinical investigation* *119*, 61-69.

Haribhai, D., Lin, W., Relland, L.M., Truong, N., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cells dynamically control the primary immune response to foreign antigen. *J Immunol* *178*, 2961-2972.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., and Weaver, C.T. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature immunology* *6*, 1123-1132.

Hazell, G.G., Yao, S.T., Roper, J.A., Prossnitz, E.R., O'Carroll, A.M., and Lolait, S.J. (2009). Localisation of GPR30, a novel G protein-coupled oestrogen

receptor, suggests multiple functions in rodent brain and peripheral tissues. *J Endocrinol* 202, 223-236.

He, D., Wu, L., Kim, H.K., Li, H., Elmets, C.A., and Xu, H. (2006). CD8<sup>+</sup> IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses. *J Immunol* 177, 6852-6858.

Herman, A.E., Freeman, G.J., Mathis, D., and Benoist, C. (2004). CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *The Journal of experimental medicine* 199, 1479-1489.

Hill, J.A., Feuerer, M., Tash, K., Haxhinasto, S., Perez, J., Melamed, R., Mathis, D., and Benoist, C. (2007). Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27, 786-800.

Hofstetter, H.H., Ibrahim, S.M., Koczan, D., Kruse, N., Weishaupt, A., Toyka, K.V., and Gold, R. (2005). Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cellular immunology* 237, 123-130.

Hooper, L.V., Midtvedt, T., and Gordon, J.I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr* 22, 283-307.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057-1061.

Hu, Y., Ota, N., Peng, I., Refino, C.J., Danilenko, D.M., Caplazi, P., and Ouyang, W. (2010). IL-17RC is required for IL-17A- and IL-17F-dependent signaling and the pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol* 184, 4307-4316.

Hue, S., Ahern, P., Buonocore, S., Kullberg, M.C., Cua, D.J., McKenzie, B.S., Powrie, F., and Maloy, K.J. (2006). Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *The Journal of experimental medicine* 203, 2473-2483.

Hueber, A.J., Asquith, D.L., Miller, A.M., Reilly, J., Kerr, S., Leipe, J., Melendez, A.J., and McInnes, I.B. (2010). Mast cells express IL-17A in rheumatoid arthritis synovium. *J Immunol* 184, 3336-3340.

Ignatov, A., Ignatov, T., Roessner, A., Costa, S.D., and Kalinski, T. (2010). Role of GPR30 in the mechanisms of tamoxifen resistance in breast cancer MCF-7 cells. *Breast cancer research and treatment* 123, 87-96.

Isensee, J., Meoli, L., Zazzu, V., Nabzdyk, C., Witt, H., Soewarto, D., Effertz, K., Fuchs, H., Gailus-Durner, V., Busch, D., *et al.* (2009). Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice. *Endocrinology* *150*, 1722-1730.

Ito, A., Bebo, B.F., Jr., Matejuk, A., Zamora, A., Silverman, M., Fyfe-Johnson, A., and Offner, H. (2001). Estrogen treatment down-regulates TNF-alpha production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice. *J Immunol* *167*, 542-552.

Ito, H., and Fathman, C.G. (1997). CD45RB<sup>high</sup> CD4<sup>+</sup> T cells from IFN-gamma knockout mice do not induce wasting disease. *J Autoimmun* *10*, 455-459.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor ROR $\gamma$  directs the differentiation program of proinflammatory IL-17<sup>+</sup> T helper cells. *Cell* *126*, 1121-1133.

Iwakura, Y., and Ishigame, H. (2006). The IL-23/IL-17 axis in inflammation. *The Journal of clinical investigation* *116*, 1218-1222.

Izcue, A., Hue, S., Buonocore, S., Arancibia-Carcamo, C.V., Ahern, P.P., Iwakura, Y., Maloy, K.J., and Powrie, F. (2008). Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* *28*, 559-570.

Jankovic, D., Kullberg, M.C., Feng, C.G., Goldszmid, R.S., Collazo, C.M., Wilson, M., Wynn, T.A., Kamanaka, M., Flavell, R.A., and Sher, A. (2007). Conventional T-bet<sup>+</sup>Foxp3<sup>-</sup> Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *The Journal of experimental medicine* *204*, 273-283.

Jensen, E.V., and DeSombre, E.R. (1973). Estrogen-receptor interaction. *Science* *182*, 126-134.

Jessup, J.A., Lindsey, S.H., Wang, H., Chappell, M.C., and Groban, L. (2010). Attenuation of salt-induced cardiac remodeling and diastolic dysfunction by the GPER agonist G-1 in female mRen2.Lewis rats. *PloS one* *5*, e15433.

Kamada, N., Hisamatsu, T., Okamoto, S., Chinen, H., Kobayashi, T., Sato, T., Sakuraba, A., Kitazume, M.T., Sugita, A., Koganei, K., *et al.* (2008). Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *The Journal of clinical investigation* *118*, 2269-2280.

- Kang, L., Zhang, X., Xie, Y., Tu, Y., Wang, D., Liu, Z., and Wang, Z.Y. (2010). Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. *Mol Endocrinol* *24*, 709-721.
- Kano, S., Sato, K., Morishita, Y., Vollstedt, S., Kim, S., Bishop, K., Honda, K., Kubo, M., and Taniguchi, T. (2008). The contribution of transcription factor IRF1 to the interferon-gamma-interleukin 12 signaling axis and TH1 versus TH-17 differentiation of CD4+ T cells. *Nature immunology* *9*, 34-41.
- Karpuzoglu, E., Fenaux, J.B., Phillips, R.A., Lengi, A.J., Elvinger, F., and Ansar Ahmed, S. (2006). Estrogen up-regulates inducible nitric oxide synthase, nitric oxide, and cyclooxygenase-2 in splenocytes activated with T cell stimulants: role of interferon-gamma. *Endocrinology* *147*, 662-671.
- Khattari, R., Cox, T., Yasayko, S.A., and Ramsdell, F. (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nature immunology* *4*, 337-342.
- Kleinschek, M.A., Boniface, K., Sadekova, S., Grein, J., Murphy, E.E., Turner, S.P., Raskin, L., Desai, B., Faubion, W.A., de Waal Malefyt, R., *et al.* (2009). Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *The Journal of experimental medicine* *206*, 525-534.
- Kleuser, B., Malek, D., Gust, R., Pertz, H.H., and Potteck, H. (2008). 17- $\beta$ -Estradiol inhibits Transforming Growth Factor- $\beta$  signalling and function in breast cancer cells via activation of Extracellular Signal-Regulated Kinase through the G protein coupled receptor 30. *Mol Pharmacol*.
- Kobayashi, T., Okamoto, S., Hisamatsu, T., Kamada, N., Chinen, H., Saito, R., Kitazume, M.T., Nakazawa, A., Sugita, A., Koganei, K., *et al.* (2008). IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut* *57*, 1682-1689.
- Kobie, J.J., Shah, P.R., Yang, L., Rebhahn, J.A., Fowell, D.J., and Mosmann, T.R. (2006). T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J Immunol* *177*, 6780-6786.
- Koch, M.A., Tucker-Heard, G., Perdue, N.R., Killebrew, J.R., Urdahl, K.B., and Campbell, D.J. (2009). The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nature immunology* *10*, 595-602.

- Kochetkova, I., Golden, S., Holderness, K., Callis, G., and Pascual, D.W. (2010). IL-35 stimulation of CD39+ regulatory T cells confers protection against collagen II-induced arthritis via the production of IL-10. *J Immunol* *184*, 7144-7153.
- Kryczek, I., Wei, S., Zou, L., Altuwaijri, S., Szeliga, W., Kolls, J., Chang, A., and Zou, W. (2007). Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *J Immunol* *178*, 6730-6733.
- Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* *75*, 263-274.
- Kuiper, G.G., Enmark, E., Peltö-Huikko, M., Nilsson, S., and Gustafsson, J.A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* *93*, 5925-5930.
- Kurosaki, T., Shinohara, H., and Baba, Y. (2009). B cell signaling and fate decision. *Annual review of immunology* *28*, 21-55.
- Langer, G., Bader, B., Meoli, L., Isensee, J., Delbeck, M., Noppinger, P.R., and Otto, C. (2010). A critical review of fundamental controversies in the field of GPR30 research. *Steroids* *75*, 603-610.
- Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., and Cua, D.J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal of experimental medicine* *201*, 233-240.
- Lavasani, S., Dzhambazov, B., Nouri, M., Fak, F., Buske, S., Molin, G., Thorlacius, H., Alenfall, J., Jeppsson, B., and Westrom, B. (2010). A novel probiotic mixture exerts a therapeutic effect on experimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. *PLoS one* *5*, e9009.
- Lee, K.A., Kang, M.H., Lee, Y.S., Kim, Y.J., Kim, D.H., Ko, H.J., and Kang, C.Y. (2008). A distinct subset of natural killer T cells produces IL-17, contributing to airway infiltration of neutrophils but not to airway hyperreactivity. *Cellular immunology* *251*, 50-55.
- Lee, Y.K., Mukasa, R., Hatton, R.D., and Weaver, C.T. (2009a). Developmental plasticity of Th17 and Treg cells. *Current opinion in immunology* *21*, 274-280.
- Lee, Y.K., Turner, H., Maynard, C.L., Oliver, J.R., Chen, D., Elson, C.O., and Weaver, C.T. (2009b). Late developmental plasticity in the T helper 17 lineage. *Immunity* *30*, 92-107.



Leithauser, F., Meinhardt-Krajina, T., Fink, K., Wotschke, B., Moller, P., and Reimann, J. (2006). Foxp3-expressing CD103+ regulatory T cells accumulate in dendritic cell aggregates of the colonic mucosa in murine transfer colitis. *Am J Pathol* 168, 1898-1909.

Lengi, A.J., Phillips, R.A., Karpuzoglu, E., and Ahmed, S.A. (2006). 17beta-estradiol downregulates interferon regulatory factor-1 in murine splenocytes. *J Mol Endocrinol* 37, 421-432.

Leppkes, M., Becker, C., Ivanov, I., Hirth, S., Wirtz, S., Neufert, C., Pouly, S., Murphy, A.J., Valenzuela, D.M., Yancopoulos, G.D., *et al.* (2009). RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* 136, 257-267.

Li, C., Corraliza, I., and Langhorne, J. (1999). A defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi chabaudi* infection in mice. *Infect Immun* 67, 4435-4442.

Li, L., Huang, L., Vergis, A.L., Ye, H., Bajwa, A., Narayan, V., Strieter, R.M., Rosin, D.L., and Okusa, M.D. (2010). IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. *The Journal of clinical investigation* 120, 331-342.

Liao, J.J., Huang, M.C., and Goetzl, E.J. (2007). Cutting edge: Alternative signaling of Th17 cell development by sphingosine 1-phosphate. *J Immunol* 178, 5425-5428.

Liu, Q., Li, J.G., Zheng, X.Y., Jin, F., and Dong, H.T. (2009). Expression of CD133, PAX2, ESA, and GPR30 in invasive ductal breast carcinomas. *Chinese medical journal* 122, 2763-2769.

Liu, V.C., Wong, L.Y., Jang, T., Shah, A.H., Park, I., Yang, X., Zhang, Q., Lonning, S., Teicher, B.A., and Lee, C. (2007). Tumor evasion of the immune system by converting CD4+CD25- T cells into CD4+CD25+ T regulatory cells: role of tumor-derived TGF-beta. *J Immunol* 178, 2883-2892.

Liverman, C.S., Brown, J.W., Sandhir, R., McCarson, K.E., and Berman, N.E. (2009). Role of the oestrogen receptors GPR30 and ERalpha in peripheral sensitization: relevance to trigeminal pain disorders in women. *Cephalalgia* 29, 729-741.

Lu, C.L., Hsieh, J.C., Dun, N.J., Oprea, T.I., Wang, P.S., Luo, J.C., Lin, H.C., Chang, F.Y., and Lee, S.D. (2009). Estrogen rapidly modulates 5-

hydroxytryptophan-induced visceral hypersensitivity via GPR30 in rats. *Gastroenterology* 137, 1040-1050.

Maggiolini, M., Vivacqua, A., Fasanella, G., Recchia, A.G., Sisci, D., Pezzi, V., Montanaro, D., Musti, A.M., Picard, D., and Ando, S. (2004). The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. *The Journal of biological chemistry* 279, 27008-27016.

Mann, M.K., Maresz, K., Shriver, L.P., Tan, Y., and Dittel, B.N. (2007). B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. *J Immunol* 178, 3447-3456.

Marie, J.C., Letterio, J.J., Gavin, M., and Rudensky, A.Y. (2005). TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *The Journal of experimental medicine* 201, 1061-1067.

Marino, M., Galluzzo, P., and Ascenzi, P. (2006). Estrogen signaling multiple pathways to impact gene transcription. *Current genomics* 7, 497-508.

Marks, B.R., Nowyhed, H.N., Choi, J.Y., Poholek, A.C., Odegard, J.M., Flavell, R.A., and Craft, J. (2009). Thymic self-reactivity selects natural interleukin 17-producing T cells that can regulate peripheral inflammation. *Nature immunology* 10, 1125-1132.

Marteau, P., Lepage, P., Mangin, I., Suau, A., Dore, J., Pochart, P., and Seksik, P. (2004). Review article: gut flora and inflammatory bowel disease. *Aliment Pharmacol Ther* 20 Suppl 4, 18-23.

Martensson, U.E., Salehi, S.A., Windahl, S., Gomez, M.F., Sward, K., Daszkiewicz-Nilsson, J., Wendt, A., Andersson, N., Hellstrand, P., Grande, P.O., *et al.* (2008). Deletion of the G protein-coupled Receptor GPR30 Impairs Glucose Tolerance, Reduces Bone Growth, Increases Blood Pressure, and Eliminates Estradiol-stimulated Insulin Release in Female Mice. *Endocrinology*.

Martin-Orozco, N., Muranski, P., Chung, Y., Yang, X.O., Yamazaki, T., Lu, S., Hwu, P., Restifo, N.P., Overwijk, W.W., and Dong, C. (2009). T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity* 31, 787-798.

Maynard, C.L., Harrington, L.E., Janowski, K.M., Oliver, J.R., Zindl, C.L., Rudensky, A.Y., and Weaver, C.T. (2007). Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nature immunology* 8, 931-941.

Maynard, C.L., and Weaver, C.T. (2008). Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. *Immunological reviews* 226, 219-233.

McClain, M.A., Gatson, N.N., Powell, N.D., Papenfuss, T.L., Gienapp, I.E., Song, F., Shawler, T.M., Kithcart, A., and Whitacre, C.C. (2007). Pregnancy suppresses experimental autoimmune encephalomyelitis through immunoregulatory cytokine production. *J Immunol* 179, 8146-8152.

McGeachy, M.J., Bak-Jensen, K.S., Chen, Y., Tato, C.M., Blumenschein, W., McClanahan, T., and Cua, D.J. (2007). TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nature immunology* 8, 1390-1397.

McGeachy, M.J., Chen, Y., Tato, C.M., Laurence, A., Joyce-Shaikh, B., Blumenschein, W.M., McClanahan, T.K., O'Shea, J.J., and Cua, D.J. (2009). The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nature immunology* 10, 314-324.

McGeachy, M.J., and Cua, D.J. (2007). The link between IL-23 and Th17 cell-mediated immune pathologies. *Semin Immunol* 19, 372-376.

McGeachy, M.J., and Cua, D.J. (2008). Th17 cell differentiation: the long and winding road. *Immunity* 28, 445-453.

McHugh, R.S., Whitters, M.J., Piccirillo, C.A., Young, D.A., Shevach, E.M., Collins, M., and Byrne, M.C. (2002). CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16, 311-323.

Meyaard, L., Hovenkamp, E., Otto, S.A., and Miedema, F. (1996). IL-12-induced IL-10 production by human T cells as a negative feedback for IL-12-induced immune responses. *J Immunol* 156, 2776-2782.

Meyer, M.R., and Barton, M. (2009). ERalpha, ERbeta, and gpER: novel aspects of oestrogen receptor signalling in atherosclerosis. *Cardiovascular research* 83, 605-610.

Michel, M.L., Keller, A.C., Paget, C., Fujio, M., Trottein, F., Savage, P.B., Wong, C.H., Schneider, E., Dy, M., and Leite-de-Moraes, M.C. (2007). Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *The Journal of experimental medicine* 204, 995-1001.

- Moore, F.D., Woodrow, S.I., Aliapoulios, M.A., and Wilson, R.E. (1967). Carcinoma of the breast. A decade of new results with old concepts. *The New England journal of medicine* *277*, 460-468 concl.
- Moore, K.W., de Waal Malefyt, R., Coffman, R.L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology* *19*, 683-765.
- Morgan, M.E., van Bilsen, J.H., Bakker, A.M., Heemskerk, B., Schilham, M.W., Hartgers, F.C., Elferink, B.G., van der Zanden, L., de Vries, R.R., Huizinga, T.W., *et al.* (2005). Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Human immunology* *66*, 13-20.
- Mosmann, T.R., and Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual review of immunology* *7*, 145-173.
- Mrabet-Dahbi, S., Metz, M., Dudeck, A., Zuberbier, T., and Maurer, M. (2009). Murine mast cells secrete a unique profile of cytokines and prostaglandins in response to distinct TLR2 ligands. *Experimental dermatology* *18*, 437-444.
- Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., and Cheroutre, H. (2007). Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* *317*, 256-260.
- Muller, R.E., Johnston, T.C., Traish, A.M., and Wotiz, H.H. (1979). Studies on the mechanism of estradiol uptake by rat uterine cells and on estradiol binding to uterine plasma membranes. *Advances in experimental medicine and biology* *117*, 401-421.
- Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C.L., Cheroutre, H., and Kronenberg, M. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nature immunology* *10*, 1178-1184.
- Muranski, P., Boni, A., Antony, P.A., Cassard, L., Irvine, K.R., Kaiser, A., Paulos, C.M., Palmer, D.C., Touloukian, C.E., Ptak, K., *et al.* (2008). Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* *112*, 362-373.
- Murphy, C.A., Langrish, C.L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R.A., Sedgwick, J.D., and Cua, D.J. (2003). Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *The Journal of experimental medicine* *198*, 1951-1957.

Murphy, K.M., and Stockinger, B. (2010). Effector T cell plasticity: flexibility in the face of changing circumstances. *Nature immunology* *11*, 674-680.

Nadal, A., Alonso-Magdalena, P., Soriano, S., Ripoll, C., Fuentes, E., Quesada, I., and Ropero, A.B. (2011). Role of estrogen receptors alpha, beta and GPER1/GPR30 in pancreatic beta-cells. *Front Biosci* *16*, 251-260.

Nakamura, K., Kitani, A., and Strober, W. (2001). Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *The Journal of experimental medicine* *194*, 629-644.

Nunes-Cabaco, H., Ribot, J.C., Caramalho, I., Serra-Caetano, A., Silva-Santos, B., and Sousa, A.E. (2010). Foxp3 induction in human and murine thymus precedes the CD4+ CD8+ stage but requires early T-cell receptor expression. *Immunol Cell Biol* *88*, 523-528.

O'Connor, W., Jr., Kamanaka, M., Booth, C.J., Town, T., Nakae, S., Iwakura, Y., Kolls, J.K., and Flavell, R.A. (2009). A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nature immunology* *10*, 603-609.

O'Connor, W., Jr., Zenewicz, L.A., and Flavell, R.A. (2010). The dual nature of T(H)17 cells: shifting the focus to function. *Nature immunology* *11*, 471-476.

O'Garra, A., Barrat, F.J., Castro, A.G., Vicari, A., and Hawrylowicz, C. (2008). Strategies for use of IL-10 or its antagonists in human disease. *Immunological reviews* *223*, 114-131.

O'Hara, A.M., and Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Rep* *7*, 688-693.

Oderup, C., Cederbom, L., Makowska, A., Cilio, C.M., and Ivars, F. (2006). Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology* *118*, 240-249.

Offner, H., and Polanczyk, M. (2006). A potential role for estrogen in experimental autoimmune encephalomyelitis and multiple sclerosis. *Ann N Y Acad Sci* *1089*, 343-372.

Ogawa, A., Andoh, A., Araki, Y., Bamba, T., and Fujiyama, Y. (2004). Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clin Immunol* *110*, 55-62.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., *et al.* (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* *13*, 715-725.

Ostanin, D.V., Bao, J., Koboziev, I., Gray, L., Robinson-Jackson, S.A., Kosloski-Davidson, M., Price, V.H., and Grisham, M.B. (2009). T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. *Am J Physiol Gastrointest Liver Physiol* *296*, G135-146.

Otto, C., Fuchs, I., Kauselmann, G., Kern, H., Zevnik, B., Andreassen, P., Schwarz, G., Altmann, H., Klewer, M., Schoor, M., *et al.* (2009). GPR30 does not mediate estrogenic responses in reproductive organs in mice. *Biology of reproduction* *80*, 34-41.

Ouyang, W., Kolls, J.K., and Zheng, Y. (2008). The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* *28*, 454-467.

Ouyang, W., and Valdez, P. (2008). IL-22 in mucosal immunity. *Mucosal immunology* *1*, 335-338.

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., *et al.* (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature immunology* *6*, 1133-1141.

Pasqualini, J.R., Gelly, C., Nguyen, B.L., and Vella, C. (1989). Importance of estrogen sulfates in breast cancer. *Journal of steroid biochemistry* *34*, 155-163.

Patey-Mariaud de Serre, N., Canioni, D., Ganousse, S., Rieux-Laucat, F., Goulet, O., Ruemmele, F., and Brousse, N. (2008). Digestive histopathological presentation of IPEX syndrome. *Mod Pathol*.

Paul, W.E., and Zhu, J. (2010). How are T(H)2-type immune responses initiated and amplified? *Nature reviews* *10*, 225-235.

Pernis, A.B. (2007). Estrogen and CD4+ T cells. *Curr Opin Rheumatol* *19*, 414-420.

Pernis, A.B. (2009). Th17 cells in rheumatoid arthritis and systemic lupus erythematosus. *J Intern Med* *265*, 644-652.

Piccirillo, C.A., Letterio, J.J., Thornton, A.M., McHugh, R.S., Mamura, M., Mizuhara, H., and Shevach, E.M. (2002). CD4(+)CD25(+) regulatory T cells can

mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *The Journal of experimental medicine* 196, 237-246.

Pickert, G., Neufert, C., Leppkes, M., Zheng, Y., Wittkopf, N., Warntjen, M., Lehr, H.A., Hirth, S., Weigmann, B., Wirtz, S., *et al.* (2009). STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *The Journal of experimental medicine* 206, 1465-1472.

Podolsky, D.K. (2002). Inflammatory bowel disease. *The New England journal of medicine* 347, 417-429.

Polanczyk, M., Yellayi, S., Zamora, A., Subramanian, S., Tovey, M., Vandenberg, A.A., Offner, H., Zachary, J.F., Fillmore, P.D., Blankenhorn, E.P., *et al.* (2004a). Estrogen receptor-1 (Esr1) and -2 (Esr2) regulate the severity of clinical experimental allergic encephalomyelitis in male mice. *Am J Pathol* 164, 1915-1924.

Polanczyk, M., Zamora, A., Subramanian, S., Matejuk, A., Hess, D.L., Blankenhorn, E.P., Teuscher, C., Vandenberg, A.A., and Offner, H. (2003). The protective effect of 17beta-estradiol on experimental autoimmune encephalomyelitis is mediated through estrogen receptor-alpha. *Am J Pathol* 163, 1599-1605.

Polanczyk, M.J., Carson, B.D., Subramanian, S., Afentoulis, M., Vandenberg, A.A., Ziegler, S.F., and Offner, H. (2004b). Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. *J Immunol* 173, 2227-2230.

Polanczyk, M.J., Hopke, C., Huan, J., Vandenberg, A.A., and Offner, H. (2005). Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. *Journal of neuroimmunology* 170, 85-92.

Polanczyk, M.J., Hopke, C., Vandenberg, A.A., and Offner, H. (2006). Estrogen-mediated immunomodulation involves reduced activation of effector T cells, potentiation of Treg cells, and enhanced expression of the PD-1 costimulatory pathway. *Journal of neuroscience research* 84, 370-378.

Polanczyk, M.J., Hopke, C., Vandenberg, A.A., and Offner, H. (2007). Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). *International immunology* 19, 337-343.

Powrie, F., and Coffman, R.L. (1993). Inhibition of cell-mediated immunity by IL4 and IL10. *Research in immunology* 144, 639-643.

Powrie, F., Leach, M.W., Mauze, S., Caddle, L.B., and Coffman, R.L. (1993a). Phenotypically distinct subsets of CD4<sup>+</sup> T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *International immunology* *5*, 1461-1471.

Powrie, F., Leach, M.W., Mauze, S., Menon, S., Caddle, L.B., and Coffman, R.L. (1994). Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4<sup>+</sup> T cells. *Immunity* *1*, 553-562.

Powrie, F., Menon, S., and Coffman, R.L. (1993b). Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. *European journal of immunology* *23*, 2223-2229.

Powrie, F., Menon, S., and Coffman, R.L. (1993c). Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. *European journal of immunology* *23*, 3043-3049.

Prossnitz, E.R., Arterburn, J.B., Smith, H.O., Oprea, T.I., Sklar, L.A., and Hathaway, H.J. (2008). Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annual review of physiology* *70*, 165-190.

Prossnitz, E.R., and Barton, M. (2009a). Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER. *Prostaglandins & other lipid mediators* *89*, 89-97.

Prossnitz, E.R., and Barton, M. (2009b). Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER. *Prostaglandins & other lipid mediators*.

Prossnitz, E.R., and Maggiolini, M. (2009). Mechanisms of estrogen signaling and gene expression via GPR30. *Mol Cell Endocrinol* *308*, 32-38.

Rachitskaya, A.V., Hansen, A.M., Horai, R., Li, Z., Villasmil, R., Luger, D., Nussenblatt, R.B., and Caspi, R.R. (2008). Cutting edge: NKT cells constitutively express IL-23 receptor and ROR $\gamma$  and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. *J Immunol* *180*, 5167-5171.

Ramirez, J.M., Brembilla, N.C., Sorg, O., Chicheportiche, R., Matthes, T., Dayer, J.M., Saurat, J.H., Roosnek, E., and Chizzolini, C. (2010). Activation of the aryl hydrocarbon receptor reveals distinct requirements for IL-22 and IL-17 production by human T helper cells. *European journal of immunology* *40*, 2450-2459.



- Read, C.P., Word, R.A., Ruscheinsky, M.A., Timmons, B.C., and Mahendroo, M.S. (2007). Cervical remodeling during pregnancy and parturition: molecular characterization of the softening phase in mice. *Reproduction* (Cambridge, England) *134*, 327-340.
- Read, S., Malmstrom, V., and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *The Journal of experimental medicine* *192*, 295-302.
- Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., and Prossnitz, E.R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* *307*, 1625-1630.
- Revankar, C.M., Mitchell, H.D., Field, A.S., Burai, R., Corona, C., Ramesh, C., Sklar, L.A., Arterburn, J.B., and Prossnitz, E.R. (2007). Synthetic estrogen derivatives demonstrate the functionality of intracellular GPR30. *ACS Chem Biol* *2*, 536-544.
- Roncarolo, M.G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., and Levings, M.K. (2006). Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological reviews* *212*, 28-50.
- Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R., Jr., *et al.* (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* *28*, 546-558.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* *155*, 1151-1164.
- Salzman, N.H., Underwood, M.A., and Bevins, C.L. (2007). Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. *Semin Immunol* *19*, 70-83.
- Samanta, A., Li, B., Song, X., Bembas, K., Zhang, G., Katsumata, M., Saouaf, S.J., Wang, Q., Hancock, W.W., Shen, Y., *et al.* (2008). TGF-beta and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. *Proc Natl Acad Sci U S A* *105*, 14023-14027.

Sanchez-Munoz, F., Dominguez-Lopez, A., and Yamamoto-Furusho, J.K. (2008). Role of cytokines in inflammatory bowel disease. *World J Gastroenterol* *14*, 4280-4288.

Sanden, C., Broselid, S., Cornmark, L., Andersson, K., Daszkiewicz-Nilsson, J., Martensson, U., Olde, B., and Leeb-Lundberg, F.L. (2010). G Protein-Coupled Estrogen Receptor 1 (GPER1)/GPR30 Localizes in the Plasma Membrane and Trafficks Intracellularly on Cytokeratin Intermediate Filaments. *Mol Pharmacol*.

Saraiva, M., Christensen, J.R., Veldhoen, M., Murphy, T.L., Murphy, K.M., and O'Garra, A. (2009). Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. *Immunity* *31*, 209-219.

Saraiva, M., and O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature reviews* *10*, 170-181.

Sauer, S., Bruno, L., Hertweck, A., Finlay, D., Leleu, M., Spivakov, M., Knight, Z.A., Cobb, B.S., Cantrell, D., O'Connor, E., *et al.* (2008). T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A* *105*, 7797-7802.

Schepers, K., Arens, R., and Schumacher, T.N. (2005). Dissection of cytotoxic and helper T cell responses. *Cell Mol Life Sci* *62*, 2695-2710.

Schumacher, A., Wafula, P.O., Bertoja, A.Z., Sollwedel, A., Thuere, C., Wollenberg, I., Yagita, H., Volk, H.D., and Zenclussen, A.C. (2007). Mechanisms of action of regulatory T cells specific for paternal antigens during pregnancy. *Obstetrics and gynecology* *110*, 1137-1145.

Sharma, M.D., Hou, D.Y., Liu, Y., Koni, P.A., Metz, R., Chandler, P., Mellor, A.L., He, Y., and Munn, D.H. (2009). Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood* *113*, 6102-6111.

Shin, H.C., Benbernou, N., Fekkar, H., Esnault, S., and Guenounou, M. (1998). Regulation of IL-17, IFN-gamma and IL-10 in human CD8(+) T cells by cyclic AMP-dependent signal transduction pathway. *Cytokine* *10*, 841-850.

Shughrue, P.J., Askew, G.R., Dellovade, T.L., and Merchenthaler, I. (2002). Estrogen-binding sites and their functional capacity in estrogen receptor double knockout mouse brain. *Endocrinology* *143*, 1643-1650.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Corbin, C.J., and Mendelson, C.R. (1993). Tissue-specific promoters regulate aromatase cytochrome P450 expression. *Clin Chem* *39*, 317-324.

Smith, H.O., Leslie, K.K., Singh, M., Qualls, C.R., Revankar, C.M., Joste, N.E., and Prossnitz, E.R. (2007). GPR30: a novel indicator of poor survival for endometrial carcinoma. *Am J Obstet Gynecol* *196*, 386 e381-389; discussion 386 e389-311.

Sollid, L.M. (2000). Molecular basis of celiac disease. *Annual review of immunology* *18*, 53-81.

Sollid, L.M., and Johansen, F.E. (2008). Animal models of inflammatory bowel disease at the dawn of the new genetics era. *PLoS Med* *5*, e198.

Song, C., Luo, L., Lei, Z., Li, B., Liang, Z., Liu, G., Li, D., Zhang, G., Huang, B., and Feng, Z.H. (2008). IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol* *181*, 6117-6124.

Spolski, R., Kim, H.P., Zhu, W., Levy, D.E., and Leonard, W.J. (2009). IL-21 mediates suppressive effects via its induction of IL-10. *J Immunol* *182*, 2859-2867.

Stark, M.A., Huo, Y., Burcin, T.L., Morris, M.A., Olson, T.S., and Ley, K. (2005). Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* *22*, 285-294.

Stock, P., Akbari, O., Berry, G., Freeman, G.J., Dekruyff, R.H., and Umetsu, D.T. (2004). Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nature immunology* *5*, 1149-1156.

Stockinger, B. (2007). Th17 cells: An orphan with influence. *Immunol Cell Biol* *85*, 83-84.

Stockinger, B., Veldhoen, M., and Martin, B. (2007). Th17 T cells: linking innate and adaptive immunity. *Semin Immunol* *19*, 353-361.

Stumhofer, J.S., Silver, J.S., Laurence, A., Porrett, P.M., Harris, T.H., Turka, L.A., Ernst, M., Saris, C.J., O'Shea, J.J., and Hunter, C.A. (2007). Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nature immunology* *8*, 1363-1371.

Subramanian, S., Yates, M., Vandenbark, A.A., and Offner, H. (2010). Oestrogen-mediated protection of experimental autoimmune encephalomyelitis in

the absence of Foxp3(+) regulatory T cells implicates compensatory pathways including regulatory B cells. *Immunology*.

Szabo, S.J., Sullivan, B.M., Peng, S.L., and Glimcher, L.H. (2003). Molecular mechanisms regulating Th1 immune responses. *Annual review of immunology* *21*, 713-758.

Tadokoro, C.E., Shakhar, G., Shen, S., Ding, Y., Lino, A.C., Maraver, A., Lafaille, J.J., and Dustin, M.L. (2006). Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *The Journal of experimental medicine* *203*, 505-511.

Takatori, H., Kanno, Y., Watford, W.T., Tato, C.M., Weiss, G., Ivanov, I., Littman, D.R., and O'Shea, J.J. (2009). Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *The Journal of experimental medicine* *206*, 35-41.

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell* *140*, 805-820.

Taleb, S., Romain, M., Ramkhalawon, B., Uyttenhove, C., Pasterkamp, G., Herbin, O., Esposito, B., Perez, N., Yasukawa, H., Van Snick, J., *et al.* (2009). Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *The Journal of experimental medicine* *206*, 2067-2077.

Taleb, S., Tedgui, A., and Mallat, Z. (2010). Interleukin-17: friend or foe in atherosclerosis? *Curr Opin Lipidol* *21*, 404-408.

Tang, Q., Adams, J.Y., Tooley, A.J., Bi, M., Fife, B.T., Serra, P., Santamaria, P., Locksley, R.M., Krummel, M.F., and Bluestone, J.A. (2006). Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nature immunology* *7*, 83-92.

Thomas, P., Pang, Y., Filardo, E.J., and Dong, J. (2005). Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* *146*, 624-632.

Thuere, C., Zenclussen, M.L., Schumacher, A., Langwisch, S., Schulte-Wrede, U., Teles, A., Paeschke, S., Volk, H.D., and Zenclussen, A.C. (2007). Kinetics of regulatory T cells during murine pregnancy. *Am J Reprod Immunol* *58*, 514-523.

Torchinsky, M.B., and Blander, J.M. (2010). T helper 17 cells: discovery, function, and physiological trigger. *Cell Mol Life Sci* *67*, 1407-1421.

- Torchinsky, M.B., Garaude, J., Martin, A.P., and Blander, J.M. (2009). Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature* *458*, 78-82.
- Tran, D.Q., Ramsey, H., and Shevach, E.M. (2007). Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood* *110*, 2983-2990.
- Tu, G., Hu, D., Yang, G., and Yu, T. (2009). The correlation between GPR30 and clinicopathologic variables in breast carcinomas. *Technology in cancer research & treatment* *8*, 231-234.
- Uhlenhake, E.E., and Feldman, S.R. (2010). Efficacy and safety of ustekinumab and etanercept for the treatment of psoriasis. *Expert opinion on biological therapy* *10*, 1105-1112.
- Uhlig, H.H., Coombes, J., Mottet, C., Izcue, A., Thompson, C., Fanger, A., Tannapfel, A., Fontenot, J.D., Ramsdell, F., and Powrie, F. (2006). Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *J Immunol* *177*, 5852-5860.
- van der Vliet, H.J., and Nieuwenhuis, E.E. (2007). IPEX as a result of mutations in FOXP3. *Clin Dev Immunol* *2007*, 89017.
- Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., and Stockinger, B. (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* *24*, 179-189.
- Veldhoen, M., and Stockinger, B. (2006). TGFbeta1, a "Jack of all trades": the link with pro-inflammatory IL-17-producing T cells. *Trends in immunology* *27*, 358-361.
- Vignali, D.A., Collison, L.W., and Workman, C.J. (2008). How regulatory T cells work. *Nature reviews* *8*, 523-532.
- Volpe, E., Servant, N., Zollinger, R., Bogiatzi, S.I., Hupe, P., Barillot, E., and Soumelis, V. (2008). A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nature immunology* *9*, 650-657.
- Voorthuis, J.A., Uitdehaag, B.M., De Groot, C.J., Goede, P.H., van der Meide, P.H., and Dijkstra, C.D. (1990). Suppression of experimental allergic

encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats. *Clinical and experimental immunology* *81*, 183-188.

Vukusic, S., Hutchinson, M., Hours, M., Moreau, T., Cortinovis-Tourniaire, P., Adeleine, P., Confavreux, C., and The Pregnancy In Multiple Sclerosis, G. (2004). Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain* *127*, 1353-1360.

Wang, C., Dehghani, B., Li, Y., Kaler, L.J., Proctor, T., Vandembark, A.A., and Offner, H. (2009a). Membrane estrogen receptor regulates experimental autoimmune encephalomyelitis through up-regulation of programmed death 1. *J Immunol* *182*, 3294-3303.

Wang, C., Dehghani, B., Magrisso, I.J., Rick, E.A., Bonhomme, E., Cody, D.B., Elenich, L.A., Subramanian, S., Murphy, S.J., Kelly, M.J., *et al.* (2008). GPR30 Contributes to Estrogen-Induced Thymic Atrophy. *Mol Endocrinol* *22*, 636-648.

Wang, C., Kang, S.G., Lee, J., Sun, Z., and Kim, C.H. (2009b). The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. *Mucosal immunology* *2*, 173-183.

Wang, J., Ioan-Facsinay, A., van der Voort, E.I., Huizinga, T.W., and Toes, R.E. (2007). Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *European journal of immunology* *37*, 129-138.

Wang, Y., Koroleva, E.P., Kruglov, A.A., Kuprash, D.V., Nedospasov, S.A., Fu, Y.X., and Tumanov, A.V. (2010). Lymphotoxin beta receptor signaling in intestinal epithelial cells orchestrates innate immune responses against mucosal bacterial infection. *Immunity* *32*, 403-413.

Ward, B. (2002). How many species of procaryotes are there? *Proc Natl Acad Sci U S A* *99*, 10234-10236.

Wei, L., Laurence, A., Elias, K.M., and O'Shea, J.J. (2007). IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *The Journal of biological chemistry* *282*, 34605-34610.

Weil, B.R., Manukyan, M.C., Herrmann, J.L., Wang, Y., Abarbanell, A.M., Poynter, J.A., and Meldrum, D.R. (2010). Signaling via GPR30 protects the myocardium from ischemia/reperfusion injury. *Surgery* *148*, 436-443.

Whitacre, C.C., Reingold, S.C., and O'Looney, P.A. (1999). A gender gap in autoimmunity. *Science* *283*, 1277-1278.

Williams, L.M., and Rudensky, A.Y. (2007). Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nature immunology* *8*, 277-284.

Workman, C.J., and Vignali, D.A. (2003). The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *European journal of immunology* *33*, 970-979.

Wostmann, B.S. (1981). The germfree animal in nutritional studies. *Annu Rev Nutr* *1*, 257-279.

Xiong, N., and Raulet, D.H. (2007). Development and selection of gammadelta T cells. *Immunological reviews* *215*, 15-31.

Yang, X.O., Pappu, B.P., Nurieva, R., Akimzhanov, A., Kang, H.S., Chung, Y., Ma, L., Shah, B., Panopoulos, A.D., Schluns, K.S., *et al.* (2008). T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* *28*, 29-39.

Yang, X.P., and Reckelhoff, J.F. (2010). Estrogen, hormonal replacement therapy and cardiovascular disease. *Current opinion in nephrology and hypertension*.

Yates, M.A., Li, Y., Chlebeck, P.J., and Offner, H. (2010). GPR30, but not estrogen receptor-alpha, is crucial in the treatment of experimental autoimmune encephalomyelitis by oral ethinyl estradiol. *BMC Immunol* *11*, 20.

Yen, D., Cheung, J., Scheerens, H., Poulet, F., McClanahan, T., McKenzie, B., Kleinschek, M.A., Owyang, A., Mattson, J., Blumenschein, W., *et al.* (2006). IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *The Journal of clinical investigation* *116*, 1310-1316.

Yu, Y., Sitaraman, S., and Gewirtz, A.T. (2004). Intestinal epithelial cell regulation of mucosal inflammation. *Immunol Res* *29*, 55-68.

Yuan, Q., Bromley, S.K., Means, T.K., Jones, K.J., Hayashi, F., Bhan, A.K., and Luster, A.D. (2007). CCR4-dependent regulatory T cell function in inflammatory bowel disease. *J Exp Med* *204*, 1327-1334.

Zarek, P.E., Huang, C.T., Lutz, E.R., Kowalski, J., Horton, M.R., Linden, J., Drake, C.G., and Powell, J.D. (2008). A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood* *111*, 251-259.

- Zhang, F., Meng, G., and Strober, W. (2008). Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nature immunology* *9*, 1297-1306.
- Zhang, G.X., Yu, S., Gran, B., Li, J., Siglienti, I., Chen, X., Calida, D., Ventura, E., Kamoun, M., and Rostami, A. (2003). Role of IL-12 receptor beta 1 in regulation of T cell response by APC in experimental autoimmune encephalomyelitis. *J Immunol* *171*, 4485-4492.
- Zhang, Z., Zheng, M., Bindas, J., Schwarzenberger, P., and Kolls, J.K. (2006). Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm Bowel Dis* *12*, 382-388.
- Zhou, L., Ivanov, I., Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D.E., Leonard, W.J., and Littman, D.R. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nature immunology* *8*, 967-974.
- Zhou, L., Lopes, J.E., Chong, M.M., Ivanov, I., Min, R., Victora, G.D., Shen, Y., Du, J., Rubtsov, Y.P., Rudensky, A.Y., *et al.* (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* *453*, 236-240.
- Zhu, J., Yamane, H., Cote-Sierra, J., Guo, L., and Paul, W.E. (2006). GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res* *16*, 3-10.
- Zou, W. (2006). Regulatory T cells, tumour immunity and immunotherapy. *Nature reviews* *6*, 295-307.
- Zou, W., and Restifo, N.P. (2010). T(H)17 cells in tumour immunity and immunotherapy. *Nature reviews* *10*, 248-256.