# CELL-SPECIFIC INTERLEUKIN-10 EXPRESSION IN THE GASTROINTESTINAL TRACT AND ITS IMPACT ON A MODEL OF ACUTE COLITIS

By

Adam Leibold

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**Abstract**

Inflammatory Bowel Diseases (IBDs) are chronic diseases characterized by aberrant inflammation in the gastrointestinal tract. They result in a significant reduction in the quality of life for 1.3 million Americans every year and are implicated in more severe pathologies like colorectal cancer. While knowledge of the gastrointestinal immune system has vastly improved recently, the etiology of IBDs like Crohn's disease and ulcerative colitis remain largely unknown. What is known, though, is that the maintenance of the epithelial layer and immunoregulatory mechanisms are pivotal toward the treatment and prevention of these diseases. Interleukin-10 (IL-10) plays an important role in immunoregulation and the induction of tolerance in the gastrointestinal tract, and has been previously shown by our lab to be constitutively expressed in intestinal epithelial cells. In order to elucidate the role of this epithelial-derived IL-10 in gastrointestinal health and the development of IBD a two-pronged approach was taken. First, an examination of how certain microbial species in the gastrointestinal tract affect IL-10 expression was performed. Then, an examination of how IL-10 signaling in intestinal epithelial cells affects the disease phenotype in a model of acute colitis was performed. It was found that the presence of bacteria in the gastrointestinal tract was important for the expression of IL-10 in both the lamina propria and epithelial layer, but the results were unable to determine whether certain species are more inclined to induce this expression than others. It was also observed that mice with impaired IL-10 signaling in their intestinal epithelial cells exhibited a less severe acute colitis phenotype when treated with dextran sulfate sodium.

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# CHAPTER 1

# BACKGROUND

## Introduction

The gastrointestinal (GI) tract serves one of the most basic mammalian functions in the absorption of nutrients and the elimination of waste. Due to the nature of the GI tract, being one of the main sites of contact with the external environment, it is constantly exposed to foreign macromolecules such as proteins and lipids, or "antigens"; not only from pathogens like bacteria, protozoa, fungi, and viruses, but also antigens from food and the natural microbiota. This creates a complex problem for the mucosal immune system in the gut, that it must tolerate these constitutive challenges. In the gut, these challenges are primarily dealt with through anatomical sequestration between the immune system and antigens in the lumen, as well as through degradation of these antigens by a broad spectrum of enzymes. In addition, pathogenic and nonpathogenic microorganisms are kept at bay by antibodies and antimicrobial peptides released into the mucosal layer. When this initial barrier breaks down, the immune system can come in contact with these antigens. The immune system is primed to react to foreign antigens in order to protect the host from infection, but for many antigens this reaction is unnecessary and potentially damaging. So the immune system must learn not to react to certain foreign antigens that break through the mucosal barrier. This hyporesponsiveness is known as tolerance. The GI tract, while performing the vital function of providing nutrients and eliminating waste, must also perform a balancing act known as homeostasis. This protects humans from harmful pathogens while tolerating antigens from food and commensal flora.

Being bathed in exogenous antigens with the goal of homeostasis requires a complex immune system with a massive amount of regulatory mechanisms. Indeed, almost 70% of the human immune system, known as the gut-associated lymphoid tissue

(GALT), resides in the GI tract for just this purpose (1). In the presence of this large portion of the immune system, the microbiota perform vital functions like digesting carbohydrates and producing vitamins. In addition to these microbes, food serves the obvious function of providing nutrients. In order for these three components—food, commensal bacteria, and the immune system—to function properly, co-evolution has resulted in the development of mechanisms for immune tolerance to many exogenous antigens (2). At the same time, in order to protect against pathogens, there remains a tightly regulated, effective inflammatory response.

Being exposed to the external environment also makes the GI tract a common entry point for many pathogens. Everything from common bacterial pathogens like *E. coli* and *Salmonella*, to more serious, life-threatening pathogens like *V. cholera* require an adequate inflammatory response. When the immune system's inflammatory response does its job and the infection is resolved, this inflammation must be subsequently controlled in order to avoid further collateral damage. The ability to regulate the inflammation caused by infection is another function of the same immunoregulatory mechanisms involved in the maintenance of tolerance. When inflammation is not properly regulated, whether it be in the steady state response to the microbiota or in the resolution of an inflammatory response to an infection, pathologies can occur caused by the immune system; a large subset of these pathologies that are specifically directed against self antigens are referred to as autoimmune diseases.

Aberrant inflammation, not sufficiently regulated and/or directed toward commensal antigens, is a hallmark of inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis (UC). These disorders have a major impact on the

quality of life in the patients they affect. They are characterized by chronic, relapsing inflammatory responses, which damage the GI tract causing severe diarrhea, pain, fatigue, and weight loss, sometimes life-threatening. Currently, in the United States, about 1-1.3 million people suffer from an IBD (3). Researchers still do not know the exact etiology of these diseases, and treatment options are lacking. Furthermore, this aberrant inflammation has been shown to enhance the risk for the development of colorectal cancer (CRC); inflammation is known to cause DNA damage, while the repair responses to the overall pathology provides a favorable environment for cancer cell proliferation (4). This is reflected in the fact that UC patients have an 18% chance of developing CRC within 30 years after their diagnosis (5). So this problem is not only limited to the immediate quality of life in patients suffering from IBD, but there are significant long-term issues that arise from unregulated inflammation. Therefore, understanding the complex mucosal immune system in the gut, how homeostasis is maintained, and what factors lead to the perturbation of this homeostasis, remains one of the most important questions in public health.

## The Microbiome

### *Commensal's contribution to homeostasis*

The GI tract is colonized by more than 100 trillion microorganisms (2), with at least 150 times more genetic information than their human host (6). These bacteria, fungi, parasites, and viruses, the majority being bacteria, are collectively known as the "microbiota." While the microbiota was once thought only to contribute to pathology, in recent years this community of microorganisms has become the focus of many research

teams attempting to elucidate how this population is shaped, what role it plays in disease, and how it contributes to the host's physiology. Each person's unique microbiome plays an intricate part in a vast range of physiological processes. From the surprising influences on the endocrine and nervous systems (6), to the expected effects on host metabolism (7), the microbiota's influence is far reaching and significant. It is important, then, to fully appreciate the symbiotic relationship between the microbiota and their human host, while also investigating the role it may play in disease.

The human microbiota is central to the digestion food and absorption of nutrients. From the fermentation of carbohydrates, to the production of vitamins, these organisms are able to carry out certain metabolic processes that their hosts are not (8). This has been shown in mouse models in which antibiotics were used to reduce the intestinal bacterial load, which was found to inhibit the production of key metabolites (9). On top of the presence of a microbiota being necessary, the composition of the microbiota has a profound impact on the host's metabolism and overall health. The use of high-throughput sequencing has allowed researchers to analyze the microbiome and determine its unique composition down to the species level; the types of bacteria that make up the microbiome are not trivial. In fact, certain perturbations in what would be considered a normal microbiota have been implicated in conditions like obesity and cancer (10). The development of intestinal tumors can even be transferred into a healthy mouse when the microbiome from a mouse with intestinal cancer on a high fat diet replaces that of the healthy mouse through a fecal transplant (11). Whether a change in the microbiome causes these conditions, or these conditions cause a change in the microbiome, is not yet

clear; neither is it clear what exactly makes a healthy microbiome in the first place. What is clear is the importance of the composition of this community of microorganisms.

In addition to the metabolic functions, the immune system in the GI tract requires the presence of a microbiome in order to fully develop and mature. Studies in germ-free mice, mice that have not been exposed to any microorganisms and do not possess a microbiota, show underdeveloped secondary lymphoid tissue in the gut and throughout the body (12). Beyond overall development of the GALT, specific types of bacteria have been implicated in the development of specific types of immune cells (13). This shows how the ontogeny of the microbiome and the functional immune system go hand in hand.

The microbiota is also important to the proper function of the GALT, and the ability of the GI tract to resist colonization by pathogens. Commensal bacteria work with their host to prevent pathogens from expanding and inducing an inflammatory response that damages the intestinal tissue. One way this is done is through direct competition for nutrients. Commensal strains with certain metabolic profiles are able to directly compete for nutrients with pathogens that contain similar metabolic profiles, and in doing so prevent the pathogen from colonizing the gut (2). Another way commensals contribute to the host's immune defense is by producing anti-microbial compounds directed against certain pathogens. Products like short chain fatty acids, a byproduct of bacterial carbohydrate fermentation, have been shown to be produced by commensal strains like *Bifidobacterium* and to mitigate infections from pathogens like *E. coli* O157:H7 (14). All of these beneficial properties highlight the microbiome's importance to the overall function of the gut, but when certain perturbations occur in this delicate ecosystem the microbiota can actually contribute to disease.

*Perturbations in the microbiota and contributions to disease*

The microbiota is a delicate ecosystem that can be easily disturbed. It is important to distinguish between acute disruptions of the microbiota and general shifts in the composition of a healthy microbiota. Studies show everything from one's age and diet to an individual's sleep schedule can have an impact on the composition of the gut microbiota (15,16). While these general shifts have been implicated in certain conditions, the more severe, acute perturbations in the microbial composition of the gut, like those caused by the use of antibiotics or infections, are much better understood. One of the most common side effects resulting from the use of antibiotics is *Clostridium difficile* infection. A recently developed treatment for this infection involves reconstituting the microbiome with a fecal transplant from a healthy individual (17). Due to the mounting evidence that disruptions in these populations significantly impact one's health, the impact of the microbiome on IBDs cannot be ignored.

The interplay between the immune system and microbiome suggests that the microbiome plays a role in IBD, and many studies have confirmed this. One example is in the Toll-Like Receptors (TLRs) of mice, receptors designed to sense and respond to stimuli from conserved antigens on microbes. The ability of mice to sense bacteria that breach the mucosal barrier through these receptors can protect against the induction of colitis (18). These results imply that the sensing of commensals in the gut plays an important role in the development of aberrant inflammation in IBD. Another factor involves dysbiosis in the microbiota, an abnormal accumulation of certain microbial populations that are normally in a very low abundance in the healthy microbiome. These strains of bacteria are referred to as pathobionts, and their role in the development of IBD

can be seen in the induction of colitis when they are purposefully colonized in immune compromised mice (2).

The presence of commensals in the gut is important for the proper development and function of the GI tract. There is a mutualistic relationship in that the commensals provide their host with proper nutrients and protect them from pathogens, while the host develops a state of tolerance that maintains a favorable environment for the commensals to colonize. In the maintenance of homeostasis it is important that the immune system develop a very specific, regulated inflammatory response, while maintaining a state of tolerance with the commensals that make up the healthy microbiome.

**Gastrointestinal Mucosal Immune System**

To maintain homeostasis in the complex environment of the gut, it is important to prevent aberrant inflammation while retaining the ability to respond to challenges when necessary. As mentioned earlier, inflammation can cause damage to the epithelial layer of the gut, pathology commonly seen in IBDs, as well as promote the development of cancer. The inflammatory response can be pathological, but it is are also a necessary part of the immune system's ability to protect the host from harmful pathogens. Maintaining homeostasis between the inflammatory and regulatory arms of the mucosal immune system is pivotal, and the GI tract has multiple unique anatomical and immunological mechanisms that help achieve this.

*Mucosal Barrier & Anatomy*

The mucosal barrier in the gut is divided into distinct subsets: the mucosal environment in direct contact with the lumen, the epithelial layer directly beneath the mucosa, and the lamina propria layer present just below the epithelial layer with a vast lymphatic system draining into the mesenteric lymph nodes (MLN). The epithelial layer consists largely of different types of epithelial cells, with immune effector and antigen presenting cells dispersed in between (**Figure 1**). The lamina propria is made of connective tissue that forms a scaffolding for immune cell trafficking and to support structures such as the epithelial cell layer, blood supply, lymph vessels, and nervous tissue (19). These two layers are separated by a thin basement membrane, forming distinct immunological compartments. Immune cells are able to travel between the epithelial layer, lamina propria, and MLNs when necessary (20).

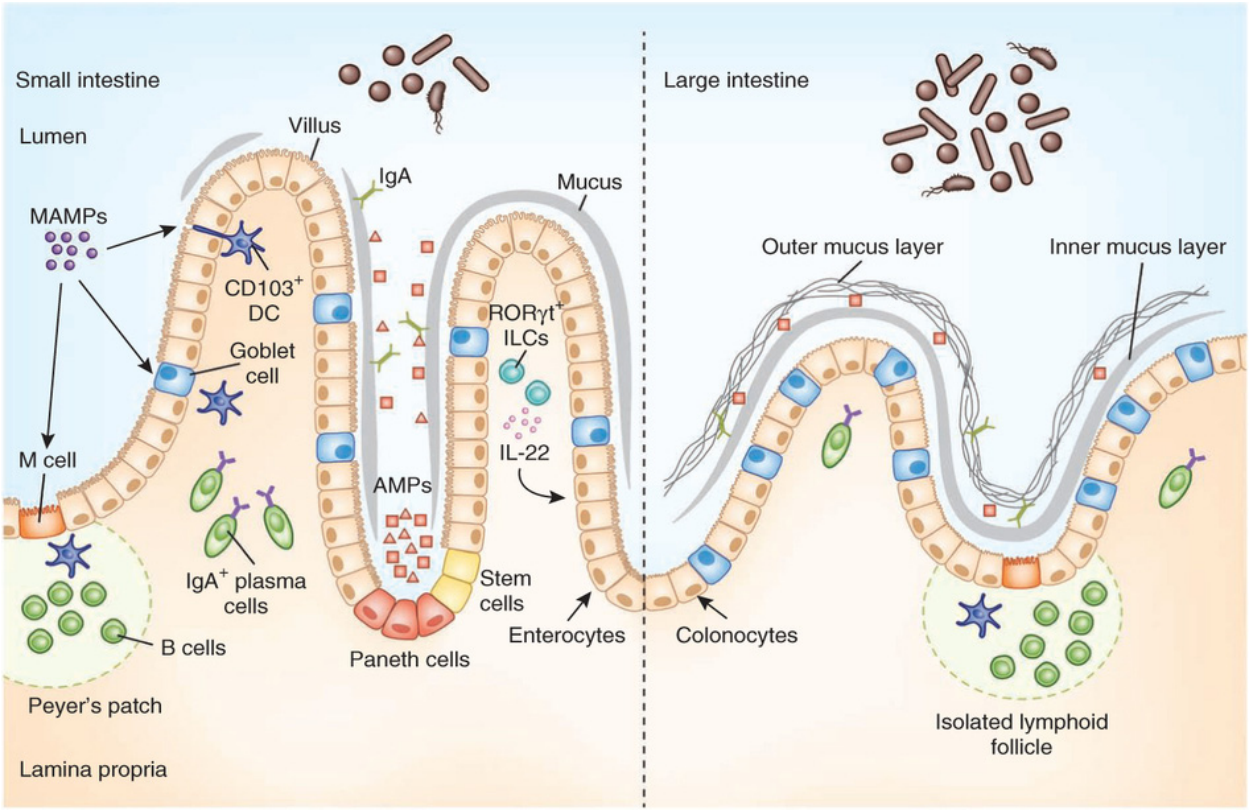


**Figure 1**: The intestinal epithelial layer containing enterocytes and specialized epithelial cells—M cells, goblet cells, Paneth cells, stem cells—as well as Peyer's patches rich in immunoglobulin secreting B-cells. Also shown is the difference in anatomy between the mucosal layers of the small and large intestine (21).

Much of the mucosal immune system's function relies on non-hematopoietic cells. The intestinal barrier is made of a monolayer of epithelial cells, or enterocytes, separated from the lumen of the gut by a thin mucosal layer. These cells form tight junctions with one another that seal off the subepithelial tissue and paracellular spaces from the outside environment. Their apical surface is coated with mucins and antimicrobial molecules that are secreted by special types of epithelial cells called goblet and Paneth cells, respectively. These cells, along with the mucosal layer, work in concert to prevent direct contact between the contents of the lumen and the large amount of immune effector cells in the sub-epithelial space (20). Recently, research has shown that

intestinal epithelial cells (IECs) do much more than simply separate immune effector cells from antigens in the lumen. IECs have been shown to express and respond to cytokines—the hormones of the immune system—directly (22). While it is not exactly clear how crucial this is to the development of immune tolerance, it is an area of ongoing research and a major focus of this thesis.

There are also stark differences between the large and small intestine, immunologically and physiologically (**Figure 1**). For example, a defining feature of the small intestine are finger-like projections extending into the lumen of the gut known as villi. This is in contrast with the colon, where the luminal surface is relatively flat. Another difference is in Paneth cells, which are mostly found in the small intestine, and goblet cells, which progressively increase in number from the small to the large intestine (19).

The focus of this thesis is the large intestine where the majority of the microbiota resides and where the IL-10 phenotype is best characterized. Though these differences may not necessarily be a focus of some studies of GI tract, it is important to keep in mind the heterogeneity of the gut from the stomach to the anus.

*Innate Immune Response in the GI Tract*

The first line of defense against commensals and pathogens that may make it past the mucosal layer is the innate immune system. It is here, where myeloid cells like macrophages and dendritic cells (DCs) rapidly phagocytize microorganisms, that the first level of regulation is seen. Intestinal macrophages, unlike most macrophages in other organ systems, do not function primarily as antigen presenting cells (APCs) to prime the adaptive arm of the immune system, but they contain greater phagocytic and bactericidal

capabilities to clear low-level challenges before adaptive responses are required (20). These tissue macrophages specific to the GI tract also show reduced expression of co-stimulatory molecules on their surface, reduced secretion of pro-inflammatory cytokines, and a propensity to express the anti-inflammatory cytokine IL-10 upon phagocytosis of apoptotic cells (20); all of these characteristics are important examples of the innate immune system's contribution to gut homeostasis.

In the same vein, lamina propria DCs, innate immune cells that are able to extend their dendrites into the lumen of the gut to sample antigens, preferentially activate a tolerogenic and anti-inflammatory immune response (23). Like macrophages, the decreased expression of costimulatory molecules and propensity to induce the expression of IL-10 shapes a tolerogenic response when presenting to effector cells of the adaptive immune response in the draining lymph nodes and lamina propria. The DC's ability to sample antigens in the gut and subsequently induce tolerance in effector cells plays a major role in the development of tolerance to food and commensal antigens. In total, these modifications to the function of the innate immune system in the gut allow for a tight regulation of the adaptive immune response.

*Adaptive Immune Response in the GI Tract*

In addition to the innate immune system, the adaptive immune system provides an extra layer of regulation and effector responses that further cultivate the balance between tolerance and inflammation. From B-cells and their secreted immunoglobulins (Ig) to T-cells and their inflammatory or anti-inflammatory responses, the adaptive immune response is the central pillar of gut homeostasis.

B-cells are responsible for the production of immunoglobulin, proteins also known as antibodies that neutralize microorganisms and other foreign antigens by binding to certain epitopes on these antigens. In the gut, these B-cells work in collaboration with secretory IECs to transport IgA, the dominant type of Ig in the gut, into the lumen to bind foreign antigens and debris. When binding to microorganisms, IgA reduces their motility and adhesive properties, limiting their ability to penetrate the mucosa (2). Because of the antigen load in the gut, massive amounts of IgA are secreted into the lumen constantly. More than 80% of human and mouse plasma cells, B-cells that specialize in the constant secretion of Ig, are located in the lamina propria of the GI tract (20).

T-cells can be categorized into two major phenotypes: $CD8^+$ T-cells and $CD4^+$ T-cells—CD4 and CD8 being co-receptors along with the T-cell receptor that recognizes antigens in the context of a major histocompatibility complex (MHC). $CD8^+$ T-cells recognize antigens in the binding cleft of MHC class I molecules present on all cell types, and are called cytotoxic T-cells because their main function is to destroy infected cells. The $CD4^+$ T-cells recognize antigens in the context of MHC class II molecules found only on APCs. $CD4^+$ T-cells can be further divided into: those responsible for supporting a more inflammatory, cytotoxic Th1 response; those responsible for a more immunoregulatory, tolerance-inducing Th2 response; and those responsible for an antimicrobial Th17 response. T cells control the development of these responses using cytokines to signal between many different immune cell types, and are controlled themselves by the cytokines in the microenvironment in which they are activated. The distinction between these responses is important. The balance between inflammation and

tolerance in the gut can largely be represented as a balance between the Th1, Th17, and Th2 responses. The disturbance of this balance is thought to be a main driver in the development of IBD (24).

As mentioned earlier, T-cells can be dispersed within the epithelial layer as intra-epithelial lymphocytes (IELs), or located in the lamina propria as lamina propria lymphocytes (LPLs). IELs are thought to be predominantly involved in local immune surveillance in the small intestine. They are phenotypically distinct from their systemic counterparts, and knowledge of their exact function remains unclear (19). During active IBD, $CD8^+$ cytotoxic T-cell levels in the epithelial layer are elevated, but in some models of colitis these $CD8^+$ T-cells are largely absent (20); so their exact function as it relates to IBD is largely enigmatic.

In contrast to the IEL $CD8^+$ cytotoxic T-cells, $CD4^+$ T-cells are present in very low levels in the epithelial layer throughout the GI tract (20). The opposite is true in the lamina propria, where $CD4^+$ T-cells play a large role in three major capacities: Th2 T-cells characterized by their secretion of anti-inflammatory cytokines like IL-4 and IL-10, Th1 T-cells characterized by their induction of a $CD8^+$ T-cell-mediated immune response and secretion of inflammatory cytokines like IFNγ and TNF, or Th17 T-cells characterized by their antibacterial response and secretion of IL-17 and IL-22 (24). During the Th2 response, an important $CD4^+$ T-cell subset called $CD4^+Foxp3^+$ regulatory T-cells or Tregs are crucial in the development of tolerance. Defects in the Treg population is a hallmark of colitis.

The $CD4^+$ T-cells in the lamina propria are often presented antigens from commensals in the lumen, but due to the local secretion of anti-inflammatory cytokines

like IL-10 and TGFβ, and the lack of costimulatory molecules on the APCs presenting these antigens, their proliferation is controlled and tolerance is induced. This mechanism has been seen to be a central pillar of homeostasis in the gut, and disturbing any facet of this regulatory control can result in disease. The best evidence for this is in mice who are genetically engineered to knockout their IL-10 gene, a well-known anti-inflammatory cytokine. These mice spontaneously develop colitis and experience an abnormally high Th1 and Th17 response in the colon (25). Though this may seem like a straightforward phenotype resulting from the lack of a key regulatory cytokine, IL-10's effects are pleiotropic, differing in the induced phenotype depending on the cell type in which it is expressed. Elucidating how each cell type expressing and responding to IL-10 affects the immune response in the gut is important in understanding the development and treatment of inflammatory bowel diseases.

**Interleukin-10**

IL-10 is a class II cytokine, and the founding member of the IL-10 family of cytokines. The IL-10 protein is a homodimer that signals through a heterodimeric cell surface complex consisting of the subunits IL-10Rα and IL-10Rβ. While IL-10Rα is specific only for IL-10, IL-10Rβ can be shared with other members of the IL-10 family and is expressed on almost all cell types. IL-10 signals through the IL-10R complex, activating Janus Kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2). These proteins self-phosphorylate then recruit and phosphorylate signal transducer and activator of transcription 3 (STAT3), which is then translocated to the nucleus to activate target genes.

What distinguishes IL-10 from other class II cytokines is its function as a potent anti-inflammatory mediator. IL-10 controls inflammation primarily by inhibiting the production of inflammatory cytokines like IFN-γ, TNF-α, IL-1β, IL-6, and IL-12, as well as through the downregulation of MHC and costimulatory molecules that are important for activation of a cell-mediated immunity. As mentioned earlier, IL-10's effects can be pleiotropic. IL-10 is able to stimulate IFN-γ expression in $CD8^+$ T-cells, and can serve as a growth factor for B-cells, mast cells, and thymocytes. This involves increased MHC class II expression on B-cells along with an increase in Ig production. So while IL-10 is the prototypical anti-inflammatory cytokine, it is important to keep in mind the cell types involved and the environment these cells are in.

*Importance in gut homeostasis*

IL-10 plays an essential role in the maintenance of gut homeostasis. IL-10 knockout mice develop spontaneous colitis (25), and, in humans, polymorphisms in the IL-10 and IL-10R genes are associated with susceptibility to IBD (26). Most hematopoietic-derived cells are able to produce IL-10, and in the GI tract some non-hematopoietic cells express IL-10, like the enterocytes lining the lumen. Studies associating IL-10 with IBD in humans, and multiple mouse models of IBD, all suggest an importance of IL-10 in the maintenance of GI homeostasis. More is known about the contribution of leukocyte-derived IL-10, while the exact role of epithelial-derived IL-10 remains elusive.

While many studies in mice have shown promising results for the use of IL-10 as a therapeutic for IBDs (27), these results have not been echoed in human clinical trials (28). This is due to a heterogeneity in the etiology of IBD, as well as the signal

transduction pathway in response to IL-10 signaling. IL-10 supplementation works best in those patients who have an IL-10 deficiency, while most patients who have normal IL-10 levels don't respond to treatment (28). The genes that are activated by STAT3 also complicate the picture regarding IL-10 in IBD, with studies showing that STAT3 can have both a pathogenic and regulatory role depending on the context in which it is activated (29).

The dynamic interaction between the different cell sources of IL-10 and the different responsive cells is important to the etiology of IBD. In T-cells, selective deletion of IL-10 expression from Tregs can lead to colitis (30). Selective deletion of the IL-10R in macrophages also exacerbates colitis, leading to a pathologic Th17 response (25). In addition to these immune cell populations, IECs have a role in IL-10 signaling in the gut. IECs can express MHC class II, allowing the epithelial cells to serve as APCs able to deliver signal one to the adaptive immune system. This IEC signaling lacks the additional costimulatory molecules, meaning IECs are able induce tolerance in potentially pathogenic T cells. Inflammatory cytokines like IFN-γ inhibit MHC class II expression in IECs, and this inhibition can be blocked through IL-10 signaling (31). Furthermore, IECs constitutively express IL-10 themselves (31).

In the chapters that follow, an attempt will be made to expand on the role of IL-10 signaling in intestinal epithelial cells. Experiments were designed to elucidate how certain microbes may affect IL-10 expression, and how a deficiency in epithelial-derived IL-10 affects a model of acute colitis. It was hypothesized that the colonization of microbes in the GI tract would induce IL-10 expression in the colonic epithelium and lamina propria. It was also hypothesized that enterocyte-derived IL-10 would play a

protective role in the development of colitis. All of this was done to further the understanding of cell-specific IL-10 expression in the GI tract, and its impact on a debilitating disease.

# CHAPTER 2

# INDUCTION OF IL-10 IN COLONIC TISSUE OF GERM-FREE MICE

## Introduction

The first objective was to tease apart the relationship between the microbiota and the innate sources of IL-10 in the gut. There are many reasons to think that the immune response to microbes in the gut begins with the epithelial cells. Beyond providing the physical barrier between the microbiota and the mucosal immune system, these cells are able to sense and respond to microbial stimuli. IECs express receptors that are specific for conserved microbial antigens called pattern-recognition receptors (PRRs), such as Toll-like receptor (TLR), NOD-like receptor (NLR), and RIG-I-like receptor families. Evidence points toward these receptors being crucial to the maintenance of homeostasis. When one of the major downstream transcription factor of these receptors nuclear factor-κB (NF-κB) is inhibited, colitis is exacerbated (32,33). These cells being so close in proximity to the microbiota and having the ability to sense them requires that they maintain a hyporesponsiveness to microbes in the lumen. Indeed, it has been shown that activation of PRRs on the apical surface of these cells, the surface facing the lumen, induces tolerance, whereas basolateral exposure to these antigens results in the activation of inflammatory pathways (34). In addition to this mechanism for IECs inducing a tolerogenic response to commensals, our lab has previously shown that these cells constitutively express IL-10.

As previously discussed, IL-10 is crucial to the maintenance of gut homeostasis. In addition to constitutive IL-10 expression, certain signaling pathways in response to microbes and inflammatory mediators have been seen to upregulate IL-10 expression in IECs. Signaling through CD1d, a surface receptor on IECs that presents microbial lipid antigens to natural killer T (NKT) cells, results in an increased production of IL-10 (35).

IFN-γ signaling in IECs has also been seen to induce IL-10 expression and subsequent barrier restitution (36). In the same vein, because APCs are constantly taking up antigens in the gut and inducing tolerance to these antigens, it would be expected that these innate cells induce IL-10 as part of this mechanism of tolerance.

The first aim of this experiment was to deduce whether IL-10 expression from IECs and APCs in the lamina propria was dependent on the presence of microbes. The second aim was to determine whether certain species of bacteria selectively induce IL-10 expression in IECs and/or APCs in the lamina propria. The first hypothesis was that the colonization of microbes in the GI tract of mice induces IL-10 expression in both IECs and APCs. The second hypothesis was that this induction of IL-10 is not specific to any of the strains of bacteria used, but that IL-10 induction would be seen across all strains.

In order to look only at innate sources of IL-10, adaptive immune cell sources were excluded. This was done using *Rag*$^{-/-}$ mice who were devoid of any B-cells or T-cells. This means that when a gene expression analysis was performed on the epithelial layer, IL-10 expressed by IELs was not included in the analysis. Similarly, in the lamina propria, LPL-derived IL-10 was not present, and the IL-10 expression that was present was primarily from APCs.

## Materials and Methods

### *Monocolonization of Mice and Isolation of Intestinal Epithelial Cells and Lamina Propria Tissue*

In collaboration with Dr. Daniel Peterson at the Johns Hopkins School of Medicine, male *Rag*$^{-/-}$ mice on a C57Bl/6J background were obtained from Dr. Peterson and maintained in germ-free housing under maintenance conditions set by the Peterson Lab. The mice were placed in individual cages, three mice per cage, within the same germ free isolator to ensure that there was no cross-contamination between mice inoculated with different strains of bacteria. The mice in each cage were then inoculated with a specific strain of bacteria, both human and mouse isolates, chosen for their known effects on the immune system in the gut. The strains used, their mouse or human origin, the amount of bacteria inoculated, and the inoculation method can all be seen in **Table 1**. Exposure to the bacteria was done by isolating bacterial colonies on agar plates, picking them with a sterile inoculation loop, and rubbing the loop on the mouse's fur. This exposure was used for all but one strain, *C. symbiosum*. For this strain an oral gavage was used to introduce the bacteria directly into the GI tract because it is anaerobic and exposure to oxygen while on the fur would kill the bacteria and lead to an unsuccessful inoculation.

| Bacterium | Isolate Type | Average cfu/ml | Inoculation Method |
|---|---|---|---|
| Germ Free | NA | NA | NA |
| Escherichia coli NC101 | Mouse | $5 \times 10^7$ | Fur exposure |
| Bacteroides thetaiotaomicron 5482 | Human | $6 \times 10^8$ | Fur exposure |
| Bacteroides caccae | Human | $6 \times 10^8$ | Fur exposure |
| Bacteroides sartorii | Mouse | $6 \times 10^4$ | Fur exposure |
| Clostridium symbiosum | Human | $2 \times 10^6$ | Oral gavage |

**Table 1**: The monocolonization of each experimental group of mice. Three mice per strain of bacteria were used.

*RNA Isolation and Real-Time PCR*

The mice were sacrificed 48 hours after exposure to allow for sufficient colonization of their GI tract. After the mice were sacrificed, their colons were harvested and subjected to lamina propria preparation methods according to the lab of Dr. Peterson. Briefly, a midline incision was performed, the colon was removed, feces were removed, the colon was cut longitudinally, and the epithelial layer was scraped off with a sterile blade. During this prep the IECs and the lamina propria tissue were each separated into different 50ml conical tubes, and kept at -80°C in Trizol in order to preserve the RNA.

These samples, along with a sample of murine brain tissue, were then subject to RNA isolation and RT-PCR. The RNA isolation was done using a chloroform extraction followed by an isopropanol extraction in order to isolate nucleic acids (RNA and DNA). For this, 200 µl of chloroform was added per 1 ml of Trizol the samples were kept in.

This mixture was incubated at room temperature for 10 minutes, centrifuged at 13200 rpm for 15 minutes at 4°C, and the top layer was carefully transferred into a fresh Eppendorf tube. 500 µl of isopropanol per 1 ml of Trizol was then added to this tube and then mixed by inversion. This solution was incubated at room temperature for 10 minutes, centrifuged at 13200 rpm for 15 minutes at 4°C, and the supernatant was aspirated. The pellet was then resuspended in 1 ml of 75% ethanol and mixed via inversion. This suspension was centrifuged at 13200 rpm for 15 minutes at 4°C, the supernatant was aspirated, and the pellet was allowed to air dry for ~10 minutes at room temperature. The dried pellet was then dissolved in a small amount of DEPC-treated water, and the RNA was then quantified and assessed for purity using a Nanodrop from Thermo Scientific.

The samples were then subject to DNAse treatment to remove the DNA, leaving pure RNA samples. All of this was done at an RNA concentration of 2000 ng/µl, and a total solution volume of 16 µl. All reagents were obtained from Invitrogen. 2 µl of DNAseI buffer and 2 µl of DNAse I was added to the RNA solution and incubated for 15 minutes at room temperature. The reaction was then terminated by adding 2 µl of 25 mM EDTA to each sample and heating the mixture to 65°C for 10 minutes.

Reverse transcription was then used to amplify the RNA in each sample. cDNA was generated using a first-strand cDNA synthesis kit obtained from Roche. First, 2 µl of random hexamers and 2 µl of 10 mM dNTPs were added to each sample of DNAse-treated RNA and incubated at 65°C for 5 minutes. The samples were chilled on ice for 1 minute and then 18 µl of a master mix containing 4 µl of 10x RT buffer, 8 µl of 25 mM $MgCl_2$, 4 µl of 0.1 M DTT, and 2 µl of RNAse out was added to each sample. This

mixture was incubated at room temperature for 2 minutes, then 2 µl of ssII RT at 50 units/µl was added to each sample. The samples were vortexed for 5 seconds, spun down, and incubated at room temperature for 10 minutes. Then each sample underwent a series of incubations at 42°C for 50 minutes and 70°C for 15 minutes. The samples were then chilled on ice, 2 µl of RNAseH was added, and then they were incubated for 20 minutes at 37°C.

After purification and reverse transcription, primers for IL-10 and β2-microglobulin were added, a RT-PCR was ran using Sybr Green, and the gene expression of each sample was assessed. IL-10 expression in each sample was normalized to the expression of the baseline gene β2-microglobulin. Two levels of analysis were performed. First, levels of IL-10 in each sample were compared to levels in the brain tissue sample in order to assess total IL-10 mRNA expression. Second, each monocolonized mouse LP or IEC sample was compared to the LP or IEC sample from the germ free mice to quantify the level IL-10 induction in response to bacteria colonization.

**Results**

In order to assess total IL-10 mRNA expression in each of the samples, a comparison to IL-10 mRNA expression in a brain tissue sample was performed. The brain tissue was used because IL-10 expression in the brain is very low, and therefore able to serve as reliable means to determine relative baseline levels of IL-10 mRNA in gut tissues. It was important to assess the presence of overall IL-10 mRNA this way as a quality control for our samples. The levels of IL-10 expression in each the LP and IECs

should be considerably higher than in the brain. If IL-10 mRNA levels in the LP or IEC samples were not higher than in the brain tissue sample, then something was wrong with the preparation and/or RT-PCR of the samples.

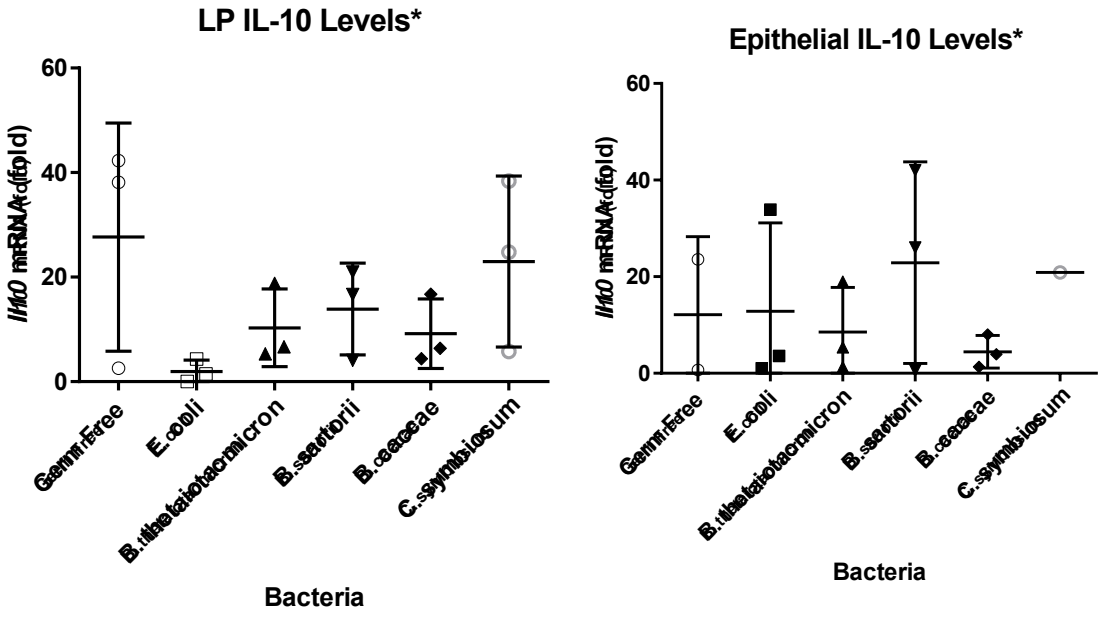


**Figure 2**: Levels of mRNA expression in comparison to brain tissue. Each strain is represented as the fold expression of IL-10 mRNA over brain IL-10 mRNA expression.

The results of this assessment can be seen in **Figure 2**. In all samples, both in the LP and IECs, the IL-10 expression was higher than in the brain. The same brain sample was used as a reference for the analysis of all LP and IEC samples. This confirms that IL-10 mRNA was able to be retrieved from the isolated tissues. This IL-10 expression did not seem to depend on the presence of microbes in the gut, as IL-10 levels in germ-free mice were similar to mice whose GI tract was colonized with bacteria.

The next step was to assess the induction of IL-10 as a result of bacterial colonization (**Figure 3**). The results shown are the levels of IL-10 mRNA expression

above that of the germ free mice, the reference level being the average of the three germ free samples. Though the results suggest that most of the bacteria strains were inducing IL-10 expression above the levels in the germ free mice, high intra-strain variation and low sample sizes led to these differences not being significant.

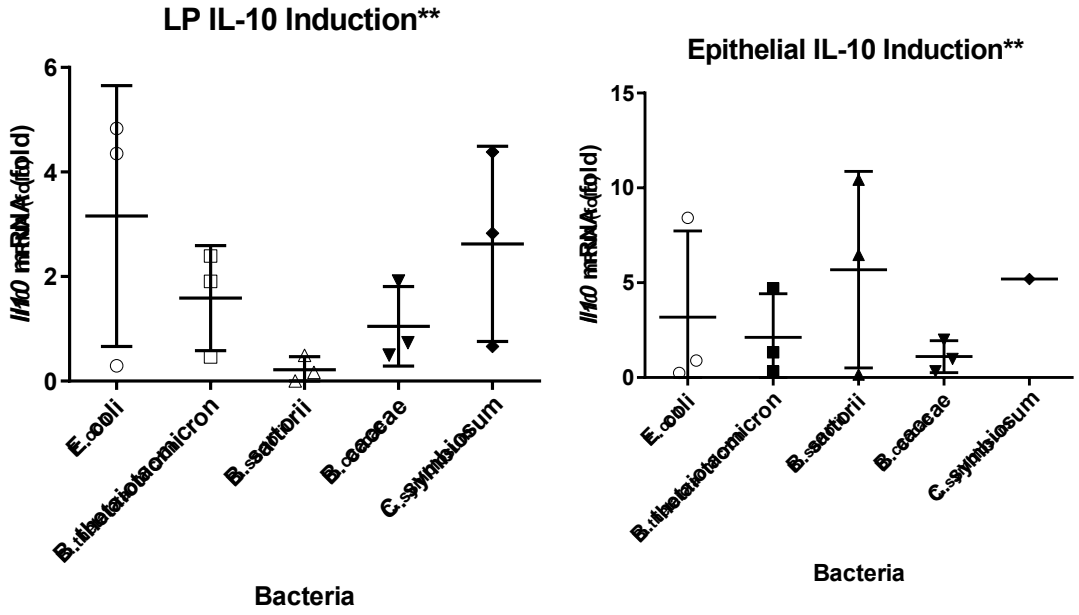


**Figure 3**: Levels of mRNA induction in monocolonized mice. Each strain is represented as the fold expression of IL-10 mRNA over that of the germ free mice.

The results in **Figure 3**, where the levels of IL-10 mRNA in germ-free mice were used as a reference, also contradict those seen in **Figure 2**, where the levels of IL-10 mRNA in the brain were used as a reference. This was likely due to multiple technical difficulties in the lamina propria and epithelial layer isolations, which led to heterogeneity in the quality of each tissue sample. Some samples contained a very small amount of tissue, while others contained large chunks of bulk tissue. Furthermore, due to the high volume of samples and limited lab personnel, some tissues were preserved in

Trizol quicker than others. All of these factors played a role in the large amount of variation seen in each experimental group.

**Discussion**

This experiment was done as a pilot study to address two questions: do epithelial cells and/or APCs express IL-10 in the absence of microbes, and do specific strains of bacteria induce IL-10 more than others. While the results were promising in that the methodology was able to assess IL-10 expression in each the lamina propria and epithelial layer, the results were not able to assess IL-10 induction as a result of bacterial colonization.

By comparing the IL-10 mRNA levels to the brain it was shown that the tissue was able to successfully be isolated and the gene expression was able to successfully be evaluated. On the other hand, when attempting to assess levels of IL-10 induction as a result of bacterial colonization, technical difficulties were encountered that hampered the ability to interpret the data and achieve significant results. The first problem came in the lack of an adequate sample size in each group. Due to germ free isolator availability, and because this was a pilot study, only three mice were used in each group. Furthermore, because of the nature of the LP prep and IEC isolation, some tissues were not able to have RNA recovered from them at all. This is evident in the fact that the sample size for IECs of *C. symbiosum* was only one.

The second problem was high variation within each experimental group, which may be the result of multiple factors. First, the mice may have been contaminated with

other bacteria. While the germ free facility is adequate for raising germ free mice, the successful monocolonization of each mouse was not confirmed. In order to do this a metagenomic analysis of the GI tract of each mouse should be performed before the tissue is isolated, during the 48 hour colonization period. The second possibly may have to do with the tissue isolation. RNA degrades quickly, so it is imperative that tissues be homogenized and frozen in Trizol as soon as possible. The tissue samples arrived from Dr. Peterson's lab largely as bulk tissue in Trizol, meaning that the lack of homogenization would leave the inner cells of each clump not exposed to the RNA-preserving solution. Because of this the integrity of the RNA was likely compromised in some samples with larger chunks of tissue.

On the other hand, some aspects of the data were consistent enough to draw some preliminary conclusions. For example, in some of the experimental groups, such as those mice colonized with *B. caccae*, it is evident that less IL-10 is induced compared to other bacterial strains. This may be important as some species of *Bacteroides* have been shown to protect against colitis in certain mouse models (37). Further assessment of the effect these bacterial strains have on IL-10 expression may be important toward elucidating another important mechanism in maintaining gut homeostasis.

In the future this experiment should be performed with a few changes to the protocol. First, an assessment of the microbiota in each mouse must be done in order to illuminate or eliminate any confounding microbes that may be present. Second, as the tissue is harvested it should be homogenized immediately to preserve the integrity of the RNA. Lastly, a larger sample size should be used in each experimental group. The differences in induction of IL-10 between each bacterial strain is most likely to be low, as

shown in **Figure 3**, and because of this any differences in induction may need a large sample size in order to obtain significant results.

# CHAPTER 3

# INTESTINAL EPITHELIAL CELL IL-10 SIGNALING IN COLITIS

## Introduction

To further examine the role of IL-10 signaling in gut epithelial cells, a controlled model of inflammation and acute colitis was used. By using this model, the importance of IL-10 signaling in a disease state and the role IL-10 plays when the epithelial layer is compromised, could be evaluated. It is important to note that one common pathology associated with inflammation of the GI tract is a disruption of the epithelial layer. Epithelial apoptosis has been shown to be a major factor in acute inflammatory bowel diseases, and there are certain models that specifically take advantage of disrupting the epithelial layer in order to induce colitis.

### *Dextran Sulfate Sodium Model of Colitis*

Some models of colitis take advantage of genetic mechanisms, like IL-10 knockout models, and some use certain chemicals in order to induce colitis. Dextran sulfate sodium, or DSS, is a chemically-induced model of colitis. DSS is a water-soluble, sulfated polysaccharide with anticoagulant properties. The DSS model is one of the most widely-used models of colitis because it is simple to employ, easily reproducible, and the course of the colitis, from acute disease to recovery, is short (38). Another convenient property is the ease with which the model can be modified. Simply by changing the concentration of DSS administered, severe or mild colitis can be induced. In addition to modifying the severity of colitis, chronic colitis can be induced that lasts long after the DSS has been removed using a cyclical administration schedule (38). This allows researchers to tailor the model to the question being asked.

Of course, this does not mean that DSS is a perfect model. One weakness is in the fact that, unlike most human IBDs, T-cells and B-cells are not required for the induction

of colitis (38). This must be kept in mind when drawing conclusions about the role of the adaptive immune system in the gut during DSS-induced colitis. However, this adds another advantage in that the model may be used in *Rag*$^{-/-}$ mice who are devoid of B-cells and T-cells, making investigation of the innate immune response possible. Like in any model of disease, the exact mechanism in which the disease is induced should be taken into account when drawing any conclusions.

The exact mechanism that makes DSS a colitogen is not known, but is suspected to be a result of disrupting the epithelial layer, inducing epithelial apoptosis and the release of damage signals and pro-inflammatory intestinal contents (38). Studies have shown that apoptosis-inducing proteins like the p53-upregulated modulator of apoptosis (PUMA) are not only upregulated in UC patient's IECs, but also induced in murine IECs as a result of DSS treatment (39). Other cellular factors that may contribute to the cytotoxicity of DSS include: the loss of tight junction protein ZO-1, which may facilitate the increase in intestinal permeability (40); a disturbance in phospholipid metabolism, which may contribute to a loss in cell membrane integrity; and a reduction in the levels of nucleotide synthesis, which may cause decreased IEC proliferation (41).

Another mechanism of DSS-induced colitis may be in the modulation of interactions between IELs, IECs, and the extracellular matrix (ECM). Lymphocytes in the gut get there through the expression of specific integrins, and perturbations in the expression of these integrins caused by DSS may contribute to an exaggerated immune response through the aggregation of intestinal lymphocytes (42). DSS also induces inflammation through modulation of the NF-κB signaling pathway and NLRP3 inflammasome. Finally, DSS has been shown to upregulate inhibitors of epithelial cell

proliferation (43). All of these results point toward DSS inducing colitis through the increase in intestinal permeability, the inhibition of IEC proliferation, and the upregulation of IEC apoptosis. Simultaneously, DSS induces inflammation through the aggregation of lymphocytes, the expression of pro-inflammatory cytokines, and the inhibition of anti-inflammatory cytokines.

*Villin-Cre System*

In order to investigate the role of IL-10 signaling solely in IECs, an intestinal epithelial cell-specific deletion of IL-10 and IL-10Rα was used. This means that, in these knockout mice, IL-10 or IL-10R function was normal throughout the mice, but was disrupted solely in the enterocytes of the GI tract. By doing this, the DSS model of colitis was able to be used in mice with either deletion and in control mice. An investigation could then be made into whether colitis was ameliorated or exacerbated by the absence of IL-10 signaling in IECs. This genetic deletion was accomplished through the use of the Villin-Cre system.

Cre-Lox recombination is a widely used technique to carry out specific deletions and insertions of genes. What this system does is take advantage of a Cre recombinase protein, which recombines two *LoxP* sequences in a genome (44). In order to carry out deletions, these *LoxP* sequences can be inserted into the genome flanking whatever the target gene may be. Then, when the Cre recombinase is expressed, the pair of *LoxP* sequences is recombined, cutting out the gene in between them (**Figure 4**). This system can be used for deletions under the control of specific promoters, allowing for cell-specific deletions. In the mice used in this experiment, whose deletions occurred only in the enterocytes of the GI tract, Cre recombinase expression was under the control of a

regulatory region of the *Villin* gene, hence the name "Villin-Cre." The *Villin* gene has been shown to be expressed homogeneously throughout the small and large intestine in only enterocytes. By placing the gene for the Cre recombinase under the control of the *Villin* gene's promoter, expression of the Cre recombinase occurs solely in the enterocytes of the GI tract (44).

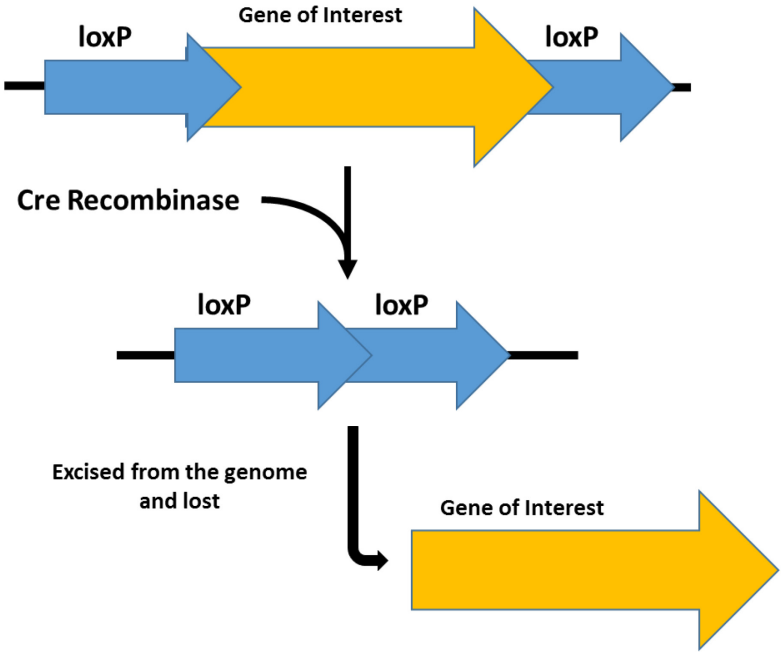


**Figure 4**: Cre-LoxP system. LoxP sites flanking the gene of choice are recombined, excising that gene.

The control mice used for the experiments outlined below had *LoxP* sequences flanking the IL-10 gene but were negative for the Cre recombinase; so the *LoxP* sequences were not actually recombined, leaving the target gene intact. These mice were used as opposed to simple wild-type mice in order to control for any extraneous effects the *LoxP* sequences may have on gene expression. The control mice were termed IL-$10^{fl/fl}$ for mice that did not express the Cre recombinase and whose target gene was intact, and

IL-$10^{\Delta IS}$ or IL-$10R\alpha^{\Delta IS}$ for mice that expressed the Cre recombinase and had an intestine-specific gene deletion of IL-10 or IL-10Rα, respectively.

## Materials and Methods

*Generation of Mice*

C57Bl/6 IL-10fl and IL-10Rαfl mice were obtained from Dr. Werner Muller at the University of Manchester, and were crossed with C57Bl/6 *Villin-Cre*$^{+}$ mice for two generations until IL-$10^{\Delta IS}$ and IL-$10R\alpha^{\Delta IS}$ mice were created. Mice were maintained through hemizygous breeding with *Villin-Cre*$^{+}$ and *Villin-Cre*$^{-}$ mice. Wild-type mice were purchased from the Jackson Laboratory (Bar harbor, ME). Male mice were used in all experiments. Maintenance conditions—humidity, temperature, light and dark cycles, feeding and water conditions—were set by the Johns Hopkins Broadway Research Building Animal Care Facility. All experimental procedures were approved by the Johns Hopkins Animal Care and Use Committee.

*Induction of Colitis by DSS*

DSS (MW 36,000-50,000) was mixed into DI water in order to obtain the desired concentration (%w/v), filtered, and then given to the mice through their drinking water. In the Johns Hopkins Broadway Research Building Animal Facility the central water feed was stopped using a plug obtained from the facility, and the DSS solution was placed in water bottles in the cage. The DSS water was given to the mice for 7 days, followed by a recovery period of 9 days in which they received water from the facility's central feed. Each day the mice were weighed, and disease severity was assessed based on weight loss, splenic weight, and colon length.

*Lamina Propria Lymphocyte and Intraepithelial Lymphocyte Isolation*

After a 16-day course of DSS the mice were euthanized via $CO_2$ asphyxiation. A midline incision was performed and the skin was retracted. The spleen was removed, along with the mesenteric lymph nodes (MLN) and intestines. The small intestine and colon were separated through a cut just above the cecum, and they were placed in separate petri dishes containing ice cold FBS-containing media, as were the spleens and MLNs. The colons were then measured, the feces were removed from the small and large intestine using tweezers to gently push the feces out, and the intestines were cut longitudinally with fine scissors. The intestines were then washed three times in petri dishes containing fresh, ice cold FBS-containing media. They were then cut into approximately 1 cm long pieces and placed in an Erlenmeyer flask containing a solution of 40 ml of FBS, 500 µl of 0.5 M EDTA, and 40 µl of DTT at a final concentration of 0.145 mg/ml solution. The solution containing the 1 cm pieces of intestine was then placed on a shaker in a warm room (37°C) for 20 minutes.

After incubation in the warm room (37°C), the contents of each Erlenmeyer flask were strained through a sterile kitchen strainer into a 500 ml beaker held on ice. The tissue left in the strainer was then placed in a 50 ml conical containing 15 ml of a pre-digestion media initially made using 45 ml of FBS-free media and 180 µl of 0.5 M EDTA. The solution was then shaken vigorously for 30 seconds and strained again. This process was repeated for each sample three times. The solution in the 500 ml beaker contained the epithelial layer.

After the pre-digestion was completed, the tissue pieces in the strainer were placed in a 200 ml beaker containing 15 ml of a digestion media that was initially made

using 25 ml FBS-free media, 2.5 mg of Liberase TL, and 12.5 mg of DNAse I. These chunks of tissue were minced using scissors in the 200 ml beaker, then each beaker was covered with parafilm and placed in the warm room on a shaker for 25 minutes. After 25 minutes, 10 ml of digestion media was added to each beaker and the warm room incubation continued for 25 additional minutes.

Meanwhile, the 500 ml beakers containing the epithelial layer was processed for isolation of IELs. The contents of each beaker were placed in separate 50 ml conicals and centrifuged at 1600 RPM for 10 minutes. After aspirating the supernatant, the pellet was suspended in 28 ml of FBS-free media and 12 ml of Percoll. The tube was then inverted multiple times and centrifuged at 1600 RPM for 20 minutes at room temperature. After this centrifugation the supernatant was aspirated, the pellet was resuspended in FBS-containing media, and the cells were counted. The IELs were cultured in a 6-well plate at a concentration of $3x10^6$ cells/ml. Half of the wells in each plate were stimulated with 50 ng/ml of Ionomycin and 20 ng/ml of PMA in the presence of Brefeldin A, while the other half remaining unstimulated. The stimulated and unstimulated cells were then incubated for 4 hours at 37°C.

After incubation of the LPLs with liberase, the tissue pieces were almost fully degraded, and 10 ml of FBS-containing media was added to the beaker to stop the reaction. The contents of each beaker were then strained through a 70 μm cell strainer into a 50 ml conical, centrifuged at 1600 RPM for 10 minutes, and resuspended in 10 ml of FBS-containing media. The cells were then counted, passed through a 40 μm cell strainer, centrifuged and the supernatant aspirated, and then cultured in a 6-well plate at a concentration of $3x10^6$ cell/ml. Like the IELs, half of the cells were stimulated with PMA

and Ionomycin, while the other half remained unstimulated as outlined above. The stimulated and unstimulated cells were then incubated for 4 hours at 37°C.

## Results

### *DSS Dosing*

The effects of DSS treatment are dose-dependent and can vary between facilities. Dosing studies in wild-type mice were first conducted in order to determine the optimal concentration that would induce moderate colitis. This was done through two consecutive experiments. The first used solutions of 1%, 2%, and 3% DSS in 15 week old C57Bl/6 mice, based on previous literature (38,41,42,43). It was found that the 1% solution was unable to induce colitis, the 2% solution was able to induce significant weight loss, and the 3% solution killed the mice at day 6 (data not shown). The second experiment used 1.5%, 2.0%, and 2.5% solutions to try narrow down the ideal DSS concentration (**Figure 5**). The 1.5% DSS solution led to no weight loss, while the 2.0% and 2.5% solutions lead to moderate and severe weight loss, respectively. It was decided that the 2.0% solution was more likely to allow for the distinguishing between more subtle changes in disease severity that may result from the genetic deletion of IL-10 or IL-10Rα in IECs.

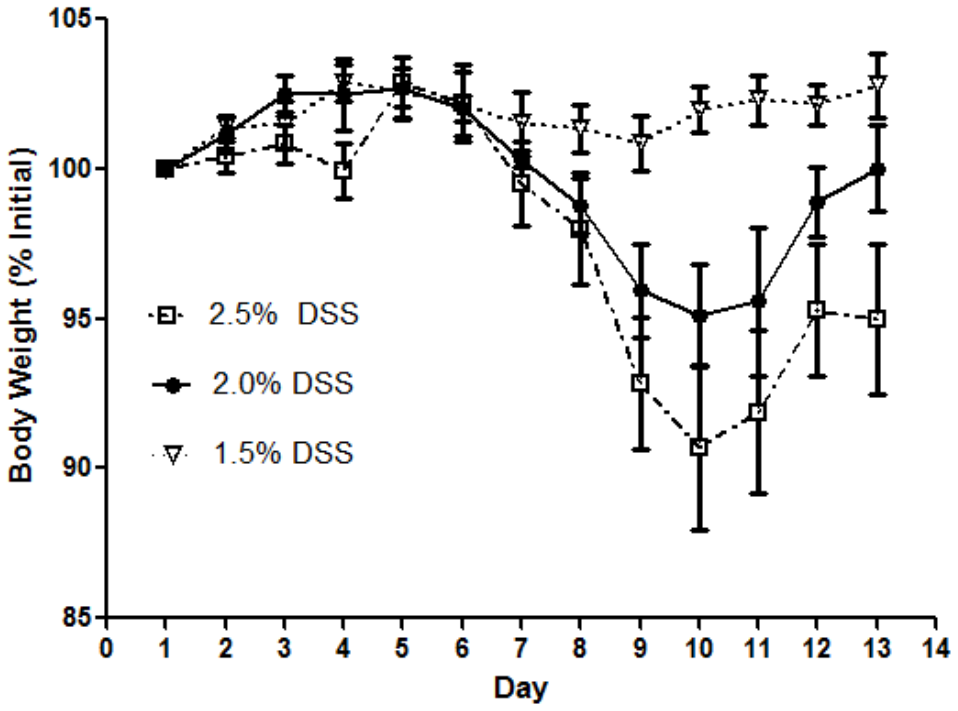


**Figure 5**: DSS dosing carried out in 3 groups (n=3) of WT-mice. DSS was given through the mice's drinking water for a period of 7 days, followed by a 7 day recovery period. The mice given the 2.0% DSS solution showed the ideal phenotype of moderate colitis, making that the concentration used in future rounds of DSS colitis.

*DSS-induced Acute Colitis in IL-10$^{\Delta IS}$ and IL-10Rα$^{\Delta IS}$ Mice*

In order to examine the role of IL-10 expression and IL-10R signaling in IECs, the phenotype of each experimental group treated with DSS was examined. This phenotype was quantified using weight loss, colon length, and splenic weight. Weight loss (**Figure 6**) was significantly lower in the IL-10$^{\Delta IS}$ and IL-10Rα$^{\Delta IS}$ mice compared to the control IL-10$^{fl/fl}$ mice. This difference in weight loss was statistically significant for the IL-10$^{\Delta IS}$ compared to the control mice on day 11, 12, 13, 15, and 16, and statistically significant for the IL-10Rα$^{\Delta IS}$ compared to the control mice on day 12 only.

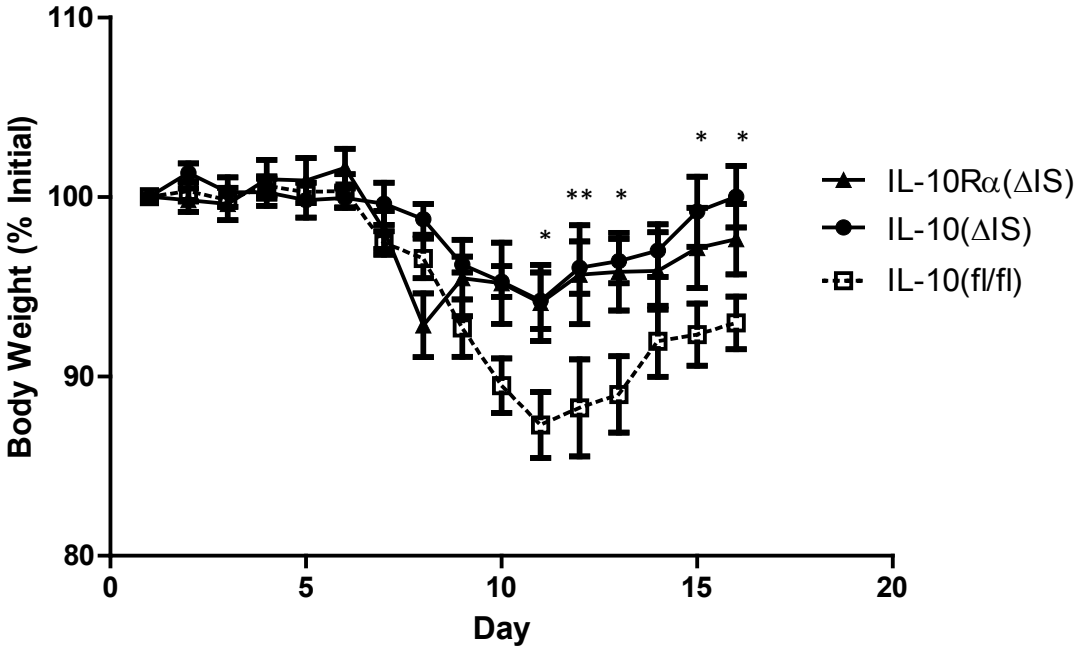


**Figure 6**: DSS colitis induced in the IL-$10^{fl/fl}$, IL-$10^{\Delta IS}$, and IL-$10R\alpha^{\Delta IS}$ mice (n=5). IL-$10^{\Delta IS}$ mice showed significantly less weight loss than the control IL-$10^{fl/fl}$ mice on days 11, 12, 13, 15, and 16, while the IL-$10R\alpha^{\Delta IS}$ mice showed significantly less weight loss only on day 12.

Colon length has proven to be a useful measurement of colitis (45), and the weight loss phenotypes seen were echoed in the measurements of colon length (**Figure 7**). Although there was a clear trend for smaller spleens in the IL-$10^{\Delta IS}$ and IL-$10R\alpha^{\Delta IS}$ groups, the splenic weights were not statistically different between groups (**Figure 8**). The lack of significance is likely a result of the low sample size, with only three mice in each group. The spleens of the IL-$10^{fl/fl}$ mice were observed to be significantly more inflamed than the IL-$10^{\Delta IS}$ and IL-$10R\alpha^{\Delta IS}$ mice spleens (data not shown). Overall, these gross measurements showed a clear phenotype, where the IL-$10^{fl/fl}$ mice had significantly worse colitis than the IL-$10^{\Delta IS}$ and IL-$10R\alpha^{\Delta IS}$ mice.

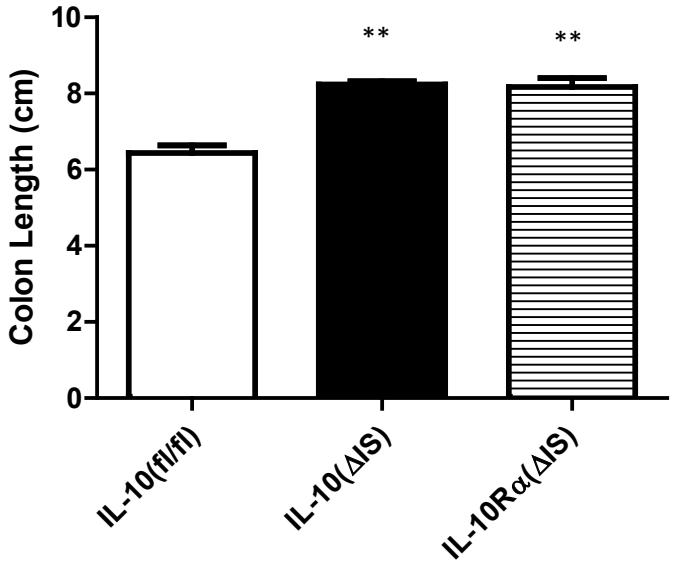


**Figure 7**: The colon length of mice in each of the experimental groups (n=3). The IL-$10^{fl/fl}$ control mice had shorter, more inflamed colons than the IL-$10^{\Delta IS}$ and IL-10R$\alpha^{\Delta IS}$ mice ($P < 0.001$).

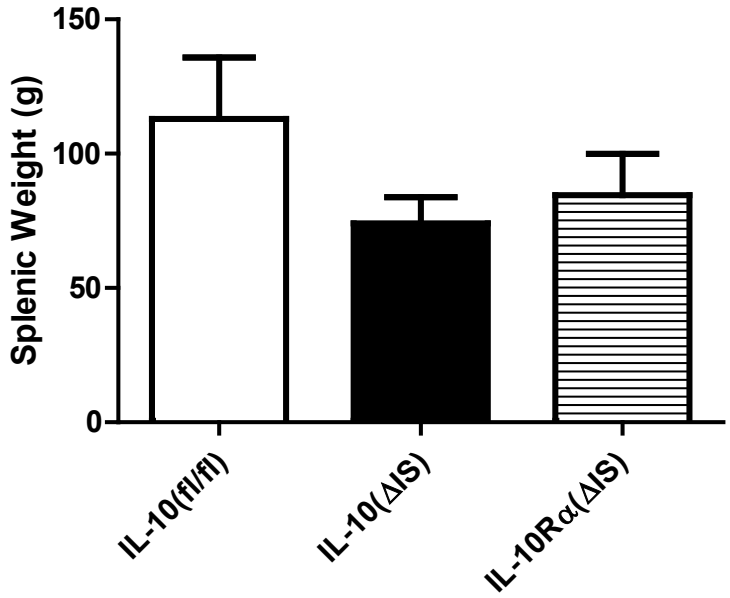


**Figure 8**: Splenic weights of each group of mice (n=3). The splenic weights were not significantly different between the groups of mice, though the VC- IL-10(FL) (IL-$10^{fl/fl}$) mice's spleens were observed to be larger and more inflamed.

*Flow Cytometry*

To determine whether IL-10 signaling affects the composition of leukocyte subsets in the recovery phase of mice treated with DSS, flow cytometry was performed. The mice were sacrificed on day 16 relative to the start of DSS treatment. LPLs, IELs, and splenocytes were then isolated for immunophenotyping. Because this analysis was done on day 16, the phenotypes of these cells pertain mostly to the recovery phase after a bout of acute colitis. In the case of the IL-$10^{\Delta IS}$ and IL-$10R\alpha^{\Delta IS}$ mice, this is when the inflammation had resolved and the mice had recovered. In the case of the IL-$10^{fl/fl}$ mice, this is when residual inflammation was still present and the mice had not yet fully recovered.

First, the phenotypes of the immune cell populations seen in each the LPLs, IELs, and spleen were analyzed. This included: proportion of $CD4^+$ and $CD8^+$ T-cells, B-cells ($CD19^+$), and T-regulatory cells ($Foxp3^+CD4^+$). In the spleen (**Figure 9**) similar levels of $CD4^+$ T-cells, $CD8^+$ T-cells, T-regulatory cells, and B-cells were seen in each of the three groups.

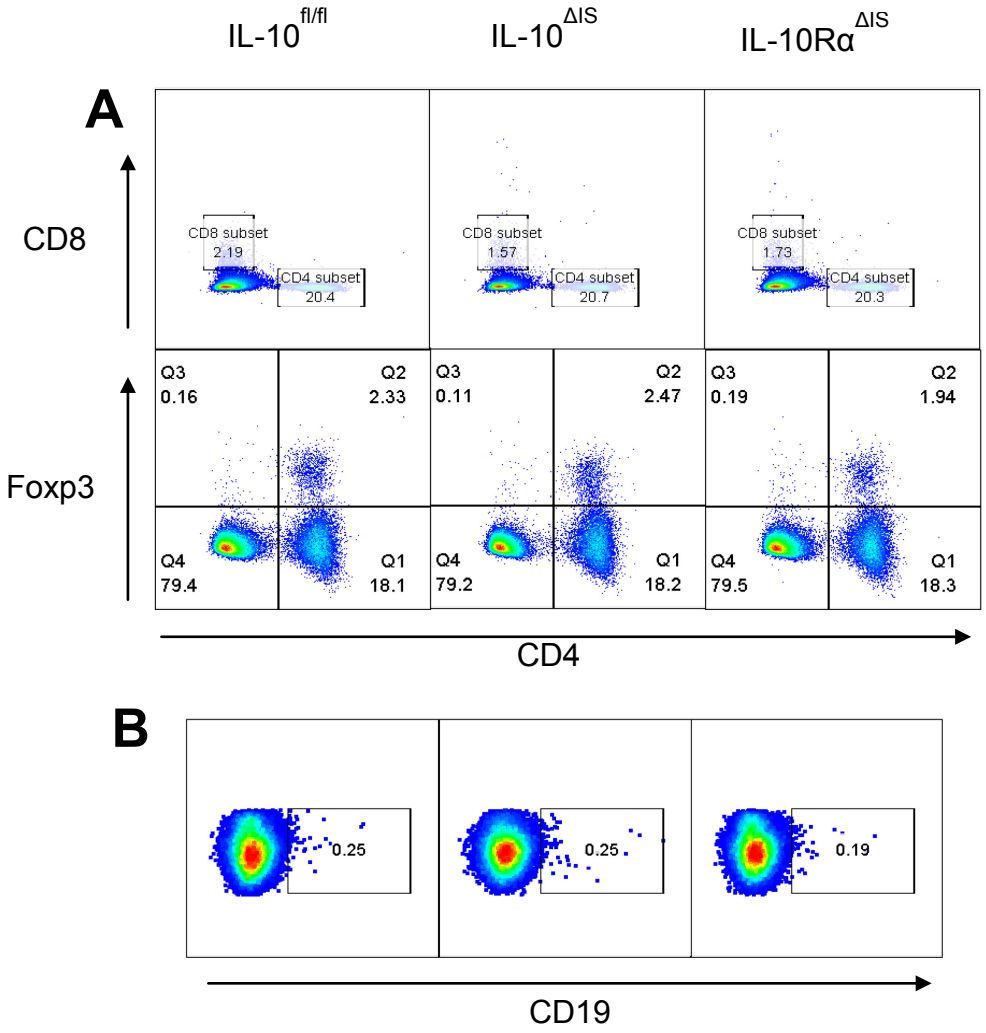


**Figure 9**: Splenic cell populations. (**A**) Cells were gated on live CD45+, then analyzed for expression of CD4, CD8, and Foxp3. (**B**) Cells were gated on live CD45+, then analyzed for expression of CD19 to detect B-cells.

In the lamina propria (**Figure 10**) more than twice as many $CD4^+$ and $CD8^+$ T-cells were seen in the IL-$10^{\Delta IS}$ and IL-10R$\alpha^{\Delta IS}$ mice compared to the IL-$10^{fl/fl}$ mice. On the other hand, the proportion of these $CD4^+$ T-cells that were $Foxp3^+$ regulatory T-cells were higher in the lamina propria of the IL-$10^{fl/fl}$ mice than the IL-$10^{\Delta IS}$ and IL-10R$\alpha^{\Delta IS}$ mice. Out of the $CD4^+$ infiltrate in the lamina propria, 42.9% were T-regulatory cells ($Foxp3^+$) in the IL-$10^{fl/fl}$ mice, compared with only 26.5% and 26.0% in the IL-$10^{\Delta IS}$ and

IL-10Rα$^{\Delta IS}$ mice, respectively. Additionally, the IL-10$^{fl/fl}$ mice had twice as many B-cells in their lamina propria compared to the IL-10$^{\Delta IS}$ and IL-10Rα$^{\Delta IS}$ mice; though, B-cell populations were low in the lamina propria in all three groups. Overall, there was less CD4$^{+}$ and CD8$^{+}$ T-cell infiltrate in the IL-10$^{fl/fl}$ mice, and a higher proportion of the CD4$^{+}$ T-cells in the IL-10$^{fl/fl}$ mice were Tregs.

The IEL populations (**Figure 11**) had a higher percentage of B-cells than both the spleen and LPLs, in all three groups. The IL-10$^{fl/fl}$ mice had, like in the lamina propria, twice as many B-cells than the IL-10$^{\Delta IS}$ and IL-10Rα$^{\Delta IS}$ mice in their intraepithelial layer. Levels of CD4$^{+}$ and CD8$^{+}$ T-cells in the intraepithelial layer were similar between the IL-10$^{fl/fl}$ mice and the IL-10$^{\Delta IS}$ mice. The IL-10Rα$^{\Delta IS}$ mice had their population skewed heavily toward a higher percentage of CD4$^{+}$ T-cell infiltrate and a lower percentage of CD8$^{+}$ T-cell infiltrate. Out of the CD4$^{+}$ IELs in these mice, 22.9% were T-regulatory cells in the IL-10$^{fl/fl}$ mice, whereas only 11.4% and 12.6% were T-regulatory cells in the IL-10$^{\Delta IS}$ and IL-10Rα$^{\Delta IS}$ mice, respectively. This proportion of Tregs is similar to the proportion seen in the LPL populations.

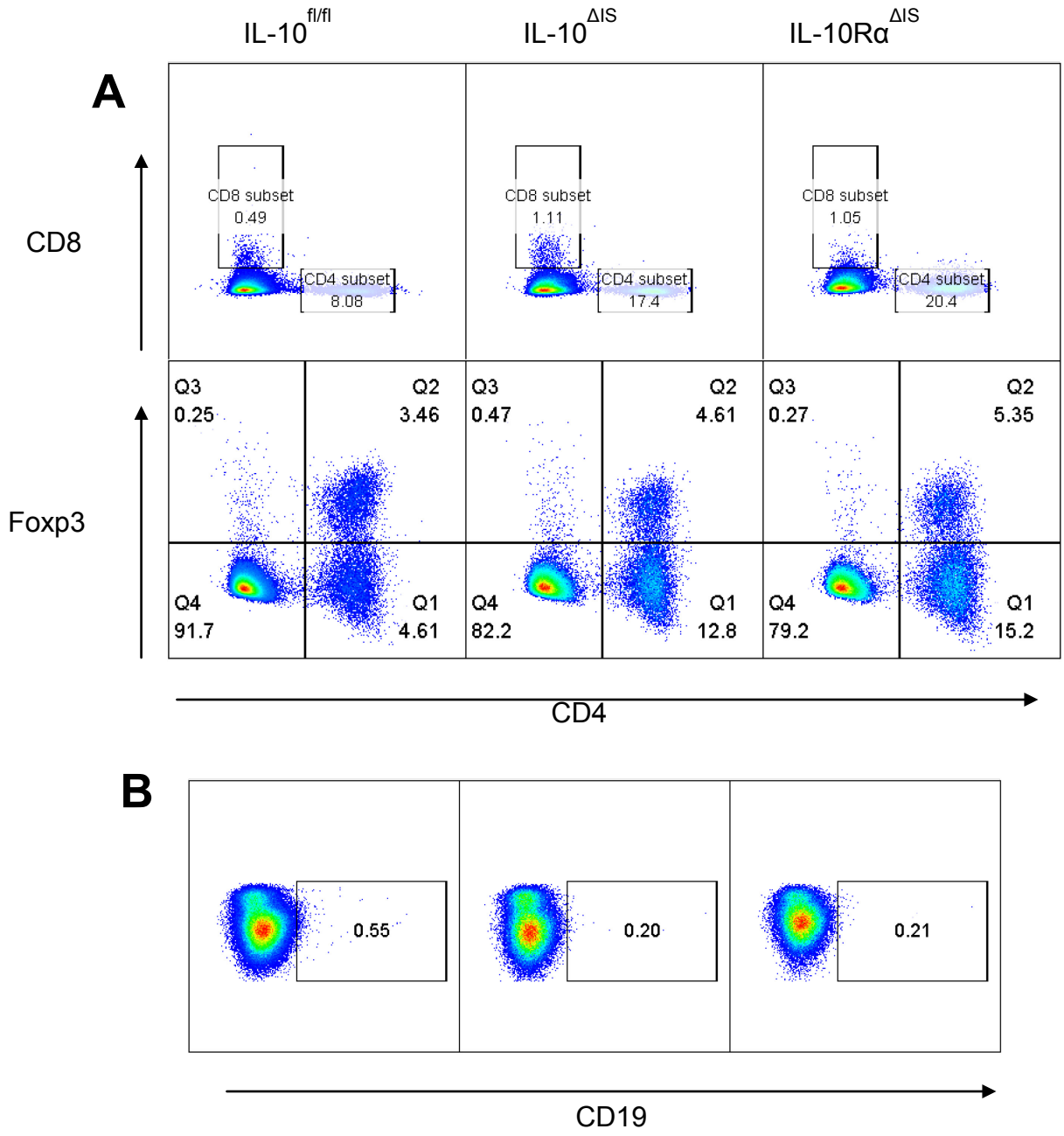


**Figure 10**: Lamina propria lymphocyte cell populations. (**A**) Cells were gated on live CD45+, then analyzed for expression of CD4, CD8, and Foxp3. (**B**) Cells were gated on live CD45+, then analyzed for expression of CD19 to detect B-cells.

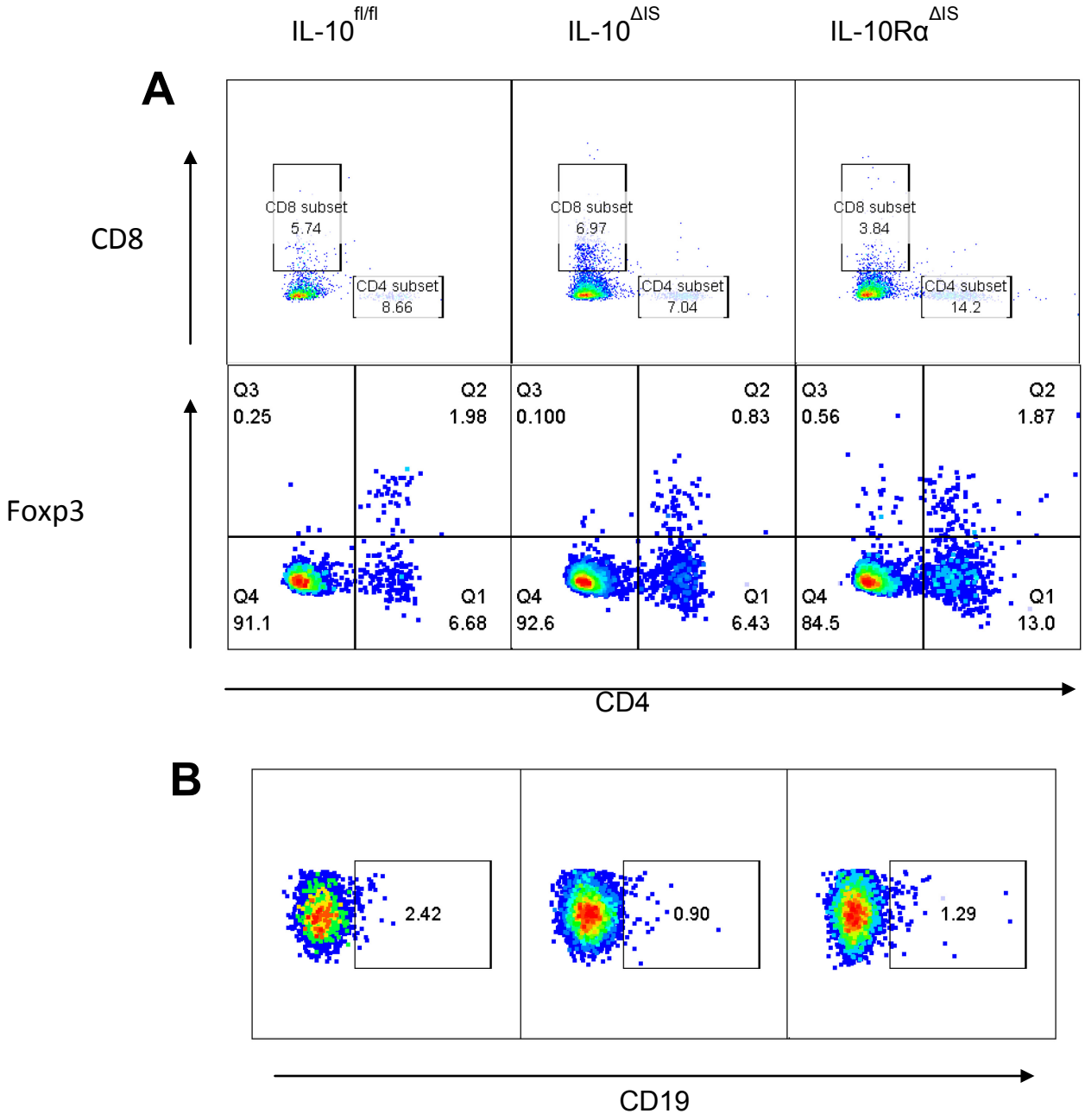


**Figure 11**: Intraepithelial lymphocyte cell populations. (**A**) Cells were gated on live CD45+, then analyzed for expression of CD4, CD8, and Foxp3. (**B**) Cells were gated on live CD45+, then analyzed for expression of CD19 to detect B-cells.

To determine the functional characteristics of these T-cell subsets, a cytokine expression profile analysis was performed. This analysis included the expression of IL-10, IL-17, IL-22, and IFN-γ in T-cells in each the lamina propria, intraepithelial layer, and spleen. **Figure 12** shows the IFN-γ expression in both $CD4^+$ and $CD8^+$ T-cells as a percent of the total $CD4^+$ or $CD8^+$ T-cells in each tissue. $CD8^+$ T-cell IFN-γ expression was similar across all groups and all tissues. Conversely, the CD4-derived IFN-γ was elevated in the IL-10Rα$^{\Delta IS}$ mice compared to the IL-10$^{fl/fl}$ and IL-10$^{\Delta IS}$ mice, in all three tissues.

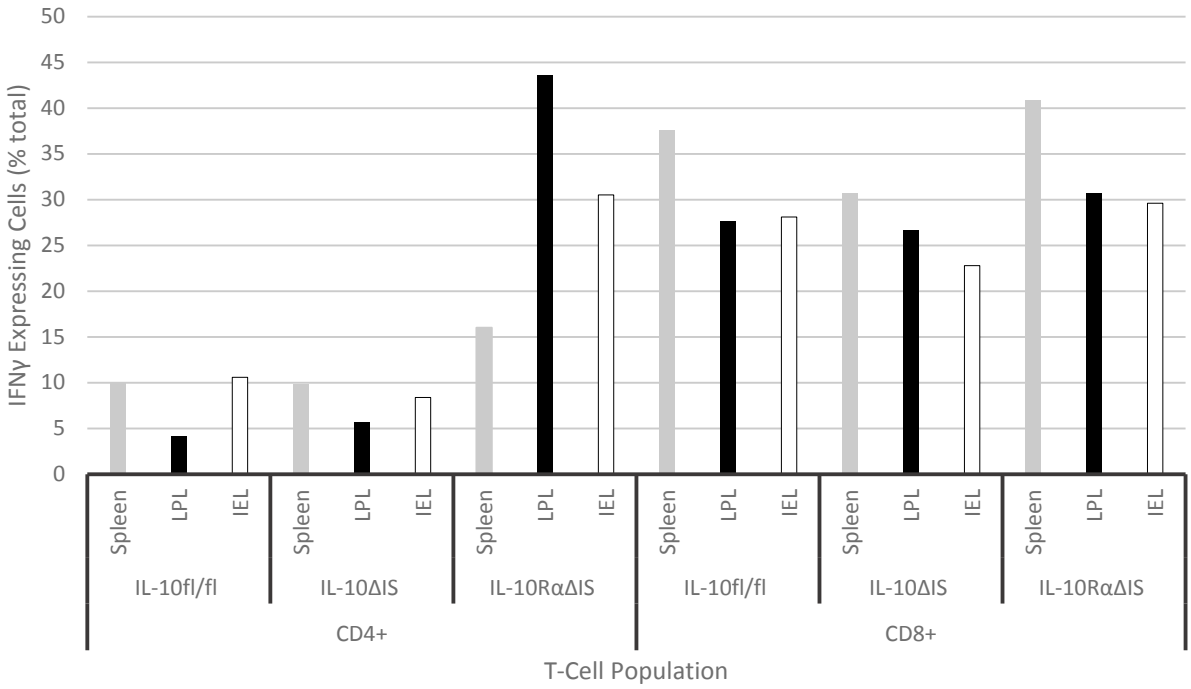


**Figure 12**: IFNγ expression in T-cells. Cells were first gated as live CD45+, then as either $CD4^+$ or $CD8^+$. Represented is the proportion (% of total) of either $CD4^+$ or $CD8^+$ cells that expressed IFNγ in each tissue.

With respect to $CD4^+$ T-cell-derived IL-17, low percentages of IL-17-expressing $CD4^+$ T-cells in the spleens of all three groups were observed (**Figure 13**). In both the

lamina propria and intraepithelial layer, similar IL-17 expression patterns in the $CD4^+$ T-cells were observed. The IL-$10^{\Delta IS}$ mice had the lowest level of IL-17 expression in these lymphocytes, which would be expected since these mice had the least severe colitis. In the same vein, IL-$10^{fl/fl}$ mice, which had the most severe colitis, had twice the $CD4^+$ T-cell-derived IL-17 in the lamina propria as the IL-$10^{\Delta IS}$ mice. Surprisingly, the level of $CD4^+$ T-cell-derived IL-17 in the IL-10R$\alpha^{\Delta IS}$ mice was more than seven times the amount of IL-17 in the IL-$10^{fl/fl}$ mice in the lamina propria, and almost four times the amount in the intraepithelial layer.

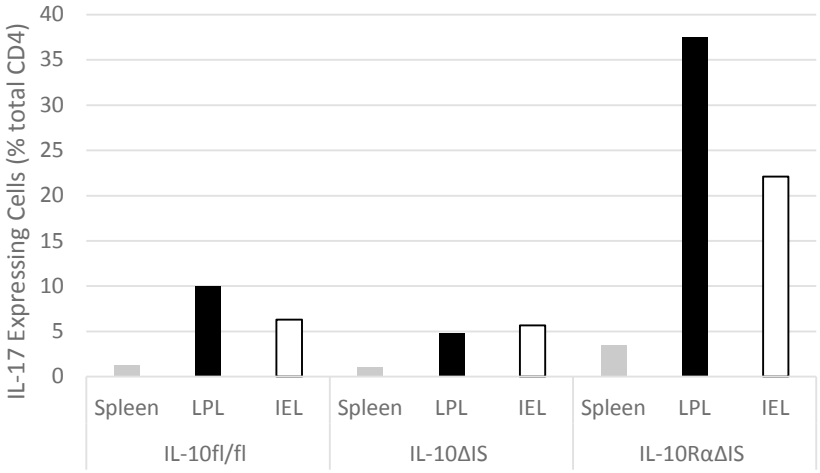


**Figure 13**: Proportion of IL-17 expressing $CD4^+$ T-cells in each the spleen, IEL, and LPL populations. Cells were initially gated on live CD45+, then as $CD4^+$ analyzed as the proportion of these $CD4^+$ cells positive for expression of each cytokine

IL-22 expression was much lower than both IL-17 and IFN-γ expression in all groups and tissues (**Figure 14**). But, similar to the IL-17 expression pattern, levels were elevated in the in the IL-10R$\alpha^{\Delta IS}$ mice. This increase was much less dramatic than the increase in IL-17 expression, and overall IL-22-expressing $CD4^+$ T-cells were sparse.

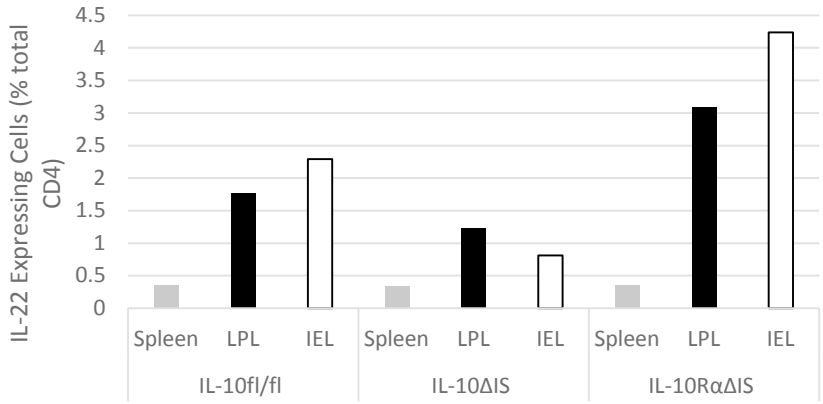


**Figure 14**: Proportion of IL-22 expressing $CD4^+$ T-cells in each the spleen, IEL, and LPL populations. Cells were initially gated on live CD45+, then as $CD4^+$ analyzed as the proportion of these $CD4^+$ cells positive for expression of each cytokine

The level of IL-10 expression in the $CD4^+$ T-cell populations can be a good indicator of the effect IL-10 signaling in IECs has in colitis. IL-10 is able to signal in a paracrine and autocrine fashion to induce T-regulatory cells and further the expression of IL-10. When the $CD4^+$ T-cells in the lamina propria and intraepithelial layer were examined (**Figure 15**), it was found that the proportion of these cells expressing IL-10 was significantly increased in the $IL\text{-}10^{fl/fl}$ mice. This may be indicative of the importance of enterocyte-derived IL-10 in the induction of IL-10 expression in IELs. This may also be an artifact of the residual inflammation still present in the $IL\text{-}10^{fl/fl}$ mice at day 16 causing there to be a need for IL-10, while in the other two groups the inflammation had already been resolved.

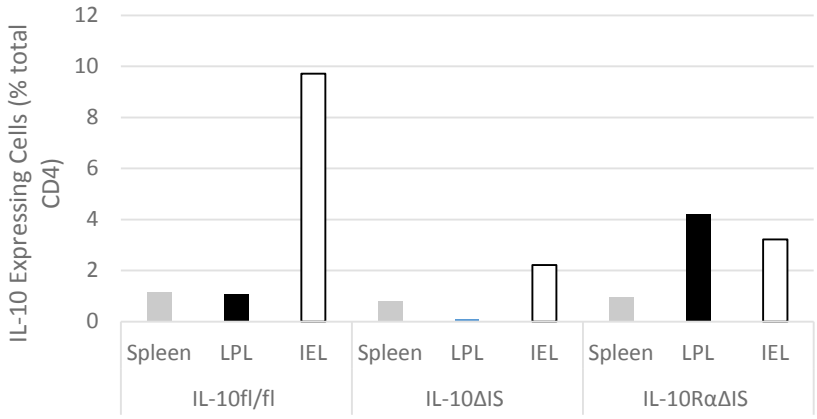


**Figure 15**: Proportion of IL-10 expressing $CD4^+$ T-cells in each the spleen, IEL, and LPL populations. Cells were initially gated on live CD45+, then as $CD4^+$ analyzed as the proportion of these $CD4^+$ cells positive for expression of each cytokine

T-regulatory cell-derived IL-17 can be a good indicator of these cells' ability to regulate inflammation. Previous research has shown a degree of plasticity between the Th17 and $Foxp3^+$ T-regulatory cell lineages, with IL-17 expression in $CD4^+Foxp3^+$ T-cells being indicative of decreased regulatory function (46). IL-10 and IL-17 expression in $CD4^+Foxp3^+$ T-cell populations was analyzed to determine if IEC-derived IL-10 had any effect on the ability of these Tregs to be efficient regulators of inflammation (**Figure 16**). The results showed that, in the lamina propria, a significantly lower proportion of the T-regulatory cells in the $IL\text{-}10^{\Delta IS}$ mice expressed IL-10, compared with the $IL\text{-}10^{fl/fl}$ and $IL\text{-}10R\alpha^{\Delta IS}$ mice. This may be indicative of the necessity of IEC-derived IL-10 in inducing efficient T-regulatory cells. The $IL\text{-}10R\alpha^{\Delta IS}$ mice showed a higher proportion of IL-10-expressing $CD4^+Foxp3^+$ T-cells, as well a higher proportion of IL-17 expressing $CD4^+Foxp3^+$ T-cells. In the lamina propria, the $IL\text{-}10^{\Delta IS}$ mice had the lowest proportion of IL-17-expressing Tregs.

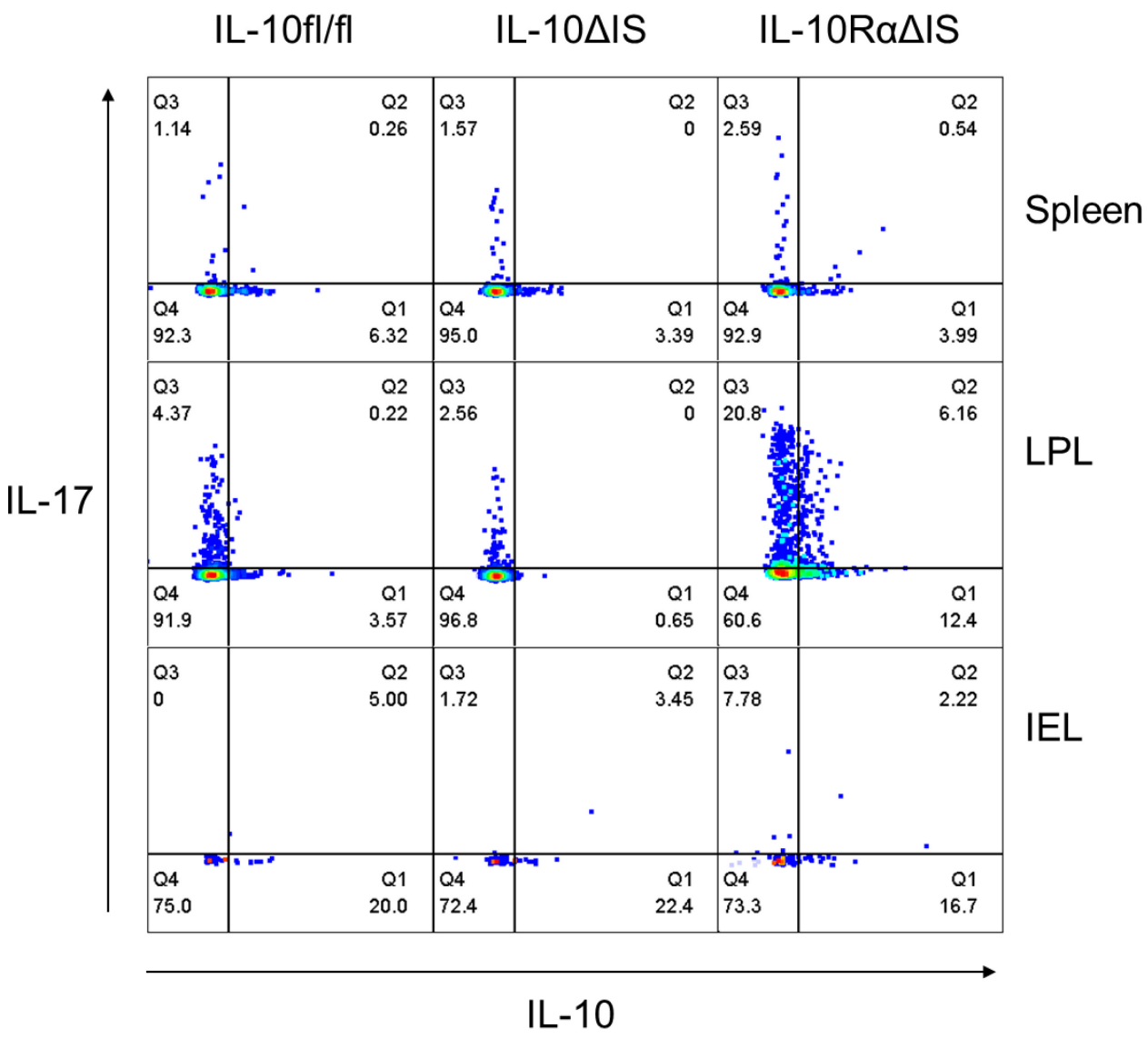


**Figure 16**: Proportion of $CD4^{+}Foxp3^{+}$ T-cells expressing IL-10 and/or IL-17 in each the spleen, LPL, and IEL populations. Cells were first gated as live CD45+ lymphocytes, then as CD4+Foxp3+ double positive cells. $IL\text{-}10^{\Delta IS}$ and $IL\text{-}10R\alpha^{\Delta IS}$ LPL populations showed a higher ratio of IL-17 expressing $CD4^{+}Foxp3^{+}$ T-cells to IL-10 expressing $CD4^{+}Foxp3^{+}$ T-cells.

## Discussion

Gastrointestinal health is one of the most rapidly advancing frontiers in medical research, and gastrointestinal diseases represent some of the largest public health issues in the world. Beyond the debilitating symptoms IBDs like Crohn's and ulcerative colitis can have on those they affect, the GI tract is central to controlling obesity, one's hormones, and even one's neurological health. At the center of this physiological mission control is a complex interface where foreign microbes are as important as one's own cells, and central to the maintenance of this interface is the limitation of the human body's own defense against these microbes. The immune system in the gut must tolerate these foreign bodies and treat them as self, while remaining vigilant in the defense against infection. Epithelial cells are the first line of this defense, not only protecting the host from foreign microbes, but defending the gut microbiota from the host's immune system. In order to coordinate the tolerance of gut microbes, cytokines like IL-10 are used to inhibit the immune system's inflammatory response. Therefore, the issue of how epithelial cells might use IL-10 to maintain a healthy GI tract was addressed.

The epithelial layer has been shown to be a target of inflammation, by both expressing and responding to cytokines. Previous studies have shown IL-10R signaling on IECs to have an antagonistic effect to IFN-γ signaling by blocking the ability of IFN-γ to inhibit IEC recovery and viability. This IL-10R signaling also blocks the upregulation of MHC class II expression caused by IFNγ signaling (47). Epithelial cells are also able to stimulate both $CD4^+$ and $CD8^+$ T-cells in the gut, but they lack the necessary co-stimulatory molecules B7-1 and B7-2 (22). Findings like these have led to the development of the concept that IECs are important inducers of suppressor cells in the

tolerogenic response in the gut. Along with studies that show IL-10 is able to inhibit inflammatory responses of the IECs themselves, IL-10 is a well-known inhibitor of the inflammatory response in lymphocytes. Furthermore, IL-10 signaling is crucial to inhibiting the development of IBD. It is curious, then, that these results showed a reduction in the intensity of DSS-induced colitis in the absence of IL-10 expressing or responding IECs.

When looking at the cell populations in the guts of these mice, the results suggest an important role for IEC-derived IL-10 in the induction of T-regulatory cells. This is seen in that a significantly higher proportion of $CD4^+$ T-cells were $Foxp3^+$ in the $IL\text{-}10^{fl/fl}$ mice. While these results echo previous studies on the role of IEC-derived IL-10 in the induction of Tregs (48), this may also be an artifact of the residual inflammation in the gut in the $IL\text{-}10^{fl/fl}$ mice. The residual inflammation being the driver of this population of cells at the point in time in which they were analyzed, instead of the presence of enterocyte-derived IL-10.

In addition to the high proportion of Tregs, the $IL\text{-}10^{fl/fl}$ mice had a significantly lower overall $CD4^+$ and $CD8^+$ T-cell infiltrate in the lamina propria. This is likely due to the higher proportion of Tregs in these mice. Tregs have been shown to suppress the proliferation of immune effector cells, and IL-10 plays a key role in their ability to suppress inflammation. One important next step would be to analyze this cell population kinetically throughout the 16-day model, looking at LPL and IEL phenotypes at multiple time points.

The inflammation induced in IL-10 KO models of colitis is mediated by inflammatory Th1 and Th17 $CD4^+$ T cells (25,24,42). Conversely, the mice in this

experiment with less severe colitis had a greater $CD4^+$ T-cell infiltrate. In analyzing the cytokine expression profiles of these lymphocytes, $CD4^+$ T-cells from IL-10R$\alpha^{\Delta IS}$ mice had significantly higher IL-17 and IFN-γ production, while the difference between IL-$10^{fl/fl}$ and IL-$10^{\Delta IS}$ mice was negligible. IL-10R$\alpha^{\Delta IS}$ mice also had a higher proportion of IL-$17^+$Foxp$3^+$ double positive T-cells, which have been implicated in IBD (46). This is interesting as the IL-10R gene has been found to be a susceptibility locus for IBD (26,25,49,50).

Previous studies have shown that the IL-10R signaling in intestinal macrophages is important in limiting the colonic Th17 response, and thus limiting the severity of colitis (25). The cytokine profiles in the $CD4^+$ T-cells of the IL-10R$\alpha^{\Delta IS}$ mice suggest that IL-10R signaling in IECs may play a similar role, but the less severe disease phenotype in these mice contradicts this conclusion. It is well known that IL-10 signaling is important, but what exact cells express and respond to IL-10, and which ones are driving the phenotypes seen, is the question that this experiment attempted to answer. IL-10R signaling in epithelial cells may play a significant role in the development of aberrant inflammation in the GI tract, but the contradictions between the lymphocyte cytokine profiles and disease phenotypes seen should be further investigated.

DSS causes colitis through direct interaction with IECs. IL-10 expression in these cells may make them more susceptible to DSS itself. When DSS damages IECs it activates the NLRP3 inflammasome and releases IL-18, which then signals through MYD88 on myeloid cells in the lamina propria to induce tissue repair and IEC proliferation (51). If there is an inhibition of this mechanism, greater bacterial infiltrate into the submucosa and persistent tissue damage is observed, which then stimulates an

inflammatory response and exacerbates disease (51). IL-18 also has inflammatory effects in the production of chemoattractants and pro-inflammatory cytokines. Previous research in arthritis has shown IL-10 to be a negative regulator of the NLRP3 inflammasome (52). Furthermore, persistent activation of the NLRP3 inflammasome has been implicated in the induction of colitis in IL-10 KO mice. It is possible, then, that autocrine IL-10 signaling in IECs regulates the NLRP3 inflammasome to delay this reaction. This would inhibit the ability of myeloid-derived IL-18 to induce IEC proliferation and repair. This mechanism would also explain the fact that the IL-$10^{\Delta IS}$ and IL-$10R\alpha^{\Delta IS}$ mice had greater T-cell infiltrate in the lamina propria, as IL-18 also induces immune cell recruitment.

The experimental approach and results shown, though preliminary, may provide important clues to the etiology of gut inflammatory diseases. Almost all cell types are able to make and respond to IL-10, and different IL-10-expressing cell types can have distinct roles in the pathogenesis of disease. This is well defined in disease models like LPS toxicity, *Leishmania* infection, and toxoplasma infection, but has yet to be clearly defined in the gut. In IBD it is important that the inflammation be resolved, and IL-10 is an important regulator of inflammation. It may be, though, that the pleiotropic effects of the cytokine make the needed IL-10 response different in certain cell types in the gut.

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**Curriculum Vitae**

# Adam T. Leibold

Address: 510 S. Collington Ave. Baltimore, MD 21231; Phone: (317) 441-4488; Email: Aleibol1@jhu.edu

## Education

- ScM, Johns Hopkins University Bloomberg School of Public Health, Department of Molecular Microbiology and Immunology, Graduated May 2015.
    - Principle Investigator: Dr. Jay Bream (410-502-2511, jbream1@jhu.edu)
    - Vaccine Science and Policy Certificate
    - Recipient of Emergent BioSolutions Fellowship ($4,700)
- B.S., Indiana University, Microbiology Major, Chemistry Minor, Graduated August 2013.

## Work Experience

- Teaching Assistant, Johns Hopkins Bloomberg School of Public Health: Dr. Jay Bream (410-502-2511, jbream1@jhu.edu), Dr. Anna Durbin (410-614-4736, adurbin1@jhu.edu)
    - Course: “Biological Basis of Vaccine Development”
- Research Assistant, Johns Hopkins Bloomberg School of Public Health Department of Epidemiology: Dr. Robert Wojciechowski (RWojciec@jhsph.edu)
- Research Assistant, Johns Hopkins Center for AIDS Research: Dr. Risha Irvin (Rirvin1@jhmi.edu)

- Certified Sterile Processing Technician, Indiana University Health Methodist Hospital: Aljean Watkins-Porter (317-963-6382), Shift Manager, Indianapolis, Indiana, December 2011-March 2013
- Research Assistant, Indiana University: Dr. Mukhopadhyay (sumukhop@indiana.edu), Bloomington, Indiana, June 2012-August 2012

**Volunteer Experience**

- Johns Hopkins Bloomberg School of Public Health Student Assembly: August 2014-June 2015
    - Served on the Social and Cultural Committee
    - Served on the Finance Committee
- Pi Kappa Phi Fraternity Philanthropy chair: September 2010- August 2012
    - Raised over $15,000 for Pi Kappa Phi's philanthropy "PUSH America"
    - Received the "Be More Involved Award" from the Mayor of Bloomington
    - Received the "Civic Engagement and Citizenship Greek Award" from Indiana University Interfraternity Council
- Timmy Global Health Fundraising Committee Chair: September 2011- May 2012