Immunomodulation of Intestinal Epithelial Cell Proliferation and Function as a Novel Host Defense Mechanism in Infectious Colitis

by

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Abstract

Although epithelial cells represent the primary site of host contact for attaching and effacing pathogens, their contribution to host defense is relatively unrecognized. Both idiopathic and infectious forms of colitis disrupt normal intestinal epithelial cell (IEC) proliferation, differentiation and function, although the mechanisms involved remain unclear. Infection by the attaching and effacing murine pathogen, *Citrobacter rodentium* leads to significant colonic hyperplasia but also a reduction in colonic goblet cell numbers (goblet cell depletion). This pathology depends on T and/or B cells as *Rag1 -/-* mice do not suffer this depletion during infection, instead suffering high mortality rates. Reconstitution studies reveal that both CD4+ and CD8+ T cell subsets greatly increase survival of *Rag1 -/-* mice. However, while mice receiving CD8+ T cells develop exaggerated colonic tissue damage and ulcers, mice receiving CD4+ T cells develop goblet cell depletion in concert with exaggerated IEC proliferation preventing deep pathogen penetration of colonic crypts. Studies with *Ifn-γ receptor -/-* mice and wildtype mice given IL-17A neutralizing antibodies identify IFN-γ signaling as a critical cytokine required for both goblet cell depletion and increased IEC proliferation. Finally, studies inhibiting notch signaling and thus vastly increasing goblet cell numbers greatly increased pathogen burdens and mortality rates. These studies thus demonstrate that goblet cell depletion reflects host immunomodulation of IEC homeostasis and reflects a novel host defense mechanism against mucosal adherent pathogens.

Preface

Chapter 2

This chapter was published in the journal Infection and Immunity. I was the primary contributor to this work, designing and carrying out experiments, as well as analyzing and presenting the data shown in the figures. Dr. Bruce Vallance provided guidance, and aided in experimental design and supervision of the research. Ms. Ganive Bhinder performed immunofluorescent staining and provided the pictures in Figure 2.7C. Dr. Ho Pan Sham and Ms. Natasha Ryz assisted in the histological scoring seen in Figure 2.2A and Dr. Kirk Bergstrom contributed to the research direction and experimental design. Finally Tina Huang performed immunofluorescent staining in various experiments.

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List of Symbols and Abbreviations

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
±	plus-minus symbol
µ	Micro
A/E	Attaching and effacing
AKT	Protein Kinase B
APC	Adenomatous polyposis coli
bHLH	Basic helix-loop-helix
BrdU	Bromodeoxyuridine
C. albicans	*Candida albicans*
C. rodentium	*Citrobacter rodentium*
cAMP	Cyclic adenosine monophosphate
CBC	Crypt base columnar
CFU	Colony forming units
CKI	Casein kinase 1
DAPI	4',6-diamidino-2-phenylindole
DBZ	Dibenzazepine
DNA	Deoxyribose nucleic acid
DSS	Dextran sodium sulfate
E. coli	*Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-Met-Leu-Phe
GALT	Gut-associated lymphoid tissues
Gfi1	Growth factor independent 1 transcription repressor

GSK3	Glycogen synthase kinase 3
H. pylori	*Helicobacter pylori*
HES	Hairy/Enhancer of Split
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intra-peritoneal
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
LPL	Lamina propria lymphocytes
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
NF-κB	Nuclear factor kappa-B
Ngn3	Neurogenin 3
NK	Natural killer
Nle	Non-Lee encoded region
P. aeruginosa	*Pseudomonas aeruginosa*
PAI	Pathogenicity island
PAS	Periodic acid-Schiff
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Rag1	Recombination activating gene 1
Reg III γ	Regenerating islet- derived protein 3 gamma

RELM-β	Resistin-like Molecule Beta
RIP	Receptor-interacting serine/threonine-protein
RPMI	Roswell Park Memorial Institute medium
S. Typhimurium	*Salmonella enterica serovar* Typhimurium
SFB	Segmented filamentous bacteria
SEM	Standard error of the mean
T3SS	Type III secretion system
TCF	T-cell factors
TCR	T cell receptor
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
Th	T helper
Tir	Translocated intimin receptor
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	T regulatory

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The last three and half years have taken me through an unforeseen series of frustrations and personal challenges but largely through the selfless care and wisdom of those who have supported me along the way, I look back at my journey not with disappointment but with triumph. My ultimate appreciation goes out to my supervisor, Dr. Bruce Vallance, whose tremendous mentoring and teaching is surpassed only by his penchant for listening, caring and understanding. I have a profound respect for Bruce as a scientist, mentor and friend and it has been my great pleasure to work with him. I also thank my graduate supervisory committee member, Dr. Kevan Jacobson, for providing unique insight and a valuable clinical approach to my studies. His motivation to care for the IBD community inspires me in the direction of my science career. Thank you to everyone who I have had the opportunity to work with in the Vallance lab. I thank Dr. Lisa Månsson and Dr. Yanet Valdez for giving me a platform to stand on early in my training; with their encouragement, smiles, and laughter I instantly felt welcome. I also thank Caixia Ma and Tina Huang for their steadfast dedication to the lab and always knowing every question I ever had about where to find or how to do anything. A special thanks to my companions and comrades Dr. Andy Sham, Natasha Ryz and Ganive Bhinder for science-talk, life-talk and everything in between. I would also like to thank Dr. Kirk Bergstrom, Dr. Deanna Gibson, Dr. Mari Montero, Dr. Maryam Zarepour, Dr. Vijay Morampudi, Dr. Martin Stahl and Kiran Bhullar for all of their knowledge, suggestions, critiques and encouragements throughout my studies. Finally, I thank my family for loving me and encouraging me to accomplish whatever I set out to do. And to Dr. Fong Huynh for her unselfish love, incredible support and believing in me even when I could not. Thank you.

To those living with mental illness in and around their lives

Chapter 1: Introduction

1.1 Intestinal architecture

The gastrointestinal tract is perhaps the most intimate interface between the outside world and the human body. It governs polar spectrums of the human body's interactions with foreign material, both the absorption of valuable and necessary nutrients and the elimination of wastes. If not daunting enough, the presence of trillions of microorganisms that reside within the gut can either aid in its function through symbiotic relationships or present severe challenges through pathogenic insult. Of course the distinction between friend and foe isn't clearly defined as even symbiotic commensal microbes can pose a threat under conditions of duress when bacterial products can leak through the normally impermeable barrier. The gastrointestinal tract, and particularly the colon, is also lined with a structural and functional mucus layer that creates a defensive matrix against intestinal pathogens. All these factors combine to create a complex intestinal ecosystem that needs to tightly discriminate nutrients from toxins and pathogens from commensals. To accomplish this, the intestinal epithelium and supporting immune cells collectively perform rigorous sampling of the luminal environment in order to orchestrate appropriate recognition and activation of both the innate and adaptive immune response. In part, these strategies reflect decisions regarding intestinal epithelial cell fate, proliferation and turnover. While the gastrointestinal tract consists of many specialized structures and regional specificities, the large intestine presents an ideal environment to study the complex relationship between the luminal environment and the host.

1.1.1 The large intestine

The human large intestine extends from the distal end of the ileum to the anus and is approximately 1.5 meters in length, filled with mucus, microorganisms, gas and compacted feces (1). The main function of the large intestine is to absorb fluids and salts (approximately 1400 ml/day) and to form feces (approximately 100 grams/day) from the chyme received from the small intestine and is thus one of the most vital homeostatic systems in the human body. The large intestine consists of several defined and continuous structures that vary in gross and histological anatomy including the cecum and ascending (proximal) colon, transverse (medial) colon, descending and sigmoid (distal) colon and the rectum (2). The cecum and the colon are virtually indistinguishable histologically and are often described as a single entity. However, subtle differences do exist including a thinner mucus layer in the cecum compared to the rectum (approximately 30 μm vs. 150 μm) and less resident bacteria. From exterior to interior, the large intestine is made up of the muscularis externa, submucosa, muscularis mucosae, lamina propria and finally the single layer of intestinal epithelial cells which surrounds the lumen (3). Of note, the muscularis externae is composed of an inner circular layer and outer longitudinal layer of smooth muscle cells, the submucosa is composed of dense, irregular connective tissue while rich in lymphatic and vascular supply, while the lamina propria resides at the base of the intestinal epithelium and is rich in lymphoid cells which help protect the intestinal lining from microorganisms. However, most pertinent to this study is the intestinal epithelium that stands between the external environment and the host.

1.1.1.1 Of mice and men

In our studies, we use the mouse as a model organism to study the intestinal epithelium and immunity. Mice mirror human biology remarkably well having only (approximately) 300 genes unique to one species or the other. Moreover the small size and relative low cost of mice, as well as the array of immunologic and genetic tools available to study the species, make the mouse the preeminent species in which to model bacterial pathogenesis (4). Anatomical and immunological discussion from this point on will refer to characteristics of the mouse colon. While nearly all discussed components are conserved between species, some differences do exist and will be highlighted within the text.

1.1.2 The colonic intestinal epithelium

The colonic intestinal epithelium is a single sheet of highly specialized cells organized in over 700,000 crypts of Lieberükhn containing a total of over 700 million epithelial cells (5). Both the size of the crypts and their compositions vary greatly depending on the region of the colon they are found within. In the cecum and proximal colon, crypts are shorter (20 vs. 30 cell positions) and consist of fewer cells (300 vs. 700 cells/crypt) compared to the distal colon (6). The colonic intestinal epithelium is made up of three specialized cell types, columnar absorptive cells, goblet cells and enteroendocrine cells (Fig. 1.1). Paneth cells, responsible for the production of antimicrobial peptides such as α-defensins/cryptdins, are also found in the intestinal epithelium of the small intestine but are absent in the colon. The most numerous cells of the colonic epithelium are the columnar absorptive cells (also known as enterocytes/colonocytes) making up about 80% of the crypt cell population (3). They are

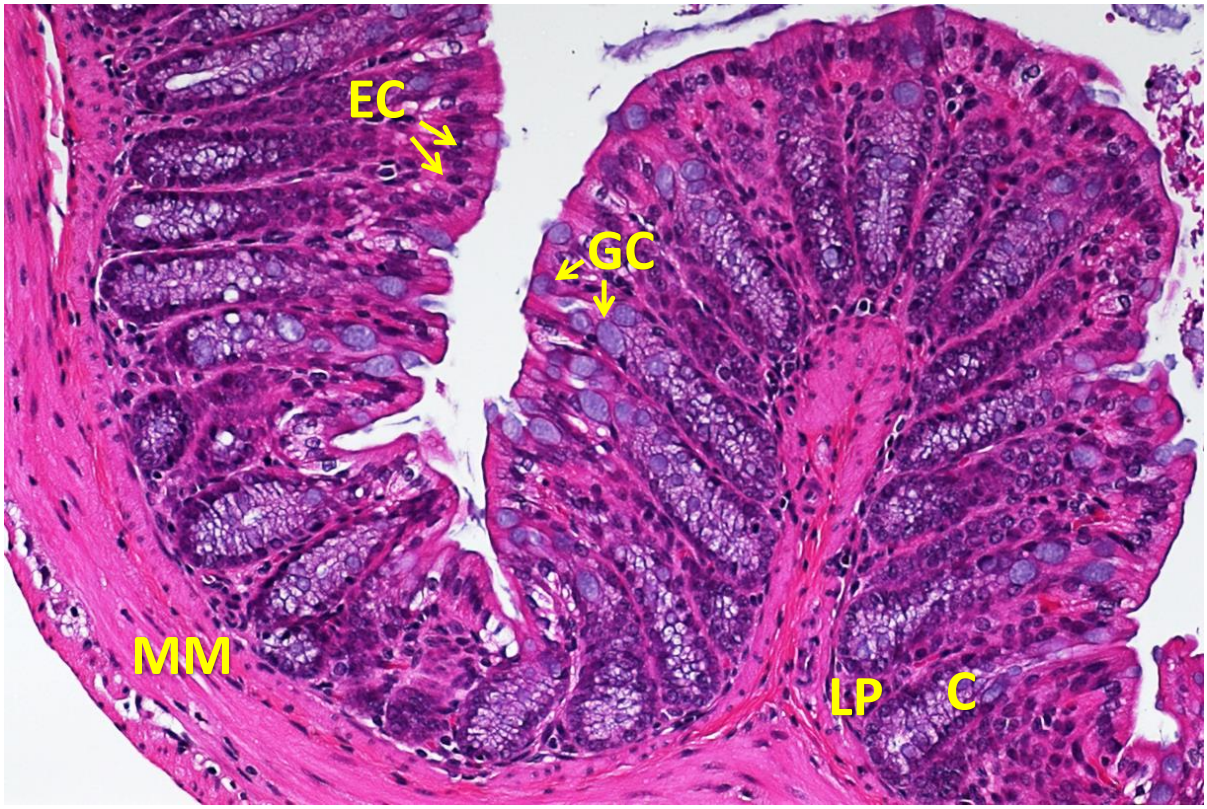

Figure 1.1: ***Cross-sectional histology of the murine colon***

The murine colonic intestinal epithelium is formed of crypts of Lieberükhn (C) which are surrounded by the lamina propria (LP) and in turn surrounded by the muscularis mucosae (MM). The colonic intestinal epithelium consists of three major cell types which are predominantly enterocytes (EC). The other two cell types are fewer in number and include goblet cells (GC) and enteroendocrine cells (not labelled). Original magnification is 200x.

tall cells of approximately 25 µm in length and have a basally located nucleus. The apical surfaces of these cells are characterized by a brush border that increases the surface area of the intestine, thereby aiding in the absorption of water and nutrients across the epithelium and into the lamina propria for distribution to the rest of the body. During maturation, these cells begin as vacuolated cells occupying the lower two-thirds of the crypt. As the cells migrate upward along the crypt, they form vacuole-like granules in their apical cytoplasm before they gradually differentiate towards the absorptive columnar phenotype. The second

most abundant cell type in the colon is the goblet cell making up to 20% of the crypt cell population (7). The number of goblet cells/crypt increases as one moves from the proximal to the distal colon. Mature goblet cells tend to be evenly distributed along the mid region of crypts but are found only sporadically at the base or luminal surface. Precursor-goblet cells, also known as oligomucous cells, are found at the base of the crypt and contain few mucous granules. Physiological maturation of goblet cells results in the enlargement of the theca as they swell with the production of mucins for example, Muc2, and other goblet cell products such as TFF3 and RELM-β, thereby giving the cells a goblet-like appearance (8). Goblet cells function mainly by secreting mucins into the lumen to make up the mucus layer. More detail on the structure and function of goblet cells will be discussed below. Finally, the least represented cell type of the colonic intestinal epithelium is the enteroendocrine cell which makes up approximately 0.4% of the epithelial cells in the colon. Enteroendocrine cells actually represent several cell lineages that are responsible for the production and release of hormones and peptides that can be released to activate the enteric nervous system (9).

1.1.3 Epithelial cell proliferation

One of the hallmark characteristics of the intestinal epithelium is its self-regenerative capacity. The intestinal epithelium is rigorously replaced every four to five days under normal conditions with cells beginning their maturation at the base of the crypt and migrating toward the intestinal lumen (10). Over the past decade, great strides have been made in the identification of the pluripotent stem cells that give rise to all other cells within the crypt. These cells, which were previously described as diminutive, constantly cycling crypt base columnar (CBC) cells, have recently been identified to express the Wnt target gene, Lgr5, in

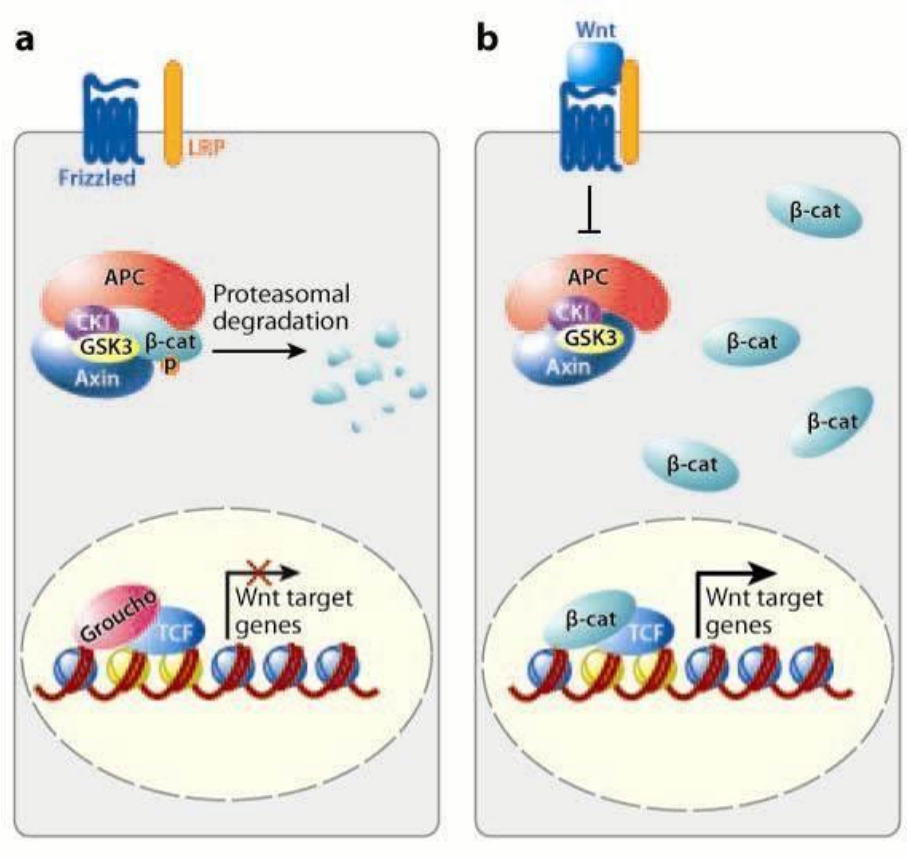

Figure 1.2: ***Schematic representation of the Wnt signaling pathway***
In the absence of Wnt (a), the destruction complex consisting of adenomatous polyposis coli (APC), casein kinase I (CKI), glycogen synthase kinase 3 (GSK3), and axin targets β-catenin for proteasomal degradation through phosphorylation. Active Wnt (b) ligands bind to Frizzled receptors inhibiting the destruction complex. β-catenin levels in the cell rises, resulting in its translocation to the nucleus and transcription of Wnt target genes. Image modified from ref (11).

both the small intestine and colon (12). The self-renewing capacity of these cells enables expansion and proliferation of the intestinal crypts. Interestingly, small intestinal Lgr5+ stem cells cycle much more rapidly than colonic Lgr5+ cells reflecting differences in the rate of epithelial turnover between the small intestine and the colon. These rare stem cells give rise to a pool of transit-amplifying cells that take half as long to cycle and are thus largely responsible for crypt proliferation (13). Transit-amplifying cells are not terminally differentiated but are partially differentiated precursors to the basic cell types undergoing

maturation. The transit-amplifying cells, like the Lgr5 stem cells, are controlled by the Wnt pathway, the primary signaling component of epithelial cell proliferation in intestinal crypts through the action of β-catenin (Fig. 1.2). In the absence of Wnt, β-catenin levels are kept low in the cell due to proteasomal degradation through the activity of the APC/CKI/GSK3/Axin destruction complex (14). However, when Wnt binds its receptor, Frizzled, β-catenin is no longer inactivated – therefore resulting in active transcription of WNT/TCF target genes which are largely responsible for the proliferative activity in the intestinal epithelium (15). Recent studies examining the expression of Wnt components indicate that WNT/TCF target genes are active in a gradient along the crypt with the highest gene activity at the base. Mice lacking the Tcf4 transcription factor and transgenic mice secreting Dickkopf-1 (a Wnt inhibitor) both suffer from a dramatic reduction in crypt proliferative activity (16, 17). However, the molecular systems that govern cell proliferation are also highly intertwined with those that govern cell differentiation.

1.1.4 Epithelial cell differentiation

Intestinal epithelial homeostasis requires the expansion of specific cell types to maintain functional balances within the gastrointestinal tract. The transit-amplifying cells undergo terminal differentiation into one of three (or four in the small intestine) cell types as described above depending on the signals they receive via the Notch pathway. The Notch pathway functions as a hierarchical signaling pathway that pushes cells towards either an absorptive lineage (enterocyte) or a secretory lineage (goblet, enteroendocrine or Paneth cells) (Fig. 1.3). It functions primarily through a cell-cell contact process which ensures an appropriate distribution of cell types. When Notch is activated through cell-cell contact of its

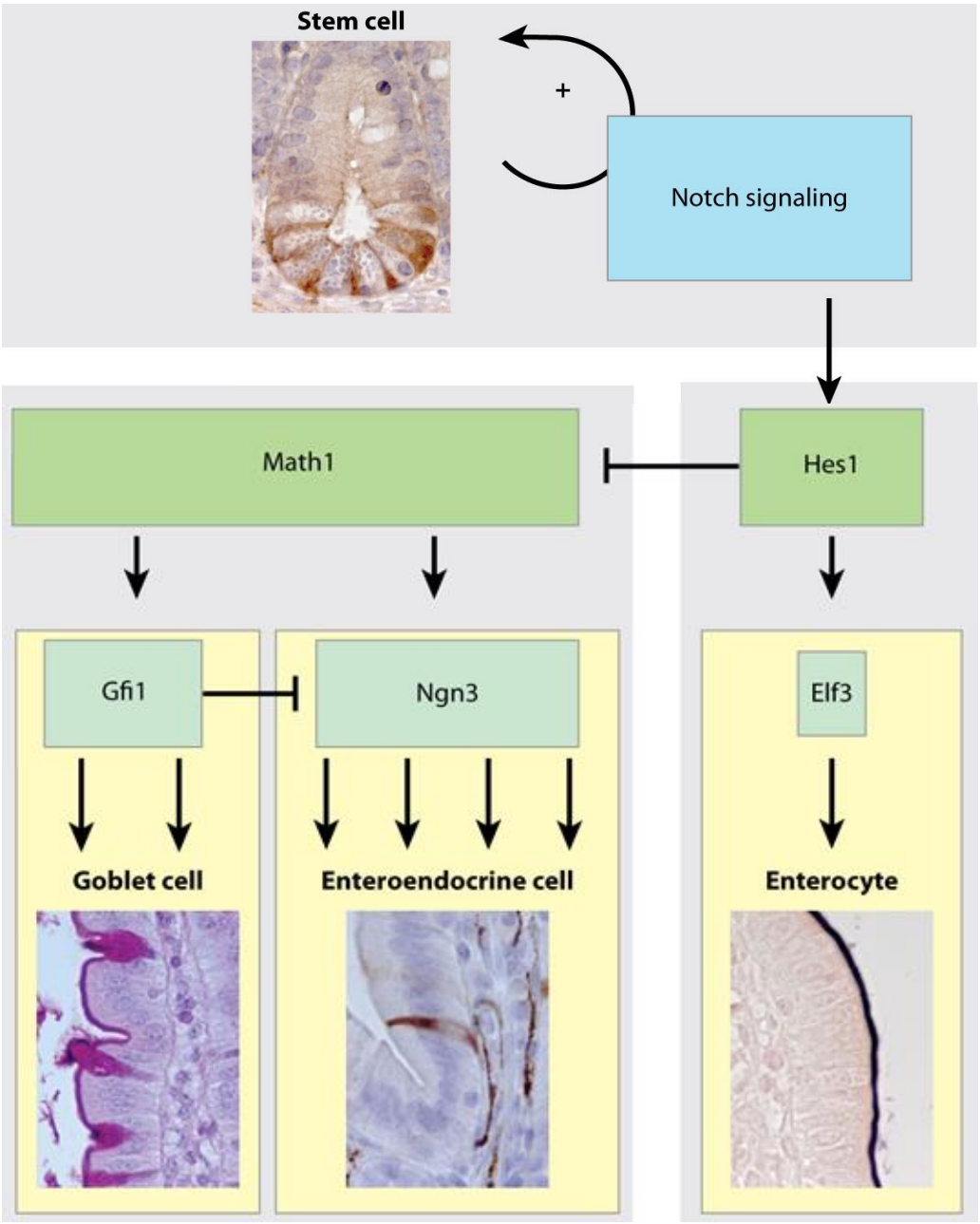

Figure 1.3: ***Schematic representation of epithelial cell lineage commitment in the colon***

Intestinal stem cells differentiate under the control of the Notch pathway, leading to the three major cell types of the colon: goblet, enteroendocrine and enterocyte. When Notch is active, Hairy/Enhancer of Split 1 (Hes1) inhibits the bHLH transcription factor, Math1, resulting in predominantly enterocyte differentiation. In the absence of Notch signaling, Hes1 is not produced and Math1 signaling results in goblet and enteroendocrine cell differentiation through Gfi1 and Ngn3 respectively. Image modified from ref (11).

transmembrane ligand, Delta, active notch results in the transcription of the Hairy/Enhancer of Split (Hes) class of transcriptional repressors that suppress basic helix-loop-helix (bHLH) transcription factors (18, 19). In the intestine, Hes1 suppresses the transcription of the bHLH transcription factor Math1, thereby leading to terminal differentiation toward an enterocyte phenotype. This is evidenced in mice lacking Math1, as they have an intestinal epithelium populated only by enterocytes (20). However, when Notch signaling is inhibited, Hes1 is not produced and the activity of Math1 results in cellular differentiation into the secretory lineage. Downstream mediators such as Gfi1 and Ngn3 control further differentiation into goblet or enteroendocrine lineages. The Wnt pathway also plays a role in cell differentiation since Wnt impairment reduces Math1 expression leading to a predominantly enterocyte containing epithelium. Therefore, crosstalk between both the Wnt and Notch pathways govern cell proliferation and differentiation under homeostatic conditions.

1.1.5 Epithelial cell turnover

As the crypt continuously undergoes epithelial cell differentiation and proliferation, terminally differentiated enterocytes also undergo apoptosis and exfoliation in a process known as epithelial cell turnover. Although the intestinal epithelium constantly undergoes cellular turnover, the mechanisms underlying this process are very poorly understood (11, 21). The maturation of enterocytes, goblet cells and enteroendocrine cells is largely dependent on the speed of their migration up the crypt and therefore relies on the level of cellular proliferation. The primary process of intestinal turnover during homeostasis is anoikis, which is a form of apoptosis resulting from loss of cellular anchorage. Tightly regulated cell sloughing is an important mechanism to preserve intestinal homeostasis since

intestinal barrier disruption can occur when the intestine undergoes accelerated epithelial cell death. It is thought that neighboring cells extend cytoplasmic extensions beneath the shedding cell, pushing the cell outwards into the lumen. Tumor necrosis factor (TNF) alpha has been shown to play a role in cellular turnover since it is able to induce mass exfoliation of epithelial cells. In addition, exposure to TNFα also reorganizes cell junction proteins, a step that may be necessary for cell extrusion under pathological conditions. This mechanism appears to rely on caspase-3 activation since inhibition of this caspase blocks cell shedding (22). However, both caspase-3 and caspase-8 knockout mice show normal gut architecture during development suggesting that they may not play a critical role during tissue homeostasis (23). Another mechanism that has been implicated in cellular turnover is necroptosis, a programmed form of necrotic cell death also driven by TNFα. Necroptosis is driven by RIP1 and RIP3 kinases and leads to rapid plasma membrane permeabilization. Differing from both apoptosis and anoikis, necroptosis is activated in response to pathological forms of cell death. Taken together, epithelial turnover is poorly understood but is likely controlled through signaling induced by a number of cytokines. Epithelial cell death and shedding under normal homeostatic conditions may simply be induced by spatial crowding as a necessity to clear out aging cells while under pathological conditions, these processes may be accelerated as a possible form of host defense through the rapid extrusion of infected cells.

1.1.6 Goblet cells

As described above, goblet cells are the second most abundant cell type in the colonic epithelium and constitute the largest number of secretory epithelial cells. They play a pivotal

role in host defense by secreting the major gel-forming mucins such as MUC2. In accordance with their secretory role, goblet cells are highly polarized and are easily recognized by their swollen theca containing secretory granules (Fig. 1.4). These secretory granules contain a variety of mucins that vary in composition depending on the region of the gut. Mucins are high molecular weight glycoproteins that are composed of a polymeric protein backbone attached to multiple oligosaccharide side-chains. In colonic goblet cells,

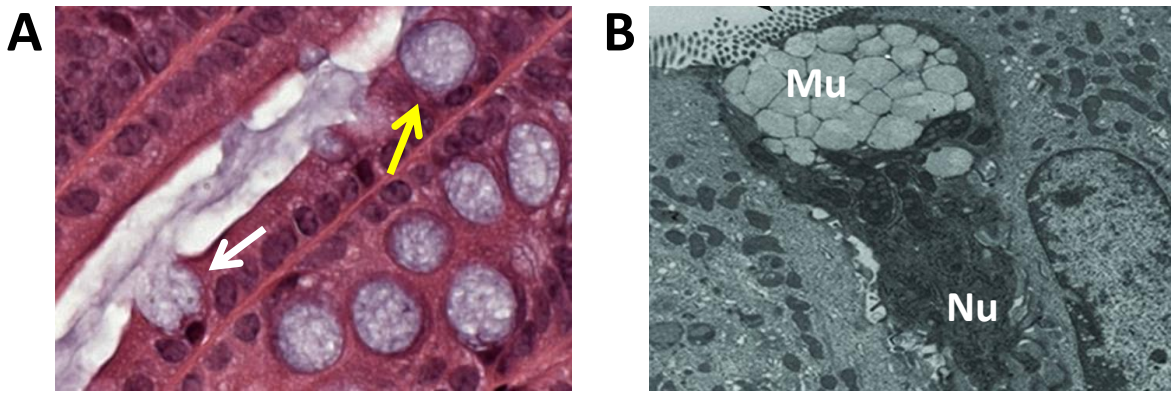

Figure 1.4: ***Histology of the colonic goblet cell***
The colonic goblet cell is easily recognized by its swollen theca containing secretory granules filled with mucins. (A) H&E staining shows mature goblet cells (yellow arrow) lining the crypt with some releasing contents into the crypt lumen (white arrow). Original magnification is 200x. (B) TEM micrograph showing an uninfected mature goblet cell characterized by apical mucin granules (Mu) and a basal nuclear compartment (Nu). Image modified from ref (24).

the primary mucin is MUC2 which contributes heavily to the formation of the mucus layer and is a key molecule in promoting host defense against both acute and chronic infections of the gut (discussed below). Baseline secretion occurs when the mucin containing secretory granules are secreted through exocytosis a single granule at a time. However, secretion can be accelerated through cholinergic or bacterial secretagogue activation. Cholinergic stimulation causes goblet cells to rapidly accelerate the discharge of mucin through stimulation by acetylcholine. This effect can be manipulated by some bacterial pathogens

through peptides such as fMLP which acts as a secretagogue, triggering the tandem fusion of many secretory granules to the apical membrane (25-27). The basal region of the goblet cell contains a compressed nucleus surrounded by numerous Golgi stacks where heavy modification and glycosylation of mucins takes place. Goblet cells also produce several important cytokines and growth factors such as the trefoil (TFF) family of proteins, which interact with and alter the properties of the mucus layer as well as promoting mucosal repair and healing. In particular, colonic goblet cells express TFF3 which is also shown to promote epithelial cell repair by promoting cell migration (28). *Tff3*-/- mice are impaired in reforming their epithelial barrier following exposure to DSS and developing colitis (29). Finally, goblet cell secreted TFF3 has been shown to bind both IgGFcγBP and MUC2 covalently to form heteropolymers that compose some of the net-like structure of mucus. Goblet cells also produce RELM-β, a protein belonging to the Resistin-like Molecule family. When induced, RELM-β can be found in high levels in the intestinal lumen with highest expression found in the colon. Most significantly, RELM-β is linked with host defense, as it plays a poorly defined but important role in clearing some types of helminth infection (30). Our lab has also found evidence that RELM-β may act as a chemo-attractant for CD4+ T cells, recruiting them to the intestine during bacterial infection (manuscript submitted).

1.1.7 The mucus layer

The mucosal epithelium of all organs is covered by mucus layers exhibiting varying degrees of thickness while the exact composition may vary in its makeup of mucin subtypes and other secreted factors. The intestinal mucus layer is specific in its composition and function as it regulates the absorption of nutrients and water while providing a barrier against

microbes and toxic compounds. In the colon, the mucus layer is at its thickest at approximately 700 µm compared to the small intestine at only 150 µm. In the colon, the mucus layer provides a physical barrier between epithelial cells and the high luminal concentration of microbes (Fig. 1.5). Under normal conditions, the mucus layer forms two defined layers that are easily identified since the inner mucus layer is almost completely devoid of microbes whereas the outer mucus layer houses numerous resident commensal bacteria. The inner mucus layer tightly adheres to the intestinal epithelium while the outer mucus layer is loose and easily washed off. Though visually distinct, the mucus layers reflect a mucin concentration gradient where the distinction between adherent and loosely adherent mucus takes place at a particular concentration threshold. The three major components of the mucus layer are (i) secreted mucins such as Muc2, (ii) nonspecific antimicrobials such as defensin proteins and (iii) antigen-specific antimicrobials such as sIgA. As described above, mucins play an important role in host defense to pathogens, with their exact role depending on their glycoforms and their functional activity. Interestingly, even adjacent goblet cells within the same crypt have been shown to produce different mucin

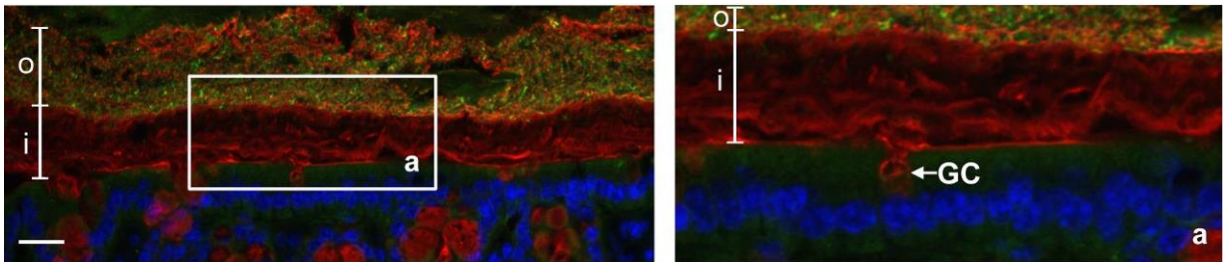

Figure 1.5: ***The outer and inner mucus layer of the colon***

The mucus layer is comprised of the microbe-free inner layer (i) and the microbe-rich outer layer (o). Cross-reaction with anti-GFP antibody labels bacteria (green), murine anti-Muc2 labels the mucus and goblet cells (red) and DAPI labels nuclei (blue) in an uninfected C57BL/6 mouse. Goblet cell is labelled GC. Original magnification is 200x. Image modified from ref (31).

glycoforms. These variations are the result of the complex array of oligosaccharides found on the glycosylated domains of these mucins. Mucin proteins undergo their extensive glycosylation within the goblet cells, through the actions of glycosyltransferases. Glycosylation leads to the formation of monomeric and multimeric glycan linkages which have a multitude of biological functions such as cell signaling, altering differentiation, adhesion and trafficking in the gut, playing a role in susceptibility and resistance to bacterial pathogens such as *Campylobacter jejuni*. Dysfunction of glycosylation is often linked to susceptibility to infectious diseases (32). Glycosylation is modulated both by the host immune system and the surrounding microbiota in a complex cross talk that has been seen in various diseases. For example, *H. pylori* is capable of varying and adapting its surface characteristics through glycosylation to aid in infection while mucin glycosylation of the host is also modulated as the infection progresses (33, 34). The mucus layer needs to be constantly replaced as it is not static due to the movement of luminal contents and cell shedding. Although outside the scope of this thesis, it is important to remark that the mucus layer closely interacts with the surrounding microbiota and recent data suggest that both the host and surrounding microorganisms together modulate mucus layer composition and function.

1.2 Intestinal bacterial pathogenesis

We live in a microbial world, surrounded and inhabited by trillions of bacteria to the point where the number of bacterial cells in and on the human body exceeds our own human cells by a factor of 10. In fact, the intestinal microbiota is often defined as an "accessory organ," functioning in immune system development, digestion, vitamin and nutrient production as

well as colonization resistance. The prevalent dogma is that bacteria occupy one of two niches in our host ecosystem. The first niche reflects a commensal or symbiotic relationship that provides benefit to the host (examples given above) while the microbe receives the benefit of a habitable ecosystem. In lay terms, these microbes are usually described as "good bacteria" because they appear to either cause no harm or alternatively, benefit the host while going about their own business. In contrast, pathogenic bacteria pose a threat to our health by causing disease, and by subverting the host to act as a platform for the pathogen to replicate and spread. The invading bacterial pathogens typically utilize a repertoire of virulence factors and toxins to subvert the host immune system, forcing their way into a suitable niche by edging out competing microbes for a habitable environment. Again in lay terms, these bacteria are labeled as "bad" because they break into the intestinal community, leaving a path of damage and destruction and then typically move on. However, stepping back and looking from a bacterial point of view, the distinction between commensal and pathogen is not so clear. In many disease states including IBD, it is suspected that leakage of bacterial products from both commensal and pathogenic bacteria may induce chronic inflammatory disease. Therefore, pathogens are defined by their strategy to colonize and not simply by their composition. Successful colonization of the intestinal tract by a pathogen represents a daunting task; since it involves survival of the harsh stomach pH, evasion of the host immune system and out-competing the resident microbiota, all of which pose unique and formidable challenges. For the pathogen, its own success comes at the cost of the host.

1.2.1 Attaching and effacing bacterial pathogens

The family of attaching and effacing (A/E) pathogens accounts for the deaths of hundreds of thousands of children each year in developing countries. These bacteria are very diverse and have the ability to infect many different hosts and regions of the gut. A/E pathogens are defined by the formation of A/E lesions, an intimate interface between the pathogen and the host epithelium resulting in reorganization of the host cytoskeleton and local destruction of microvilli. A hallmark of the A/E pathogen strategy is non-invasive attachment to the apical surface of the host epithelium, thereby residing on the apical surface of the epithelium, and within the gut lumen. A/E pathogens accomplish this colonization strategy through the use of a type III secretion system (T3SS), a needle-like apparatus that allows for the translocation of bacterial virulence factors into the host. These factors then act to subvert and utilize host cell processes for the benefit of the pathogen. Coding for these virulence factors occurs within a genetic pathogenicity island (PAI) found within the genomes of these microbes. This PAI contains the locus of enterocyte effacement (LEE) responsible for encoding many of the translocated effector proteins expressed by A/E pathogens, including the translocated intimin receptor, tir. Overall, studies have identified 21 core effectors encoded within both LEE and non-LEE regions that are shared by all A/E pathogens and work in concert to enable bacterial colonization of the host, and ultimately to cause disease. Of the known A/E pathogens, enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *Escherichia coli* (EHEC) are the most prevalent human pathogens. However, since EPEC and EHEC are unable to infect mice effectively, *Citrobacter rodentium*, a murine A/E pathogen that causes pathologically identical lesions to EPEC and EHEC, is our model of choice for studying these host-pathogen responses *in vivo*.

1.2.2 The Citrobacter rodentium mouse model

Citrobacter rodentium has been used as an *in vivo* model for transmissible murine colonic hyperplasia since 1970, providing an excellent platform to study host-pathogen interactions. Following oral administration, *C. rodentium* preferentially infects the mouse cecum,

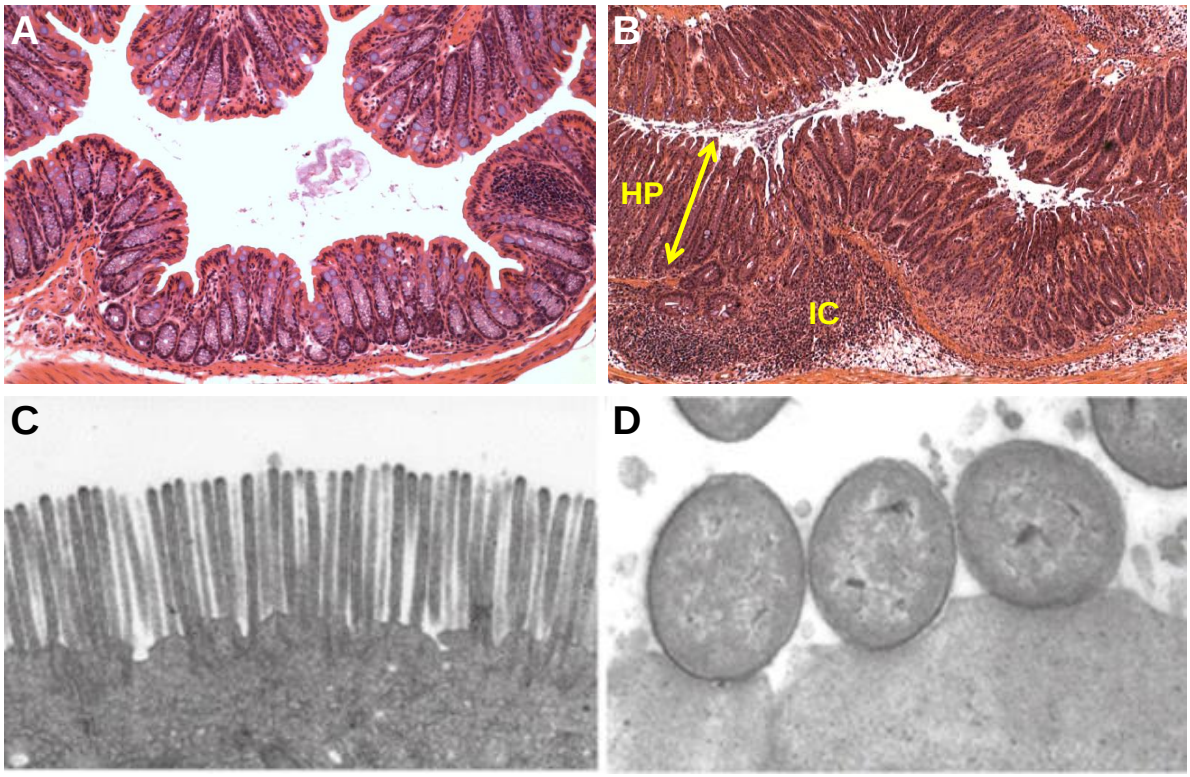

Figure 1.6: ***The murine C. rodentium transmissible infectious colitis model***
Infection with *C. rodentium* (B) compared to uninfected control (A) results in crypt hyperplasia (HP), infiltration of immune cells (IC) and loss of mature goblet cells. H&E stained distal colons of control and infected C57BL/6 mice, original magnification 200x. (C) Intact microvilli are effaced during infection with *C. rodentium* (D) through the formation of A/E lesions as shown in TEM micrographs. Image modified from ref (35).

followed by the distal colon 2 to 3 days later. Between 5 and 14 days post-infection, colonic *C. rodentium* CFU/gram peaks at 10^9. By day 10 post-infection, the adaptive immune system becomes activated, involving Th1 and Th17 CD4+ T cells which are required for bacterial clearance. Clearance of the infection is complete between 21 and 28 days post-infection. The

hallmark pathologies associated with *C. rodentium* infection include dramatic crypt hyperplasia (crypt lengths increase up to 3 times their original length), infiltration of immune cells (including IFN-γ secreting Th1 cells (as well as Th17 cells)) and goblet cell depletion (phenotypically distinct loss of mature goblet cells) (Fig. 1.6). Most mouse strains including C57BL/6 do not succumb to *C. rodentium* infection but C3H/HeJ mice show high mortality by day 10. However, reported mortality rates for this strain vary among labs, possibly due to differences in the commensal microbiota carried by the infected mice. Rag1 -/- mice, which are lacking T and B cells, also fail to clear the infection. Interestingly, Rag1 -/- mice also show reduced pathology, exhibiting less crypt hyperplasia and goblet cell depletion. Although not well understood, the adaptive immune system plays an important role in the clearance of and host response to *C. rodentium* infection.

1.3 The adaptive immune system

All cells of the adaptive immune system originate in the bone marrow, the site where many of them also mature. Through a variety of mechanisms, these cells migrate to peripheral tissues such as the gastrointestinal tract through circulation in blood and the lymphatic system. Within the bone marrow reside the hematopoietic stem cells (HSCs) that give rise to all cellular elements in the blood. Characterization of HSCs has evolved dramatically in recent years and now includes a range of potency and regenerative capacities. Before leaving the bone marrow, the pluripotent HSCs differentiate into common myeloid progenitor cells or common lymphoid progenitor cells, the two main branching paths of bone marrow derived cell fate. Examples of myeloid cells include granulocytes, macrophages, specific subsets of dendritic cells and mast cells while examples of lymphoid cells include natural killer cells as

well as T and B lymphocytes. T and B cells make up the bulk of the adaptive immune system; T cells are primarily responsible for cell-mediated immunity whereas B cells are responsible for antibody production. While the scope of my project includes elements of both innate and adaptive immune responses, the focus is certainly on the role of T lymphocytes and their response to infectious disease in the gut.

1.3.1 Intestinal T cells

In terms of the adaptive immune system, the intestine is often described as being divided into the inductive gut-associated lymphoid tissue (GALT) and immune effector sites. GALT includes Peyer's patches of the small intestine, mesenteric lymph nodes and lymphoid aggregates of the colon, which house largely naïve T and B lymphocytes. Upon stimulation, active T cells migrate to effector sites such as the intestinal lamina propria which houses lamina propria lymphocytes (LPL) and the epithelium which houses intraepithelial lymphocytes (IEL). Nearby dendritic cells and macrophages sample the local environment and present antigens to the T cells allowing for a specific and strong immune response. Intestinal T cells carry unique properties over traditional blood circulating lymphocytes. At their most basic level, intestinal T cells can be split into two major subsets based on their T cell receptor (TCR) and unique co-receptors (Fig. 1.7). The first subset contains the traditional "type a" cells which include TCR$\alpha\beta^{+}$ MHC class II activated CD4+ (CD4+ T cells) and MHC class I activated CD8$\alpha\beta^{+}$ T cells (CD8+ T cells) and have classical T helper, cytotoxic, memory and regulatory roles. The second subset known as "type b" cells express TCR$\alpha\beta$ or TCR$\gamma\delta$ while expressing CD8$\alpha\alpha$ but without the CD4 or CD8$\alpha\beta$ co-receptors. These cells are not MHC restricted and are often found to be self-reactive, but they also play

protective roles in maintaining barrier integrity and immune homeostasis. These cells are found predominantly as IELs while “type a” cells are found primarily as LPLs which will be the focus of the following section and my thesis. Many different T cell subtypes exist at these effector sites, each with their own specificity and function, which will be discussed below.

1.3.2 CD4+ T cells

CD4+ T cells predominantly exist as T helper cells and are responsible for potentiating activity in target cells such as B cells, macrophages and cytotoxic CD8+ T cells through the release of cytokines. Following signal activation by MHC class II antigen presentation, T helper cells proliferate and mature into effector, memory or regulatory cells. Effector cells combat the spread of pathogens, while memory cells guard against subsequent infections and regulatory cells suppress immune responses when necessary. As first described by Mosmann and Coffman, these T helper cells primarily exist as either Th1 or Th2 subtypes, specialized to bolster the host immune system against intracellular bacteria and multicellular parasites respectively. T cell receptor signaling in coordination with IL-12 and IL-4 receptor signaling potentiate gene expression, promoting the cells into either subtype. More recently, Th17 and T regulatory (Treg) subtypes have also been defined. Th17 cells are thought to play a role in host defense against bacterial pathogens such as *C. rodentium* and fungal pathogens such as *C. albicans* by initiating protective pro-inflammatory responses. Tregs are essential for immunological suppression, protecting against autoimmune diseases by suppressing costimulatory molecules on dendritic cells, inducing anti-inflammatory pathways in T cells and releasing immunoregulatory cytokines such as TGF-β and IL-10 (36). In the intestinal lamina propria, all classical CD4+ Th subsets are present. *C. rodentium* infection has been

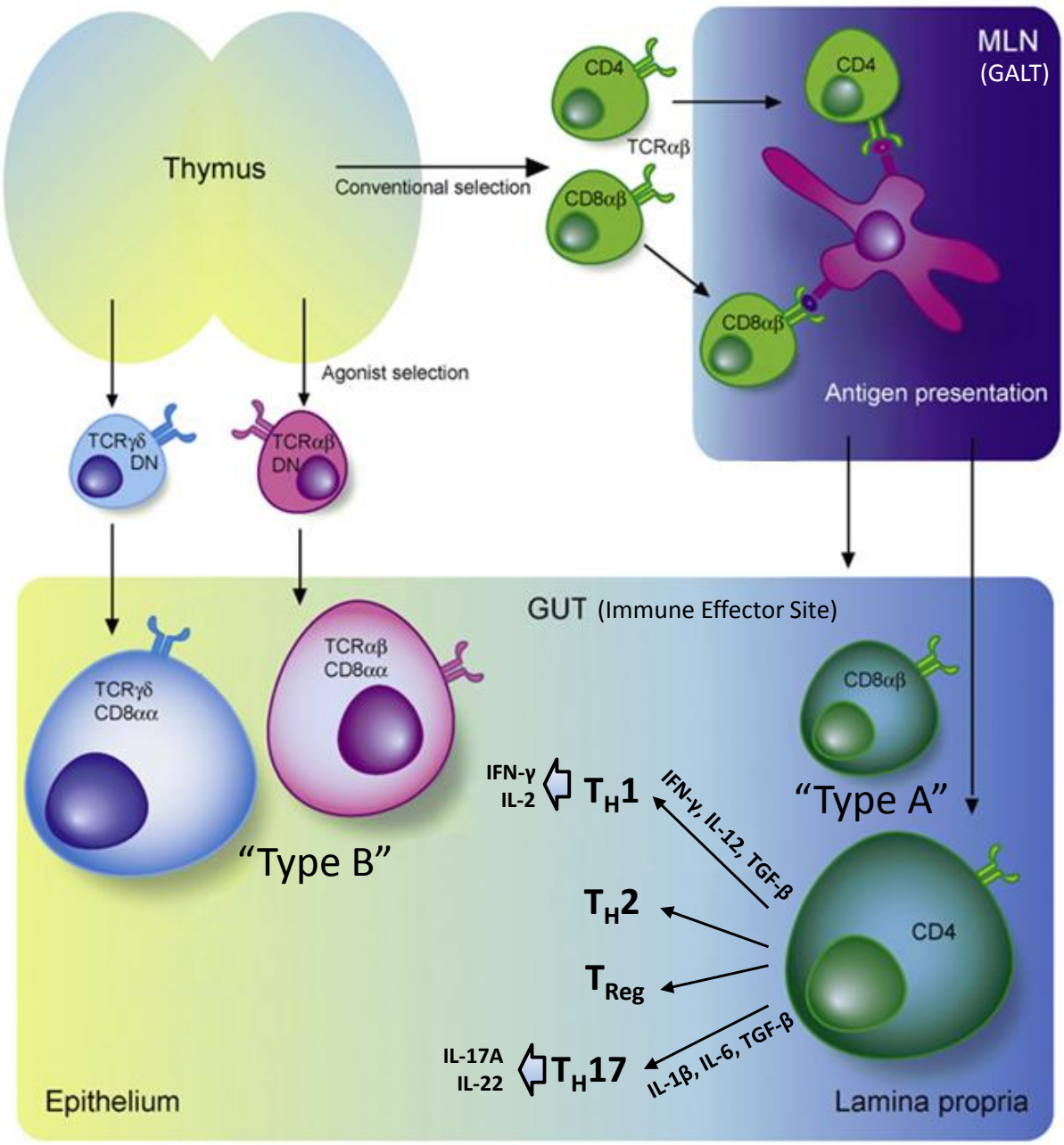

Figure 1.7: ***Intestinal T cell subsets***

Intestinal T cells originate from the thymus and either develop into "type a" cells such as CD4+ and CD8αβ+ T cells, which when found in the intestine, reside mostly in the lamina propria or "type b" cells such as the TCRγδ and TCRαβ CD8αα T cells which reside predominantly in the epithelium. Following activation, CD4+ T cells further specialize as T helper cells (Th1, Th2 and Th17) or T regulatory cells of which Th1 and Th17 play an important role in *C. rodentium* host defense. Image modified from ref (39).

shown to preferentially activate Th1 and Th17 responses and therefore will be the focus in the following sections (37, 38).

1.3.2.1 Th1 immune response

The inflammatory lymphocyte infiltrate found in the colon during *C. rodentium* infection consists predominantly of CD4+ T cells with a Th1 phenotype (40). Differentiation into a committed Th1 subtype requires stimulation of the T cell by IFN-γ, IL-12 and TGF-β. The source of these signals may be other antigen-specific T cells; however, during early stages of infection when these cells are low in number, macrophages and NK cells may be the required sources of the cytokines IL-12 and IFN-γ respectively. Th1 cells mainly produce IL-2 and IFN-γ as their effector cytokines. INF-γ has been intensely studied and is well recognized as a chief pro-inflammatory cytokine. Some of the pro-inflammatory activity of IFN-γ include its ability to induce the expression of inducible nitric oxide synthase (iNOS) by macrophages as well as epithelial cells, causing an increase in production of IgG from activated B cells and increasing Th1 differentiation in T cells while suppressing Th2 cell differentiation (41-43). Of note, IFN-γ has also been proposed to regulate intestinal epithelial homeostasis, since it was recently shown to regulate intestinal epithelial cell proliferation and apoptosis through serine-threonine protein kinase AKT/β-catenin and Wnt/β-catenin signaling pathways (44). However, it is important to note that while IFN-γ is produced primarily by CD4+ Th1 cells, it is also produced by macrophages, dendritic cells, NK cells and CD8+ T cells.

1.3.2.2 Th17 immune response

The Th17 immune response is characterized by the production of the cytokines IL-17A and IL-22, the former a potent recruiter of neutrophils that provide host defense against extracellular bacteria. Like Th1 cells, Th17 cells require antigen presentation and stimulation by cytokines, in this case by IL-1β, IL-6 and TGF-β. The Th17 immune response has been shown to be important in host defense against bacteria although Th17 cells also play a pathogenic role in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis and even have an apparently detrimental effect on host defense to viral and parasitic infections. IL-17A has been shown to have a potent pro-inflammatory function *in vitro* (45). The importance of the Th17 response during *C. rodentium* infection is highlighted by a reliance on Th17 cells to successfully clear the pathogen; IL-17A and IL-17F increase expression of β-defensin proteins in the infected colon, while mice with impaired Th17 responses display reduced survival rates (37, 46). As well, IL-22 plays an essential role in maintaining immune barrier function of the epithelium during infection with *C. rodentium* although the primary source of IL-22 during infection is dendritic cells (47). IL-22 has also been shown to induce LPS-binding protein, which both neutralizes LPS and activates mononuclear phagocytes in host defense (48).

1.3.3 CD8+ T cells

In addition to CD4+ T cells, the "type a" cells also include cytotoxic CD8+ T cells ($CD8\alpha\beta^{+}$, $CD8\alpha\alpha^{-}$). CD8+ T cells residing in the lamina propria are found in much smaller populations compared to CD4+ T cells in the same tissues, between 4 to 10 fold less. Most of these CD8+ T cells exist as memory cells which respond directly to antigen challenge and provide

a cytotoxic response at the local site of infection. These cells exhibit a restricted T cell receptor diversity compared to circulating T cells suggesting specificity due to repeated stimulation in the gut. CD8+ T cells combat infection by producing IFN-γ and TNF-α as well as chemokines that directly recruit and/or activate macrophages and neutrophils (49). In addition, activated CD8+ T cells can induce cytolysis either through perforin or through up-regulation of FasL, both leading to apoptosis which can act to clear damaged and/or infected IECs. However, CD8+ T cells have not been shown to play a major role in controlling *C. rodentium* infection (38). Instead, CD8+ T cells generally respond to intracellular bacterial as well as viral infections. Also present at low numbers in the colon but in high numbers in the small intestine are "type b" TCRγδ and TCRαβ CD8αα^{+} cells. These cells are often considered "unconventional" and little is known about their behavior and function. From what is known, TCRγδ cells regulate IEC homeostasis by promoting IEC turnover and down-regulating the expression of MHC class II molecules (50). *In vitro*, activated TCRγδ cells express keratinocyte growth factor which may function in repairing damaged epithelial tissue (51). On the other hand, TCRαβ CD8αα^{+} cells share similar but unique "unconventional" function as TCR γδ cells. TCRαβ CD8αα^{+} cells are able to prevent CD4+CD45RB^{high} T cell driven colitis in SCID mice which suggest they may have a regulatory role in the mucosa (52). Overall, "unconventional" T cells, while present, fall outside the focus of this thesis but are important to keep in mind as we move forward.

1.4 Research Objectives

Thus far, I have outlined the unique physiology and components of the intestinal epithelium that allow for not only intestinal absorptive function but also for efficient barrier function

against foreign microbes and antigens. As well, I have discussed the intestinal adaptive immune cells, particularly T cells which provide host defense through the release of cytokines and by immunomodulating the function of other cell types. Functioning as the site of direct interface between the host and any invading pathogens, the intestinal mucosa tries to sequester antigens and microbes within the gut lumen, by maintaining the health of the luminal commensal microbiota, as well as by promoting epithelial barrier integrity and innate and adaptive immune responses. Throughout this introduction, I have highlighted the dramatic capability of intestinal epithelial cells to modulate their structure and function through rapid reorganization, cell differentiation, proliferation and changes in cell function. While these mechanisms play a primary role in intestinal homeostasis, their role in host defense is currently understated. Infection of the intestine by intestinal pathogens can cause massive reorganization of IECs. For example, *C. rodentium* infection causes both crypt hyperplasia and the loss of mature goblet cells (goblet cell depletion) in mice. Despite their unique appearance and role in the gut, little is known about whether goblet cells change their function during infectious colitis and IBD. Although the mucus layer has been shown to be an effective physical barrier between IECs and intestinal microbes, it seems paradoxical that the host response during infection would result in goblet cell depletion, and thus reduce mucin production. As well, increasing intestinal IEC turnover has been implicated as an effective mechanism of host defense in helminth parasite expulsion from the GI tract. Cliffe et. al demonstrated that an IL-13 driven increase in epithelial cell turnover can act like an "epithelial escalator" that is capable of expelling *Trichuri*s out of the intestine. It is possible that the increased IEC turnover seen during *C. rodentium* infection may act in a similar way.

The major objective of this thesis is to explore changes in intestinal epithelial cell proliferation and differentiation, focusing in particular on goblet cells. Our studies are some of the first to explore how intestinal goblet cells change their function, what signaling pathways are involved and the potential impact of these changes on intestinal host defense.

Chapter 2: CD4+ T cells drive goblet cell depletion during Citrobacter rodentium infection

2.1 Introduction

The intestinal epithelium is comprised of several distinct epithelial cell types, each defined by its individual structure and function and contributing to the overall absorption and secretory capability of the gut. To maintain intestinal health, the gastrointestinal tract requires its epithelial layer to be selectively permeable to allow efficient digestion and absorption of nutrients (53). It also needs to provide a stringent defense and discriminating barrier to ward off potential opportunistic pathogens or even commensal microbes from damaging the underlying mucosa (54, 55). The intestinal epithelium can be delineated into two specialized cell types, absorptive enterocytes that make up the majority of the epithelium, and the less abundant secretory cells that include enteroendocrine cells, Paneth cells and goblet cells (6). Of these epithelial subtypes, goblet cells are the most numerous in the colon and rectum, and play a key role in maintaining intestinal barrier function through the release of structural mucins such as Muc2 as well as proteins that modulate epithelial repair and function, such as Trefoil factor 3 (TFF3) and RELM-β (28, 56, 57).

Another hallmark of the intestinal epithelium is its rapid turnover, with its entire replacement in humans occurring within 3-5 days under normal conditions. Such rapid turnover highlights how important the proper regulation of epithelial cell differentiation, migration and luminal sloughing is to the maintenance of intestinal health and homeostasis (11, 58). Moreover, the typical cellular distribution and arrangement of cell types within the epithelial layer

undergoes dramatic changes during many forms of gastrointestinal disease. For example human inflammatory bowel diseases (IBD) as well as many forms of infectious colitis are associated with the depletion (or loss) of goblet cells, and or a functional diminishment in their secretory profile (59, 60). While goblet cell depletion can also be modeled in murine models of chemically induced colitis (DSS) as well as the infectious colitis caused by the attaching/effacing (A/E) bacterial pathogen *Citrobacter rodentium,* at this time it is unclear why goblet cells are depleted, how the depletion is mediated and what effect their depletion may have on colitis progression and/or host defense (24, 61, 62).

Goblet cells most notably generate the mucus layer that lines the intestinal epithelium through the secretion of the heavily O-glycosylated mucin protein, Muc2. Several groups have shown that the addition of mucus (Muc2) to epithelial cells in culture inhibits the ability of bacterial pathogens to adhere to the epithelial cells and/or invade them (63, 64). Conversely, mice lacking Muc2 show increased susceptibility to colitis and suffer increased contact between their intestinal epithelium and luminal microbes, thereby promoting intestinal inflammation (31). In addition, bacterial pathogens frequently express proteases that are able to cleave Muc2, suggesting that the mucus layer presents a protective barrier that needs to be overcome for successful pathogenesis (65, 66). In addition to the protective mucus layer, goblet cell derived proteins such as TFF3 and RELM-β promote epithelial repair and/or anti-microbial function after their secretion into the neighboring lumen (67-69). Despite these protective actions, it is unclear whether goblet cell function is always protective since the mucus layer may also provide a nutrient source for pathogenic microbes (70).

We recently showed that *C. rodentium* infection of mice results in the depletion of colonic goblet cells and their mucins, and interestingly the depletion was not seen in infected mice lacking T and B cells (24). This immune cell requirement led our group to investigate the role of CD4+ versus CD8+ T cells in modulating goblet cell depletion, and how this may relate to epithelial turnover and ultimately to protection against *C. rodentium* induced colitis. In the current study we demonstrate that CD4+ T cells drive *C. rodentium* induced intestinal epithelial cell hyper-proliferation and goblet cell depletion, likely through a Th1 driven response that depends on IFN-γ. The epithelial cell hyper-proliferation and goblet cell depletion are clearly associated with survival of the host and ultimately clearance of the pathogen, whereas use of the Notch inhibitor DBZ increased goblet cell numbers, impairing host defense and leading to poor survival of infected hosts.

2.2 Experimental Procedures

2.2.1 Mice

6 to 8 week old C57BL/6 mice were obtained from the UBC Center for Disease Modeling (BC, Canada) and *Rag1 -/-* (on a C57BL/6 background) and *Ifn-γ receptor -/-* mice with appropriate controls were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were kept in sterilized, filter-topped cages and fed autoclaved food and water while being routinely monitored and tested for common pathogens. The protocols employed were approved by the University of British Columbia's Animal Care Committee and were in direct accordance with guidelines drafted the Canadian Council on the Use of Laboratory Animals.

2.2.2 Bacterial strains and infection of mice

Mice were orally gavaged with 100 uL of wild-type *C. rodentium* (formerly known as *Citrobacter freundii* biotype 4280 strain DBS100) culture grown in Luria broth overnight at 37 degrees Celsius and at a concentration of 2.5 x 10^8 colony forming units (CFU).

2.2.3 Tissue collection

Mice were anesthetized with isofluorane and euthanized at 12-15 days post-infection or after losing approximately 15% of initial their bodyweight and showing signs of significant morbidity (piloerection, hunching and/or shaking). Colons, ceca, spleens, mesenteric lymph nodes and livers were all excised and stored either in 10% neutral buffered formalin (Fisher) or in 4% paraformaldehyde. Formalin fixed tissues were paraffin embedded and sectioned by the histology laboratory at the Child and Family Research Institute (CFRI). The paraformaldehyde-fixed tissues were washed in phosphate-buffered saline (PBS), then embedded in Shandon Cryomatrix embedding medium (Thermoelectron Corporation) and subsequently frozen by partial immersion in liquid N2-precooled 2-methylbutane. Additional tissue samples were stored in RNA later (Qiagen) at -80 degrees Celsius. To enumerate bacterial load, colon and cecum tissue were collected separately, homogenized in PBS, serially diluted, plated onto LB agar dishes and colonies enumerated.

2.2.4 RNA extraction and quantitative RT-PCR

Colon tissues stored in RNAlater (Qiagen) at −86°C were thawed on ice and weighed, and total RNA was extracted using a Qiagen RNeasy kit following the manufacturer's instructions. Total RNA was quantified using a Bio-Rad SmartSpec (Bio-Rad), and 1 to 2 μg

of RNA was reverse transcribed using a Qiagen Omniscript reverse transcription (RT) kit (Qiagen) according to manufacturer's instructions. Agarose gels were stained with SYBR Safe DNA gel stain (Molecular Probes) and visualized with a Chemi Doc XRS system (Bio-Rad). For quantitative PCR, Bio-Rad Supermix was used at a 1:2 dilution, and real-time PCR was carried out using a Bio-Rad MJ Mini-Opticon according to manufacturer's instructions. Quantitation was carried out using GeneEx Macro OM 3.0 software.

2.2.5 Histological staining

Briefly, 5-μm paraffin sections were deparaffinized by heating them at 55 to 65°C for 10 min, cleared with xylene, and rehydrated through an ethanol gradient to water. For periodic acid-Schiff (PAS) staining, standard histological techniques were used. Rat antisera against *C. rodentium* Tir (1:500; a gift from W. Deng), anti-muc2 (H-300, 1:100), rabbit anti-CD4 (GK 1.5, 1:200), CD3 (ab5690, 1:100), CD8 (53.67, 1:200) and anti-ki67 (CP249B, 1:100) were used as primary antibodies, and were diluted in PBS containing 1% bovine serum albumin. Following, 0.2% Triton X-100 (Sigma) permeabilization, immunofluorescent labeling for all stains was carried out with the appropriate secondary antibody using AlexaFluor 488-conjugated goat anti-rat IgG, AlexaFluor 568-conjugated goat anti-rabbit IgG, or AlexaFluor 568-conjugated goat anti-rat IgG (Invitrogen). Tissues were mounted using ProLong Gold Antifade + DAPI (Invitrogen) for DNA staining. Sections were captured with a Zeiss AxioImager microscope equipped with an AxioCam HRm camera operating through AxioVision software (version 4.4).

2.2.6 Histopathological scoring

To assess tissue pathology, paraffin-embedded colonic tissue sections (5 μm) were stained with haematoxylin and eosin, and then examined by two blinded observers. For *C. rodentium* infection, tissue sections were assessed for submucosal edema (0 = no change; 1 = mild; 2 = moderate; 3 = profound), epithelial hyperplasia (scored based on percentage above the height of the control where 0 = no change; 1 = 1–50%; 2 = 51–100%; 3 = > 100%), epithelial integrity (0 = no change; 1 = < 10 epithelial cells shedding per lesion; 2 = 11–20 epithelial cells shedding per lesion; 3 = epithelial ulceration; 4 = epithelial ulceration with severe crypt destruction) and neutrophil and mononuclear cell infiltration (0 = none; 1 = mild; 2 = moderate; 3 = severe), as previously described. The maximum score that could be obtained with this system is 13 points

2.2.7 Reconstitution of Rag 1 -/- Mice with CD4+ and CD8+ T cells

The adaptive immune system was partially reconstituted in *Rag1 -/-* mice using splenic and mesenteric lymph node (MLN) populations of CD4+ or CD8+ T cells. In brief, wild-type immunocompetent mice were euthanized, and their spleens and MLNs were aseptically removed. Spleens and MLNs were placed in RPMI medium with 10% fetal bovine serum, mashed to a pulp with the rubber end of the plunger from a 1.0-ml syringe, and then forced through a 70-μm-pore-size filter (BD Biosciences), generating a single-cell suspension. Following two washes with RPMI medium, cells were pelleted and then resuspended in PBS. Cells were then bound to biotinylated primary antibodies specific for either CD4 or CD8 which in turn were bound to streptavidin coated magnetic beads and separated using the Miltenyi MiniMACs apparatus. Cells were then counted, and viability was analyzed by

trypan blue exclusion. Recipient *Rag1* -/- mice were then inoculated via the tail vein with 2 × 10^8 viable T cells. Mice were left for 8 weeks and then tested for the success of reconstitution by staining colonic tissue sections for the presence of T lymphocytes using the marker CD3.

2.2.8 IL-17A neutralization

C57BL/6 mice were treated with three injections at days 5, 7 and 10 post-infection with monoclonal anti-IL-17A antibody (R&D). Each intra-peritoneal (IP) injection consisted of 100 µg per mouse diluted in 100 µl PBS.

2.2.9 Notch inhibition

Notch processing in infected mice was inhibited using the γ-secretase inhibitor dibenzazepine (DBZ) obtained from Axon MedChem (Cat. No. Axon 1488). Each mouse was given three IP injections of 10µM/kg at day 9, 10 and 11 post-infection. DBZ was delivered in a vehicle consisting of 10% DMSO in PBS.

2.2.10 Statistical analysis

Statistical significance was calculated by using either a two-tailed Student t test or the Mann-Whitney t test as indicated, with assistance from GraphPad Prism software (version 4.00; GraphPad Software, San Diego, CA) (www.graphpad.com). A P value less than 0.05 was considered significant. The results were expressed as means and standard errors of the means unless indicated otherwise.

2.3 Results

2.3.1 CD4+ and CD8+ T cell reconstitution reduces infection-induced mortality

As outlined previously, C. rodentium infection is known to promote goblet cell depletion in the colon of mice, whereas this pathology is not seen in mice lacking T and B cells. We confirmed this observation, finding that immunocompetent C57BL/6 mice develop an approximately 60% decrease in intestinal goblet cell numbers in the distal colon by day 10 post-infection (24). In contrast this goblet cell depletion is not seen in infected mice lacking T and B cells (Rag1 -/- mice). Moreover Rag 1 -/- mice are known to carry significantly heavier C. rodentium burdens than C57BL/6 mice, as well as rapidly succumb to infection (71). To address whether these readouts were attributable to the absence of either CD4+ or CD8+ T cells, we reconstituted Rag1 -/- mice with CD4+ and CD8+ T cells isolated from C57BL/6 mice and after 8 weeks orally infected these mice with C. rodentium. Throughout the infection, all mice progressively lost weight but non-reconstituted Rag1 -/- mice began to succumb to the infection by day 8 and by day 15 only 53% of the mice survived (Fig. 2.1A). In contrast, CD4+ and CD8+ T cell reconstitution greatly improved the outcome of infected mice, such that 86% and 93% respectively survived ($p = 0.004$ and $p = 0.003$), while 100% of C57BL/6 mice survived. Interestingly, this change in survival was not accompanied by a change in pathogen burdens. Regardless of survival, there was no difference in *C. rodentium* CFU/gram of tissue among non-reconstituted, versus CD4+ and CD8+ T cell reconstituted *Rag1 -/-* mice at day 12 post-infection (Fig. 2.1B). However, control C57BL/6 mice carried comparatively less cecal and colonic *C. rodentium* burdens. Therefore, while the presence of CD4+ and CD8+ T cell reduced mortality among infected *Rag1 -/-* mice, it was not due to changes in pathogen burdens.

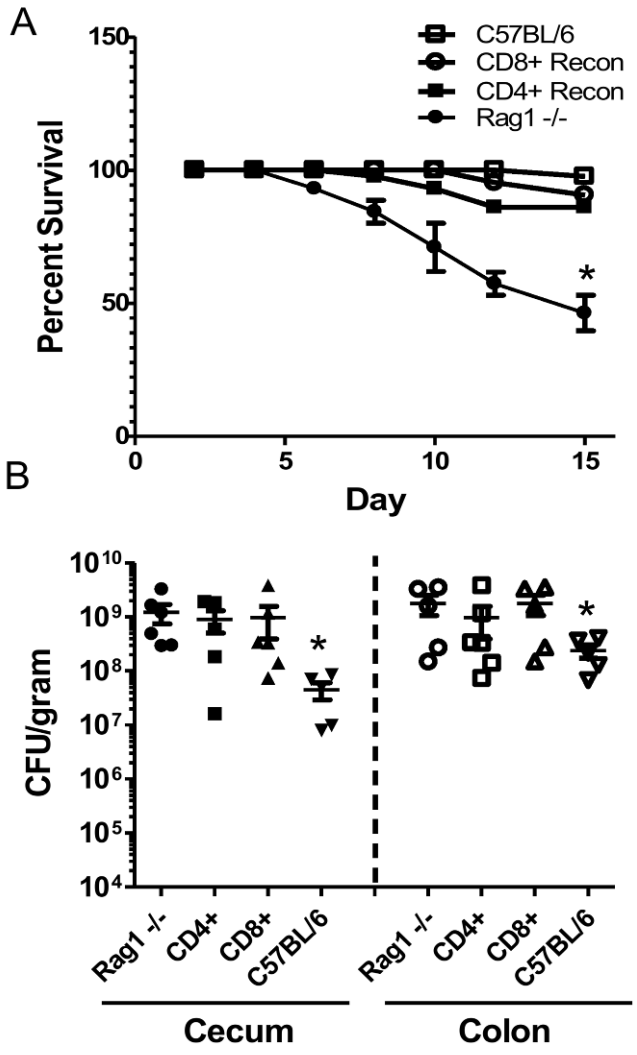

Figure 2.1: ***CD4+ and CD8+ T cells reconstitution reduces mortality in Rag 1 -/- mice***

(A) Percent survival of *Rag 1 -/-* mice during infection is greatly reduced compared to reconstituted CD4+, CD8+ and C57BL/6 control mice. Each symbol represents the mean of three independent infections. Error bars indicate ± 1 SEM. Asterisk shows significance, with $P < 0.05$. (B) No differences were identified among *C. rodentium* CFU/gram of tissue in the cecae and distal colons from *Rag 1 -/-*, CD4+ and CD8+ T cell reconstituted mice at day 12 p.i. Cecae and distal colons from control C57BL/6 mice yielded significantly lower bacterial burdens compared to *Rag 1 -/-* counterparts. Error bars indicate SEM from at least six mice. Asterisk shows significance, with $P < 0.05$

2.3.2 CD4+ and CD8+ T cell reconstitution increases intestinal pathology

We next addressed what role the specific T cell subsets might play in controlling colonic tissue pathology during infection. Our initial assessment included mononuclear cell infiltration, epithelial integrity, crypt hyperplasia and submucosal edema. Interestingly, both CD4+ and CD8+ T cell reconstitution of *Rag 1-/-* mice dramatically worsened histological damage in the distal colon compared to non-reconstituted *Rag 1 -/-* mice (Fig. 2.2A/B). A detailed examination of the resulting pathologies revealed very different outcomes following CD4+ versus CD8+ T cell reconstitution. CD4+ T cell reconstitution led to exaggerated crypt hyperplasia compared to CD8+ T cell reconstituted *Rag1 -/-* mice as reflected in their histological scores (2.89 ± 0.11 vs. 2.2 ± 0.15 respectively, $p = 0.002$). However, this was offset by severe epithelial damage and mucosal ulceration found in the colons of CD8+ T cell reconstituted mice but not in those receiving CD4+ T cells (2.89 ± 0.56 vs. 0.67 ± 0.17 respectively, $p = 0.002$). In further analysis, we immunofluorescently stained serial colon sections for CD3 expression to determine the localization of T cells in each group of reconstituted mice. We found that CD4+ T cells tended to accumulate in clusters within the lamina propria and at the base of hyperplastic crypts whereas CD8+ T cells were found surrounding regions of mucosal ulceration (Fig. 2.2C/D).

2.3.3 Only CD4+ T cell reconstitution restores goblet cell depletion, crypt hyperplasia and proliferation in Rag1 -/- mice

Our next step was to determine whether CD4+ and CD8+ T cell reconstitution affected goblet cells by examining colon tissue sections processed for H&E staining. Goblet cell numbers, their morphology and their distribution in the colons as well as proliferation found

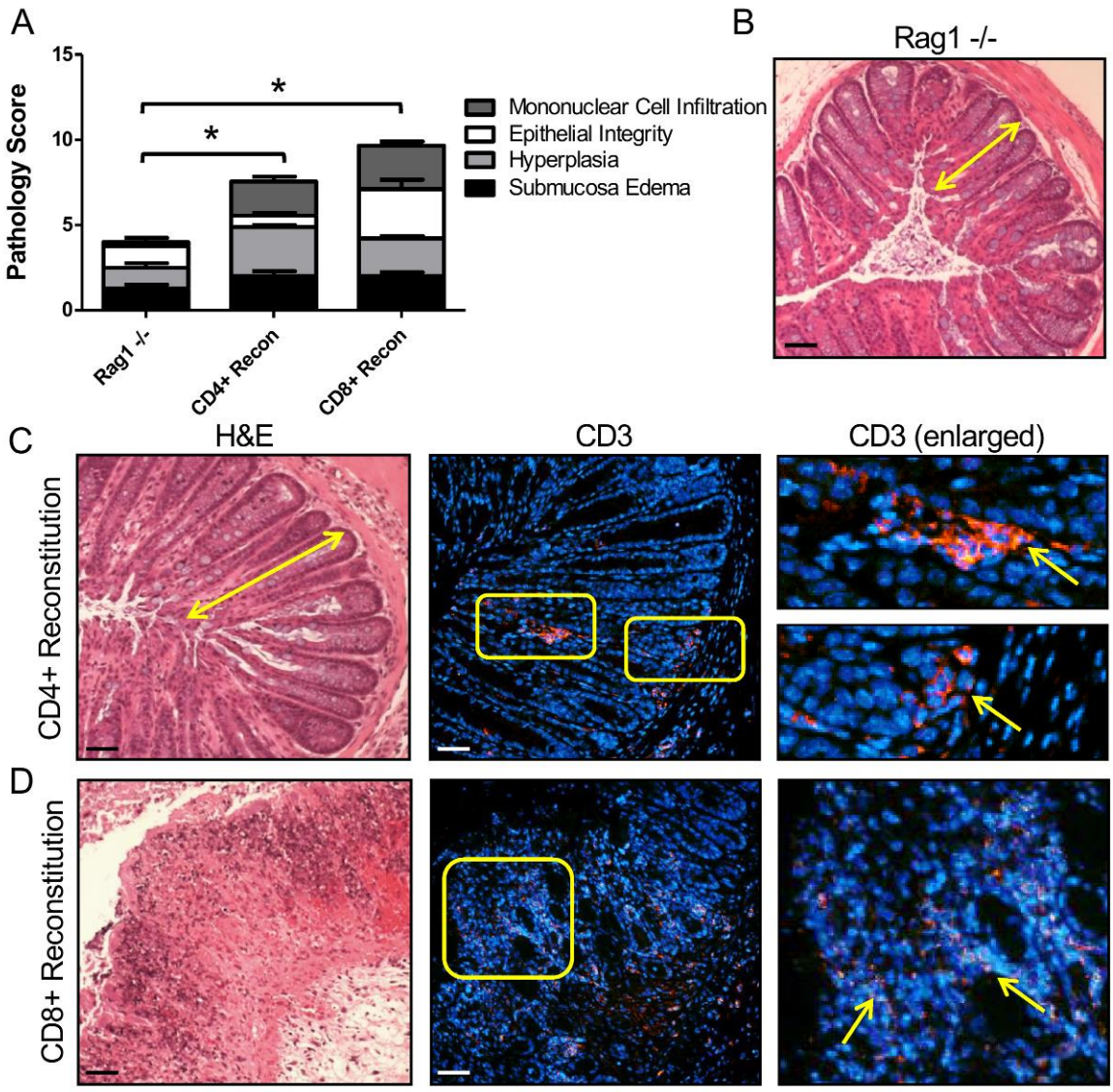

Figure 2.2: ***Increased pathology in Rag1 -/- mice reconstituted with CD4+ or CD8+ T cells***

(A) Comparative pathological scores of control Rag1 -/-, CD4+ and CD8+ reconstituted mice. CD4+ reconstitution greatly increased hyperplasia while CD8+ reconstitution resulted in worsened epithelial integrity. Bars represent the average pathology scores of at least 3 experiments, each with 5-8 mice. Error bars indicate SEM. Asterisk shows significance, with $P < 0.05$. (B) Representative H&E staining of distal colonic tissues removed on day 12 p.i. from non-reconstituted Rag1 -/-, (C) CD4+ T cell reconstituted, and (D) CD8+ T cell reconstituted mice exhibiting different associated pathologies. Double-headed arrows highlight increased hyperplasia in CD4+ while CD8+ T cell reconstitution exhibits severe focal ulceration. (C and D) Anti-CD3 staining (red) in serial sections showing aggregates of CD3-positive cells (yellow arrows) in the lamina propria and regions of mucosal ulceration. Original magnification was x200. Scale bars = 50 µm.

in *Rag1 -/-* mice following *C. rodentium* infection were markedly different than that seen in infected wildtype C57BL/6 mice. Furthermore, reconstitution of CD4+ and CD8+ T cells yielded dramatically different results (Fig. 2.3A). We enumerated goblet cells per 100 enterocytes, and as noted previously, *Rag1 -/-* mice maintain baseline-like distribution and numbers of goblet cells in the distal colon by day 12 post-infection (21.8 ± 2.6 goblet cells) whereas C57BL/6 mice showed significant depletion of mucin-filled goblet cells (3.5 ± 1.3 goblet cells, $p < 0.001$) (Fig 3B). Strikingly, reconstitution of *Rag1 -/-* mice with CD4+ T cells led to partial but significant restoration of goblet cell depletion by day 12 post-infection (11.0 ± 2.4 goblet cells, $p = 0.005$). However, the restored goblet cell depletion was patchier than that seen in wildtype mice; since although most crypt regions showed significant goblet cell depletion, in other crypt regions, numerous mature goblet cells remained. On the other hand, CD8+ T cell reconstitution, regardless of mucosal damage, resulted in no overt change in goblet cell numbers (20.8 ± 2.6 goblet cells). While focal ulcerated regions displayed high levels of goblet cell depletion due to eradication of crypt architecture, over all, CD8+ T cell reconstitution had no effect on goblet cell numbers. Next, we measured colonic crypt lengths in these mice and found CD4+ T cell reconstitution increased crypt lengths by 54.4% ± 6.2%, which was not seen following CD8+ T cell reconstitution (Fig. 2.3C). Restoration of crypt lengths was also accompanied by significantly increased numbers of ki67 positive cells per crypt in mice reconstituted with CD4+ T cells (Fig. 2.3D). The expression of ki67 protein is strictly associated with cell proliferation and used by many groups as a putative marker for proliferation (72). Interestingly, this appears to be a partial restoration of the massive proliferative response seen in C57BL/6 mice, again showing a patchy appearance. These

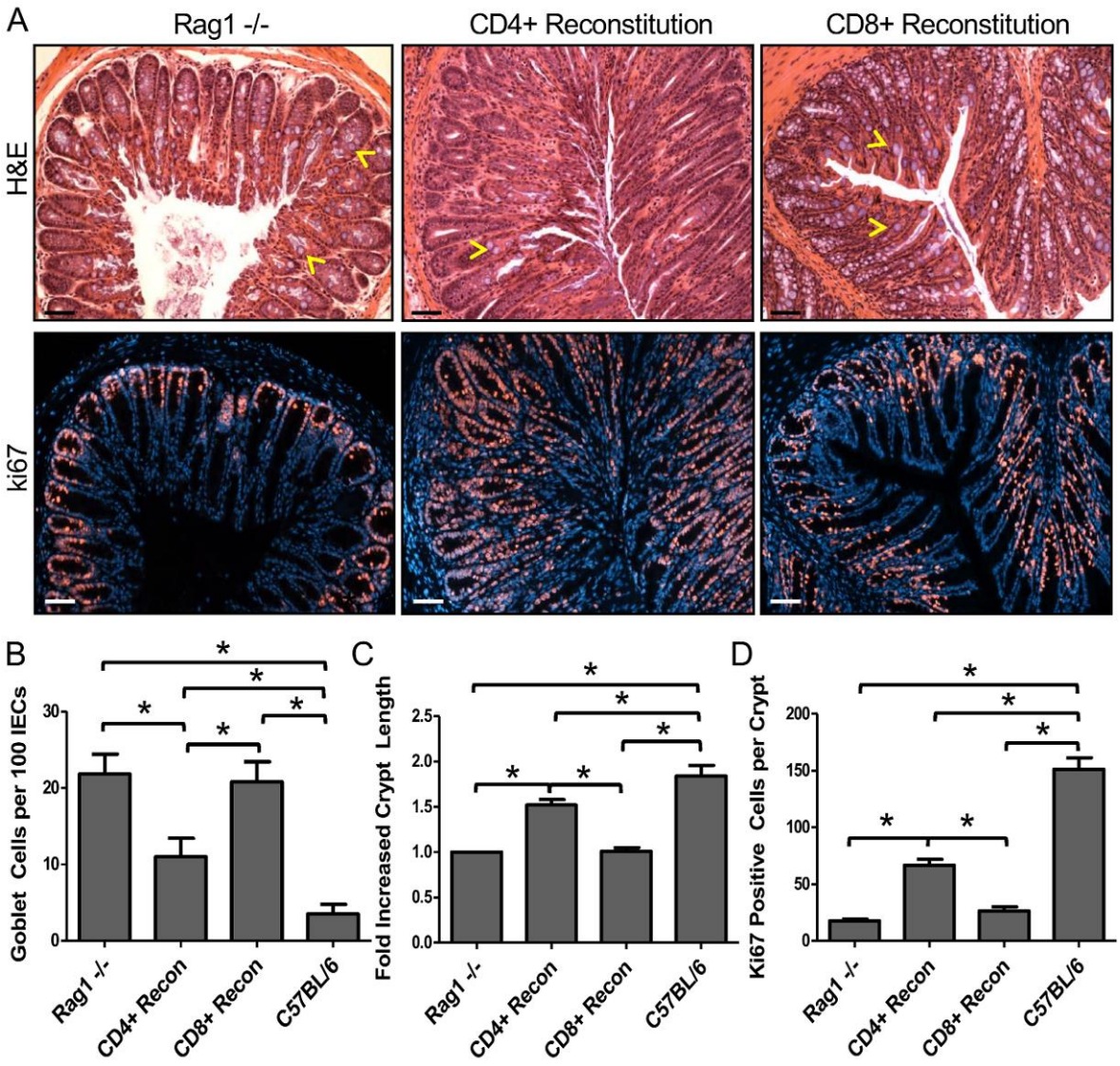

Figure 2.3: ***Restored hyperplasia, proliferation and goblet cell depletion in CD4+ reconstituted Rag1 -/- mice*** **(A) Representative H&E and anti-ki67 (red) staining of day 12 p.i. distal colons from non-reconstituted Rag1 -/-, CD4+ and CD8+ T cell reconstituted mice. Arrowheads indicate representative mature goblet cells. Original magnification was x200. Scale bars = 50 µm. (B) Quantification of goblet cells per 100 intestinal epithelial cells (IECs) in distal colons of mice at day 12 p.i. Bars represent the means of 3 experiments, each with 5-8 mice, accounting for 20 sections. (C) Fold increase in crypt lengths in distal colons of mice at day 12 p.i. Bars represent mean fold increase of crypt length relative to Rag1 -/- mice. (D) Enumeration of ki67 positive cells per crypt in distal colons of mice at day 12 p.i. Bars represent the mean number of ki67 positive cells per crypt. Error bars indicate SEM. Asterisk shows significance, with $P < 0.05$.**

results demonstrate that CD4+ T cells, but not CD8+ T cells regulate goblet cell depletion, crypt hyperplasia and IEC proliferation during *C. rodentium* infection.

2.3.4 CD4+ T cell reconstituted Rag1 -/- mice show decreased Muc2, Tff3 and Relm-β but increased IFN-γ and IL-17A

Further investigation of goblet cell depletion led us to assess alterations in the mRNA transcript levels of goblet cell mediators. We measured differences in Muc2, Tff3 and Relm-β mRNA levels by quantitative PCR. Non-reconstituted mice displayed high levels of Muc2, Tff3 and Relmβ while reconstitution with CD4+ significantly decreased Muc2 levels by 10 fold (Fig. 2.4). As well, a similar trend of decreased mRNA levels was found for Tff3 and Relmβ in CD4+ reconstituted mice. In addition, we measured mRNA transcript levels in IFN- *γ* and IL-17A**,** and found an increase in both of these cytokines in mice reconstituted with CD4+ T cells.

2.3.5 IFN-γ receptor regulates goblet cell depletion during C. rodentium infection

C. rodentium is widely known to induce a Th1 and Th17 immune response resulting in increased populations of IFN-γ and IL-17A producing CD4+ T cells found within the infected colonic mucosa (37, 46). To determine whether IFN-γ signaling promotes the goblet cell depletion seen during infection, we analyzed goblet cell numbers in *Ifn-γ receptor -/-* mice under both uninfected and infected conditions. Interestingly, under uninfected conditions, *Ifn-γ receptor -/-* mice had higher numbers of mature goblet cells compared to uninfected C57BL/6 mice (16.0 ± 0.7 vs. 11.2 ± 0.4 goblet cells/100 enterocytes respectively, $p < 0.001$) (Fig. 2.5A). Furthermore, after 12 days of infection, *C. rodentium* induced neither

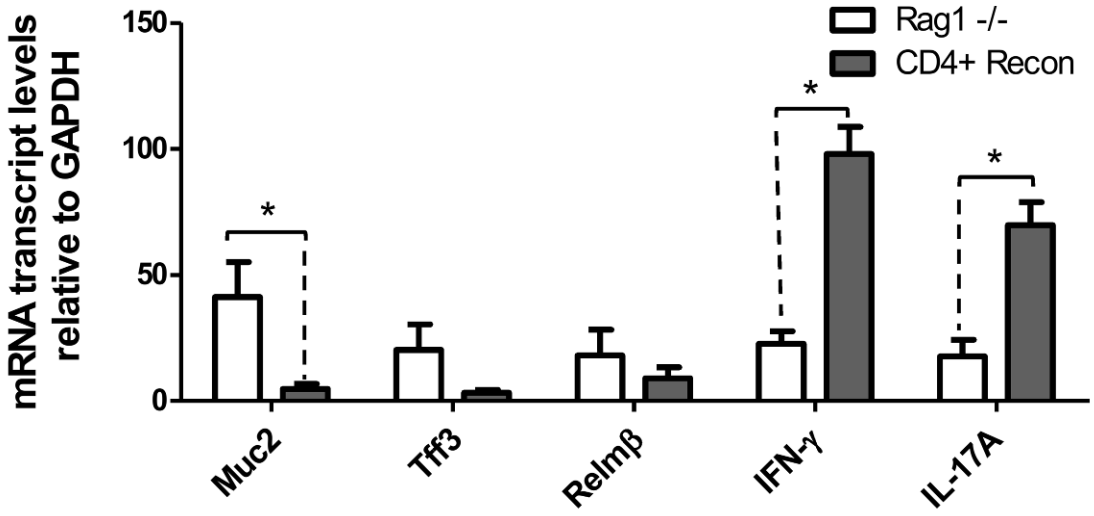

Figure 2.4: ***CD4+ T cell reconstitution affects mRNA transcript levels during C. rodentium infection***
Bars represent mean mRNA transcript levels of Muc2, Tff3, Relm-β, IFN- γ and IL-17A relative to control GAPDH by quantitative RT-PCR from 3 independent experiments. Significant differences in gene expression with CD4+ reconstitution were found in Muc2, IFN- γ and IL-17A. Error bars indicate SEM. Asterisk shows significance, with P < 0.05.

crypt hyperplasia nor goblet cell depletion in *Ifn-γ receptor -/-* mice compared to wildtype (17.4 ± 1.9 vs. 2.0 ± 0.3 goblet cells/100 enterocytes, p <0.001). Interestingly, while loss of IFN-γ dependent signaling abrogated infection induced crypt hyperplasia and goblet cell depletion (Fig. 2.5B), the induction of other pathologies (mononuclear cell infiltration, loss of epithelial integrity and submucosal edema) associated with *C. rodentium* infection was not impaired in *Ifn-γ receptor -/-* mice (data not shown).

2.3.6 Mice treated with IL-17A neutralizing antibody still exhibit goblet cell depletion

In addition to our investigation of IFN-γ, we also examined whether IL-17A modulates intestinal goblet cell numbers and architecture during *C. rodentium* infection. IL-17A is a

critical factor in the development of Th17 responses, as well as providing mucosal protection against *C. rodentium* (73). By neutralizing IL-17A in infected C57BL/6 mice using a neutralizing antibody against IL-17A, we found a significant five-fold increase in *C. rodentium* burdens at 12 days post-infection ($1.16 \pm 0.51 \times 10^8$ vs. $5.00 \pm 1.32 \times 10^8$ CFU/gram, $p = 0.03$) (Fig. 2.5C). However, the crypt hyperplasia and other pathology readouts in mice given the neutralizing antibody were markedly similar to those of our vehicle injected control C57BL/6 mice. Furthermore, there was significant goblet cell depletion seen in these mice, such that the colonic tissues from the IL-17A depleted mice were visually indistinguishable from those of mice not receiving IL-17A antagonist (4.0 ± 0.9 vs. 2.9 ± 0.6 goblet cells/100 enterocytes) (Fig. 2.5D). Taken together, these data suggest that IL-17A does not play a major role in goblet cell depletion and crypt hyperplasia. Instead these mechanisms appear to be governed by IFN-γ dependent signaling.

2.3.7 Goblet cell depletion is not associated with changes in notch pathways

Our results show that infection-induced goblet cell depletion is driven by CD4+ T cells and IFN-γ dependent signaling. However it remains unclear how the host immune system alters the intestinal epithelium to induce the loss of mature goblet cells. A potential candidate is the notch pathway, as it governs the differentiation of intestinal stem cells, by promoting progenitor epithelial cells towards a goblet cell lineage (74). Since notch signaling regulates HES1 expression, which suppresses the downstream factor Math1 (20), we examined gene expression of these notch signaling components using qPCR. Notably, we found no significant differences in the expression of HES1 and Math1 mRNA levels between uninfected and infected C57BL/6 mice (Fig. 2.6). In addition, we performed

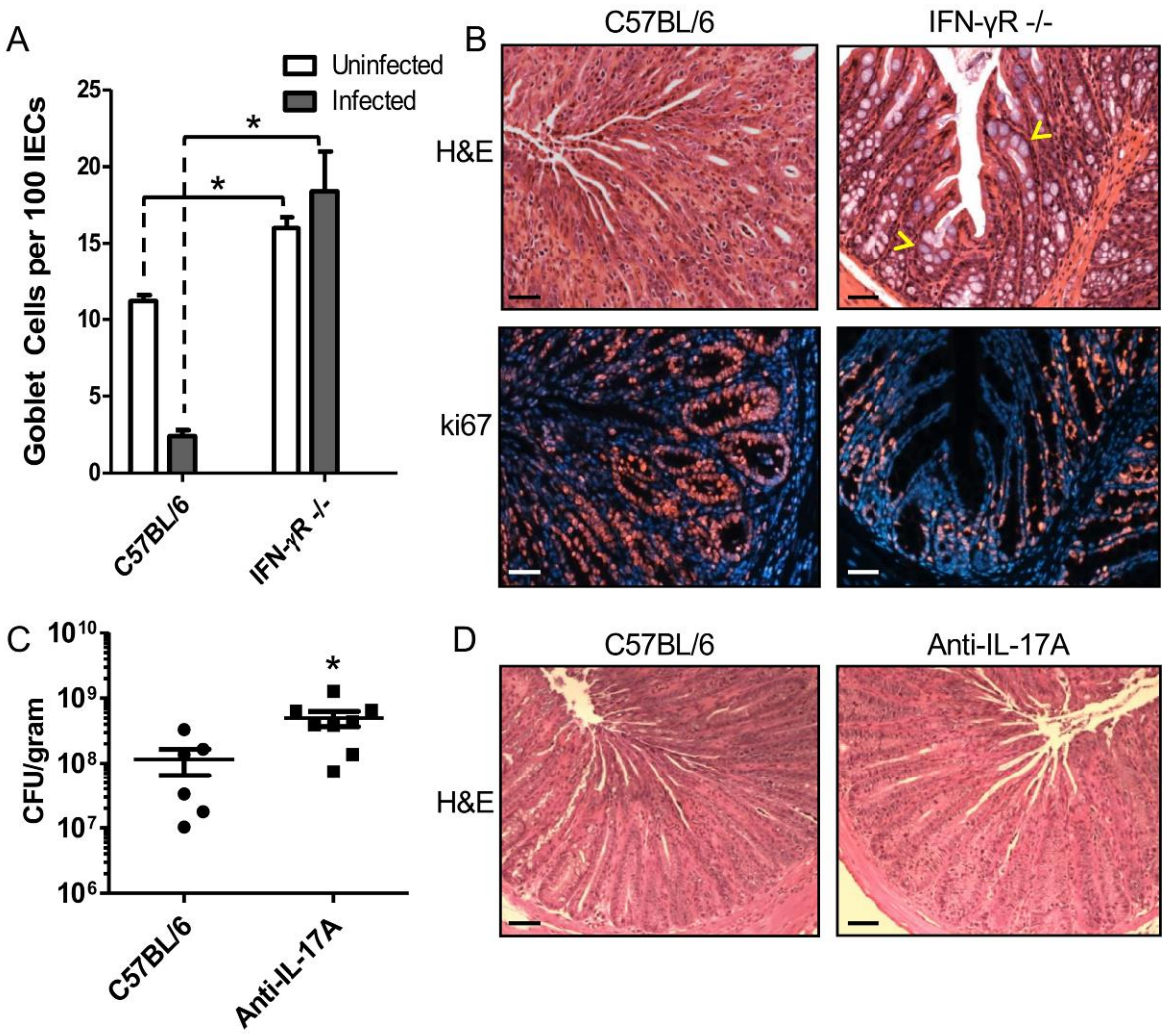

Figure 2.5: ***IFN- γ signaling impacts goblet cell depletion and IEC proliferation during infection***

(A) Quantification of goblet cells per 100 IECs in C57BL/6 and Ifn-γ receptor -/- mice with or without C. rodentium infection. Error bars indicate SEM. Asterisk shows significance, with $P < 0.05$. (B) H&E and anti-ki67 (red) stained distal colons showing high numbers of goblet cells (H&E) and reduced proliferation (ki67). Arrowheads indicate representative mature goblet cells. Original magnification was x200. Scale bars = 50 µm. (C) C57BL/6 mice treated with IL-17A neutralizing antibody (anti-IL-17A) carry heavier C. rodentium burdens at day 12 p.i. compared to control mice. Error bars indicate SEM from at least six mice. (D) H&E staining of control and IL-17A neutralized distal colons of mice at day 12 p.i. No changes in goblet cell number or hyperplasia were observed. Original magnification was x200. Scale bars = 50 µm.

immunofluorescent staining for HES1 in the distal colon, and found that *C. rodentium* infection caused no differences in the localization or abundance of the protein (data not shown). These results indicate that the changes in goblet cell numbers seen during *C. rodentium* infection are not governed at the level of notch dependent differentiation.

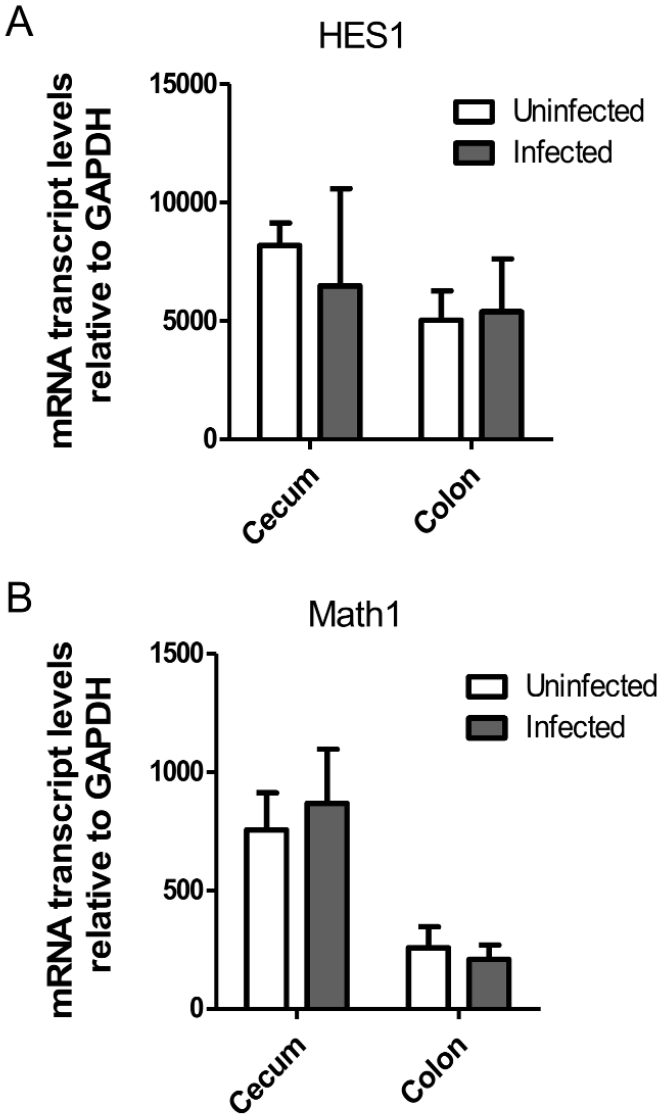

Figure 2.6*: mRNA transcript levels of HES1 and Math1 during C. rodentium infection*

Bars represent the mean expression of HES1 (A) and Math1 (B) relative to control GAPDH by quantitative RT-PCR from 3 independent experiments. No significant differences were found between comparative (colon vs. colon, cecum vs. cecum) tissues. Error bars indicate SEM.

2.3.8 Goblet cell depletion and crypt hyperplasia protect against C. rodentium infection

The dramatic crypt hyperplasia seen only in CD4+ T cell reconstituted mice led us to next examine whether increased IEC proliferation (and their more rapid turnover) might be responsible for the goblet cell depletion. Through detailed analysis using serial sections of both H&E and ki67 stained tissue, we determined that 89% of crypts exhibiting goblet cell depletion also exhibit increased proliferation and hyperplasia (Fig. 2.7A, yellow area). On the other hand, crypts exhibiting few ki67 positive cells and decreased hyperplasia contain mature goblet cells in high numbers (Fig. 2.7A, green area). We next examined whether *C. rodentium* preferentially localized to either goblet cell depleted or goblet cell filled crypts. Through co-staining for the *C. rodentium* translocated effector Tir and the proliferative marker Ki67, we determined that *C. rodentium* penetrated deeply into crypts with little hyperplasia and high numbers of goblet cells (such as those in non-reconstituted *Rag1 -/-* mice). Interestingly, crypts displaying high levels of goblet cell depletion such as those found in *Rag1 -/-* mice reconstituted with CD4+ T cells only showed *C. rodentium* colonization on the most apical epithelial cells at the luminal surface (Fig. 2.7B).

As previously noted, colonic goblet cells produce large amounts of the mucin Muc2. To address the functional impact of goblet cell depletion, we fluorescently stained Muc2 in infected tissues and found not only decreased numbers of Muc2 expressing goblet cells in the crypts of CD4+ T cell reconstituted mice but also decreased Muc2 staining within the colonic lumen compared to non-reconstituted mice (Fig. 2.7C). Interestingly, *C. rodentium*, visualized by staining tissues for Tir, appeared to be highly co-localized to those crypts filled

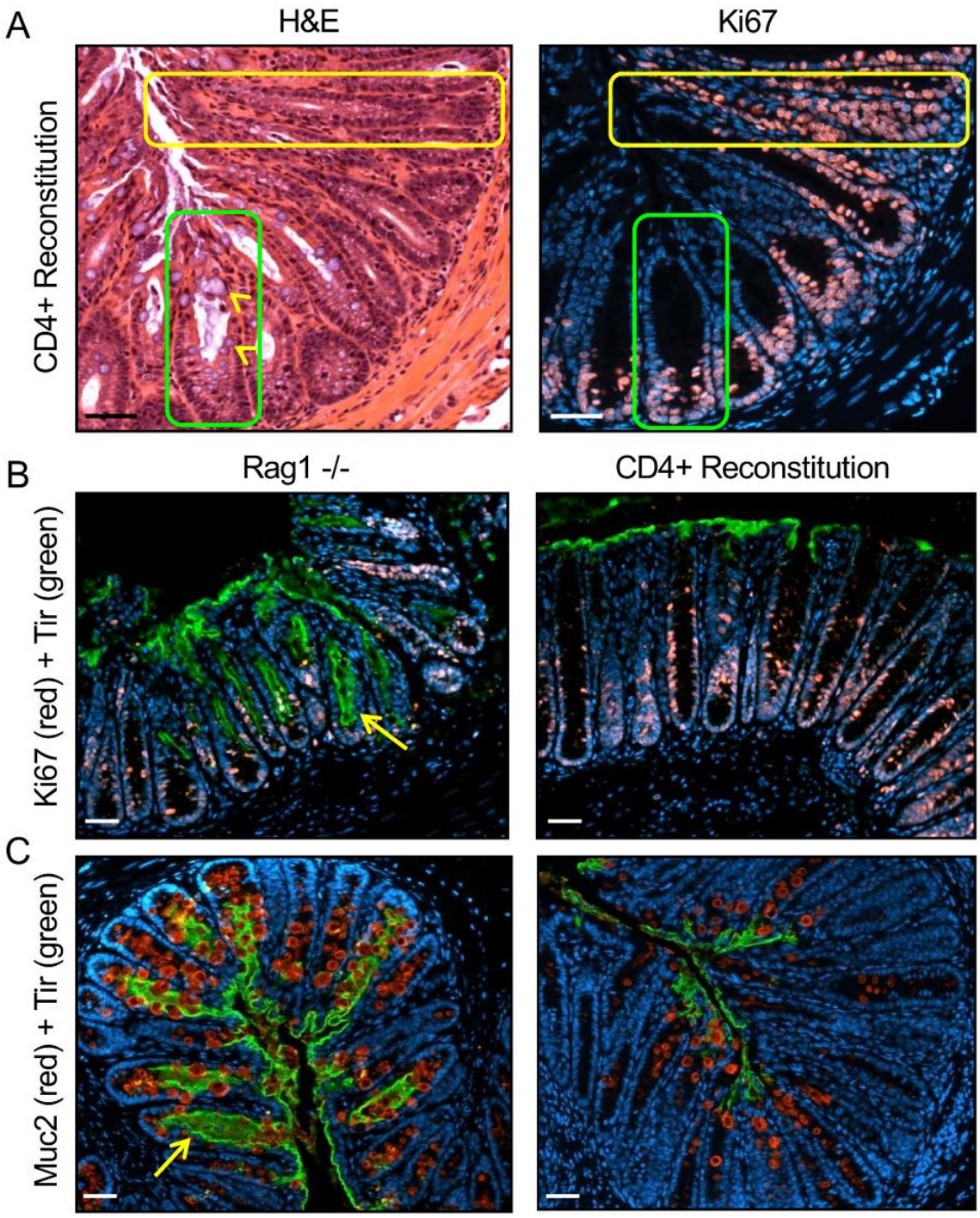

Figure 2.7: ***Impaired goblet cell depletion is associated with deep penetration of crypts by C. rodentium***

(A) H&E and ki67 (red) stained serial sections exhibiting patchy goblet cell depletion in the distal colons of CD4+ T cell reconstituted mice at day 12 p.i. Crypts displaying goblet cell depletion also display high numbers of ki67 positive cells (yellow area) while crypts with mature goblet cells (arrowheads) exhibit

low numbers of ki67 positive cells (green area). Original magnification, x200. Scale bars = 50 µm. (B) Immunofluorescence staining for C. rodentium translocated effector Tir (green), ki67 (red, top panels), Muc2 (red, bottom panels) and DNA (blue) in distal colonic tissues at day 12 p.i. Non-reconstituted Rag1 -/- mice have deeply filled crypts containing C. rodentium (arrows), few ki67 positive cells (top-left panel) but increased Muc2 expression (bottom-left panel). CD4+ T Cell reconstitution prevents C. rodentium from associating deep within crypts while dramatically increasing numbers of ki67 positive IEC (top-right panel) but decreasing Muc2 expression (bottom-right panel). Original magnification was x200. Scale bars = 50 µm.

with Muc2 and lined with large numbers of Muc2 positive goblet cells. 95% of crypts deeply colonized with Tir also contained more than 10 mature goblet cells. These results suggest that either *C. rodentium* preferentially colonizes these Muc2 containing crypts, or that the pathogen is at least partially cleared from crypts that undergo increase epithelial cell proliferation and goblet cell depletion.

2.3.9 Over-differentiation of goblet cells increases pathogen burdens and mortality rates

While our studies indicate that goblet cell depletion and increased epithelial cell proliferation are driven by CD4+ T cells and associated with protection against *C. rodentium*, the other actions of CD4+ T cells complicates our assessment of the role of these processes in providing host defense against *C. rodentium*. Though we already determined that notch signaling is not overtly altered in this model, notch inhibition has been shown to dramatically increase goblet cell numbers and mucus production in the intestine (75). We

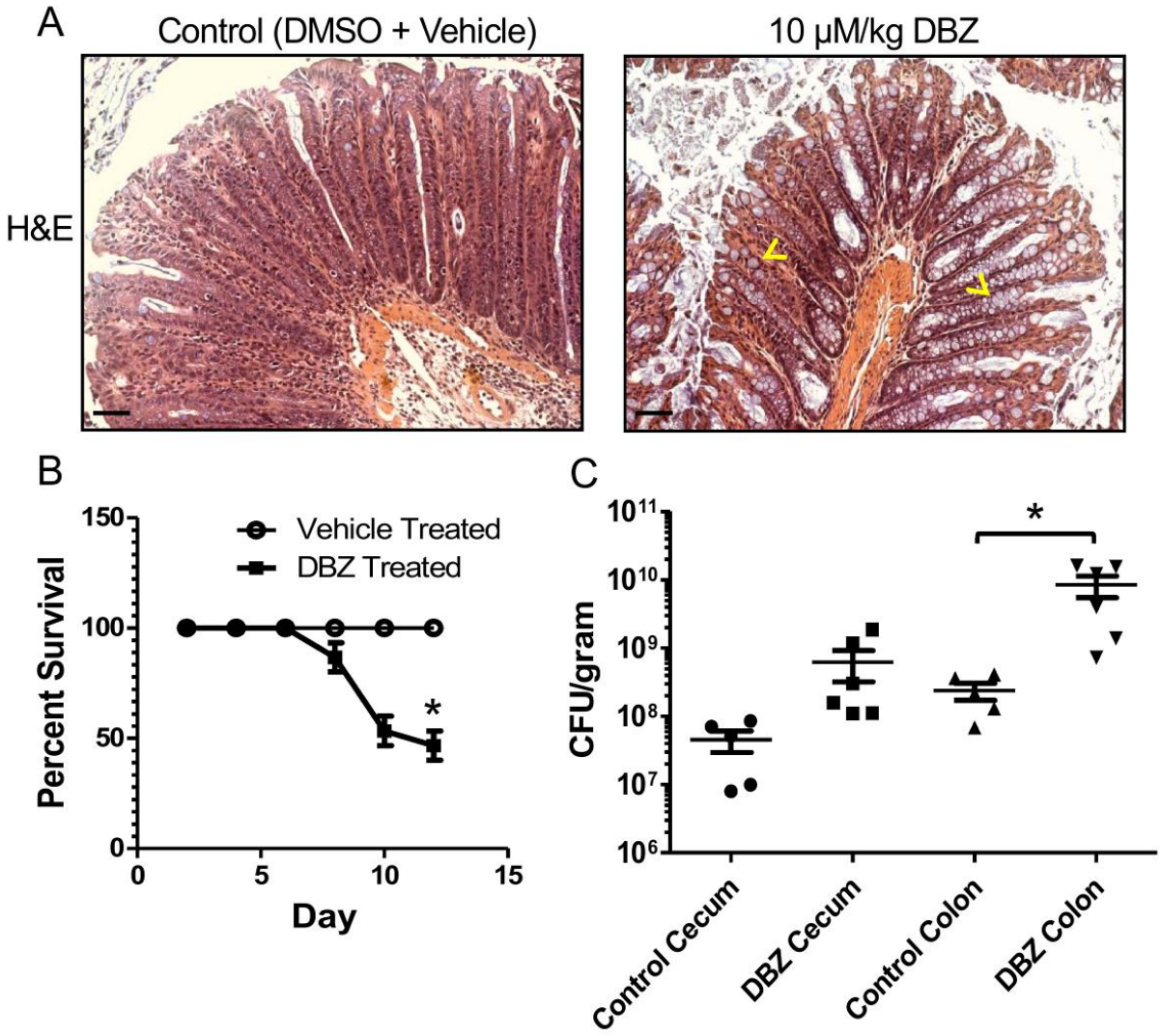

Figure 2.8: ***Treatment with γ-secretase inhibitor DBZ increases mortality and C. rodentium burdens***

(A) Representative H&E stained images from infected distal colons showing increased goblet cell numbers in DBZ treated C57BL/6 mice. Arrowheads indicate representative mature goblet cells. Original magnification was x200. Scale bars = 50 μm. (B) Percent survival of DBZ treated mice is reduced beginning at day 9 p.i. Each symbol indicates the mean of three independent infections. Error bars indicate SEM. Asterisk shows significance, with $P < 0.05$. (C) DBZ treated mice carry increased C. rodentium CFU/gram of tissue in distal colons at day 12 p.i. Error bars indicate SEM from at least six mice. Asterisk shows significance, with $P < 0.05$.

therefore tested the impact of notch inhibition and increasing intestinal goblet cell numbers on the course of *C. rodentium* infection. Notch was inhibited in mice using serial injections of γ-secretase inhibitor dibenzazepine (DBZ, Axon Medchem) at days 9, 10 and 11 post-infection. DBZ-treated mice exhibited a dramatic 6-fold increase in mature goblet cells, a 65% reduction in crypt length as well as a thickened mucus layer (Fig. 2.8A). Moreover, only 46.7% ± 6.7% of mice treated with DBZ survived to day 12 post-infection compared to 100% of vehicle treated mice surviving (Fig. 2.8B). Treatment with DBZ also led to significantly greater pathogen burdens in the colon but not the cecum (Fig. 2.8C) again indicating that infection induced goblet cell depletion and increased epithelial proliferation play an important role in protecting the host during *C. rodentium* infection.

2.4 Discussion

Our previous work determined that *C. rodentium*-induced goblet cell depletion depends on the presence of T and B cells, although the specific cell types and mechanisms driving this pathology were not determined. Here we examined the key components and mechanisms that govern goblet cell depletion by reconstituting immunodeficient *Rag1 -/-* mice with either CD4+ or CD8+ T cells. Our findings show that it is CD4 + T cells that drive goblet cell depletion, potentially through the actions of IFN-γ, since loss of IFN-γ signaling (but not IL-17A) abrogated the infection induced depletion. Our results also indicate that it is increased IEC proliferation, rather than alterations in Notch signaling, that cause goblet cell depletion. In addition, by staining tissue sections for *C. rodentium*, we determined that this pathogen preferentially co-localizes to crypts containing large numbers of goblet cells rather than to hyperplastic, goblet cell depleted crypts. These data suggest that goblet cell depletion and

increased IEC proliferation protect against *C. rodentium* infection and correspondingly, when infected mice were given a Notch inhibitor that dramatically increased goblet cell numbers and reduced IEC proliferation, the treated mice showed increased susceptibility to infection, carrying significantly heavier pathogen burdens and suffering high mortality rates. In our reconstitution studies, we confirmed that *Rag1 -/-* mice frequently succumb to *C. rodentium* infection, whereas immunocompetent C57BL/6 mice do not. Previous studies infecting mice lacking T and B cells with *C. rodentium* typically found such infections to be fatal, which was attributed to heavier pathogen burdens and to an inability to clear the infections (71, 76). Moreover, we previously showed that reconstituting *Rag1 -/-* mice with both T and B cells reduced *C. rodentium* burdens as well as mortality rates (24). Interestingly, we found that reconstituting *Rag 1 -/-* mice with either CD4+ or CD8+ T cells reduced their infection-induced mortality rates, but did not alter pathogen burdens. This suggests that it is B cells that likely control pathogen burdens whereas T cell reconstitution reduces mortality in this model through other mechanisms. Without a change in pathogen load, we focused our attention towards the effects of reconstitution on colon architecture. Overall, we demonstrated that CD4+ and CD8+ T cell reconstitution led to distinct colonic pathologies, not found in non-reconstituted *Rag1 -/-* mice. Reconstitution with either T cell subtype led to grossly enhanced histological damage, with CD8+ T cell reconstituted mice suffering patchy ulceration in their mid and distal colon regions. Associated with these focal ulcers were aggregates of CD8+ T cells. While it is unclear whether these ulcers were directly the result of the actions of the CD8+ T cells, there was no evidence of goblet cell depletion in this group of mice.

In contrast, reconstitution with CD4+ T cells led to increased IEC proliferation as well as dramatic goblet cell depletion and crypt hyperplasia, similar to, albeit patchier than the colonic pathologies seen in infected C57BL/6 mice. Aside from a reduction in goblet cell numbers, we also noted a reduction in Muc2 mRNA levels as well as reduced expression of immunoreactive Muc2 protein within goblet cells and within the colonic lumen. Since the goblet cell depletion was patchy, we wondered whether it might reflect regions where intestinal epithelial cell proliferation and turnover was greatest. Indeed, we did find a correlation between goblet cell depletion and regions showing significant crypt hyperplasia and increased IEC proliferation as revealed by Ki67 staining. Notably, the localization of *C. rodentium* was altered by the CD4+ T cell reconstitution since although *C. rodentium* was noted to penetrate deeply into the colonic crypts of *Rag1 -/-* mice, it was predominantly limited to the luminal surface of those hyperplastic crypts showing goblet cell depletion in the CD4+ T cell reconstituted mice. These findings suggest that increased IEC proliferation and/or goblet cell depletion may play a host-protective role during these infections.

C. rodentium has been widely shown to induce a strong Th1 and Th17 immune response in infected mice. While there are many inflammatory cytokines upregulated in this model, we elected to study IFN-γ and IL-17A (73, 77), as these cytokines play critical host protective roles against *C. rodentium* (78). While our results confirm that IL-17A limits *C. rodentium* burdens, neutralizing this cytokine had little impact on the colonic pathology suffered during infection. In contrast, much of the infection induced IEC proliferation, crypt hyperplasia and goblet cell depletion was dependent on IFN-γ signaling, since these responses were largely abrogated in infected *Ifn-γ receptor -/-* mice. IFN-γ is typically produced in large amounts by

CD4+ T cells, and indeed we observed that CD4+ T cell reconstituted mice expressed much higher mRNA levels of IFN-γ over non-reconstituted *Rag1 -/-* mice. Interestingly, goblet cell function has also been shown to be altered in *Salmonella* Typhimurium infection, albeit much more rapidly than that seen in the *C. rodentium* model (79). Studies by Songhet et al, established a role for IFN-γ receptor signaling in regulating the loss of goblet cell mucin production. While the exact mechanisms involved remain unclear, they showed that it was expression of the IFN-γ receptor by non-hematopoietic cell types (such as goblet cells) that mediated the goblet cell depletion.

The concept that loss of goblet cells and their function would protect the host from an intestinal pathogen is of course surprising, considering that many studies, including those from our lab, have shown that intestinal mucus plays a protective role in host defense (31, 80). For example, mice deficient in Muc2 and therefore lacking an intestinal mucus layer are highly susceptible to *C. rodentium* colonization, frequently succumbing to infection. Moreover, many bacterial pathogens are equipped with flagella or mucin-cleaving proteolytic enzymes that aid microbes in passing through the intestinal mucus barrier (65, 81). In fact, the success of pathogens in bypassing the intestinal mucus layer, and perhaps even using it as a nutrient source, could explain why goblet cell depletion may prove beneficial. As we noted, the goblet cell depletion is overt by days 10-12 post-infection, long after the pathogen has successfully colonized its host. Although the mucus layer may have delayed and limited the extent of initial pathogen colonization, by this later stage of infection, the mucus layer has failed in its role of preventing pathogen colonization. We hypothesize that in the face of heavy pathogen colonization of the intestinal mucosal surface, the exaggerated microbial

stimulation of the mucosal immune system drives increased IEC proliferation as a generalized host defense mechanism. The rapid turnover of IEC, as well as the sloughing of infected IEC into the intestinal lumen may aid in clearing mucosal adherent pathogens such as *C. rodentium*. Moreover, the loss of mature goblet cells and the reduced production of mucus may reduce nutrient availability for *C. rodentium*, again promoting its clearance.

Our findings suggest that *C. rodentium* induced goblet cell depletion does not rely on Notch signaling mediated changes in goblet cell differentiation. This was somewhat unexpected, since the notch pathway and bHLH transcription factors such as math1 are known to play a key role in controlling the fate of intestinal epithelial progenitor cells, determining whether they differentiate into secretory lineages such as goblet cells (20). Moreover, studies have shown that notch inhibition can prevent goblet cell depletion during dextran sulphate sodium-induced colitis (82). Instead, our findings indicate that the goblet cell depletion we observe reflects increased IEC proliferation. Our analysis of crypts displaying goblet cell depletion identified the presence of small and weakly PAS stained cells, which we hypothesize are immature goblet cells. This observation leads us to believe that IEC proliferation may reduce the opportunity for goblet cells to mature, hence the observed goblet cell depletion in highly proliferating crypts. Proliferation, as opposed to differentiation, controls rapid expansion of the cellular pool, giving rise to increased cell numbers and accelerated IEC turnover kinetics. IEC proliferation is governed by the canonical Wnt pathway and functions both independently and cooperatively with the notch pathway (83). Other groups have provided insight into the role played by changes in IEC turnover kinetics on goblet cell maturation. For example, deletion of Kruppel-like factor 4 can result in up-regulation of genes in the Wnt

pathway, increasing IEC proliferation, thereby leading to quicker migration of goblet cells along the crypt-axis ultimately leading to their shedding into the lumen (84). While more work is required to determine the interplay and potential interdependence between IEC proliferation and goblet cell depletion, our study shows a clear association and protective role for both processes.

In conclusion, we demonstrate that the goblet cell depletion seen during *C. rodentium* infection reflects the immunomodulation of the intestinal epithelium by CD4+ T cells. Dependent on IFN-γ signaling, the goblet cell depletion appears to reflect increased IEC proliferation and turnover, thereby limiting the ability of goblet cells to reach maturity. Ultimately, the goblet cell depletion and increased IEC proliferation are associated with protection against *C. rodentium*, and may reflect a generalized host response against mucosal pathogens. Defining the exact mechanisms by which these responses help clear *C. rodentium* infection from the mucosal surface may offer insights into the development of new approaches to combat mucosal adherent bacterial pathogens.

Chapter 3: Discussion

3.1 The role of immunomodulation in host defense

Our studies on the immunomodulation of goblet cell function and its impact on host defense present a novel perspective on the ability of the host to counter bacterial pathogens. While shedding light on the effect of IEC proliferation, intestinal turnover and goblet cell depletion on mucosal adherent bacterial clearance, they also raise many questions regarding our current understanding of intestinal host defense. Most notably, a dichotomy seemingly exists in that immunomodulation in the gut appears to enhance pathology but at the same time aid in survival. Traditionally, increased pathology is used as a determinant of the severity of disease and in fact this is most often the case. However, our studies suggest that survival and reduced pathological damage are not always clearly linked. For example, we have shown that CD4+ T cells drive IEC proliferation in *C. rodentium* infection which likely drives an increase in IEC turnover, aiding in bacterial clearance and ultimately improving host survival. Of course, the usefulness of these pathological responses to the host greatly depends on the strategy of infection used by the pathogen as well as the current physiology of the host. In some cases, it is suspected that increased IEC turnover results in the creation of gaps in the intestinal barrier that may be used by pathogens to subvert the normally impenetrable seal between IEC (85). On the other hand, increased IEC turnover has been shown to play a valuable role in helminth parasite expulsion from the gut and in our studies, defense against enteric attaching and effacing pathogens. There is evidence that some bacterial pathogens have developed countermeasures to prevent epithelial turnover. For example, *Shigella* utilizes IpaB, a type 3 secretion system translocated protein which results in cell cycle arrest in rabbit intestinal crypt progenitors (86). Kim et. al have also shown that

Shigella deploys another T3SS effector, OspE which interacts with integrin-linked kinase at the basement membrane of IEC and increases the strength of focal adhesions preventing the shedding of infected epithelial cells (87). Considering that many mucosal pathogens (bacterial, viral and parasites) directly infect their host's epithelial cells, the exfoliation of these infected epithelial cells is problematic for pathogens that replicate on or within the gastrointestinal tract, urinary tract, or respiratory tract (88). With respect to host defense, my studies alert the need to consider multiple perspectives when defining host-driven pathological responses. At this time, the current dogma in understanding pathological disease and host response cannot tell us whether IEC shedding/turnover is good or bad.

The same dichotomy exists in the role played by goblet cells in mucosal defense against bacteria. Goblet cells clearly play a pivotal host protective role through their release of antimicrobials and effector proteins but primarily through the release of Muc2 and their maintenance of the intestinal mucus layer. Mucin production is clearly protective since the absence of Muc2 leads to dramatically increased susceptibility to *C. rodentium* as well as enteric parasitic infection (31, 89). However, our study shows a host driven response that dramatically reduces the number of mature goblet cells and the levels of secreted luminal Muc2, yet protects the host by reducing deep bacterial penetration of colonic crypts. Does this mean that goblet cells are detrimental in host defense against *C. rodentium*? My experiments suggest that goblet cell depletion may either be an artifact of increased epithelial turnover and/or an intentional mechanism to reduce goblet cell numbers but at this point it is unclear if either or both are true. Since overexpression of goblet cells alone through γ-secretase/notch inhibition which made mice more susceptible to *C. rodentium* induced

mortality, perhaps it is not in the best interest of the host to restore mature goblet cell numbers during accelerated IEC turnover. Other studies also support the detrimental effect of mucin production during infection. Excessive mucus levels have been shown to be detrimental in the lung, potentially leading to obstruction of airflow and favoring chronic microbial colonization. It is possible that excessive mucus could have similar ramifications in the gut. There are well characterized mucin secretagogues produced by bacterial pathogens, such as the cholera toxin of *Vibrio cholerae* which triggers a cAMP-dependent mucin release. In addition, exposure to *E. coli* increases MUC2 gene expression in mucin-producing NCIH292 epithelial cells while the airways pathogen *P. aeruginosa* activates MUC2 gene transcription in both lung and colonic goblet cells (90, 91). It is also likely that the contribution of goblet cells is relative to the stage of infection (early vs. late). Goblet cells and their secreted products prevent pathogens from direct contact with IECs but during late stage infection, when the host is attempting to clear bacteria, secreted antimicrobials may be ineffective (especially against invasive bacteria) and mucus may trap or even act as a nutrient source for pathogens. These findings highlight the need for further studies to clarify both the adverse and beneficial biological roles of mucus and goblet cells in enteric host defense.

3.2 Future directions regarding the immunomodulation of gut function

As stated above, my study raises many questions concerning how the immune system promotes intestinal host defense, and how immune mediated changes in gut function may promote unexpected changes in host-pathogen interactions within the GI tract. While there are always mechanistic questions that need to be answered, I am also interested in several

new avenues and techniques that I've encountered/explored during the course of my training. In the following sections, I will share both my personal interests and potential future studies that may be followed up by my lab mates or other collaborators.

3.2.1 Determining a mechanism for goblet cell depletion

So far, our group has established that goblet cell depletion due to *C. rodentium* infection is CD4+ T cell dependent and likely relies on the actions of IFN-γ. Although some groups have postulated that changes in notch signaling may be responsible for controlling goblet cell numbers during gut inflammation, we have shown this is not the case during *C. rodentium* infection. It is instead likely that Wnt signaling plays a larger role in goblet cell depletion than previously thought since our studies confirm an individual crypt association between increased IEC proliferation and goblet cell depletion. We postulate that the massive cell proliferation that we observe decreases the time taken for epithelial cells to turnover, leaving insufficient time for goblet cells to reach maturity and thereby resulting in the goblet cell depletion phenotype. In order to confirm this, we would need to establish a clearer marker for mature goblet cells. Prime candidates to study are Kruppel-like factor 4, shown to be responsible for terminal differentiation of goblet cells (92, 93). Another question that remains is the role of IFN-γ in the process. While our studies suggest that CD4+ T cells are the primary source of IFN-γ, there are other cell types that may contribute to the IFN-γ production occurring during infection. More importantly, it is unclear whether IFN-γ is acting directly upon IEC, or if acts on other cell types which then drive the changes in IEC proliferation. This can be determined by using crossing floxed IFN-γ receptor knock-out

mice with mice expressing the enzyme cre in a cell-specific such as villin-cre, which is IEC specific.

3.2.2 From proliferation to IEC turnover

The most striking features of the intestinal pathology we observed in the *C. rodentium* infected mouse were increased epithelial cell proliferation and crypt hyperplasia. Although it is generally accepted that increased proliferation is indicative of increased intestinal turnover, this may not always be the case. In this study, we define intestinal turnover as the physical extrusion of IEC as a result of the combination of proliferation of new cells and death of old cells. However, the term intestinal turnover has been used loosely in scientific literature as some groups consider increased proliferation alone sufficient for the term. Since intestinal turnover is poorly defined, it is difficult to measure its relative contribution to host defense although recent studies suggest its potential importance (58). One of the roadblocks to successfully defining intestinal turnover is that the concept requires rapid proliferation of cells, migration up the crypt and finally extrusion into the lumen. While proliferation is relatively easy to measure using the putative marker, ki67, and migration by pulse chase BrdU, it is difficult to measure cell sloughing, the final stage of IEC turnover. The difficulty primarily stems from the complexity of cell death pathways that lead to cell sloughing that are stimuli dependent. For example, epithelial cell extrusion that leads to barrier breaching is thought to be due to increased caspase-1 activated epithelial pyroptosis (94). On the other hand, the intestinal epithelium undergoes cell shedding and gap formation following cell detachment, which occurs under normal physiological conditions. Finally, *Shigella* induces a necrosis type of cell death in nonmyleoid cells such as IECs which is Nod1, NFκB, and

RIP2-dependent (95). In our studies, we do not assess cell death pathways but this would be an important area for further focus.

3.2.3 Intestinal turnover and gut microflora

Although I do not specifically address the contribution of the gut microflora in my studies, the resident commensal bacteria undoubtedly play a large role in regulating both the microenvironment and overall physiology of the gut. Already, it is well established that the composition of gut commensal bacteria greatly influences susceptibility to a multitude of infectious organisms by altering host immune and epithelial responses in the gut. At a basic level, germ-free mouse studies have revealed that commensal bacteria prime the immune system and provide a baseline responsiveness that allows the host to successfully defend against pathogens (96). As well, our lab has shown that commensal bacteria promote colonization resistance through direct or indirect interactions with pathogens (97, 98). Recent studies also suggest commensal microbes have immunomodulatory effects on the host. Savage et al. show that epithelial cells migrate from the base of the crypt to the villus tip twice as fast as germ-free mice (99). Transcriptome profiling studies by Chowdhury et al. reveal that the resident microbiota are required for the expression of IFN-γ inducible genes that contribute to intestinal cell turnover and mucus biosynthesis (100). However, due to their nature, it is difficult to assess the direct contribution of the resident microflora during pathogenic insult; most pathogens eliminate or displace resident commensal microbes shortly after infection. Because of this, it may be more valuable to investigate the effect of microbial communities on effector T cells that we have shown responsible for immunomodulation during *C. rodentium* infection. Recently, segmented filamentous bacteria (SFB) have been

shown to have the ability to skew CD4+ T helper subsets. Colonizing germ-free mice with SFB leads to an accumulation of Th17 cells (101). Even conventional C57BL/6 mice can have upwards of a five-fold increase in Th17 cells after being colonized with SFB (102). This being the case, it is possible for resident commensal microbes to change the way the host may respond to *C. rodentium* and warrants considering the contribution of the commensal microflora when discussing host immunomodulation.

3.2.4 Multiphoton microscopy

During my training, I had the valuable opportunity to investigate and optimize novel *in vivo* imaging techniques using multiphoton microscopy in the gut of a live mouse through collaboration with Tim Murphy's lab (Brain Research Center, UBC). Multiphoton microscopy is a non-invasive technique and was first described regarding the study of live cells in the brain (103). It allows for cellular level resolution while allowing structure and physiology to remain intact. The basis of two-photon microscopy is the combinatory excitation of two low energy photons at the focal plane. In single photon microscopy (i.e. confocal), the absorption of a high energy photon (i.e. blue) is required for the emission of a low energy wavelength (i.e. green) but in multiphoton microscopy, two photons can carry only half the energy required for traditional excitation (104). Simultaneous absorption of these 2 low energy photons (infrared) results in a single higher energy wavelength (i.e. green). Therefore, the advantage of multiphoton microscopy over traditional optical microscopy methods, particularly in thick, living tissue include: (i) reduced effects of light scattering and phototoxicity because longer, low energy wavelengths are used to penetrate

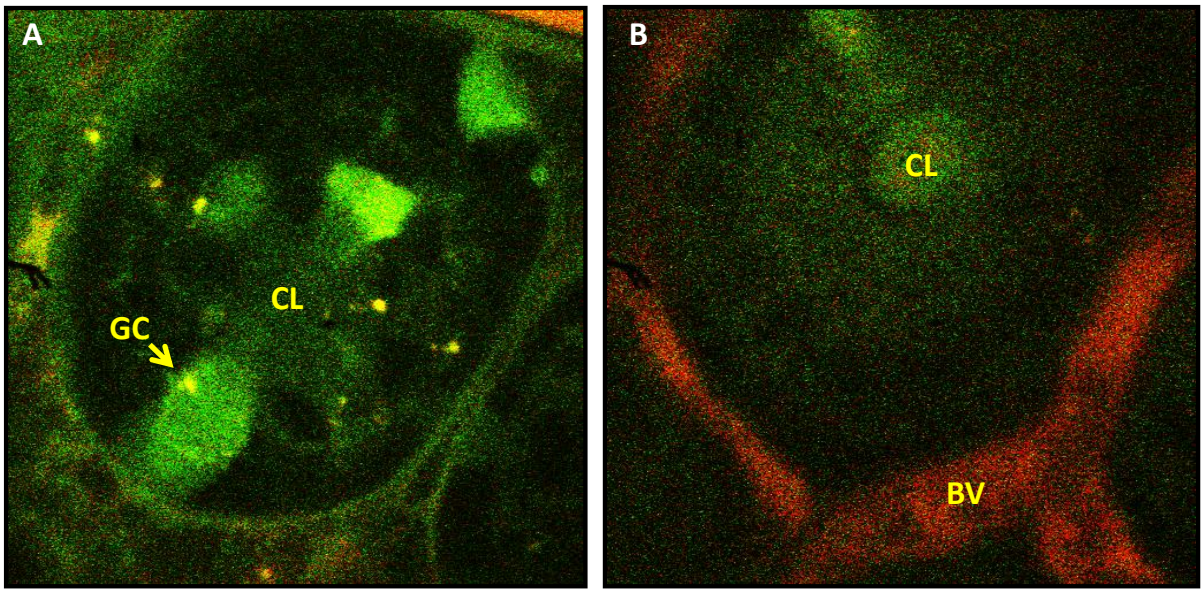

Figure 3.1: ***In vivo multiphoton images of the mouse cecum***

(A) Multiphoton capture of the mouse cecum showing autofluorescent mucus (green) filling a goblet cell (GC) and being secreted into the crypt lumen (CL). (B) 100 microns deeper, the crypt lumen is still visible by autofluorescent mucus (green) and the crypt is surrounded by a blood vessel (BV) labelled with injected red dextran.

tissues at depths up to 1 mm (105), (ii) minimal photo-bleaching because excitation only occurs at the point of focus where both photons can combine (106), and (iii) increased excitation signal since a pin-hole aperture is not required because only in-focus excitation takes place (107). Multiphoton microscopy has already been used for high-resolution image capturing of tumor vascularization, kidney physiology, embryonic development, and T-cell behavior via *in vivo* lymph node imaging (106, 108, 109). Recently, multiphoton imaging has been used to study host-pathogen interactions. For example, bacterial interactions with the kidney and small bowel have been imaged real-time using GFP+ expressing bacteria (110).

My work with multiphoton imaging involved evaluating the potential of this technique for studying bacterial host interactions in our models, and optimizing it for the gut. Imaging the gut brought many unique challenges to overcome. For example, gut peristalsis made it difficult to maintain focus in the live animal, exteriorization of the gut was required for imaging, a cecal loop was required to prevent the dispersion of injectable fluorescent markers, the fluorescent eGFP bacteria were difficult to detect and finally the regular mouse diet contained autofluoresencent chlorophyll which complicated the imaging. However, despite these barriers, we were able to successfully visualize the cecal wall at a depth of 200 microns using a combination of Hoechst and fluorescent dextran staining that allowed for striking real-time images and videos. We also developed approaches to control gut movement by submerging the tissue in the calcium channel blocker, nifedipine, allowing us to mount the tissue more effectively. Interestingly, mucus also appeared to have autofluorescent properties and although this may be a barrier to overcome later, it aided in our ability to visualize both goblet cells and colonic crypts (Fig. 3.1). Though further optimization would be required, this technique provides many opportunities to study intravital mucin dynamics and secretion in real time during infection.

3.3 Final Remarks

The findings in this thesis present a novel perspective on the role of immunomodulation of gut function in promoting host defense against enteric pathogens. Rapid intestinal proliferation and goblet cell depletion are hallmarks of *C. rodentium* infection and although originally considered pathologies, they may reflect a potent host defense mechanism driven by CD4+ T cells that promotes bacterial clearance. Although increased IEC turnover has

already been recognized as an important host defense mechanism against parasitic infection, my studies suggest a similar importance in defense against mucosal adherent bacterial pathogens. My studies also exemplify the importance of balance; as the host attempts to maintain intestinal homeostasis in the face of enteric infection, goblet cell depletion is surprisingly protective. Goblet cells, already shown to be critical in managing infection through release of its products, have the potential to be detrimental. Taken together, these studies support the potential for therapeutics and understanding host defense through the immunomodulation of intestinal epithelial cells.

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