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THE ROLE OF INTESTINAL EPITHELIAL CELLS AND THE REGULATION OF
THE POLYMERIC IMMUNOGLOBULIN RECEPTOR IN HOMEOSTASIS AND
INFLAMMATION

DISSERTATION

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy in the
Department of Microbiology, Immunology, and Molecular Genetics
College of Medicine
at the University of Kentucky

By
Aubrey Leigh Frantz

Lexington, Kentucky

2012

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ABSTRACT OF DISSERTATION

THE ROLE OF INTESTINAL EPITHELIAL CELLS AND THE REGULATION OF THE POLYMERIC IMMUNOGLOBULIN RECEPTOR IN HOMEOSTASIS AND INFLAMMATION

The mammalian intestine harbors an estimated 100 trillion microorganisms, which normally maintain a mutually beneficial relationship with the host. The intestinal epithelium consists of a single layer of intestinal epithelial cells (IECs) that provides a physical barrier as well as innate immune defense, preventing this vast community of microbes from entering host tissues. Secretory immunoglobulin A (SIgA) acts as the first line of antigen-specific immunity at the interface between the gut microbiota and the intestinal epithelium. Polymeric IgA secreted by plasma cells in the intestinal *lamina propria* is transported across IECs by the polymeric immunoglobulin receptor (pIgR). Defects in epithelial barrier and immune functions can lead to infections with opportunistic and pathogenic microbes and contribute to the etiology of inflammatory bowel disease (IBD). Here we investigate the ability of IEC biomarkers to define the mechanism and severity of intestinal inflammation, as well as provide insight into the function of IEC in regulating intestinal homeostasis and inflammation. Importantly, down-regulation of pIgR expression was a common feature in human IBD and mouse models of experimental colitis. One molecule of pIgR is consumed for every molecule of SIgA transported, thus high expression of pIgR is required to maintain sufficient supply of SIgA. Accordingly, we investigate the mechanisms by which IECs regulate pIgR expression in response to colonic bacteria. Cross-talk between the microbiota and IECs is mediated by pattern recognition receptors, including Toll-like receptors (TLR), leading to expression of gene products that enhance epithelial barrier function and innate immunity. The cytoplasmic adaptor protein MyD88 transduces signals from TLRs that recognize bacterial products. We show that pIgR induction by colonic bacteria is dependent on TLR4-MyD88 activation of NF- κ B signaling. We examined the role of epithelial-specific MyD88 signaling in antibacterial immunity and epithelial expression of key gene products that participate in innate immunity in the gut by generating mice with an IEC-targeted deletion of the *Myd88* gene (*MyD88^{ΔIEC}*). *MyD88^{ΔIEC}* mice display immunological and antimicrobial defects resulting in increased susceptibility to experimental colitis. We conclude that cross-talk between bacteria and IECs via MyD88-dependent signaling is crucial for maintenance of gut homeostasis.

KEYWORDS: Intestinal epithelial cell (IEC), polymeric immunoglobulin receptor (pIgR), MyD88, IgA, Inflammatory Bowel Disease (IBD)

Aubrey Frantz

Student's Signature

April 20, 2012

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This dissertation is dedicated to the 2011-2012 University of Kentucky's Men's Basketball Team and their soon-to-be National Championship.

“The best *DEFENSE* is a good offense”

Go CATS.

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Chapter One: Introduction

“Good fences make good neighbors.”~ Robert Frost

This mid 17th –century proverb was used by Robert Frost in his 1914 poem “Mending Wall”, in which Frost questions whether rebuilding a stone wall each spring between his and his neighbor’s property is actually necessary to maintain good will between neighbors. While it seems that Frost doubts a physical barrier is required to maintain these relationships, in the intestine, this proverb can be taken literally. The intestinal epithelium, composed of a single layer of intestinal epithelial cells (IECs), provides a biological “fence” that separates the vast number of bacteria in the intestinal lumen from host tissue. In addition to this physical barrier, IECs must also provide immunological defenses to maintain a symbiotic relationship between microbiota and host. When these fences and active defenses fail, intestinal inflammation can result. In this work, the critical role of IECs in regulating intestinal homeostasis and inflammation is investigated.

Structure and Function of the Intestinal Epithelium – “The Good Fence”

Epithelial surfaces, including the skin, respiratory tract, gastrointestinal tract and urinary tract, make up the main interfaces between the host and external environment¹. The primary function of these epithelial surfaces is to provide a physical and immunological barrier to prevent invasion by microorganisms, with which these surfaces are in constant contact. The quantity and composition of these microorganisms vary based on the anatomical site². In particular, the gastrointestinal tract (GI) is the largest reservoir of commensal microorganisms in the mammalian body. It is estimated that the

mammalian gut is home to 10^{14} bacteria, with at least 1,000 different bacterial species³. Bacterial concentrations within the GI tract increase exponentially as it descends from the stomach to the distal colon⁴. The upper GI tract, including the stomach, duodenum, jejunum and proximal small intestine contains minimal bacterial loads of fewer than 10^4 per gram of luminal contents⁵. The bacterial concentrations increase towards the lower small intestine, reaching levels of about 10^7 and 10^8 cells per gram, while bacterial loads increase to approximately 10^{11} bacteria per gram of stool in the colon⁴. A single layer of intestinal epithelial cells, known collectively as the intestinal epithelium, provides a physical barrier that separates the vast number of bacteria in the intestinal lumen from the underlying *lamina propria*¹. The intestinal epithelium consists of four major cell lineages that arise from a pluripotent stem cell progenitor. These include absorptive enterocytes, which make-up the majority of intestinal epithelial cells (IECs), the mucus-producing goblet cells, enteroendocrine cells, which are specialized hormone-producing endocrine cells in the gastrointestinal tract, and Paneth cells, which are the main producers of antimicrobial peptides³ (**Figure 1-1**). IECs are structurally and functionally polarized, with an apical surface facing the intestinal lumen and a basolateral surface facing the underlying *lamina propria*⁶. These epithelial cells constitute a large surface area, approximately 100m^2 for an average human, which is structurally organized by the crypt-villus axis. The turnover rate of IECs, which is approximately 3-5 days, is governed by the rate of cell proliferation in the crypt as well as the cell shedding at the villus surface³. Intestinal stem cells located in the crypt regions undergo proliferation and differentiation into enterocytes, enteroendocrine cells, or goblet cells and migrate upward to the top of the villus where cellular apoptosis and epithelial shedding occurs. Intestinal stem cells

can also differentiate into Paneth cells that migrate downward to the bottoms of the crypts (**Figure 1-1**). During intestinal epithelial cell differentiation and migration, the formation of epithelial tight junctions (TJ) occurs at cell-cell contact sites to ensure a virtually impermeable barrier. Accordingly, in a healthy gut, the proper expression and formation of TJ proteins are the limiting factor that regulates intestinal permeability⁵. Therefore this well organized structure of the intestinal epithelium creates a secure fence that separates the neighboring microbiota from the neighboring *lamina propria*.

Neighbors to the North – The Intestinal Microbiota

While the primary function of IECs is to maintain spatial separation between bacteria and host tissue, thus preventing bacterial invasion and infection, the majority of intestinal bacteria maintain a symbiotic relationship with the host. The colonic microbiota break down complex carbohydrates that cannot be metabolized by the host⁷; bacterial fermentation generates short-chain fatty acids that serve as energy sources for colonic ECs⁷; colonic bacteria produce antimicrobial peptides that promote the maintenance of a symbiotic community⁸; and the presence of commensal bacteria protect against pathogen colonization by competing for nutrients and space⁹. Additionally, communication between commensal bacteria and IECs has been shown to control the rate of epithelial turnover and differentiation, enhance epithelial barrier function and shapes the developing mucosal immune system^{3,10}.

Interestingly, humans are born germ-free but the GI tract is rapidly colonized after birth¹¹. The gut microbiota is highly diverse, with 15,000-36000 identified species and at least 1,000 different species present in the microbiota of each individual³. However, it is

believed that only 30-40 bacterial species represent greater than 99% of an individual's microbiota¹². The four major bacteria phyla found in the colonic mucus include *Firmicutes* (~64%), *Bacteroidetes* (~23%), *Proteobacteria* (~8%) and *Actinobacteria* (~5%). While the microbiota generally lives in a symbiotic relationship with the host, these bacteria can contribute to disease. Accordingly, the presence of bacteria is required for the development of disease in at least 20 well characterized mouse models of experimental colitis^{13,14}. While techniques to analyze the composition of the microbiota are variable, a general "dysbiosis," or imbalance in the composition of the microbiota, is observed in patients with inflammatory bowel disease (IBD)¹⁵. Increases in adherent and invasive *Escherichia coli* along with reduced colonization of members of *Firmicutes* and *Bacteroidetes* have been found in IBD patients compared to healthy individuals¹⁵⁻¹⁷. However, whether this dysbiosis is a cause or an effect of intestinal inflammation is still under investigation^{14,18}.

Neighbors to the South- Immune Cells of the *Lamina Propria*

Beneath the intestinal epithelium and separated from the microbiota, a layer of mucosal tissue, known as the *lamina propria*, is a major mucosal effector site that functions to recruit T and B cells emerging inductive sites¹⁹. The *lamina propria* is composed of stromal cells (mostly myofibroblasts) and immune cells including B cells, T cells, macrophages and dendritic cells (DCs) (**Figure 1-2**)⁶. As mentioned, the intestinal epithelial barrier in a healthy gut does not allow penetration of commensal bacteria into the lamina propria, thus preventing excessive immune responses. However, limited bacteria-immune cell interactions occur in a control manner in specialized lymphoid

structures, including Peyer's patches (PPs) of the small intestine and isolated lymphoid follicles located throughout the length of the intestinal tract¹⁹. These inductive sites are located beneath specialized cells within the epithelium known as microfold (M) cells. M cells are capable of transporting luminal contents, including commensal bacteria, to resident antigen presenting cells (mostly DCs) within the PP or lymphoid follicle (**Figure 1-2**)⁶. Bacterial-laden DCs can then migrate to PP and mesenteric lymph nodes (MLNs) where they can induce B and T cells to undergo clonal expansion and differentiation upon activation by antigen. The presence of the microbiota also has a strong influence on the development of these secondary lymphoid structures that make-up the mucosal associated lymphoid tissue (MALT) and gut-associated lymphoid tissue (GALT)²⁰. Germ-free mice exhibit defective MALT and GALT development, including reduced numbers of PPs and smaller PPs, smaller and fewer cellular mesenteric lymph nodes (MLNs) and less cellular *lamina propria*, as compared to conventionally raised mice²¹⁻²³.

Toll-like Receptor Signaling Provides Communication Between Neighbors

Cross talk between bacteria and host cells is mediated by pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are a family of PRRs that recognize pathogen-associated molecular patterns (PAMPs)⁶. While the general term PAMP is used for these TLR ligands, the molecular patterns that are actually recognized by these TLRs are not just from pathogens, but commensal bacteria, viruses and endogenous proteins as well. There are 13 characterized TLRs, 10 of which are homologous between mice and humans (**Figure 1-3**)⁶. The TLRs that are primarily responsible for recognizing bacterial products, including TLR1 (bacterial lipoproteins), TLR2 (bacterial lipoproteins,

peptidoglycans), TLR4 (lipopolysaccharide (LPS), TLR5 (flagellin) and TLR6 (LTA, diacyl lipoproteins), are generally expressed on the cell surface^{6,24}. Alternatively, the TLRs that are primarily responsible for the recognition of viral components, including TLR3 (dsRNA), TLR7 (ssRNA), TLR8 (ssRNA), and TLR9 (bacterial/viral unmethylated CpG DNA), are generally expressed on intracellular endosomal membranes⁶. TLRs are inducible or constitutively expressed, at various levels and combinations, in all cell-types throughout the intestine²⁴. However, given the close association between IECs and the microbiota, TLR signaling by IEC provide the first line of communication between bacteria and host.

All TLRs are type 1 transmembrane receptors that contain an N-terminal leucine-rich repeat domain (LRR), a transmembrane region and a C-terminal cytoplasmic domain^{6,25}. The C-terminal domain shares high homology to the type-1 IL-1 receptor and is thus termed the Toll/IL-1R (TIR) domain²⁶. Upon ligation of TLRs with an appropriate ligand, signaling cascades are activated, resulting in the innate immune effector responses as well as the initiation of adaptive immune responses. Stimulation of TLRs leads to the recruitment of cytoplasmic adaptor proteins²⁵. Five adaptor proteins have been identified; myeloid differentiation factor 88 (MyD88), MyD88-adaptor like protein (Mal/TIRAP), TIR-containing adaptor protein inducing interferon- β (TRIF), Trif-related adaptor molecule (TRAM) and sterile α - and armadillo-motif containing protein (SARM)²⁵. MyD88 is utilized by all TLRs, except TLR3. TRIF is utilized by TLR3 as well as TLR4. Accordingly, unlike other TLRs, TLR4 utilizes both MyD88 and TRIF via recruitment of the bridging adaptors Mal and TRAM (**Figure 1-4**). These adaptor proteins initiate signaling cascades that ultimately lead to the activation of the nuclear

factor κ B (NF- κ B)²⁷ signaling pathways and parallel activation of several mitogen-activated protein kinases (MAPK) including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase ERK (ERK), and p38 MAPK²⁶. Activation of these signaling pathways leads to the induction of pro-inflammatory chemokines, cytokines and antimicrobial peptides^{6,17}. Accordingly, NF- κ B and MAPK activation is significantly reduced in MyD88-deficient mice²⁸. Along with the activation of NF- κ B and MAPK, the MyD88-independent, Trif-dependent signaling pathway leads to the phosphorylation and translocation of IRF3 to the nucleus where it regulates the expression of type 1 interferons (IFN)²⁶ (**Figure 1-4**). TLR signaling has been shown to enhance epithelial barrier function by inducing expression of tight junction proteins, mucin production, antimicrobial peptides and protection against epithelial apoptosis⁶. Intestinal epithelial cells of germ-free mice have reduced expression of TLRs, as compared to conventionally raised mice²⁹, suggesting a role for the microbiota in maintaining innate immune function.

The transcription factor NF- κ B is a central regulator of inflammatory and immune responses in most organ systems. Inactive NF- κ B is localized to the cytosol, complexed to the inhibitory protein I κ B³⁰. Upon stimulation, including TLR signaling, I κ B is phosphorylated by I κ B Kinase (IKK) and degraded, allowing NF- κ B translocation to the nucleus and induction of gene transcription^{30,31}. In the intestine, NF- κ B signaling has diverse functions that govern both protective and destructive immune responses. Whether these effects are beneficial or harmful depend on the cell type involved, the level of NF- κ B activation as well the specific environmental and pathophysiological

conditions. While excessive NF- κ B signaling in immune cells has been implicated as a major contributor to intestinal inflammation, recent studies have indicated that NF- κ B signaling in IEC is required to maintain intestinal homeostasis^{30,32,33}. Epithelial deletion of NEMO, the common regulatory subunit of the NF- κ B IKK complex, causes excessive intestinal epithelial apoptosis and epithelial barrier defects leading to the translocation of commensal bacteria³⁴. Accordingly, a balance of NF- κ B signaling must be maintained to achieve a protective level of “physiological” inflammation.

Mucus Layer Provides a Preliminary Line of Defense

To support this epithelial barrier, a mucus layer covers the single layer of intestinal epithelial cells within the small intestine and colon. This mucus layer is composed of transmembrane molecules known as mucins³⁵. The main mucin protein and primary structural component of mucus is mucin-2 (MUC2), a large gel-forming protein (>100 MDa) produced by goblet cells. Once released from the apical side of the goblet cell, MUC2 molecules dimerize and trimerize to form a large network, which can be further cross-linked to form a well organized and stable structure³⁵. Interestingly, the small intestine and colon contain very distinct mucus layer properties. The colon is protected by an inner and outer mucus layer³⁶. The thick inner mucus layer is relatively sterile and firmly attached to the epithelium. This inner layer is continuously converted to a less dense outer mucus layer that is permeable to commensal bacteria³⁵. In contrast, the mucus layer of the small intestine is thin and is relatively permeable to the sparse bacterial loads found in this area³⁵. These differences in mucus properties are consistent with the difference in bacterial concentrations as well as anatomical functions of these

two organs⁵. For example, a dense, tightly bound mucus layer would be detrimental for the small intestine, whose primary function is to chemically digest and absorb nutrients from ingested food. On the other hand, the presence of the compacted inner mucus layer in the colon facilitates the effective passage of waste and prevents bacteria association with the epithelial surface. Accordingly, mice deficient in Muc2 demonstrate increase epithelial permeability and bacteria penetration of the intestinal epithelium^{37,38}.

An Epithelial Immune Barrier Reinforces the Physical Barrier

In addition to forming an impermeable physical barrier, IECs also actively maintain an immunological barrier to prevent bacterial penetration. IECs secrete a broad range of antimicrobial peptides (AMPs), including defensins, cathelicidins and calprotectins that exhibit broad-spectrum antibiotic activity against bacteria, fungi, viruses and yeast³. These small cationic peptides are secreted into the lumen of the gut in response to microbial stimulation¹. Once in the mucus layer, the majority of AMPs can insert themselves into bacterial membranes, resulting in bacterial cell lysis¹. Analyses of antimicrobial activity in luminal contents indicate that AMPs are retained in the mucus layer and are not found in the lumen³⁹. Accordingly, a diffusion barrier is created that concentrates levels of AMPs near the epithelial surface, increasing the effectiveness of these proteins in the protection of the epithelium from bacterial adherence and penetration⁴⁰. All IEC lineages produce and secrete AMPs, although Paneth cells located in the epithelial crypts are known to be the main source of AMPs^{1,35}. Several AMPs are constitutively expressed, including many defensins, while some subsets of AMPs require TLR activation by specific bacterial subsets⁴⁰. MyD88-deficient mice have undetectable

levels of the AMP regenerating islet-derived protein 3 γ (RegIII γ)⁴¹ and it has recently been shown that RegIII γ expression was reduced in the small intestine of mice with an epithelial deletion of MyD88⁴². Furthermore, in the absence of MyD88 or Paneth cell development there is an increase in the translocation of commensal and pathogenic bacteria⁴³. Taken together, these results suggest that bacterial-IEC cross-talk mediated by MyD88 signaling is crucial for the expression of AMPs that limit the invasion of commensal and pathogenic bacteria. However, the role of epithelial MyD88 signaling in regulating antimicrobial activity in the colon has not been investigated.

SIgA Provides the First Line of Antigen-Specific Defense

Secretory antibodies of the IgA class (SIgA) provide the first line of antigen-specific immune protection in the gut against potential pathogens⁴⁴. SIgA antibodies in mucosal secretions contribute to intestinal homeostasis by limiting access of microbial and environmental antigens to the host tissue, maintaining the integrity of the epithelial barrier and shaping the composition of the commensal microbiota^{45,46}. IgA is the predominant antibody isotype in mucosal tissues and comprises approximately 75% of the total antibody production in the mammalian body. However, IgA antibodies are a minor component of serum (systemic) antibodies, which highlights the importance of the mucosal immune system in human immunity⁴⁷. There is a clear association between the commensal microbiota and the induction of IgA responses. IgA levels in mucosal secretions and the number of IgA-producing plasma cells in the *lamina propria* are increased several orders of magnitude between colonized and germ-free mice of the same

strain⁴⁸. Thus, the significant induction of intestinal IgA in the presence of commensal bacteria supports a major role for IgA in protection at these mucosal sites.

The production of IgA-specific for commensal bacteria can occur via several well orchestrated interactions between bacteria and immune cells within the GALT⁴⁹ (**Figure 1-5**). DCs in the PPs and lymphoid follicles capture bacteria internalized by M cells and migrate to the interfollicular region of the PPs or MLNs where they present antigen to CD4⁺ T cells. Antigen-activated T cells can induce B cells to differentiate into IgA⁺ plasma cells that home, by means of α 4B7, CCR9 and/or CCR10 expression, to the *lamina propria*^{1,4}. T-cell independent IgA responses have also been demonstrated^{47,50}. The intestinal *lamina propria* also contains IgM⁺ B cells that are thought to be a precursor of IgA-producing plasma cells⁵¹. *Lamina propria* DCs can directly present antigen to these B cells⁵², and provided stimulation with additional B-cell stimulating factors occurs, can induce B cell differentiation into IgA⁺ plasma cells within the *lamina propria*⁵⁰. Lastly, IECs and DCs can directly initiate the class switch recombination from IgM to IgA by releasing B-cell activating factors, such as BAFF and APRIL, in response to TLR signaling^{4,19}. Both BAFF and APRIL are recognized by the BAFF receptor and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) receptor on B cells, which utilize the MyD88-dependent activation of NF- κ B to initiate class switch recombination⁵³.

IgA secreted by plasma cells in the *lamina propria* must be transported across the epithelial layer in order to protect the epithelial surface from colonization and invasion by pathogenic bacteria. This transport process is mediated by the polymeric immunoglobulin receptor (pIgR)⁴⁴ (**Figure 1-6**). pIgR is a mucosal epithelial specific

protein that is synthesized as an integral membrane protein and is targeted to the basolateral side of the epithelial cell. At the basolateral surface, pIgR can bind dimeric IgA secreted by IgA+ plasma cells in the *lamina propria*. pIgR and bound IgA is then endocytosed and transported to the apical side of the epithelial cell. At the apical side, non-specific proteolytic cleavage of pIgR occurs, resulting in the release of the secretory component (SC) of pIgR bound to IgA. This complex is known as SIgA. This transport process provides multiple levels of immune protection (**Figure 1-7**): pIgR has been shown to excrete potentially harmful IgA-containing immune complexes from the *lamina propria* into the lumen⁵⁴. Transcytosis of SIgA has been shown to neutralize viruses and microbial products within IECs⁵⁵. At the apical surface, release of SIgA and SC, both of which have mucus-binding properties that facilitate the trapping of bacteria within the mucus layer, enhances epithelial barrier function and limits bacterial adherence and translocation^{44,46,56}. In the absence of dimeric IgA, constitutive transcytosis and cleavage still occurs, releasing free SC into mucosal secretions⁴⁶. Free SC has been shown to contribute to innate immunity by acting as a non-specific bacterial scavenger and limiting inflammatory immune response⁵⁴. pIgR deficient mice display reduced levels of IgA in mucosal secretions and elevated levels of serum IgA⁵⁷. Moreover, while IgA deficient and pIgR deficient mice are more susceptible to experimental colitis, as compared to wild-type mice, pIgR deficient mice develop more severe intestinal inflammation than IgA deficient mice, indicating a protective role for SIgA and SC in intestinal inflammation⁵⁸.

Regulation of pIgR Expression and Transport of SIgA

One molecule of pIgR is consumed for every molecule of SIgA transported into the lumen, thus the rate of pIgR transcription controls the rate of SIgA transcytosis and high expression of pIgR is required to maintain sufficient supply of SIgA and SC^{44,46}. Accordingly, steady-state levels of pIgR mRNA in mouse and human IECs are very high. pIgR expression has been shown to be regulated *in vitro* by a range of factors, including cytokines, hormones, dietary factors, microbial factors and viral factors⁵⁹ (**Table 1-1**). Our lab has shown that expression of pIgR is much higher in the colon than in the ileum of both healthy controls and Crohn's disease (CD) patients, which is likely due to enhanced bacterial stimulation of IECs due to greater numbers of commensal bacteria⁶⁰. A direct role for commensal bacteria in inducing pIgR expression *in vivo* was first suggested by demonstrating that germ-free mice monocolonized with the bacterium *Bacteroides thetaiotamicron* resulted in upregulation of pIgR expression⁶¹. Importantly, our lab has recently shown that pIgR expression in the colonic mucosa and IgA levels in colonic mucus were reduced in MyD88 deficient mice, suggesting that TLR-dependent MyD88 signaling may also regulate pIgR expression and IgA transport *in vivo*⁶². However, in that study it was not determined whether pIgR was regulated directly by MyD88 signaling in IECs or indirectly by immune cells in the intestinal *lamina propria*. *The work presented here investigates the hypothesis that cross-talk between the commensal microbiota and IECs has a central role in the induction of pIgR expression and the maintenance of intestinal homeostasis.*

When Intestinal Epithelial Fences and Defenses Fail- Intestinal Inflammation

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and Ulcerative colitis (UC), are relapsing, chronic inflammatory diseases of the intestine. Although the causes of these diseases remain incompletely understood, IBD is thought to result from inappropriate inflammatory responses to normal components of the intestinal microbiota in genetically susceptible individuals¹⁷. The adaptive immune system, in particular a heightened effector T cell response, has traditionally been the focus of investigations into the pathogenesis of IBD⁶³. However, numerous recent reports have demonstrated that a defective innate immune response within the intestinal mucosa is a primary contributor⁶⁴⁻⁶⁸. In particular, dysfunction of the intestinal epithelium has been shown to be a dominant pathway in IBD⁶⁵. Genetic defects, as well as experimentally-induced damage, restricted to the epithelium can initiate and promote intestinal inflammation in the presence of healthy underlying immunity³⁴. Thus given their anatomical and functional position, IECs are central to the process of intestinal homeostasis. IEC regulate innate immune responses by controlling bacterial-epithelial interactions, as well as regulate adaptive immune responses by controlling epithelial-*lamina propria* immune cell cross-talk⁶⁵.

Defective epithelial barriers or dysregulation of these epithelial defenses have been shown to lead to a breakdown in intestinal homeostasis and contribute to intestinal inflammation⁶⁹. There are several lines of evidence that support the idea that epithelial permeability defects precede the onset of intestinal inflammation^{5,69}. IBD patients have an increase in intestinal paracellular permeability and defects in the regulation of tight junctions⁷⁰. Mice with a constitutively active myosin light chain kinase (MLCK)

transgene display increased permeability that is quantitatively and qualitatively similar to individuals who are high risk for developing IBD⁶⁹. Furthermore, this defect in tight junction permeability is shown to be sufficient to accelerate the onset and enhance the severity of intestinal inflammation⁷¹. However, the presence of inflammation has also been indicated as a cause of epithelial barrier dysfunction, creating a vicious cycle for the aggravation and perpetuation of inflammation^{3,17}.

Many TLR genetic variants have been associated with human IBD phenotypes and susceptibility. Polymorphisms in TLR1, TLR2 and TLR9, resulting in dysfunctional epithelial TLR signaling, are associated with increased risk of developing colitis and more severe disease^{72,73}. TLR4 variants that result in hyporesponsive LPS-induced TLR signaling are associated with more severe CD phenotype^{74,75}. Mice deficient in individual TLRs that recognize bacterial components, including TLR2, TLR4, TLR5, and TLR9, as well as MyD88-deficient mice, are more susceptible to DSS colitis^{6,76-79}. Deletion of TLR5 alone results in the development of spontaneous colitis⁸⁰. Furthermore, cell type-specific deletions and bone marrow chimera studies have demonstrated that epithelial-specific TLR signaling has a protective role against the development of and response to intestinal inflammation^{32,42,43,79,81,82}. Notably, epithelial deletion of the downstream NF-κB regulatory component NEMO caused severe spontaneous colitis in mice³⁴. Alternatively, there is evidence to suggest that hyperactivation of epithelial TLR signaling is associated with disease. Epithelial-specific deletion of the single immunoglobulin IL-1 receptor-related molecule (SIGIRR), a negative regulator of MyD88-dependent TLR signaling, was shown to increase susceptibility of mice to DSS colitis, characterized by constitutive upregulation of pro-inflammatory genes⁸². In

humans, TLR4 expression is upregulated in primary epithelial cells from CD and UC patients with active disease, indicating a state of TLR hyperactivation⁸³. Hyperactivity to flagellins, a TLR5 ligand, is observed in sera from patients with CD^{84,85}. Taken together, these studies suggest that regulated bacteria-epithelial cell cross-talk is essential for maintenance of intestinal homeostasis and prevention of intestinal inflammation.

The mucus layer has a crucial role in maintaining intestinal homeostasis and a breakdown in mucus-related defenses are associated with intestinal inflammation. Loss of goblet cells, resulting in reduced mucin secretion, is a hallmark of IBD^{1,79}. Additionally, Muc2-deficient mice, which lack a defined mucus layer, develop severe spontaneous colitis^{37,38}. Several studies have suggested that impaired Paneth cell development and function and defective IEC secretion of AMPs, in particular α -defensins, may contribute to CD susceptibility^{86,87}.

Our lab has recently revealed that reduced expression of pIgR and dysregulated transport of SIgA in the colonic mucosa is commonly observed in CD patients⁶⁰. Additionally, reduced pIgR expression in a subset of CD patients resulted in accumulation of IgA deposits in the *lamina propria*. It is likely that this accumulation of IgA in the *lamina propria* could leak into systemic circulation, resulting in the elevated serum concentrations of IgA observed in these patients as well as pIgR-deficient mice⁶⁰. Thus, reduction of pIgR expression during colitis would be predicted to diminish not only the antimicrobial function of luminal SIgA, but also the removal of IgA-bound antigens from the *lamina propria*. These defects would exacerbate local inflammation and increase the level of IgA immune complexes, a potential risk factor for development of IgA nephropathy, which has been linked with IBD⁸⁸. IgA deficiency, independent of

pIgR expression, is estimated to be 10 times more frequent among patients with intestinal diseases than in the general public, and it has been proposed that dysregulation of IgA responses to commensal bacteria results in excessive innate pro-inflammatory responses, propagating intestinal inflammation¹⁹. Mice deficient in either pIgR or IgA are more susceptible to DSS induced colitis⁵⁸.

IBD is a significant and growing health problem in the U.S., resulting in four million prescriptions, over 200,000 hospitalizations, and approximately 1,000 annual deaths. Although progress has been made in the treatment of IBD, many therapeutic options include general immunosuppressants and immunomodulatory agents that hardly address the diversity of colonic inflammatory phenotypes. These biological drugs block key molecules that are involved in the induction and maintenance of inflammation in several signaling pathways, all of which have different contributions in different patients⁸⁹. Therefore considering the complex nature and the heterogeneous clinical presentation of IBD, the need to identify molecular biomarkers that can predict disease behavior is critical for personalized therapy and effective clinical management of disease. Significant attempts have been made to identify genetic markers of IBD. The information that is available include several categories of potential biomarkers, including mucosal cytokines and chemokines, adhesion molecules, activation markers, serological markers, as well as immune and non immune cell subsets⁸⁹⁻⁹³. Considering the culminating evidence that IECs are central to the maintenance of intestinal homeostasis and intestinal epithelium dysfunction is associated with IBD pathogenesis, potential IEC biomarkers are continually being investigated. Various studies have shown that IECs inappropriately express the class II antigen HLA-DR, as well as the co-stimulatory molecule B7 in

actively inflamed mucosa of IBD patients^{94,95}. Down-regulation of TLR3 and defensin expression and upregulation of TLR4 expression was found in active CD patients^{83,96,97}. However, since IBD is clearly a multifactorial disease that results in a range of inflammatory phenotypes, multifactorial approaches are being utilized to investigate potential IBD biomarkers. Accordingly, Arsenescu *et al.* recently identified signature mRNA biomarkers in IECs from CD patients that were predictive of clinical characteristics and responses to therapy⁶⁰. These studies analyzing human IBD patients are essential for identifying clinical phenotypes and have potential to significantly advance diagnostics. However, due to the limitations associated with working with patients, many of these studies do not, or cannot, investigate the underlying mechanisms of disease. By understanding the association between the expression of potential biomarkers with mechanisms of intestinal inflammation and disease severity, it may be possible to evaluate and tailor therapeutic options to address the patient's specific disease course.

Summary and Significance

Intestinal epithelial cells have the unique and complex job of maintaining physical barriers and immunological defenses in order to not only maintain a symbiotic relationship with their microbial neighbors, but to prevent these neighbors from causing serious harm in the rest of the host's intestinal and systemic neighborhood (**Figure 1-8**). The overall aim of this work is to investigate the hypothesis that IECs are central regulators of intestinal homeostasis and inflammation. By using a multifactorial approach to analyze epithelial gene expression during experimental colitis, patterns of

epithelial biomarkers were identified that define underlying mechanisms of inflammation and are predictive of disease severity. Importantly, this study revealed that the downregulation of pIgR expression and altered transport of IgA are common features in human IBD as well as mouse models of experimental colitis, suggesting that maintaining pIgR expression is required for intestinal homeostasis. These results led to the investigation into the regulation of pIgR expression by colonic bacteria. By performing a series of *in vitro* experiments utilizing inhibitors and siRNAs targeted against specific components of innate immune signaling pathways in IECs, the MyD88-dependent activation of NF- κ B was identified to be a major regulator of pIgR induction by commensal bacteria. To determine whether this regulatory mechanism was functional *in vivo*, a novel mouse model employing an epithelial-specific deletion of the *Myd88* gene was generated. Loss of MyD88 signaling in IECs caused significantly reduced expression of pIgR, leading to impaired SIgA transport; reduced expression of Muc-2, leading to altered mucus production; and reduced expression of AMPs, leading to defective mucus-associated antimicrobial activity. Taken together, these results suggest a model in which MyD88-dependent bacterial-epithelial cell cross-talk is crucial for maintenance of physical and immunological barrier function in the intestine.

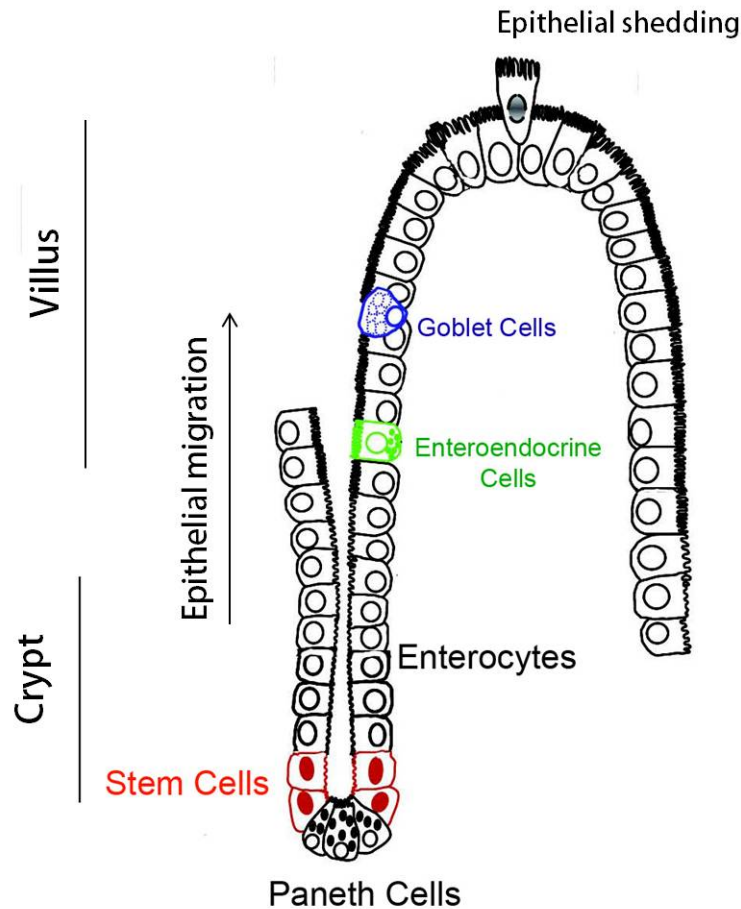


Figure 1-1 Intestinal epithelial cell lineages and the formation of the crypt-villus

axis. The intestinal epithelium consists of four major cell lineages that arise from a pluripotent stem cell progenitor located in the epithelial crypt region. These include absorptive enterocytes, which make-up the majority of intestinal epithelial cells (IECs), the mucus-producing goblet cells, enteroendocrine cells, which are specialized hormone-producing endocrine cells in the gastrointestinal tract, and Paneth cells, which are the main producers of antimicrobial peptides. Intestinal stem cells differentiate into enterocytes, enteroendocrine cells, or goblet cells and migrate upward to the top of the villi where cellular apoptosis and epithelial shedding occurs. Intestinal epithelial stem cells can also differentiate into Paneth cells that migrate downward to the bottoms of the crypts. (Adapted with permission from Yu *et al.* WJGP 2012)

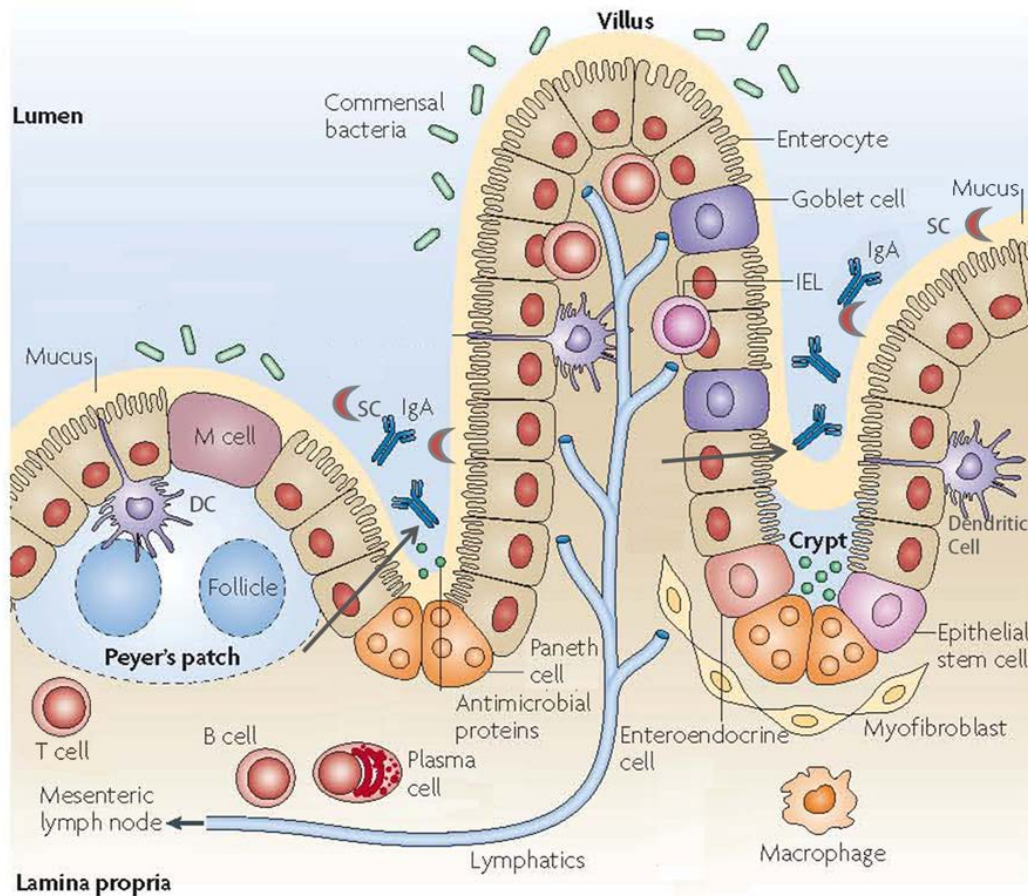


Figure 1-2 Overview of the structure of the intestinal immune system. The intestinal epithelium separates the commensal bacteria in the lumen from the immune cells of the *lamina propria*. A mucus layer containing secreted antimicrobial peptides, SIgA and free secretory component (SC) reinforces the physical epithelial barrier. Below the epithelium, the *lamina propria* contains myofibroblasts, B cells, T cells, dendritic cells (DCs) and macrophages. Specialized lymphoid structures, including Peyer's patches, allow for sampling of luminal contents. Antigen-loaded DCs can travel via the lymphatics to the mesenteric lymph nodes where they can induce B-cell differentiation into IgA-secreting plasma cells. (Adapted with permission from Abreu *et al.* Nat. Rev. Immuno. 2010)

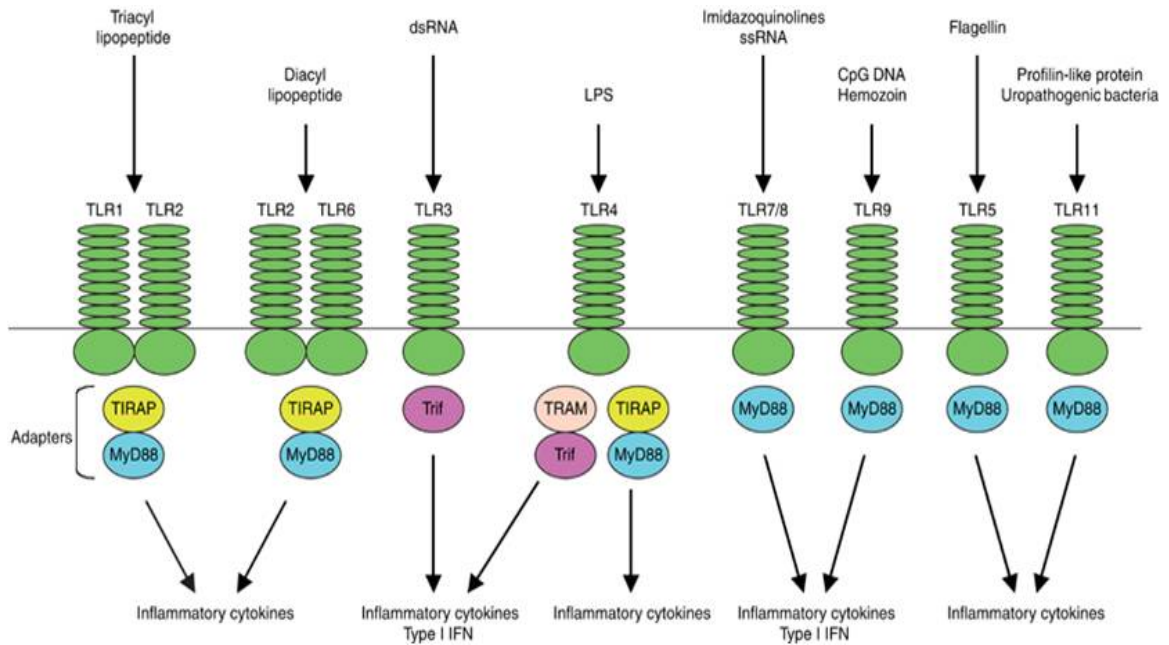


Figure 1-3 Toll-like receptors (TLR), PAMPs, and Adaptor proteins. All TLRs that predominantly recognize bacterial components utilize the adaptor protein MyD88. TLR4 is unique in its ability to signal through MyD88 and Trif. (Adapted with permission from Kawai and Akira, *Cell Death and Differentiation* (2006) 13, 816–825)

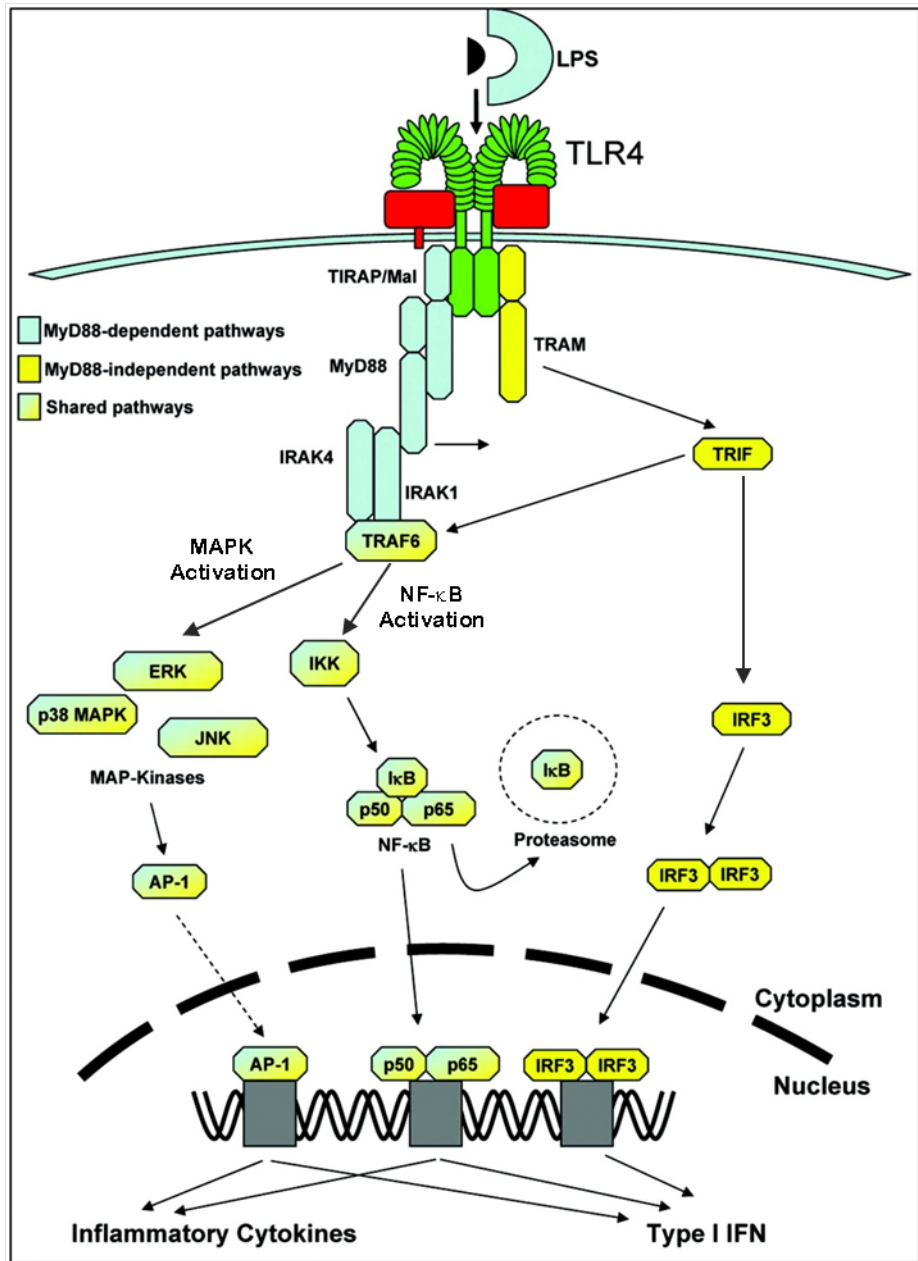


Figure 1-4. TLR4 signaling pathway. LPS binds to the TLR4 complex on the apical surface of IEC, initiating MyD88-dependent (blue) and MyD88-independent (yellow) signaling pathways. Both MyD88-dependent and independent signaling pathways can lead to the activation of NF-κB and MAPK, resulting in the transcription of inflammatory cytokines. (Adapted with permission from Gribar *et al.* Mol. Med. 2008)

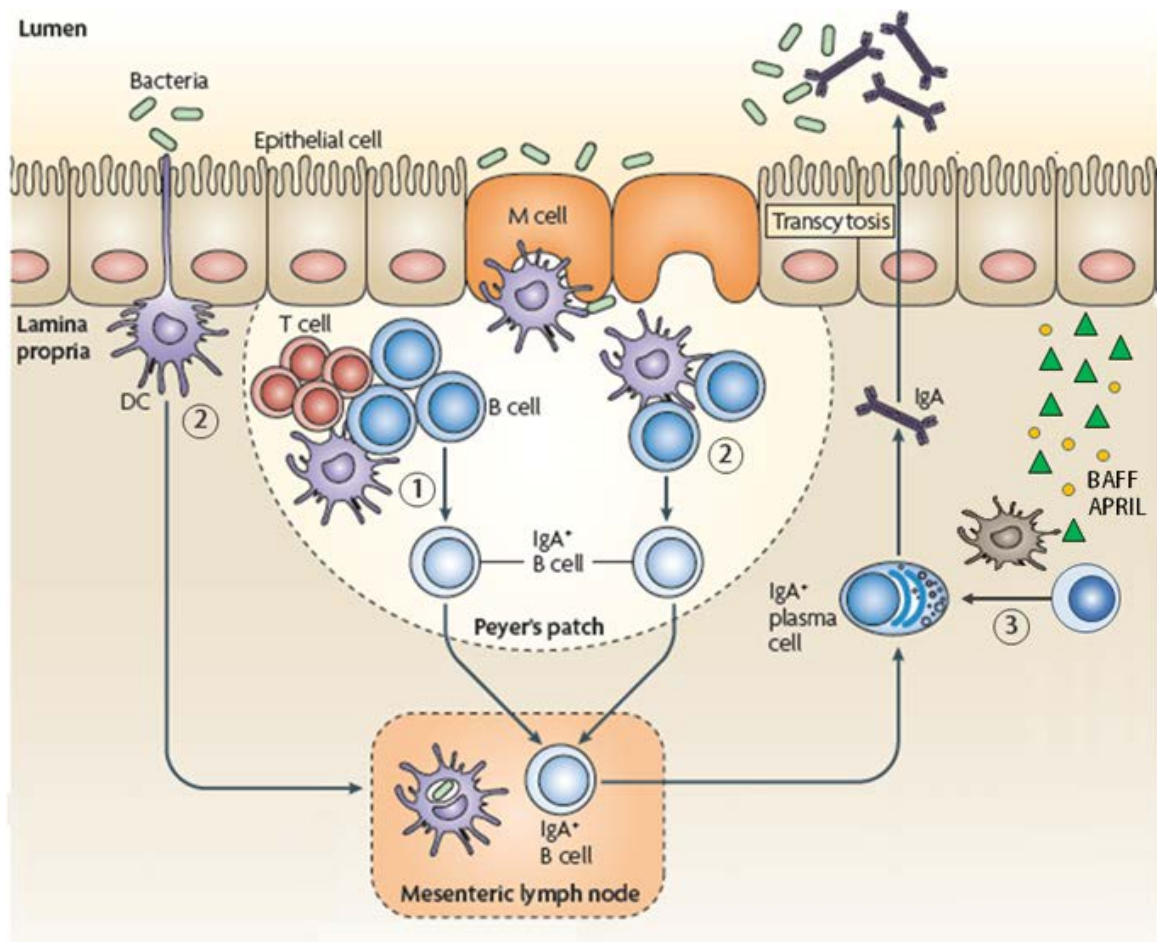


Figure 1-5 Production of IgA specific for intestinal bacteria. 1) Dendritic cells (DC) in the Peyer's Patches (PP) and lymphoid follicles capture bacteria internalized by M cells and migrate to the interfollicular region of the PP or mesenteric lymph nodes (MLN) where they present antigen to CD4⁺ T cells. Antigen-activated T cells can induce B cells to differentiate into IgA⁺ plasma cells that home to the *lamina propria* and secrete dimeric IgA. 2) *Lamina propria* DCs can directly present antigen to these B cells, and when provided with additional B-cell stimulating factors, can induce B cell

differentiation into IgA⁺ plasma cells within the *lamina propria*. **3)** IECs can deliver IgA-inducing signals to *lamina propria* B cells by releasing B-cell activating factors in response to TLR signaling. (Adapted with permission from Hooper *et al.* Nat. Rev. Immunol. 10(3):159-69 2010)

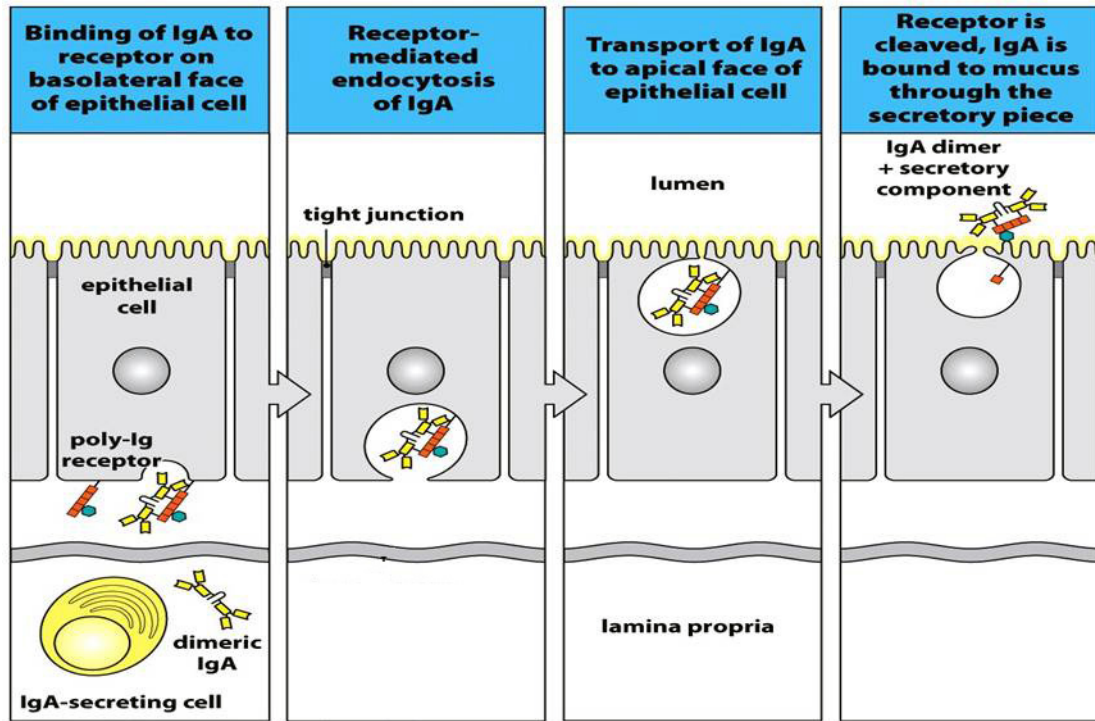


Figure 9.22 The Immune System, 3ed. (© Garland Science 2009)

Figure 1-6 pIgR mediated transcytosis of IgA. The polymeric-Ig receptor (pIgR) is synthesized by IECs and targeted to the basolateral side of the epithelial cell. Dimeric IgA secreted by plasma cells in the *lamina propria* is bound by pIgR on the basolateral side. This complex is transported via receptor-mediated endocytosis to the apical side of the epithelial cell. Here the receptor is cleaved to leave the extracellular secretory component (SC) of pIgR bound to IgA. This complex, known as SIgA, binds to mucus while the nonfunctional residual component of pIgR is degraded (not shown). (Adapted from Janeway *et al.* Immunobiology: the immune system in health and disease. Third Edition. New York: Garland Sciences; 2001)

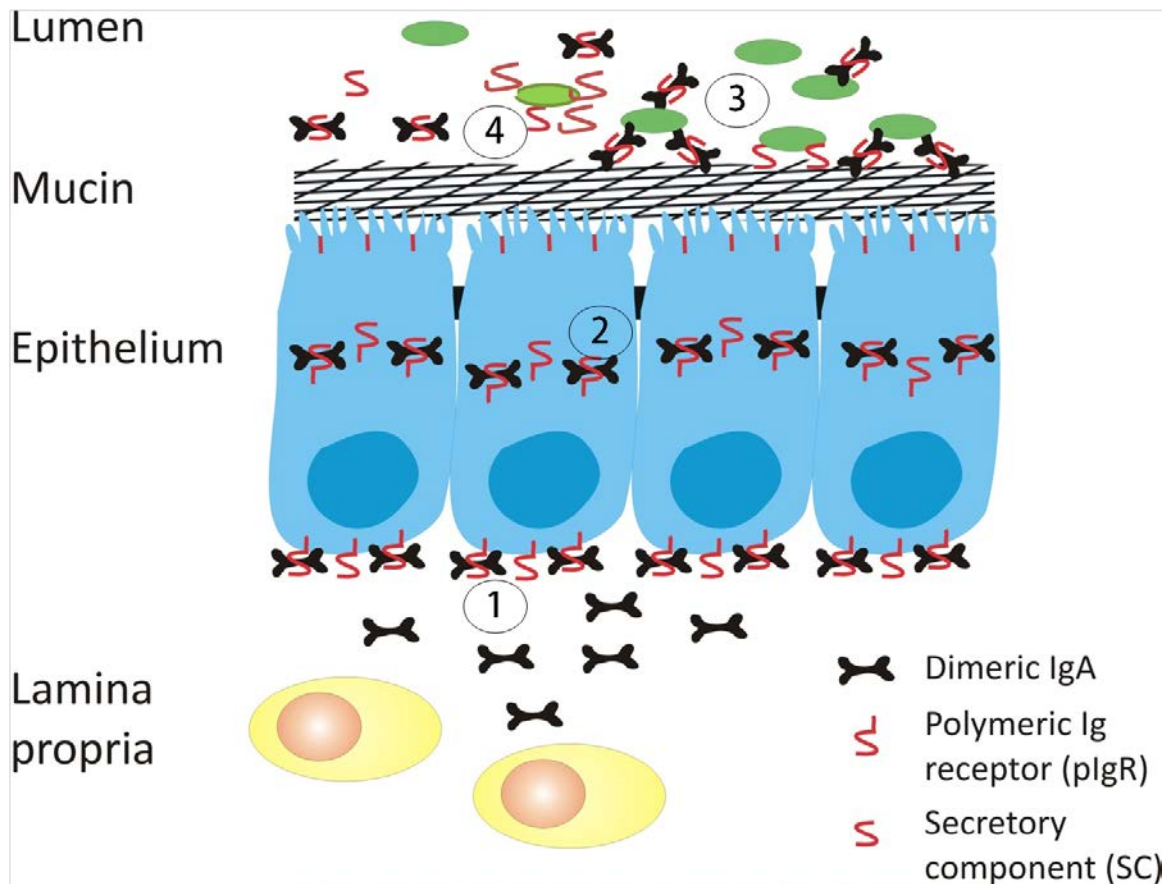


Figure 1-7 Mucosal immune defenses mediated by pIgR and SIgA. The pIgR mediated transcytosis of IgA provides several levels of immune protections. **1)** Dimeric IgA in the *lamina propria* binds to and excretes antigen back into the lumen via pIgR-mediated transcytosis across IEC. **2)** polymeric IgA bound to pIgR can inhibit intracellular virus production and neutralize pro-inflammatory antigens and factors. **3)** SIgA released in to the intestinal lumen binds and neutralizes bacteria and bacterial products, preventing adhesion to and penetration of the epithelium. **4)** Free secretory component (SC) acts as a non-specific scavenger, neutralizing potential pro-inflammatory antigens.

Table 1-1 Regulators of plgR expression in the intestine	
Cytokines	Nature of Regulation
IFN- γ	Upregulates plgR in human intestinal epithelial cell line; synergistic with TNF, IL-1, and IL-4
TNF	Upregulates plgR in human intestinal epithelial cells; synergistic with IFN- γ , IL-1, and IL-4
IL-1	Upregulates plgR in human intestinal epithelial cells; synergistic with IFN- γ and TNF
IL-4	Upregulates plgR in human intestinal epithelial cells; synergistic with IFN- γ and TNF
Microbial Factors	
Butyrate	Upregulates plgR in a human intestinal epithelial cell line
<i>Bacterioides thetaiotamicron</i>	monocolonization if germ-free mice upregulates plgR expression in IECs
<i>Saccharomyces boulardii</i>	Treatment of rats upregulates plgR expression in IECs
<i>E. coli</i> LPS	Upregulates plgR in a human intestinal epithelial cell line
Viral Factors	
Reovirus	Upregulates plgR in a human intestinal epithelial cell line
double stranded RNA	Upregulates plgR in a human intestinal epithelial cell line
Additional Factors	
Glucocorticoids	Upregulates plgR in a human intestinal epithelial cell line
Retinoic acid	Enhances upregulation of plgR by IL-4, IFN- γ , and TNF in a human intestinal epithelial cell line

Table 1-1 Regulation of pIgR expression in the intestine.

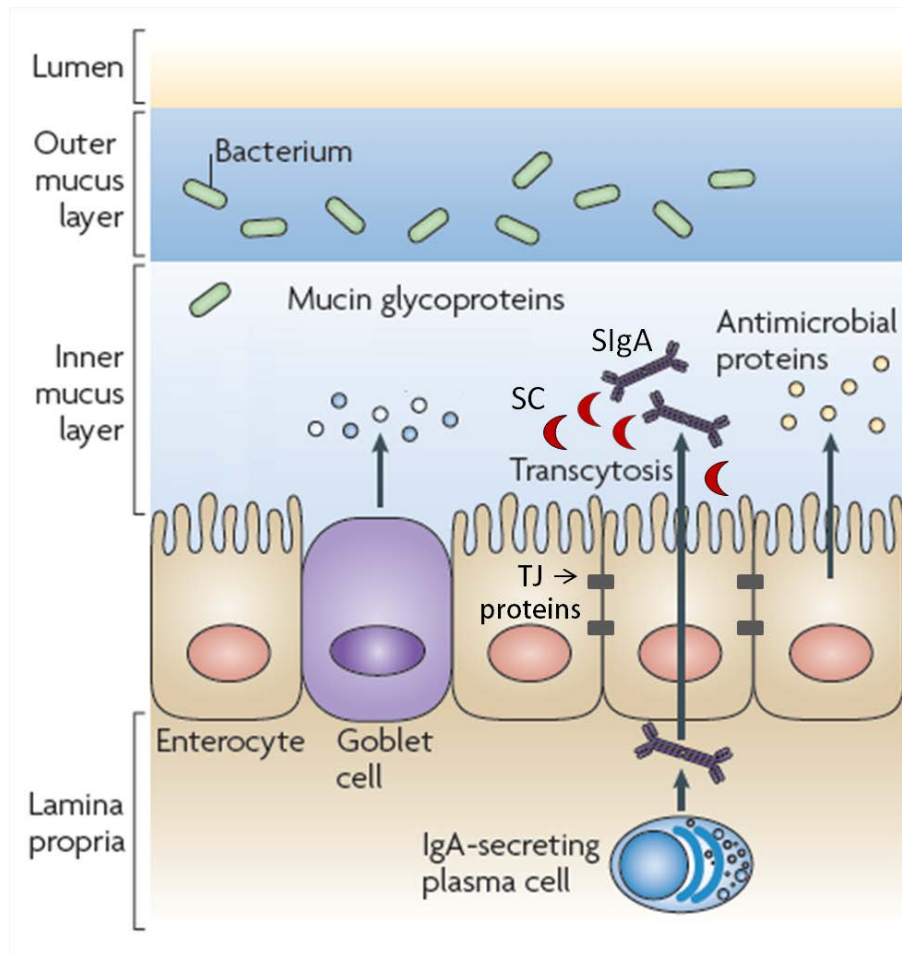


Figure 1-8 Colonic epithelial immune defenses. A single layer of intestinal epithelial cells, joined together by tight junction (TJ) complexes, creates a physical barrier between the intestinal microbiota and the *lamina propria*. To reinforce this physical barrier, IECs also create an immunological barrier. Goblet cells secrete mucin proteins that complex to create a well organized mucus layer. IECs secrete antimicrobial peptides that are retained in the mucus layer. Additionally, IgA secreted by plasma cells in the *lamina propria* is transported by pIgR across the epithelium. SIgA and free secretory component (SC) are released into the mucus layer where they can bind bacteria and bacterial products. (Adapted with permission from Hooper *et al.* Nat. Rev. Immunol. 2010)

Chapter Two: Materials and Methods

Animals

Six-week old female wild-type C57BL/6 and 8-week old female *Rag1*^{-/-} C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mouse strains used in the generation of MyD88^{ΔIEC} mice were on the C57BL/6 background and purchased from Jackson Laboratories (Bar Harbor, ME). Mice of the B6.129P2-*MyD88*^{tm1Defr/J} strain were intercrossed to generate mice homozygous for *loxP*-flanked *Myd88* (MyD88^{Flox}). The B6.SJL-Tg (Vil-cre) 997Gum/J strain, which is transgenic for *Cre* recombinase under control of the intestinal epithelial-specific mouse *Vill* gene, was intercrossed with MyD88^{Flox} mice to generate mice with a targeted deletion of the *Myd88* gene in epithelial cells in the small and large bowel (MyD88^{ΔIEC}). Experiments were conducted with littermates generated by crossing MyD88^{ΔIEC} mice hemizygous for the *Vill-Cre* transgene with MyD88^{Flox} mice. All mice were housed in microisolator cages with sterile bedding, food and water in AAALAC-accredited facility. All procedures were conducted in accordance with guidelines set forth by the University of Kentucky Institutional Animal Care and Use Committee.

DSS Model of Acute Colitis

Acute colitis was induced by oral administration of dextran sulfate sodium (DSS), as described⁹⁸. DSS (molecular weight 36,000 –50,000; MP Biomedicals, Aurora, OH) was dissolved in water at indicated concentration and given *ad libitum* to mice for 8 days in place of normal drinking water. Water intake was measured daily to ensure consistency

in DSS intake among mice. A disease activity index (DAI) was calculated daily for each mouse during DSS treatment, on a 0-4 scale that assessed weight loss, stool consistency, and fecal blood (visible and occult). Weight loss scores were determined as 0 = no weight loss, 1 = 1–5% weight loss, 2 = 6–10% weight loss, 3 = 11–15% weight loss, and 4 ≥ 15% weight loss. Stool scores were determined as 0 = normal stools, 2 = loose stools, 4 = diarrhea. Bleeding scores were determined as 0 = no bleeding, 1 = positive guaiac occult blood test (minimal color change to green), 2 = positive guaiac occult blood test (maximal color change to blue), 3 = blood visibly present in the stool, no clotting on the anus, and 4 = gross bleeding from the anus with clotting present. ColoScreen-ES testing kits (Helena Laboratories, Beaumont, TX) were used to detect occult blood. ColoScreen-ES testing kits (Helena Laboratories, Beaumont, TX) were used to detect occult blood.

T cell Transfer Model of Chronic Colitis

Chronic colitis was induced by adoptive transfer of naïve T cells from wild-type mice into *Rag1*^{-/-} recipients, which lack B and T lymphocytes, as described⁹⁹. To purify T-effector (Teff) and T-regulatory (Treg) cell populations for adoptive transfer, single cell suspensions from spleens of 10 week-old wild-type C57BL/6 mice were prepared in phosphate-buffered saline with 4% fetal bovine serum. In two of the three independent experiments, CD4⁺CD45RB^{hi} and CD4⁺CD45RB^{lo} cell populations were isolated by fluorescence-activated cell sorting. Spleen cells were labeled with anti-CD4 antibodies (eBiosciences, San Diego CA) and anti-CD45RB (Biolegend, San Diego CA) and sorted into CD4⁺CD45RB^{hi} (as a source of Teff) and CD4⁺CD45RB^{lo} fractions (as a source of natural Treg), defined as the uppermost 30% of cells with the brightest CD45RB staining

and the lowest 15% of cells with the dimmest CD45RB expression, respectively (**Figure 3-1A, left panel**). The Teff and Treg fractions were subsequently demonstrated to differ dramatically in cell surface expression of CD25, a marker for natural Treg (Figure **3-1A, right panel**). In one of the three independent experiments, CD4⁺ splenic T cells were separated into CD25⁻ (Teff) and CD25⁺ (Treg) populations by magnetic bead sorting using the Regulatory T cells kit (Miltenyi, Auburn CA). Nine-week-old *Rag1*^{-/-} C57BL/6 mice received a single intraperitoneal injection of 4 x 10⁵ purified Teff, with or without 1 x 10⁵ purified Treg. Following adoptive transfer, body weight was monitored every 3 days and mice were sacrificed at 10 weeks post-transfer. No significant differences were observed in clinical parameters, tissue histology or colonic EC gene expression that could be attributed to the different methods of purification of Teff and Treg populations (immunofluorescence vs. magnetic beads).

Tissue Histology

Colons were dissected from euthanized mice and the length of the colon from the anus to the cecal junction was measured. For T cell transfer colitis, colons were weighed and the weight/length ratio was calculated. Colons were cut lengthwise, and half of each colon was prepared in a “Swiss roll”¹⁰⁰ and fixed in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ). Fixed tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and evaluated in a blinded fashion for evidence of inflammation and epithelial damage. Tissue damage for DSS colitis was quantified on a scale from 0–4 as previously described⁹⁸, using the following criteria: 0, no change from normal tissue; grade 1, mild inflammation present in the mucosa, comprised mainly of

mononuclear cells, with little epithelial damage; grade 2, multifocal inflammation greater than a grade 1 score, including mononuclear cells and some neutrophils, detachment of crypt glands from the muscularis mucosae, mucin depletion from goblet cells, and occasional detachment of the epithelium from the mucosa; grade 3, multifocal inflammation greater than a grade 2 score, including mononuclear cells and neutrophils infiltrating the submucosa, crypt abscesses present with increased mucin depletion, and significant epithelial disruption, including some ulceration; grade 4, crypts no longer present, severe mucosal inflammation mainly composed of neutrophils, and epithelium no longer present or completely detached. An average of 4 fields of view per colon were evaluated for each mouse to obtain a composite score. For T cell transfer colitis, a total histopathological score was calculated by combining the scores for each of the seven following parameters for a maximum score of 17: degree of inflammation in the lamina propria (score 0-3), goblet cell loss (score 0-3), abnormal crypts (score 0-3), presence of crypt abscesses (score 0-1), mucosal erosion and ulceration (score 0-1), submucosal spread to transmural involvement (score 0-3), and number of neutrophils counted at 40x magnification (score 0-3).

Colonic Epithelial Cell Isolation

CEC were isolated as previously described¹⁰¹. Colons were opened longitudinally, cut into 1 mm pieces, and the mucus layer was removed by incubation for 20 min with agitation at 37°C in a solution of 1mM dithiothreitol (Sigma, St. Louis MO) in DMEM:Ham's F-12 (1:1) tissue culture medium (Lonza, Walkersville MD), supplemented with 5% fetal bovine serum and antibiotics (100 U/ml penicillin, 100

μg/ml streptomycin, and 250 ng/ml fungizone) (Invitrogen Life Technologies, Carlsbad CA). The supernatants were discarded, and CEC were stripped from the colon pieces by incubation for 1 h at 37°C in a solution of 0.5 M EDTA in supplemented tissue culture media. The suspension was vortexed vigorously, and the remaining colon fragments were allowed to settle at the bottom of the flask. The upper layers were centrifuged at 2000 rpm for 10 minutes at 4°C to collect ECs. Further purification of the CEC fraction was accomplished by resuspension of the cell pellets in a 10 ml solution of 30% w/v Percoll (Sigma) and centrifugation at 1300 rpm for 20 min at room temperature. The top layer containing floating CEC was removed and transferred into a 15 ml conical tube. The volume was brought to 10 ml with supplemented tissue culture medium, and the CEC solution was pelleted by centrifugation at 2000 rpm for 10 min at 4°C. CEC viability, assessed by trypan blue exclusion, was typically > 90 %. Flow cytometry analysis of representative CEC preparations indicated that contamination with CD45⁺ hematopoietic cells was approximately 0.4 % for control mice and 1.3 % for DSS-treated mice (**Figure 3-1B**).

Analysis of mRNA Levels by Real-time PCR and Nanostring nCounterTM

Hybridization

mRNA levels for individual genes were analyzed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) as previously described¹⁰². Total RNA was purified from isolated CEC using the Qiagen RNeasy Protect mini kit (Qiagen, Germantown MD). RNA was reverse-transcribed to generate cDNA templates using the TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA). Specific mRNA

levels were quantified by quantitative real-time polymerase chain reaction, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) as previously described¹⁰². The level of β 2-microglobulin mRNA, which we found did not vary with experimental treatments, was used to normalize mRNA for tested genes according to the following formula: $(2^{-(C_{T \text{ test}} - C_{T \text{ } \beta 2\text{-microglobulin}})}) \times 100\%$. For mRNA analysis using the Nanostring nCounterTM hybridization technique, colonic epithelial cells were isolated from freshly dissected colons as previously described¹⁰¹. Abundance of mRNA transcripts was analyzed by Nanostring nCounterTM hybridization¹⁰³, and normalized using an algorithm developed by NanoString, Technologies (Seattle, WA).

Immunofluorescence Microscopy

Sections of formalin-fixed, paraffin-embedded colon tissues were dewaxed and rehydrated using standard protocols. Antigen retrieval was performed by incubating slides for 20 min at 95°C in 25 ml of 10 mM sodium citrate buffer, pH 6. Endogenous peroxidase activity was quenched by incubation for 10 min at room temperature in 25 ml of 3% (v/v) H₂O₂. Cell-associated pIgR and free secretory component (the cleaved extracellular domain of pIgR) were detected by overnight incubation of tissue sections at 4°C with a polyclonal goat antibody to mouse secretory component (2.5 μ g/ml) (R&D Systems, Minneapolis, MN). Sections were washed with PBS and incubated for 30 min at room temperature with a horseradish peroxidase-conjugated rabbit antibody to goat IgG (1 μ g/ml) (Invitrogen, Camarillo CA). Bound peroxidase was revealed with TSA Plus-TMR Reagent (Perkin-Elmer, Waltham MA). IgA was detected in the same tissue sections by subsequent incubation for 1 hour at room temperature with FITC-conjugated

antibody to mouse IgA (1 μ g/ml) (eBiosciences, San Diego CA). MyD88 was detected with a rabbit antibody (Abcam, Cambridge, MA) followed by horseradish peroxidase-conjugated antibody to rabbit IgG (Invitrogen, Camarillo CA) and TSA Plus-TMR Reagent (Perkin-Elmer, Waltham MA). All antibodies were diluted in Blocking Reagent (Perkin-Elmer). Sections were mounted with VectaShield (Vector Laboratories, Burlingame CA) containing DAPI to visualize nuclei. Stained tissue sections were imaged on a Zeiss Axiophot confocal microscope (Carl Zeiss Microscopy LLC, Thornwood, NY) with Axiovision image software.

Statistical Analysis

Statistical differences in disease parameters mRNA levels, CFU, and disease activity were determined by analysis of variance and Fisher's protected least significant difference test. Correlation Z test was used to test for correlations among mRNA levels for different biomarkers. Factor analysis was used to reduce the 5 mRNA biomarkers into two principal components (PCs), as previously described¹⁰⁴. To assign scores for PC1 and PC2, the mRNA level for each of the five biomarkers for each mouse was first normalized to the mean for that biomarker for all mice within the DSS protocol or the T cell transfer protocol. Individual scores for PC1 and PC2 were calculated as the sum of the normalized value for each biomarker multiplied by the weight for that biomarker. Linear regression analysis was used to compare PC1 or PC2 values with % initial weight, colon length or the ratio of colon weight/length, and histological score. For MyD88 ^{Δ IEC} and MyD88Flox mice with or without DSS treatment, nineteen genes from a total of 36 were selected for PCA, based on ranking of P values for the comparison of mRNA

transcript levels in MyD88^{ΔIEC} and MyD88^{Flox} mice, with and without DSS treatment (n = 31) (see **Tables 5-2, 5-3 and 5-4**). Factor analysis by the principal component method³ detected a significant relationship among the 19 transcripts ($\chi^2 = 816.7$, $p < 0.0001$), and reduced the data to 4 principal components (PCs) (**Figure 5-14**). PC1 and PC2 together accounted for 68.8% of the overall variance in gene expression, whereas PC3 and PC4 only contributed minimally (variance proportion < 0.1). Scores for PC1 and PC2 for each individual mouse were calculated as the sum of normalized expression levels for each gene multiplied by the corresponding coefficient for PC1 and PC2. A one-tailed Student's *t* test was used to compare TER values for colon tissues. Permutational multivariate ANOVA was utilized to compare the fecal microbiota between mouse genotypes, based on distance matrices of OTUs. All statistical analyses were performed using StatView Software (SAS Institute, Cary, NC).

Cell Culture

The HT-29v20 subclone of the human colon adenocarcinoma cell line HT-29 was cultured as described.¹⁴ Where indicated, cells were stimulated with human recombinant TNF (R & D Systems, Minneapolis, MN) at 10 ng/ml, purified *Escherichia coli* O26:B6 LPS (Sigma-Aldrich, St Louis, MO) at 1 μg/ml, pIC (Sigma-Aldrich) at 100 μg/ml. The human acute myeloid leukemia cell line THP-1 (Tsuchiya et al., 1980) (a generous gift of Dr. Sarah D'Orazio, University of Kentucky) were cultured in DMEM Ham's F-12 (1:1) media supplemented with 5% fetal bovine serum and antibiotics (100 U/ml penicillin 100 ng/ml fungizone) at 37°C and 5% CO₂. All cell culture reagents were from Lonza Walkersville (Walkersville, MD) or Invitrogen Life Technologies (Carlsbad, CA). THP-1

cells were maintained in suspension culture, and were diluted to a density of 1×10^6 cells/well in 24-well dishes immediately before stimulation. Additionally, HT-29 and THP-1 cells were treated with the following TLR ligands at a final concentration of 1 $\mu\text{g/ml}$: N-palmitoyl-S-[2,3 bis(palmitoyloxy)- propyl]-Cys-Ser-Lys trihydrochloride (Pam3CSK4) (InvivoGen, San Diego, CA), a synthetic tripalmitoylated lipopeptide that acts as a ligand for TLR2; or highly purified lipopolysaccharide (LPS) from *E. coli* O26:B6 (Sigma-Aldrich, St. Louis, MO), which acts as ligand for TLR4.

To knockdown MyD88, HT-29 cells were transfected with SureSilencingTM plasmids encoding shRNA specific for human MyD88 or an irrelevant control shRNA (SABiosciences, Frederick, MD, cat no. KH00911P), and puromycin-resistant subclones were isolated as described¹⁰⁵. For RelA or RelB knockdown experiments, HT-29 cells were previously transfected with the pSIREN-RetroQ expression plasmid (BD Biosciences, San Jose, CA) encoding RelA, RelB, or an irrelevant control siRNA, selected for stable integration of the plasmid by resistance to puromycin, and cloned by limiting dilution. The sequence of the RelB siRNA was previously reported. 26 The sequence of the RelA siRNA (5'–3') was

GATCCGATCAATGGCTACACAGGATTCAAGAGATCCTGTGTAGCCATTGATC
TTTTTTTG and the sequence of the control siRNA (5'–3') was GATCCGCGGGCTG
ATGCTGCACCAATTCAAGAGATTGGTGCAGCATCAGCCCGTTTTTTTG.

Bacterial Culture from Mouse Feces and Tissues

Fecal pellets were gently released from dissected colons and homogenized in PBS. Luminal washes were collected by flushing colons with 5 ml of PBS, and mucus

was collected by gentle scraping. Aliquots of fecal homogenates, luminal washes and mucus were cultured for 48 h on LB agar at 37° under anaerobic conditions. For analysis of bacterial translocation, MLNs were homogenized in PBS and cultured anaerobically for 48 h. *E. coli* strain Nissle 1917 (a generous gift of Dr. Ulrich Sonnenborn, Ardeypharm GmbH, Herdecke, Germany) was transformed to kanamycin resistance, and Kan^R *E. coli* were delivered to the mice in the drinking water at a dose of 2.5 x 10⁷ CFU/ml for 7 days. Feces, luminal washes and colonic mucus were collected as described above and cultured anaerobically in 50 µg/ml kanamycin to enumerate CFU.

Measurement of Transmucosal Electrical Resistance (TER)

Segments of mouse colon were mounted in 0.3 cm² aperture Ussing chambers (Physiologic Instruments, San Diego, CA), and TER was measured as previously described¹⁰⁶. Apical and basolateral recording buffers contained 118 mM NaCl, 5 mM KCl, 2 mM NaH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, 10mM HEPES, 10mM glucose, and 22 mM NaHCO₃, pH 7.4, and were bubbled with 95% O₂ and 5% CO₂ at 37 °C.

Analysis of Mucus-Associated Antimicrobial Activity

Colonic mucus was isolated as described above, diluted 1:3 in PBS, and concentrated by centrifugation at 2500 rpm for 10 min. 10⁶ CFU of Kan^R *E. coli* from a mid-log phase culture was added to the mucus, then centrifuged together at 1500 rpm for 15 min at room temperature. Serial dilutions of bacterial suspensions before and after incubation with colonic mucus were cultured anaerobically for 24 h in 50 µg/ml kanamycin to enumerate CFU.

Bacterial Invasion Assays

Binding and invasion of HT-29 cells was assessed for *E. coli* (strain Nissle 1917), *Klebsiella pneumoniae* (strain isolated from MLNs of MyD88^{ΔIEC} mice) and *Salmonella typhimurium* (strain SL1344) as described¹⁰⁷. Approximately 10⁶ CFU were added to monolayers of HT-29 cells at an MOI of 10:1. After 2 h at 37°, cell monolayers were washed and lysed with 1% Triton X-100 to enumerate total cell-associated bacteria. Parallel co-cultures of bacteria and HT-29 cells were treated with gentamicin (100 μg/ml) for 90 min to kill extracellular bacteria, washed and cultured to enumerate intracellular bacteria. Membrane-associated bacteria were calculated by subtracting internalized CFU from total cell-associated CFU.

Analysis of the Fecal Microbiota

Fecal samples were collected from littermate MyD88^{Flox} and littermate MyD88^{ΔIEC} mice, and frozen samples of fecal DNA (extracted with the QIAamp DNA stool kit, Qiagen, Germantown, MD) were shipped to Second Genome, Inc. (San Bruno, CA) for analysis of microbial richness and diversity by the PhyloChipTM microarray method¹⁰⁸ (www.secondgenome.com). Bacterial 16S rRNA genes were amplified by PCR using the forward primer 5'-AGRGTTCGATCMTGGCTCAG-3' and reverse primer 5'-GGTACCTTGTTACGACTT-3', fragmented, biotin labeled, and hybridized to the PhyloChipTM Array, version G3. Stained arrays were scanned with a GeneArray® scanner (Affymetrix), and analyzed by Affymetrix software (GeneChip® Microarray Analysis Suite). All profiles are inter-compared in a pair-wise fashion to determine a

UniFrac distance metric, which utilizes the phylogenetic distance between OTUs to determine the dissimilarity between communities¹⁰⁹. For weighted UniFrac, (WUniFrac), both the abundance and dissimilarities among OTUs were considered. The phylogenetic tree shown in **(Figure 5-9)** was generated using the “Interactive Tree of Life” software tool¹¹⁰. Based on 14,193 OTUs present in at least one of the mice, a Welch *t*-test was performed across the two genotypes using abundance metrics. Principal Component Analysis (PCA) was used to position points on two-dimensional ordination plots based on dissimilarity values **(Figure 5-10A , left panel)**. Significant OTUs whose abundance characterizes each mouse genotype **(Figure 5-10A, right panel)** were identified by Prediction Analysis for Microarrays, which utilizes a nearest shrunken centroid method¹¹¹.

Analysis of fecal IgA and albumin by ELISA

Freshly collected feces were diluted in 50 mM Tris, pH 7.4, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, and analyzed by ELISA as previously described¹¹ using mouse IgA (Cat. No. E90-103) and mouse albumin (Cat. No. E90-134) ELISA Quantification Sets (Bethyl Laboratories, Montgomery TX).

Quantification of Bacteria by qRT-PCR

Fecal homogenates, luminal washes and mucus were collected as described above. Bacterial DNA was extracted by column purification using the QIAamp DNA stool mini kit (Qiagen Cat. No. 51504), and the 16S rRNA gene was amplified by real-time PCR using universal primers as described¹¹². To quantify total numbers of bacteria,

C_T values from individual samples were compared to regression lines of C_T values generated using defined quantities of bacterial DNA from representative species from the four major phyla of colonic bacteria.

Identification of *K. pneumoniae* in mesenteric lymph nodes

Mesenteric lymph nodes (MLNs) were dissected under sterile conditions, homogenized in sterile PBS, and cultured for 48 h on LB agar at 37° under anaerobic conditions. Approximately 25% of the bacterial colonies had a large, mucoid morphology. To identify these bacteria, 12 colonies derived from two different MyD88^{ΔIEC} mice were harvested and cultured overnight in LB broth at 37° under anaerobic conditions. Bacterial DNA was extracted by column purification, and the 16S rRNA gene was amplified by real-time PCR using universal primers¹¹². The PCR products were ligated into the pGEM-T vector (Promega, Madison, WI), then used to transform frozen competent *E. coli* DH5α (Promega) to ampicillin resistance using standard protocols. Two ampicillin-resistant colonies were harvested and cultured in LB broth containing 100μg/ml ampicillin. Bacterial DNA was purified using the Qiagen miniprep kit (Qiagen, Germantown, MD), and shipped to ACGT, Inc. (Wheeling, IL) for sequence analysis. Identical sequences of the unknown 16S rRNA gene (which were identical for the 2 colonies analyzed; **Figure 5-4A**) were compared with the sequences of known bacterial genomes by BLAST analysis of the National Center for Biotechnology Information database (<http://blast.ncbi.nlm.nih.gov/Blast>). The highest alignment scores were observed with 16S rRNA genes from 5 strains of *Klebsiella pneumoniae*, and slightly lower scores were observed with 16SrRNA genes from other species in the

family *Enterobacteriaceae*, including *Escherichia coli* (**Figure 5-4B**). The original bacterial colonies cultured from the MLNs of MyD88^{ΔIEC} mice were positively identified as *K. pneumoniae* by biochemical tests using differentiation media², including Indole, Simmons citrate and Christensen's urea agar, according to the manufacturer's protocol (Remel, Lenexa, KS) (**Figure 5-4C**).

Chapter Three: Multifactorial Patterns of Gene Expression in Colonic Epithelial Cells Predict Disease Phenotypes in Experimental Colitis

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are thought to result from inappropriate inflammatory responses to normal components of the intestinal microbiota in genetically susceptible individuals¹¹³⁻¹¹⁵. Recent evidence has implicated both ineffective innate immune responses and overactive adaptive immune responses in the pathogenesis of IBD^{63-68,113,114,116}. Colonic epithelial cells (CEC) bridge innate and adaptive immune responses through cross-talk with luminal bacteria and with lamina propria immune cells^{65,113,114}. There is a critical need to identify cellular and molecular biomarkers that can predict disease behavior and guide personalized therapy, and a number of candidates have been proposed⁸⁹⁻⁹³. Considering the complex etiology and the heterogeneous clinical presentation of IBD, we propose that a multifactorial approach offers significant advantages over the use of single biomarkers for diagnosis and treatment of IBD.

Our group recently identified signature biomarkers in the colonic mucosa of CD patients that could be used in a multifactorial analysis to classify patients into molecular phenotypic subsets that were predictive of clinical characteristics and responses to therapy⁶⁰. These biomarkers comprised 5 genes: the RelA subunit of NF- κ B; A20, an ubiquitin-modifying enzyme that negatively regulates the NF- κ B activation pathway; the polymeric immunoglobulin receptor (pIgR), which mediates epithelial transcytosis of IgA antibodies; the pro-inflammatory cytokine tumor necrosis factor (TNF), and the pro-

inflammatory chemokine interleukin (IL)-8. The aim of the present study was to define patterns of biomarker gene expression that reflect the underlying mechanism and severity of intestinal inflammation. To this end we utilized two mouse models of colitis with very different etiologies. Oral administration of dextran sulfate sodium (DSS) induces acute colitis that is mediated primarily by effectors of innate immunity and causes damage to the epithelial barrier¹¹⁷. In contrast, adoptive transfer of naïve T cells into immunodeficient mice causes chronic colitis initiated by effector T cells and characterized by epithelial hyperplasia, goblet cell depletion and transmural inflammation^{99,118-120}. We found different patterns of biomarker expression in these two models of experimental colitis that were predictive of disease severity. Importantly, down-regulation of expression and altered cellular localization of pIgR was a common feature of both colitis models. Furthermore, these defects were associated with failure to transport IgA. These results provide insights into the functions of CEC in regulation of intestinal inflammation, and provide proof of principle for the use of principal component analysis in the molecular diagnosis of intestinal inflammation.

Results

Assessment of disease severity in DSS and T cell-mediated colitis

Acute colitis induced by oral administration of DSS resulted in significant weight loss, disease activity (diarrhea and blood loss) and pathological findings (colon shortening), with more severe symptoms in mice administered 3% DSS than 2% DSS (**Figure 3-2 A, B**). Tissue damage, including edema, goblet cell loss, ulcerations, epithelial cell destruction, and infiltration of inflammatory cells, was observed in all

DSS-treated mice and was more severe in mice treated with 3% DSS (**Figure 3-2 C**). Chronic colitis induced by adoptive transfer of CD4⁺CD45RB^{hi}CD25⁻ Teff cells (**Figure 3-1A**) into *Rag1*^{-/-} recipients resulted in a different clinical picture involving weight loss and an increased ratio of colon weight/length (**Figure 3-2 D,E**), but no symptoms of acute colitis such as diarrhea or blood loss (data not shown). Co-administration of CD4⁺CD45RB^{lo}CD25⁺ Treg with Teff corrected the clinical symptoms of T cell-mediated colitis. Interestingly, colon tissues from control *Rag1*^{-/-} mice exhibited a significantly shorter epithelial crypt length than did wild-type mice (compare **Figure 3-2 B and 2E**) suggesting that the absence of B and T lymphocytes impaired normal development of the colonic epithelium. Adoptive transfer of Teff, with or without Treg, resulted in extensive crypt lengthening (**Figure 3-2 F**). Histological evidence of Teff-mediated colitis included infiltration of lymphocytes and mononuclear cells into both the mucosa and submucosa, but fewer neutrophils. Some mucosal edema and epithelial damage was observed, but this was less severe than in acute DSS-mediated colitis mice (compare **Figure 3-2 C and F**). Co-administration of Treg with Teff resulted in a moderate reduction in the inflammatory infiltrate and lessened epithelial damage and mucosal edema.

Differential expression of “signature” biomarkers in colonic epithelial cells in acute DSS-mediated and chronic T cell-mediated colitis

We used these different models of experimental colitis to examine the relationship between the etiology of intestinal inflammation and gene expression in colonic ECs. Flow cytometry analysis of representative CEC preparations indicated that contamination

with CD45⁺ hematopoietic cells was on average less than 1% (**Figure 3-1B**). RNA was extracted from isolated ECs, and levels of mRNA were analyzed for 5 “signature” biomarkers that we have used to classify IBD patients into molecular phenotypic subsets⁶⁰, i.e., RelA, A20, pIgR, TNF and MIP-2 (a functional homolog of human IL-8) (**Figure 3-3**). While the overall patterns of biomarker expression varied considerably between DSS and T cell-mediated colitis, the one common feature was down-regulation of pIgR mRNA. Interestingly, co-administration of Treg with Teff prevented down-regulation of pIgR. These data are consistent with the notion that mucosal inflammation, independent of its cause, is sufficient to impair normal expression of pIgR. By contrast, down-regulation of the RelA subunit of NF-κB was a more consistent feature of T cell-mediated colitis than DSS colitis. Down-regulation of RelA in mice adoptively transferred with Teff was correlated with reduced expression of A20, a RelA target gene that is induced by the classical NF-κB pathway and acts as a negative feedback regulator of NF-κB activation¹²¹. Co-administration of Treg with Teff did not restore RelA mRNA to control levels, but was sufficient to prevent down-regulation of A20. Finally, acute DSS colitis was characterized by strong up-regulation of the pro-inflammatory genes TNF and MIP-2 in CEC, which was not seen in chronic T cell-mediated colitis.

Multifactorial patterns of biomarker expression predict disease phenotype

It is clear that synergistic interactions among multiple gene products are involved in inflammatory responses, thus we used a multifactorial approach to identify patterns of gene expression that are associated with different etiologies of colitis. Factor analysis by the principal component (PC) method is a statistical approach that is used to reduce the

number of variables in a complex data set and to classify relationships between those variables. We previously used PC analysis, based on the 5 “signature” biomarkers described in Figure 2, to classify CD patients into molecular phenotypic subsets that were predictive of clinical disease⁶⁰. Here we demonstrate different patterns of gene expression associated with DSS and T cell-mediated experimental colitis in mice (**Figure 3-4**). Chi-square analysis indicated that variability in the gene expression data sets for both DSS colitis and T cell transfer colitis could be reduced to two PCs ($p < 0.0001$) (**Figure 3-4A**). Eigen values indicated that the proportion of variance among mice in expression of these 5 variables that could be described by PC1 and PC2 together was 72.5% for DSS colitis and 75.1% for T cell transfer colitis. The coefficients for each variable describe its relative contribution to PC1 and PC2. For both DSS colitis and T cell transfer colitis, PC1 was strongly weighted toward RelA, A20 and pIgR, with a negative weight for MIP-2. Interestingly, TNF had a minimal contribution to PC1 for DSS colitis, but contributed strongly to PC1 for T cell transfer colitis. PC2 was strongly weighted toward TNF and MIP-2 for both DSS colitis and T cell colitis. The relative contributions of RelA and A20 to PC2 differed for the two models, and pIgR did not contribute significantly to PC2 for either model. We conclude that the different patterns of epithelial gene expression reflect the different etiologies of DSS and T cell-mediated colitis and may be useful for predicting disease phenotype. To this end, each mouse was assigned a score for PC1 and PC2 based on the sum of the weighted expression levels for all 5 biomarkers (see Materials and Methods for details) (**Figure 3-4B**). For the DSS model, PC1 scores did not vary significantly among treatment groups, whereas the score for PC2 increased significantly with increasing DSS dose. Interestingly, the opposite pattern held

for T cell transfer colitis. Adoptive transfer with Teff resulted in a significantly lower PC1 score, which was prevented by co-administration of Treg, whereas no significant differences in PC2 scores were observed.

To investigate whether these multifactorial PCs could predict disease severity, we performed linear regression analysis to examine the relationship between PC1 and PC2 scores and clinical measures of disease (**Figure 3-5**). In the DSS colitis model, increased PC2 scores were significantly correlated with weight loss, decreased colon length (a measure of disease severity in DSS colitis),¹²² and tissue damage (quantified by histological score). A different pattern was observed for the T cell transfer colitis model, in which decreased PC1 scores were significantly correlated with weight loss and an increased ratio of colonic weight/length (a measure of disease severity in T cell transfer colitis).^{99,118} However, tissue damage in this model was significantly correlated with increased PC2 scores, similar to the pattern seen in DSS-induced colitis. Taken together, these results suggest that a multifactorial approach combining the expression of multiple biomarkers in colonic EC can be used to discriminate among different mechanisms of inflammation and to predict disease severity.

Colonic inflammation results in altered localization of pIgR and reduced epithelial transcytosis of IgA

We previously reported that expression of pIgR and epithelial transcytosis of IgA were dysregulated in the colonic mucosa of Crohn's disease patients⁶⁰. Because down-regulation of pIgR mRNA in CEC was a common feature of both DSS and T cell-mediated colitis (**Figure 3-3A, B**), we hypothesized that epithelial transcytosis of IgA

would also be altered. Visualization of pIgR and IgA in sections of colonic tissue revealed reduced expression and aberrant localization of pIgR in the colonic epithelium in both DSS-induced (**Figure 3-6A**) and T cell-mediated colitis (**Figure 3-6B**). In untreated wild-type mice (**Figure 3-6A**, left panel), pIgR expression was abundant and predominantly localized to the lower two-thirds of the epithelial crypts. Significant co-localization of pIgR and IgA was observed, consistent with normal transcytosis of IgA. However, in the colons of DSS-treated mice, pIgR staining was clearly reduced, consistent with reduced pIgR mRNA levels (**Figure 3-3A**), and was not concentrated in the lower section of the crypts. Importantly, we also observed less co-localization of IgA and pIgR in DSS-treated mice, although the presence of IgA-producing plasma cells in the lamina propria was increased. Similar to wild-type mice, pIgR was predominantly localized to the lower two-thirds of the epithelial crypts in control *Rag1*^{-/-} mice (**Figure 3-6B**). No IgA was detected in the colons of these mice, since they lack B cells and cannot produce IgA. Total pIgR staining was markedly reduced in the *Rag1*^{-/-} mice adoptively transferred with Teff, consistent with down-regulation of pIgR mRNA (**Figure 3-3B**), and concentration of pIgR in the lower section of the crypts was not seen. Expression of pIgR was substantially restored when Treg cells were co-transferred with Teff (**Figure 3-6B**), consistent with the restoration of pIgR mRNA levels (**Figure 3-3B**). However, concentration of pIgR localization in the lower section of the crypts was not fully restored.

Discussion

Molecular classification of IBD patients based on multifactorial patterns of biomarker expression offers the potential to personalize diagnosis and clinical management of this chronic and debilitating disease. Experimental mouse models of IBD have proven to be valuable tools for investigating mechanisms of IBD pathogenesis and for identifying potential therapeutic targets^{117,123}. Oral administration of DSS causes concentration- and time-dependent cytotoxicity of intestinal epithelial cells, thus disrupting intestinal barrier function¹²⁴. Because acute colitis could be induced by DSS in mice with severe combined immunodeficiency, which lack B and T lymphocytes¹²⁵, it has long been thought that intestinal inflammation in this model is driven mainly by innate immune responses. However, more recent studies comparing DSS-induced colitis in immunodeficient and wild-type mice suggested that the presence of lymphocytes may exacerbate inflammation, particularly at low doses of DSS¹²⁶. In the T cell transfer model, chronic colitis develops as a result of enteric antigen-driven activation, polarization and homeostatic expansion of naïve T cells to produce colitogenic Th1 and/or Th17 effector cells^{117, 118}. Co-administration of T regulatory cells with T effector cells prevents induction of colitis, verifying a significant protective role for T regulatory cells in IBD pathogenesis^{99,127}.

Here we have focused on a limited set of 5 “signature” epithelial biomarkers that we previously validated for prediction of disease severity in CD patients⁶⁰. Given the heterogeneous clinical presentation in IBD, it is likely that a classification scheme based on multiple biomarkers will be more informative than the use of individual biomarkers. It is clear that the expression of many genes may change during intestinal inflammation.

However, by focusing on only 5 highly informative genes, we have provided proof of principle that a multifactorial approach can transform a limited amount of data into a potentially powerful diagnostic tool. We have now identified distinctive expression patterns of these 5 biomarkers expression in two models of experimental colitis with different etiologies (**Figure 3-3**). Principal component analysis reduced the 5 biomarkers into 2 multifactorial PCs that were specific for each model (**Figure 3-4**) and were predictive of disease severity (**Figure 3-5**). We propose that PC1 is a composite biomarker reflecting homeostatic epithelial barrier function, characterized by NF- κ B-stimulated physiological inflammation. The individual biomarkers RelA, A20 and pIgR were dominant contributors to PC1 in both colitis models. We previously reported that high expression of PC1 was associated with mild disease activity and good responses to therapy in CD patients⁶⁰. We found that high expression of pIgR was correlated with robust epithelial transcytosis of IgA. While excessive NF- κ B signaling in immune cells has been implicated as a major contributor to intestinal inflammation and the development of IBD, NF- κ B signaling in epithelial cells has been shown to promote intestinal homeostasis¹²⁸⁻¹³⁰. Our finding of a potential protective role for NF- κ B signaling is consistent with the report of Eckmann *et al.*³³ in which ablation of the NF- κ B-activating kinase IKK β in intestinal epithelial cells resulted in an exacerbation of DSS-induced colitis. However, these investigators found that loss of epithelial IKK β had no impact on chronic colitis that occurs spontaneously in IL-10-deficient mice. Thus the protective functions of epithelial-specific NF- κ B signaling likely depend on specific etiology of intestinal inflammation. We found that epithelial expression of the pro-inflammatory genes TNF and IL-8 (or mouse MIP-2, a functional homolog of human IL-

8) contributed strongly to PC2. While a normal range of PC2 values is consistent with NF- κ B-stimulated physiological inflammation, high PC2 values may indicate an excessive and damaging inflammatory response. Here we found that disease severity in acute DSS colitis was correlated with elevated PC2 values, with no significant alterations in PC1 values. These results are consistent with our previous findings in CD patients, where a subset of recently-diagnosed patients with elevated PC2 values and variable PC1 values tended to present with acute inflammation that responded well to immunosuppressive and anti-TNF therapy⁶⁰. In a recent analysis of these biomarkers in colonic biopsies of patients with UC, we observed a similar correlation between elevated PC2 values and acute inflammation (Bruno *et al.*, unpublished data). These findings are significant in that many features of DSS colitis, such as macroscopic appearance and histological injury, more closely resemble acute inflammation in UC than CD¹³¹. In contrast, we found that some measures of disease severity in chronic T cell-mediated colitis (weight loss, colon weight/length ratio) were correlated with low PC1 values, whereas tissue damage in the colon was associated with high PC2 values. This pattern is similar to that observed in subsets of CD and UC patients with low PC1 and variable PC2 values, characterized by chronic inflammation and poor responses to immunosuppressive and anti-TNF therapy⁶⁰ (Bruno *et al.*, unpublished data). The results of our current study suggest that acute colitis mediated by innate immune responses is associated with increased epithelial expression of pro-inflammatory genes, whereas chronic colitis driven by adaptive T cell responses is associated with diminished expression of genes associated with homeostatic epithelial barrier function. These findings provide insights into the role of epithelial cells in the regulation of colonic inflammation, and support the use of these

epithelial biomarkers for monitoring disease course and guiding therapy in IBD patients.

We propose that multifactorial analysis of CEC gene expression may be valuable for monitoring the transition from acute to chronic colitis in IBD patients. This approach may enhance our understanding of immunological mechanisms underlying inflammation at different stages, which may require different therapeutic approaches, and may also help to identify patients at risk for sequelae associated with chronic inflammation, such as colitis-associated cancer.

A novel finding that emerged from this study was that expression of pIgR in CEC was down-regulated in both acute DSS and chronic T cell colitis (**Figure 3-3**). Furthermore, DSS-induced inflammation was associated with reduced epithelial transcytosis of IgA (**Figure 3-6A**). These results were consistent with our findings in CD patients with either acute or chronic colitis, in which expression of pIgR and transcytosis of IgA in CEC was reduced compared to normal individuals or CD patients with mild disease⁶⁰. The importance of pIgR in prevention of intestinal inflammation is supported by the report that pIgR-deficient mice were more susceptible to DSS-induced colitis than were wild-type mice⁵⁸. pIgR transports polymeric IgA from the basal to the apical surface of intestinal epithelial cells, where proteolytic cleavage of the extracellular domain of pIgR (secretory component, SC) releases secretory IgA (SIgA) into the intestinal lumen^{44,132}. Because one molecule of pIgR is synthesized for every molecule of IgA transported, the level of pIgR expression controls the rate of epithelial transcytosis of IgA. SIgA antibodies play a critical role in shaping the composition of the gut microbiota and in maintenance of intestinal homeostasis⁴⁵. SIgA binds to intestinal mucus via glycan-mediated interactions, thus forming an immunological barrier to penetration of the

epithelial layer by microbes and environmental antigens, and can also neutralize antigens and microbial products intracellularly during pIgR-mediated epithelial transcytosis. In addition, pIgR can transport IgA-containing immune complexes across polarized IEC monolayers, thus disposing of antigens through a non-inflammatory process¹³³. Thus, reduction of pIgR expression during colitis would be predicted to diminish not only the antimicrobial function of luminal SIgA, but also removal of IgA-bound antigens from the lamina propria. These defects would exacerbate local inflammation and increase the level of IgA immune complexes, a potential risk factor for development of IgA nephropathy, which has been linked with IBD⁸⁸.

It should be noted that a protective role for pIgR in the T cell transfer model of colitis could not involve transcytosis of SIgA, since B cell-deficient recipient *Rag1*^{-/-} mice do not produce IgA. However, it is well known that free SC, which is released when pIgR that is not associated with IgA is cleaved at the apical surface of epithelial cells, contributes to intestinal homeostasis by inhibiting bacterial adherence to the intestinal mucus layer and neutralizing of potential pro-inflammatory factors^{54,134}. We found that expression of pIgR mRNA was reduced (Fig. 2B) and the cellular localization of pIgR protein was aberrant (Fig. 5B) in *Rag1*^{-/-} mice adoptively transferred with Teff. Importantly, co-administration of Treg prevented both of these defects. Taken together, our results suggest that inflammation in the colon, whether due to innate immune responses in the DSS model or adaptive immune responses in the T cell model, creates a local environment that is not conducive to optimal expression and cellular trafficking of pIgR.

We found that epithelial expression of TNF was significantly increased in acute DSS colitis (Fig. 2A) and contributed strongly to pro-inflammatory PC2, but not to homeostatic PC1 values (**Figure 3-4A**). In contrast, altered epithelial expression of TNF was not a feature of T cell-mediated chronic colitis (**Figure 3-3B**) and contributed to both PC1 and PC2 values (**Figure 3-4A**). Although TNF expression was not elevated in CEC of mice with T cell-mediated colitis, we cannot rule out the possibility that production of TNF by other cell types contributes to intestinal inflammation in this model. We previously reported that expression of TNF in mucosal biopsies (which contain stromal and bone marrow-derived cells in addition to epithelial cells) was actually reduced in a large subset of CD patients with longstanding, chronic inflammation⁶⁰. We recently reported that stimulation with TNF enhances expression of pIgR through a RelA-dependent mechanism in a human CEC line¹³⁵, suggesting that physiological production of TNF in the colonic epithelium may contribute to intestinal homeostasis by promoting pIgR-mediated transcytosis of SIgA and innate immune functions of SC. However, failure to regulate TNF production clearly contributes to the pathogenesis of IBD. Biological therapies designed to neutralize TNF have been extremely useful in managing severe colitis¹³⁶, and a recent report suggests that anti-TNF therapy may promote the activation and expansion of T regulatory cells in CD patients¹³⁷. However, neutralization of TNF can be associated with significant adverse effects, including exacerbation of disease, serious infection and malignancies. Furthermore, anti-TNF therapies are only effective in about half of patients, and there are as yet no clear clinical guidelines regarding which anti-TNF drug to use first or how to manage patients who experience recurrence of symptoms¹³⁸. Our findings in CD patients and in experimental models of

colitis suggest that monitoring TNF expression in the colonic mucosa may be useful for understanding disease mechanisms and identifying those patients most likely to benefit from anti-TNF therapy.

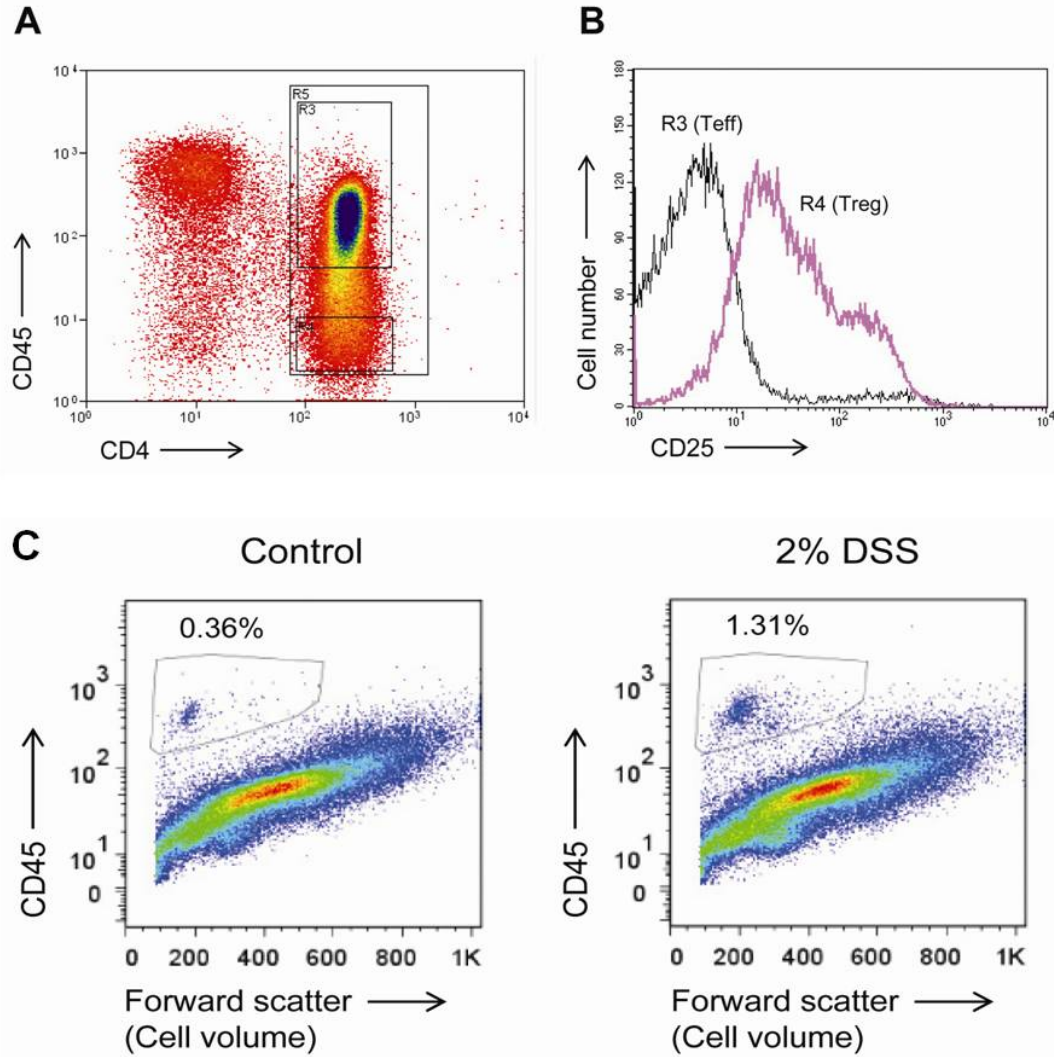


Figure 3-1 Purification of T cells and colonic epithelial cells (CECs). T effector and T regulatory cell populations for T cell transfer model of experimental colitis. **(A)** Single cell suspensions of spleens from wild-type mice were separated into CD4⁺CD45RB^{hi} (fraction R3) and CD4⁺CD45RB^{lo} fractions (fraction R4) by fluorescence-activated cell sorting. **(B)** The histogram shows intensity of cell surface CD25 staining in the CD4⁺CD45RB^{hi} (black) and CD4⁺CD45RB^{lo} (purple) fractions. **(C)** Purity of colonic epithelial cells (CEC). Isolated CECs were analyzed for cell surface CD45 by flow

Figure 3-1 (continued) cytometry. The major peak in each plot represents ECs, and the gated area represents contaminating small CD45^{hi} hematopoietic cells. (Frantz *et al.* Multifactorial Patterns of Gene Expression in Colonic Epithelial Cells Predict Disease Phenotypes in Experimental Colitis. *Inflammatory Bowel Disease in press* 2012.)
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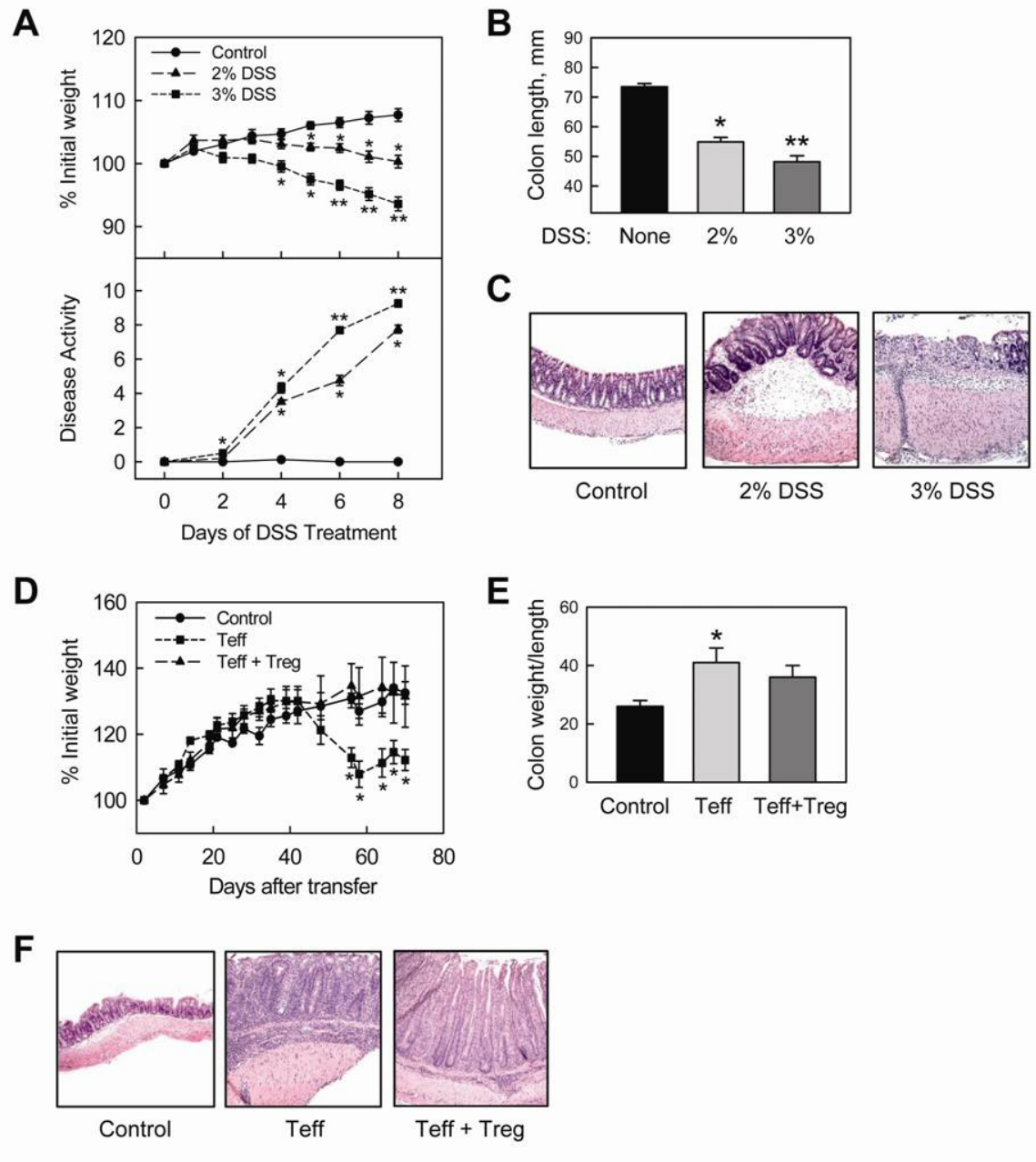


Figure 3-2 Assessment of disease severity in DSS and T cell transfer colitis. DSS

colitis. Mice were left untreated or administered 2% or 3% DSS in the drinking water for 8 days. (A) Changes in body weight (expressed as a percentage of weight at the beginning of the experiment) and disease activity (see Materials and Methods for an

Figure 3-2 (continued) explanation of the disease activity index). Data from 1 of 3 independent experiments with similar results are expressed as mean \pm SEM (n = 8). **(B)** Colon lengths were measured on day 8; data combined from 3 independent experiments are expressed as mean \pm SEM (n = 16). **(A, B)** One asterisk indicates that the mean is significantly different from the mean for control mice, and two asterisks indicate that the mean is significantly different from the mean for both control mice and mice treated with 2% DSS (p < 0.05). **(C)** Colon histology from representative mice at day 8. Images of formalin-fixed colon tissues stained with hematoxylin and eosin were captured with a 20X objective. **(D-F)** T cell transfer colitis. *RagI*^{-/-} mice were left untreated (control) or adoptively transferred with 4 x 10⁵ CD4⁺CD45RB^{hi}CD25⁻ T-effector cells (Teff), with or without 1 x 10⁵ CD4⁺CD45RB^{lo}CD25⁺ T-regulatory cells (Treg). **(D)** Changes in body weight; data from 1 of 3 independent experiments with similar results are expressed as mean \pm SEM (n = 8). **(E)** Colons were measured and weighed at 10 weeks post-transfer; data are expressed as mean \pm SEM (n = 10). **(D, E)** An asterisk indicates that the mean is significantly different from the mean for control *RagI*^{-/-} mice (p < 0.05). **(F)** Colon histology from representative mice at 10 weeks post-transfer. Images of formalin-fixed colon tissues stained with hematoxylin and eosin were captured with a 20X objective. (Frantz *et al.* Multifactorial Patterns of Gene Expression in Colonic Epithelial Cells Predict Disease Phenotypes in Experimental Colitis. *Inflammatory Bowel Disease in press* 2012.) Reprinted with permission.

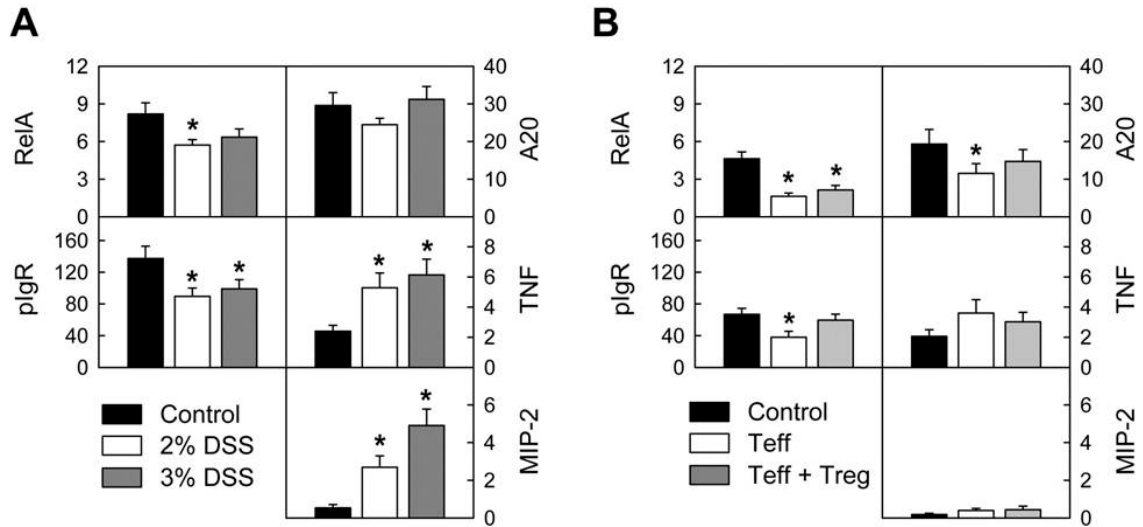


Figure 3-3 Comparison of biomarker expression in colonic ECs in experimental

colitis: (A) DSS colitis; (B) T cell transfer colitis. Colitis was induced as described in

Figure 1, and colonic ECs were isolated at the end of the experiments. mRNA levels were

measured by qRT-PCR and normalized to β 2-microglobulin mRNA. For each model,

data from 3 independent experiments were combined and expressed as mean \pm SEM (n =

16 for DSS colitis and n = 10 for T cell transfer colitis). Asterisks indicate that the mean

for treated mice is different from the mean for control mice ($p < 0.05$). (Frantz *et al.*

Multifactorial Patterns of Gene Expression in Colonic Epithelial Cells Predict Disease

Phenotypes in Experimental Colitis. *Inflammatory Bowel Disease in press* 2012.)

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A DSS colitis

Factor Analysis Summary	
Number of Variables	5
Number of Factors	2
Number of Cases	43
Degrees of Freedom	14
Bartlett's Chi Square	58.591
P-Value	<.0001
Factor Extraction Method:	Principal Components

Eigenvalues	Magnitude	Variance Proportion
PC1	2.17	0.433
PC2	1.44	0.288

Variables	Coefficients	
	PC1	PC2
RelA	0.907	0.020
A20	0.813	0.337
plgR	0.750	-0.166
TNF	0.099	0.857
MIP-2	-0.335	0.752

T cell transfer colitis

Factor Analysis Summary	
Number of Variables	5
Number of Factors	2
Number of Cases	30
Degrees of Freedom	14
Bartlett's Chi Square	62.697
P-Value	<.0001
Factor Extraction Method:	Principal Components

Eigenvalues	Magnitude	Variance Proportion
PC1	2.61	0.522
PC2	1.14	0.229

Variables	Coefficients	
	PC1	PC2
RelA	0.841	-0.239
A20	0.919	0.047
plgR	0.745	0.008
TNF	0.667	0.554
MIP-2	-0.246	0.882

B

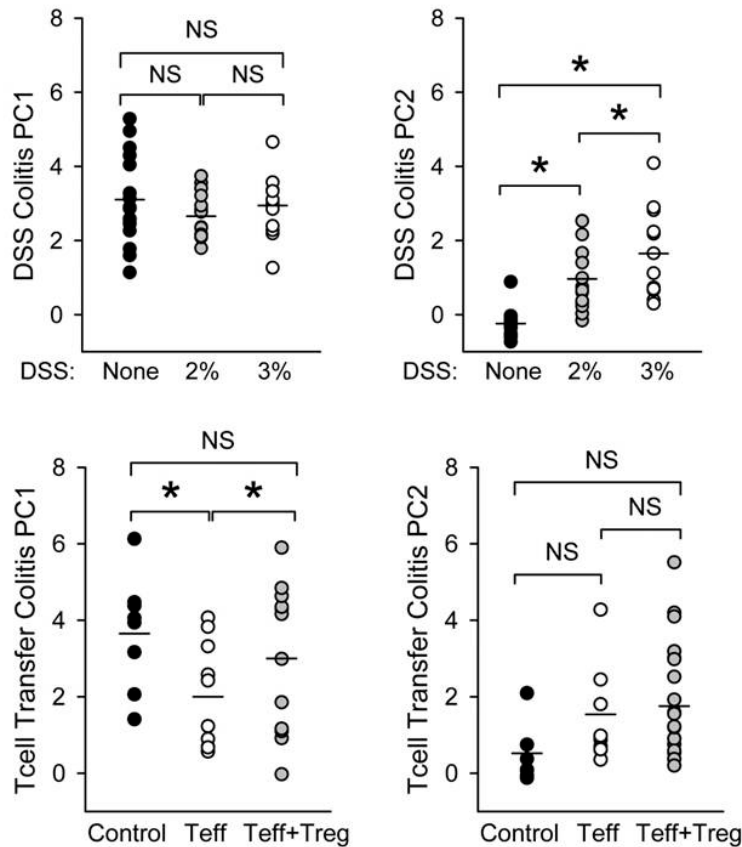


Figure 3-4 Multifactorial analysis of biomarker expression patterns in experimental colitis, using data described in Figure 2 for mRNA levels in colonic ECs. (A) Factor analysis was used to reduce data for 5 variables, NF- κ B, RelA, A20, pIgR, TNF and MIP-2, into two principal components (PCs). Eigen values indicate the proportion of variance among mice in expression of these 5 variables that can be described by PC1 and PC2. Weighted coefficients are listed for each variable comprising PC1 and PC2. **(B)** PC1 and PC2 scores were calculated for each individual mouse based on the sum of normalized expression levels for each variable multiplied by its corresponding coefficient for PC1 and PC2 (see Materials and Methods). Combined data from 3 independent experiments for each mouse model are expressed as mean \pm SEM ($n \leq 14$ mice/group for DSS colitis and $n = 10$ mice/group for T cell transfer colitis). Asterisks indicate that the mean PC values for the indicated comparisons are significantly different ($p < 0.05$); NS = not significant. *Reprinted with permission.*

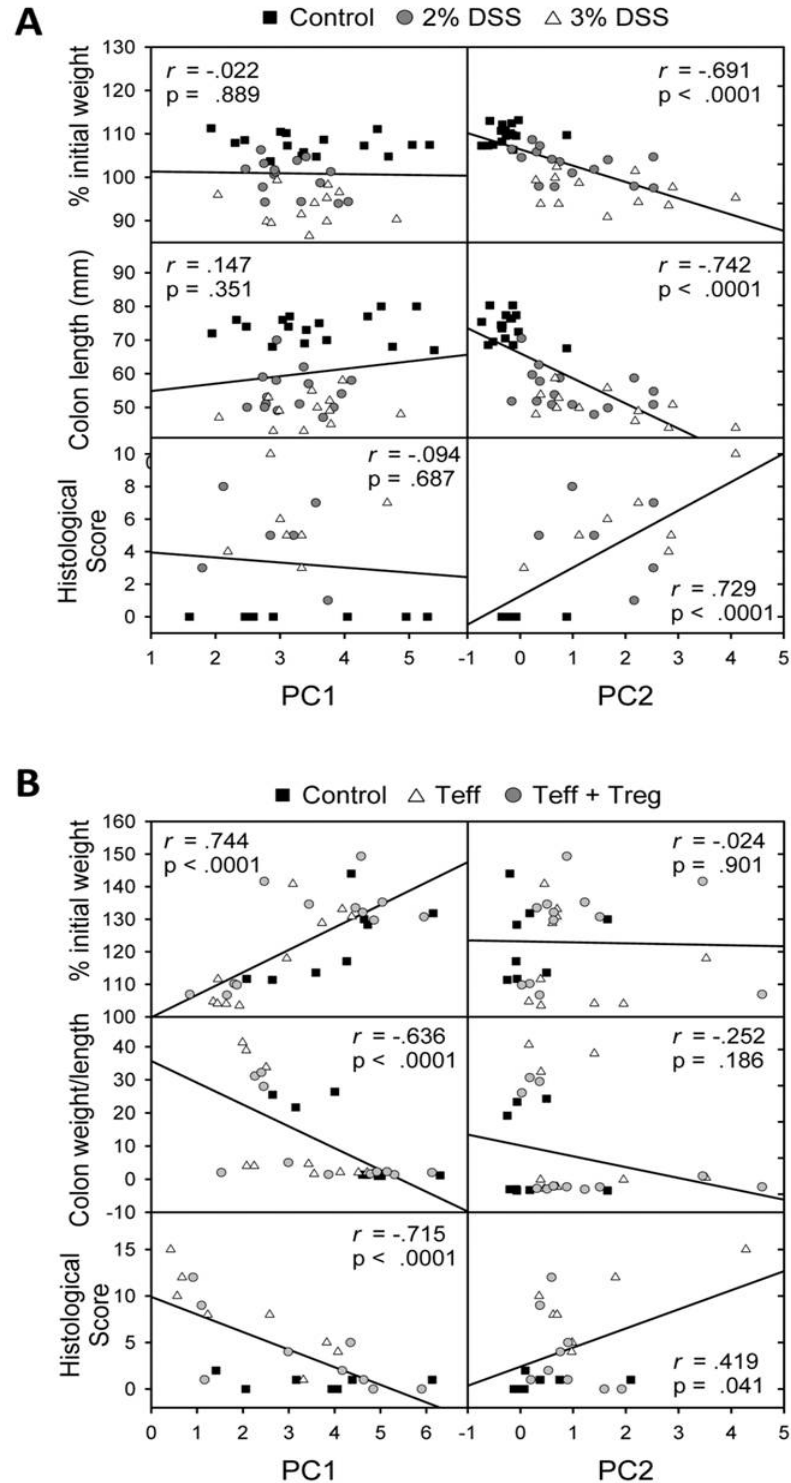


Figure 3-5 Expression of colonic epithelial biomarkers predicts disease severity in experimental colitis: (A) DSS colitis; (B) T cell transfer colitis. Plots depict linear

regression comparing PC1 and PC2 scores for each mouse to body weight at the end of

Figure 3-5 (continued) the experiment, and colon length (DSS colitis) or the ratio of colon weight to length (T cell transfer colitis). Correlation coefficients (r) and P-values (p) are listed for each regression line. (Frantz *et al.* Multifactorial Patterns of Gene Expression in Colonic Epithelial Cells Predict Disease Phenotypes in Experimental Colitis. *Inflammatory Bowel Disease in press* 2012.) *Reprinted with permission.*

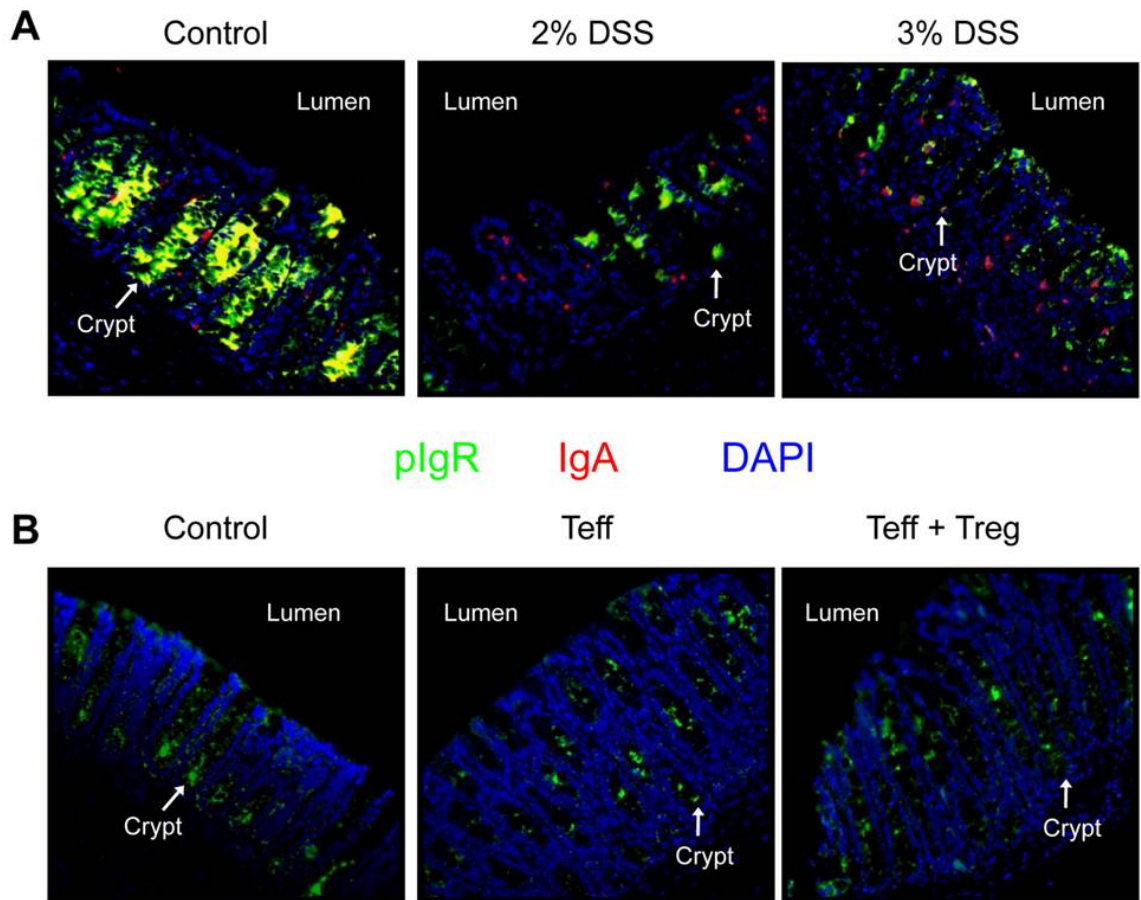


Figure 3-6 Localization of pIgR and IgA in colonic mucosa of mice with experimental colitis: (A) DSS colitis; (B) T cell transfer colitis. Sections of formalin-fixed colons were stained by immunofluorescence for pIgR (green), IgA (red) and nuclei (DAPI; blue). Representative images are shown for each treatment group. Images were captured with a 25X objective. (Frantz *et al.* Multifactorial Patterns of Gene Expression in Colonic Epithelial Cells Predict Disease Phenotypes in Experimental Colitis. *Inflammatory Bowel Disease in press* 2012.) Reprinted with permission.

Chapter Four: Regulation of pIgR expression by commensal bacteria in human intestinal epithelial cells

Introduction

The mammalian gut is home to an estimated one trillion bacteria and it is the responsibility of the intestinal epithelium to separate this vast number of bacteria from host tissue⁶. Secretory IgA antibodies, transported across intestinal epithelial cells (IEC) by the polymeric immunoglobulin receptor (pIgR), provide the first line of defense against potentially harmful bacteria^{44,132}. This transport process provides multiple levels of immune protection: Excretion of IgA-containing immune complexes; transcytosis of SIgA has been shown to neutralize viruses and microbial products within IEC⁵⁵; release of SIgA at the apical surface of the epithelium serves as a physical barrier, limiting bacterial adherence translocation^{44,56}; and secretory component, the cleaved extracellular domain of pIgR, unbound to IgA has been shown to contribute to innate immunity by acting as a non-specific bacterial scavenger and limiting inflammatory immune response⁵⁴. One molecule of pIgR is consumed for every molecule of SIgA transported into the lumen, thus the rate of pIgR transcription controls the rate of SIgA transcytosis and high expression of pIgR is required to maintain sufficient supply of SIgA and SC⁴⁴. Accordingly, steady-state levels of pIgR mRNA in mouse and human IEC are very high. We have shown that expression of pIgR is much higher in the colon than in the ileum of both healthy controls and CD patients, which is likely due to enhanced bacterial stimulation of IECs due to greater numbers of commensal bacteria⁶⁰. However, the mechanisms by which IEC maintain high levels of pIgR expression while limiting

inflammatory immune responses in the face of the vast microbial community are not completely understood. Therefore the aim of this chapter is to dissect the molecular mechanism by which pIgR expression is regulated in IEC by commensal bacteria and compare this to the regulation of the pro-inflammatory response.

Cross-talk between commensal bacteria and IEC is mediated by pattern-recognition receptors, including Toll-like receptors (TLRs)⁶. While TLR signaling is capable of triggering inflammation in response to microbial insult, these innate immune receptors establish intestinal homeostasis by discriminating between symbiotic commensal bacterial and harmful pathogens⁶. Accordingly, IEC TLR signaling identifies the composition of the local microbiota and instructs the underlying effector and regulatory immune cells, with the ultimate function to dampen inflammation while providing immune protection^{6,139,140}. This process involves production and transport of large quantities of IgA in order to maintain this balance¹⁴⁰. Previous studies from our lab have demonstrated that stimulation of TLR3 and TLR4 with purified ligands induces pIgR expression in IEC *in vitro*¹⁴¹. TLRs that sense microbial components require the cytoplasmic adaptor protein, MyD88 to activate downstream signaling pathways. MyD88 deficiency results in significant impairment of intestinal IgA responses and homeostasis^{62,142}. However, the role of IEC MyD88-dependent TLR signaling in the regulation of pIgR expression is unclear.

MyD88-dependent TLR signaling leads to the activation of nuclear factor κ B (NF- κ B)²⁷ signaling pathways and parallel activation of several mitogen-activated protein kinases (MAPK) including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase ERK (ERK), and p38 MAPK²⁶. Accordingly, NF- κ B and MAPK activation is

significantly reduced in MyD88-deficient mice²⁸. However, the contributions of these pathways in the induction of pIgR by bacterial stimulation have not been investigated. The NF- κ B family of transcription factors consists of five members in mammals, RelA, RelB, c-Rel, p50 and p52³¹. These NF- κ B proteins can form homo- or heterodimers that bind DNA consensus sequences on target genes to regulate gene transcription. Inactive NF- κ B is localized to the cytoplasm in association with I κ B inhibitory proteins. However, upon stimulation, phosphorylation and degradation of I κ B proteins occurs, resulting in the nuclear translocation of NF- κ B dimers³⁰. Two NF- κ B signaling pathways have been identified. The classical RelA-dependent pathway is activated primarily by pro-inflammatory cytokines and TLR ligands, leading to the degradation of I κ B α and the nuclear translocation of RelA/p50 heterodimers³¹. The translocation of RelA/p50 into the nucleus leads to the activation of gene transcription, including the rapid induction of several pro-inflammatory chemokines and cytokines, such as IL-8 and TNF, which comprise the early pro-inflammatory response¹⁴³. This early pro-inflammatory response is quickly downregulated by negative regulators within hours of induction¹⁴⁴. In contrast, the induction of pIgR by pro-inflammatory cytokines and TLR ligands is slow and sustained, peaking at 24 hours and remaining high^{141,143}. The alternative NF- κ B pathway is primarily activated by a limited number of stimuli including lymphotoxin B and B-cell activating factor (BAFF) and involves the nuclear translocation of RelB/p52 dimers^{31,145}. Although NF- κ B signalling in the intestine has been studied extensively, the differential roles of the classical RelA-dependent or the alternative RelB-dependent pathway are not fully understood due to its complex nature and the intrinsic variability in different cell types¹⁴⁶. Our lab has previously

demonstrated that transcription of the PIGR gene, in response to TNF and TLR signaling, requires a κ B element in the first intron that is a perfect match to the RelA consensus sequence¹⁴⁷. Additional studies using siRNA have shown that de novo synthesis of RelB is required for optimal TNF-mediated induction of pIgR¹⁴⁸. This chapter will investigate the contributions of the classical and alternative NF- κ B pathways in the induction of pIgR transcription under the hypothesis that these factors may have differential roles in the maintenance of pIgR expression and the induction of pro-inflammatory immune responses. By utilizing chemical inhibitors and intestinal epithelial cell lines in which the expression of critical components of these signaling pathways are knocked down, stimulus-specific mechanistic differences in the regulation of pIgR and pro-inflammatory gene expression in intestinal epithelial cells are examined.

Results

Activation of the NF- κ B pathway by TNF and TLR signaling is required to induce pIgR expression.

To determine if the RelA-dependent NF- κ B pathway is required for the induction of pIgR expression, the human IEC line HT-29 cells was stimulated with TNF or ligands for TLR4 (lipopolysaccharide (LPS)) or TLR3 (polyinosinic:polycytidylic acid (pIC)), in the presence or absence of BAY 11-7082, an inhibitor of I κ Ba phosphorylation (**Figure 4-1**). Consistent with previous reports from our lab using this cell line, IL-8 mRNA levels rapidly increased following 3 hours of stimulation with TNF or TLR ligands, indicative of the early pro-inflammatory response. Likewise, induction of IL-8 by all

three ligands was inhibited by BAY11-7082, suggesting that this early pro-inflammatory response induced by TNF and TLR signaling in IECs involves the classical NF- κ B pathway. The varying magnitude of IL-8 induction by TNF, LPS and pIC, as well as the varying degree of IL-8 inhibition in the presence of BAY 11-7082, suggests that NF- κ B activation may be stimulus-specific and/or there may be additional contributions from other signaling pathways in the regulation of the early pro-inflammatory response. Alternatively, induction of pIgR was delayed until 24 hours of TNF or TLR stimulation, consistent with previous published results from our lab, and the magnitude of induction was similar for all three stimuli. Additionally, pIgR induction in response to all three stimuli was completely inhibited in the presence of BAY 11-7082B, indicating that pIgR induction by TNF and TLR signaling requires activation of the classical NF- κ B pathway. These results are not specific to the HT-29 cell line because similar results were observed when using another human IEC line, LoVo cells (**Figure 4-2**). Other colon carcinoma cell lines, including CaCo2, SW480 and LS174T, were also tested (data not shown); however, while similar NF- κ B-dependent gene expression was observed in these cell lines, pIgR mRNA levels were extremely variable. Therefore, since HT-29 cells are a well established model for studying IEC responses and the regulation of pIgR, all additional *in vitro* cell culture experiments have been performed using HT-29 cells. Our lab has previously demonstrated that changes in mRNA levels in response to various stimuli are directly proportional to changes in protein expression for pIgR and IL-8 in HT-29 cells. Taken together, these results suggest that the early pro-inflammatory response and the delayed induction of pIgR expression in human IECs require activation

of the classical NF- κ B pathway, yet the contributions of the RelA-dependent NF- κ B pathway in these responses may be different.

Differential contributions of the classical NF- κ B pathway to the induction of early inflammatory response genes and the induction of pIgR expression

To investigate the mechanisms that regulate the early induction of pro-inflammatory genes and the delayed induction of pIgR in IEC by TNF and TLR signaling, control, RelA and RelB knockdown cells were stimulated with TNF, LPS or pIC for 3 and 24 hours (**Figure 4-3**). The early pro-inflammatory response, characterized by the induction of IL-8 and TNF expression, was significantly inhibited in the RelA knockdown cells, particularly in response to TLR signaling. Knockdown of RelA also inhibited the induction of A20, the cytoplasmic ubiquitin-editing enzyme that acts as a negative feedback regulator of the classical NF- κ B pathway. While, knockdown of RelB had little effect on the induction of gene expression in response to LPS or pIC stimulation, RelB knockdown partially inhibited TNF-stimulated induction of early response genes, indicating an involvement of the alternative NF- κ B pathway.

Knockdown of RelA inhibited the late induction of pIgR in response to TNF stimulation but not to stimulation by TLR ligands. Interestingly, the steady-state levels of pIgR mRNA were significantly higher in RelB knockdown cells, as compared to control and RelA knockdown cells, indicating a potential inhibitory effect of RelB on pIgR expression. Accordingly, RelB knockdown did not inhibit the up-regulation of pIgR expression in response to any stimuli, but further increases in pIgR mRNA were observed

upon stimulation. Taken together, these results suggest that the classical RelA-dependent NF- κ B pathway is required for the regulation of pIgR expression by TNF signaling, however additional signaling pathways may be able to compensate to promote the induction of pIgR in response to TLR signaling. To ensure that inhibition of RelA by siRNA did not have indirect effects on the MAPK signaling pathway, the expression of MAPK-phosphatase 1 (MKP-1) was analyzed. MKP-1, the phosphatase that downregulates MAPK signaling, contains multiple MAPK-responsive elements in its promoter region, but it does not contain any κ B-binding sites and should not be directly affected by NF- κ B inhibition. Accordingly, only minor changes in MKP-1 levels were observed in RelA or RelB knockdown cells, suggesting the NF- κ B knockdown did not significantly affect MAPK signaling.

Differential contributions of the MAPK signaling pathway in the regulation of the early pro-inflammatory response and the polymeric immunoglobulin receptor

It has been well established that stimulation of TNF and TLR signaling leads to the activation of not only NF- κ B signaling, but additional signaling pathways, including MAPK signaling. To investigate the role of MAPK signaling in regulating expression of early pro-inflammatory genes and the late induction of pIgR in response to TNF and TLR signaling, HT-29 cells were stimulated with TNF, LPS or pIC in the presence of individual MAPK inhibitors (inhibitors to p38, JNK, and ERK) or a combination of all three inhibitors (**Figure 4-4**). Inhibition of p38 and ERK signaling significantly reduced the induction of IL-8 in response to TNF and pIC, while inhibition of JNK had no effect

on this early pro-inflammatory response. Likewise, the combination of all three MAPK inhibitors prevented the induction of IL-8 in response to all three stimuli, suggesting that these MAPK pathways work synergistically to regulate the early pro-inflammatory response. Alternatively, inhibition of MAPK signaling did not inhibit the induction of pIgR, rather, inhibition of p38 as well as a combination of all three inhibitors actually enhanced induction of pIgR in response to TNF and pIC. These results are consistent with previous work that demonstrated that TNF-induced expression of pIgR in HT-29 cells was enhanced when ERK activation was inhibited. Induction of pIgR by LPS was not affected by any MAPK inhibitors¹⁴⁹. Taken together, these results suggest that MAPK signaling, while stimulus-specific, plays different roles in regulating the early pro-inflammatory response and the late induction of pIgR. Specifically, MAPK signaling pathways act synergistically to enhance expression of pro-inflammatory genes, while inhibiting the late induction of pIgR.

pIgR expression is regulated by TLR4 signaling in intestinal epithelial cells

These results indicate that multiple signaling pathways may function to maintain pIgR expression in IECs, particularly in response to bacterial-induced TLR signaling. However, it is generally accepted that IEC are “hyporesponsive” to constant stimulation with intestinal bacteria, maintaining immune tolerance to prevent excessive inflammation^{6,17}. This idea is supported by the observation from our lab that when stimulated with various species representing the four major phyla of colonic bacteria, HT-29 cells only responded to stimulation from bacteria in the family *Enterobacteriaceae*⁶². To investigate the mechanism underlying the hyporesponsiveness

of intestinal epithelial cells to commensal bacteria and the maintenance of pIgR expression, HT-29 human intestinal epithelial cell line and THP-1 human monocyte cell line were stimulated with purified *E. coli* (*Enterobacteriaceae* family) LPS, a ligand for TLR4, and Pam3CSK4, a ligand for TLR2. These ligands were chosen due to their ligand preferences and spatially restricted expression by IECs. While TLR2, TLR4 and TLR5 all recognize bacterial components and are expressed on the epithelial surface of IECs, TLR4 and TLR2 have been shown to be expressed on the apical surface of epithelial cells, as opposed to TLR5 which is only expressed on the basolateral surface of IECs⁶. Consistent with previous published work, pIgR expression was induced upon activation of TLR4 (**Figure 4-5A**). However, induction of pIgR was not observed in response to TLR2 activation. The unresponsiveness to PamCSK4 stimulation was not gene specific because IL-8 expression was not induced upon TLR2 signaling, but was significantly induced in response to TLR4 signaling in HT29 cells (**Figure 4-5B**). In contrast, THP-1 monocytes induced very high levels of IL-8 mRNA in response to either TLR2 or TLR4 signaling. One possible explanation for the hyporesponsiveness of HT-29 cells to TLR2 stimulation is the observation that THP1 cells express 2000-fold higher levels of TLR2 mRNA than HT-29 cells. Alternatively expression of TLR4, as well as the cytoplasmic adaptor protein MyD88, was relatively similar between the IEC and monocyte cell-lines (**Figure 4-5**). To determine whether the hyporesponsiveness of IEC was due to the inhibition of TLR signaling, the expression of TLR signaling inhibitors, the TLR decoy receptor, Single Ig IL-1-related receptor (SIGIRR) and the Toll-interacting protein (TOLLIP), a cytoplasmic protein that binds to the intracellular domains of TLRs to inhibit IRAK phosphorylation and downstream signaling, were analyzed. The basal level

of SIGIRR was 3-fold higher in HT-29 cells, compared to THP-1 cells, and expression of this TLR inhibitor was not downregulated in response to TLR signaling in HT-29 cells, as observed in THP-1 cells. Likewise, while the basal level of TOLLIP was slightly lower in HT-29 cells, expression of this TLR inhibitor was significantly upregulated in response to TLR stimulation, which was not observed in THP-1 cells. Taken together, these results suggest that the hyporesponsiveness of IEC to colonic bacteria may be a result of reduced TLR2 signaling as well as the upregulation of TLR signaling inhibitors.

To investigate the hypothesis that pIgR induction by commensal bacteria is dependent on TLR4 signaling, HT-29 cells were stimulated with *E.coli* strain Nissle (*EcN*) for 24 hours in the presence of increasing concentrations of polymixin B, an antibiotic that neutralizes LPS, inhibiting activation of TLR4 (**Figure 4-6**). Accordingly, while *EcN* stimulation significantly induced pIgR expression, this induction was inhibited in a dose-dependent manner in the presence of polymixin B. These results indicate induction of pIgR by colonic bacteria is primarily dependent on the activation of TLR4 signaling by LPS.

Gene expression induced by colonic bacteria is dependent on intestinal epithelial MyD88 signaling

Ligation of TLR4 leads to the recruitment of the cytoplasmic adaptor protein MyD88. We have recently reported that the steady-state levels of pIgR mRNA was significantly reduced in MyD88 deficient mice⁶². However, in this study it was not determined whether pIgR expression was regulated directly by MyD88 signaling in IEC

or indirectly by immune cells in the *lamina propria*⁶². To investigate the hypothesis that MyD88 signaling in IEC regulates pIgR expression in response to colonic bacteria, HT-29 cells were stably transfected with shRNA directed against MyD88, or an irrelevant control. Significant knockdown of MyD88 expression was achieved in shRNA transfected cells (**Figure 4-7**). Stimulation with *EcN* for 3 hours upregulated MyD88 expression in both control and MyD88 knockdown cells, but the expression of MyD88 in the knockdown cells was less than unstimulated control cells. Likewise, *EcN* induced expression of IL-8, which peaked at 3hrs in control transfected cells, but this induction was significantly inhibited in MyD88 knockdown cells. Similarly, *EcN* induced pIgR expression by 24 hours in control transfected cells, but induction of pIgR was significantly inhibited in MyD88 knockdown cells. Furthermore, correlation analysis revealed a strong positive correlation between MyD88 expression and IL-8 and pIgR expression. These results suggest that the induction of both IL-8 and pIgR in response to commensal bacteria is dependent on intestinal epithelial MyD88 signaling.

Induction of pIgR by *E. coli* is dependent on NF- κ B signaling, but not MAPK signaling

Activation of MyD88-dependent TLR signaling leads to the activation of the NF- κ B and MAPK signaling pathways, resulting in the induction of gene expression. To investigate the contribution of these downstream signaling pathways to the induction of early pro-inflammatory genes and late pIgR expression in response to colonic bacteria, HT-29 cells were stimulated for 3 or 24 hours in the presence or absence of Bay 11-7082, an inhibitor of NF- κ B, or inhibitors to the MAPK signaling pathways. Inhibition of NF-

κ B activation completely inhibited the induction of both IL-8 and pIgR in response to *E. coli* stimulation (**Figure 4-8A**), suggesting NF- κ B signaling is required to induce IL-8 and pIgR expression in response to colonic bacteria. To determine the contributions of RelA and RelB in the induction of IL-8 and pIgR in response to colonic bacteria, RelA and RelB knockdown cells were stimulated with *EcN* for 3 or 24 hours (**Figure 4-8B**). Inhibition of RelA significantly reduced the induction of IL-8, while inhibition of RelB had no effect. Alternatively, inhibition of either RelA or RelB had no effect on the upregulation of pIgR in response to *EcN*. These results indicate that while IL-8 induction is dependent on the classical RelA pathway of NF- κ B activation, RelA and RelB may be interchangeable for the induction of pIgR by colonic bacteria. Inhibition of the p38 MAPK pathway, but not the ERK or JNK MAPK pathway, significantly reduced the induction of IL-8 by *EcN* (**Figure 4-9**). However, none of the MAPK inhibitors had any effect on the induction of pIgR by *EcN* (**Figure 4-9**). Taken together, these findings suggest that while both the early pro-inflammatory response and the late pIgR response requires MyD88-dependent TLR4 signaling in IEC, these responses have distinct downstream signaling requirements. The activation of the early pro-inflammatory genes in response to *EcN* is dependent on activation of p38 and the classical RelA-dependent NF- κ B pathway, while the late induction of pIgR requires NF- κ B activation, but is independent of MAPK signaling.

Discussion

The function of NF- κ B signaling in the intestine epithelium is complex. A balance of inflammatory and anti-inflammatory immune responses must be maintained to

ensure adequate protection against potential pathogens while preventing collateral damage to the intestinal epithelium³⁰. Several mouse models support the idea that hyperactivation of NF- κ B contributes to intestinal inflammation. In particular, mice lacking epithelial-specific expression of SIGIRR, the negative regulator of TLR-dependent NF- κ B activity, develop much more severe DSS-induced colonic inflammation⁸². These results suggest that bacterial-induced hyperactivation of NF- κ B could be sufficient for promoting intestinal inflammation and maintaining a level of NF- κ B hyporesponsiveness may be beneficial in response to significant bacterial stimulation. On the other hand, ablation of NF- κ B in the intestinal epithelium is also associated with inflammation. Loss of epithelial-specific NF- κ B activity by deletion of epithelial-NEMO causes severe spontaneous colitis in mice³⁴. Mice with a targeted deletion of *Rela* in IECs exhibited reduced expression of pro-restitution genes, increased epithelial apoptosis and increased susceptibility to DSS-induced acute colitis³⁰. Furthermore, while individual ablation of IKK1 or IKK2 in IECs is insufficient to cause spontaneous inflammation, mice deficient in both IKK1 and IKK2 develop colitis similar to epithelial-specific NEMO-deficient mice³⁴; thus, the classical NF- κ B pathway, or at least functional IKK1 or IKK2, is essential in maintaining intestinal homeostasis. Accordingly, these new findings presented here suggest that regulation of pIgR expression in IECs may be an important mechanism by which NF- κ B maintains intestinal homeostasis during pro-inflammatory immune responses. Here we show that while the induction of pIgR by soluble ligands, including TNF and TLR ligands, is dependent on NF- κ B signaling, the specific contributions of the classical and alternative NF- κ B pathways are stimulus specific. In particular, by inhibiting *RelA* expression, we demonstrated that the TNF-

stimulated upregulation of pIgR expression requires the classical NF- κ B signaling pathway. These results are supported by the abolishment of transcriptional activity of a PIGR reporter gene in these RelA knockdown HT-29 cells in response to TNF stimulation¹⁰⁵. Alternatively, the regulation of pIgR expression by TLR signaling appears to involve both the classical and alternative NF- κ B pathways. Similar to TNF stimulation, inhibiting I κ B α phosphorylation significantly inhibited pIgR induction in response to LPS and pIC, which is supported by the observation that the transcriptional activity of a PIGR reporter gene was significantly reduced in RelA knockdown HT-29 cells¹⁰⁵. However, knockdown of either RelA or RelB did not inhibit the induction of pIgR mRNA levels following LPS or pIC stimulation. These results suggest that the classical and alternative NF- κ B signaling pathways, as well as additional pathways, may be able to compensate for the loss of either RelA or RelB in order to maintain pIgR expression in the presence of bacterial stimulation.

Although our results suggest that additional signaling pathways may contribute to the regulation of pIgR expression, inhibition of MAP kinases, which are known to be activated by TNF and TLR signaling, did not block induction of pIgR. In particular, inhibition of ERK activation actually increased pIgR expression, which is consistent with previous reports¹⁴⁹. In contrast, MAPK activation was required for the early induction of IL-8 expression, highlighting the mechanistic differences in the regulation of the early pro-inflammatory response and the slow and sustained induction of pIgR expression. Taken together, these results suggest that the regulation of pIgR expression in intestinal epithelial cells may be an important mechanism by which NF- κ B maintains intestinal homeostasis during pro-inflammatory responses. Furthermore, by utilizing signaling

pathways that are distinct from those involved in regulating the early pro-inflammatory response, IECs are able to upregulate pIgR expression in response to microbial and host stimuli while suppressing a potentially damaging pro-inflammatory response.

This hyporesponsiveness, or suppression of pro-inflammatory responses, by IECs in response to bacterial stimulation is highlighted by the observation that HT-29 cells respond to purified LPS and whole *EcN*, although not as robustly as the THP-1 monocyte cell lines. Alternatively, HT-29 cells were unresponsive to TLR2 stimulation, unlike THP-1 cells. Consistent with these results, we found that while IECs express very low levels of TLR2, expression of TLR4 and MyD88 is similar between cell types and are upregulated by pro-inflammatory stimuli. Furthermore, induction of pIgR by whole *E. coli* was primarily dependent on the TLR4-activation of MyD88 signaling. Interestingly, studies examining the structural requirements for TLR4-mediated signaling indicate that LPS molecules from only a limited group of Gram-negative bacteria, specifically those of the family *Enterobacteriaceae*, have the ability to act as a TLR4 ligand^{150,151}. LPS molecules from other Gram-negative commensal bacteria signal through TLR2 rather than TLR4¹⁵². Unlike TLR2 expression, which remained low following stimulation with PAM3CSK4, TLR4 expression was upregulated in response to *E. coli* LPS. Moreover, it has previously been demonstrated that IgA can neutralize *E. coli* LPS within IECs⁵⁶. Taken together, these results suggests a negative-feedback, anti-inflammatory role for intracellular SIgA in response to TLR4 signaling. Accordingly, induction of pIgR expression by a subset of commensal bacteria may be a mechanism by which IECs maintain pIgR expression while limiting pro-inflammatory responses in the face of continuous interactions with gut bacteria. Consistent with this idea, our lab demonstrated

that pIgR expression was selectively upregulated in response to bacteria from the family *Enterobacteriaceae* of the phylum *Proteobacteria*, suggesting that different types of colonic bacteria differentially regulate pIgR expression in IEC.⁶²

Similar to results using purified ligands, we demonstrate that induction of the pro-inflammatory chemokine, IL-8, by EcN in HT-29 cells requires both NF- κ B and p38 MAPK signaling. Consistent with previous results, the increase in IL-8 was rapid and transient, returning to near base-line levels following 24 hours of EcN stimulation. In contrast, the slow and sustained induction of pIgR expression by EcN required NF- κ B signaling, but not MAPK signaling. In support of these results, our lab has shown that EcN induced expression of a negative regulator of MAPK signaling, MKP-1, in HT-29 cells. Also, we have shown that MKP-1 levels are 16-fold higher in the colon than in the small intestine of mice, consistent with the increase bacterial load. Furthermore, these levels are significantly reduced in MyD88 deficient mice⁶². Accordingly, these results suggest that limiting MAPK signaling may be an important role of MyD88-dependent TLR signaling by a subset of commensal bacteria.

Overall, the results presented here demonstrate that IEC utilize distinct mechanisms to regulate the expression of the early pro-inflammatory response and the late and sustained pIgR response in efforts to maintain intestinal homeostasis. Specifically, stimulation of pIgR expression by a subset of commensal bacteria or host stimuli would enhance pIgR-mediated transport of protective IgA antibodies, as well as innate immune functions mediated by SC. Transient induction of moderate levels of IL-8 by IECs, at much lower levels than induced by byproducts of viral replication or other non-bacterial stimuli¹⁴¹, would promote influx of neutrophils in sufficient quantities to

enhance innate immunity without excess inflammation. This early pro-inflammatory response is rapidly followed by the induction of negative regulatory molecules, such as MKP-1, Tollip and Sigirr, that would prevent excess activation of NF- κ B and MAPK signaling. Therefore, cross-talk between commensal bacteria and IECs is crucial for regulating the levels of NF- κ B activity and maintaining of intestinal homeostasis.

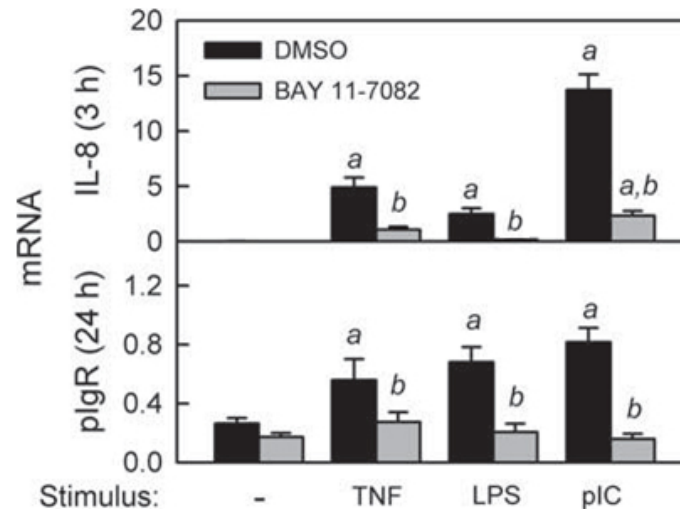


Figure 4-1 Activation of the classical NF- κ B pathway by TNF, LPS, and pIC in intestinal epithelial cells. HT-29 cells were stimulated with TNF (10 ng/ml), LPS (1mg/ml), or pIC (100mg/ml) for 3 or 24 h, in the presence or absence of 10 mM BAY11-7082, an inhibitor of I κ B α phosphorylation. Cells cultured in the absence of BAY 11-7082 were treated with an equivalent volume of vehicle (DMSO). mRNA levels for IL-8 and pIgR were quantified by quantitative reverse transcriptase-PCR (qRT-PCR) and normalized to GAPDH mRNA. Data from three independent experiments were combined and expressed as mean \pm s.e.m. ($n = 9$): *a*, the mean for stimulated cells is significantly greater than the mean for unstimulated cells ($P < 0.05$); *b*, the mean for cells treated with BAY 11-7082 is significantly different from the mean for cells given the same stimulus in the absence of BAY 11-7082 ($P < 0.05$). (Bruno, M.E., Frantz, A.L., Rogier, E.W., Johansen, F.E. & Kaetzel, C.S. Regulation of the polymeric immunoglobulin receptor by the classical and alternative NF-kappaB pathways in intestinal epithelial cells. *Mucosal Immunol* **4**, 468-478(2011)) Reprinted with permission.

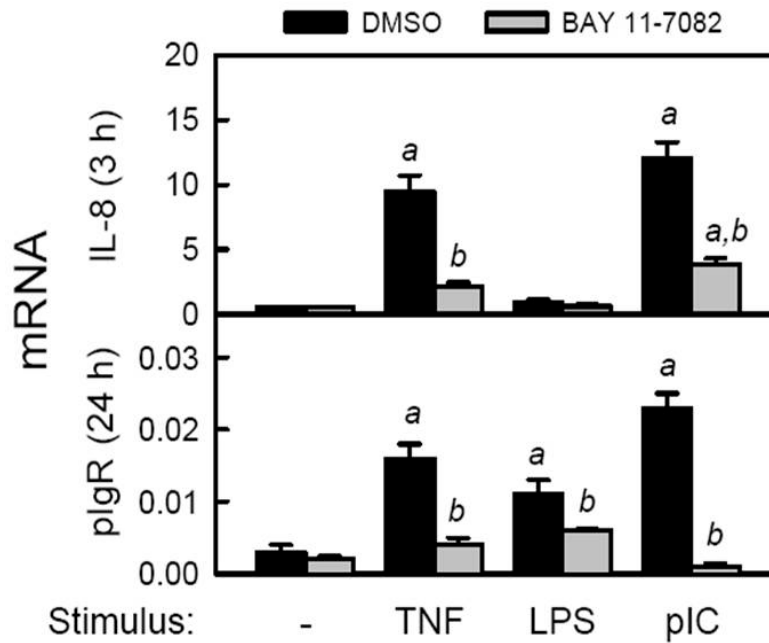


Figure 4-2 Effect of NF- κ B inhibition on the response of the LoVo human IEC line to LPS, pIC and TNF. LoVo cells were stimulated with TNF (10ng/ml), LPS (1mg/ml) or pIC (100 mg/ml) for 3 or 24 h, in the presence or absence of 10 μ M BAY 11-7082, an inhibitor of I κ B α phosphorylation. Cells cultured in the absence of BAY 11-7082 were treated with an equivalent volume of vehicle (DMSO). mRNA levels for IL-8 and pIgR were quantified by qRT-PCR and normalized to GAPDH mRNA. Data are expressed as mean \pm SEM (n = 4): *a*, the mean for stimulated cells is significantly greater than the mean for unstimulated cells ($p < 0.05$); *b*, the mean for cells treated with BAY 11-7082 is significantly different from the mean for cells given the same stimulus in the absence of BAY 11-7082 ($p < 0.05$). (Bruno, M.E., Frantz, A.L., Rogier, E.W., Johansen, F.E. & Kaetzel, C.S. Regulation of the polymeric immunoglobulin receptor by the classical and alternative NF-kappaB pathways in intestinal epithelial cells. *Mucosal Immunol* **4**, 468-478(2011)) *Reprinted with permission.*

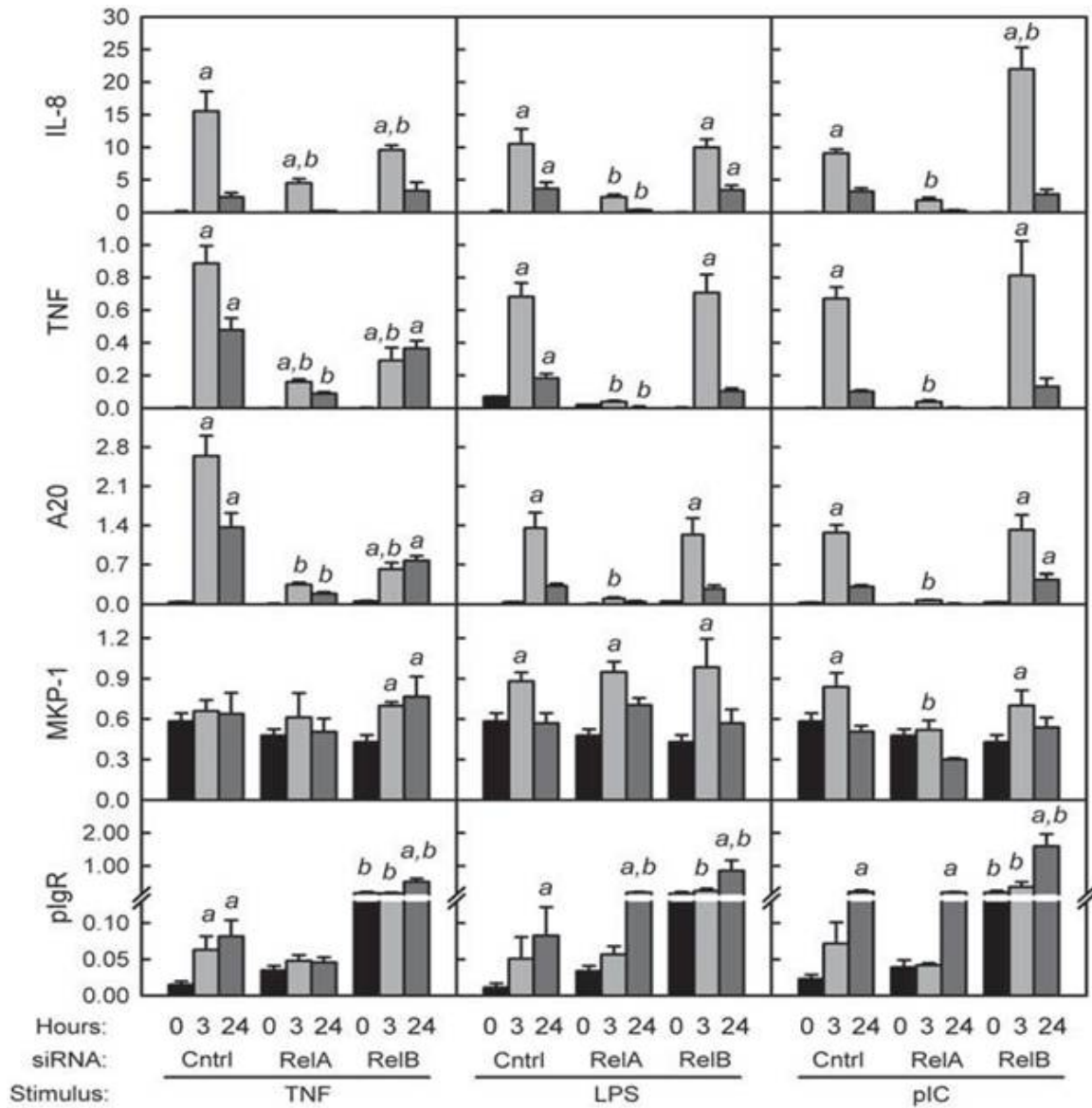


Figure 4-3 Effect of RelA and RelB knockdown on TNF, LPS, and pIC stimulated gene expression. mRNA levels were analyzed by quantitative reverse transcriptase-PCR (qRT-PCR) in HT-29 cells stably transfected with control, RelA, or RelB small inhibitory RNAs (siRNAs) and treated with TNF (10 ng/ml), LPS (1 mg/ml), or pIC (100 mg/ml) for 3 or 24 h. Data from two to three independent experiments were combined and expressed as mean \pm s.e.m. ($n = 8 - 12$): *a*, the mean for stimulated cells is significantly

Figure 4-3 (continued) greater than the mean for unstimulated cells expressing the same siRNA ($P < 0.05$); b , the mean for cells expressing RelA or RelB siRNA is significantly different from the mean for cells expressing control siRNA given the same stimulus for the same length of time ($P < 0.05$). (Bruno, M.E., Frantz, A.L., Rogier, E.W., Johansen, F.E. & Kaetzel, C.S. Regulation of the polymeric immunoglobulin receptor by the classical and alternative NF-kappaB pathways in intestinal epithelial cells. *Mucosal Immunol* **4**, 468-478(2011)) *Reprinted with permission.*

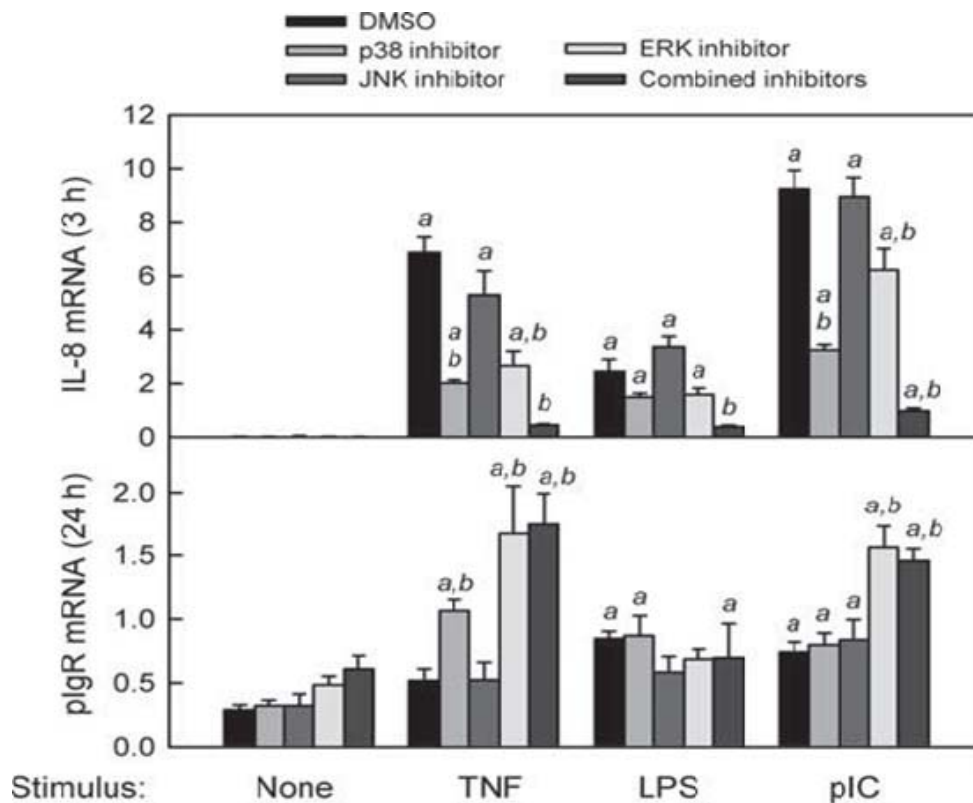


Figure 4-4 Effect of MAPK inhibition on the response of HT-29 cells to tumor necrosis factor TNF, LPS, and pIC. HT-29 cells were stimulated with TNF (10 ng/ml), LPS (1mg/ml), or pIC (100 mg/ml) for 3 or 24 h, in the presence or absence of individual MAPK inhibitors or a combination of the three inhibitors at the same concentrations (10 mM SB20358, p38 inhibitor; 10 mM SP600125, c-Jun N-terminal kinase (JNK) inhibitor; 10 mM PD98059, extracellular-regulated kinase (ERK) inhibitor). Cells cultured in the absence of MAPK inhibitors were treated with an equivalent volume of vehicle DMSO. mRNA levels for IL-8 and pIgR were quantified by quantitative reverse transcriptase-PCR (qRT-PCR) and normalized to GAPDH mRNA. Data from two independent experiments were combined and expressed as mean \pm s.e.m. ($n = 8$): *a*, the mean for stimulated cells is significantly greater than the mean for unstimulated cells ($P < 0.05$);

Figure 4-4 (continued) *b*, the mean for cells treated with MAPK inhibitors is significantly different from the mean for cells given the same stimulus in the absence of MAPK inhibitors ($P < 0.05$). (Bruno, M.E., Frantz, A.L., Rogier, E.W., Johansen, F.E. & Kaetzel, C.S. Regulation of the polymeric immunoglobulin receptor by the classical and alternative NF- κ B pathways in intestinal epithelial cells. *Mucosal Immunol* **4**, 468-478(2011)) *Reprinted with permission.*

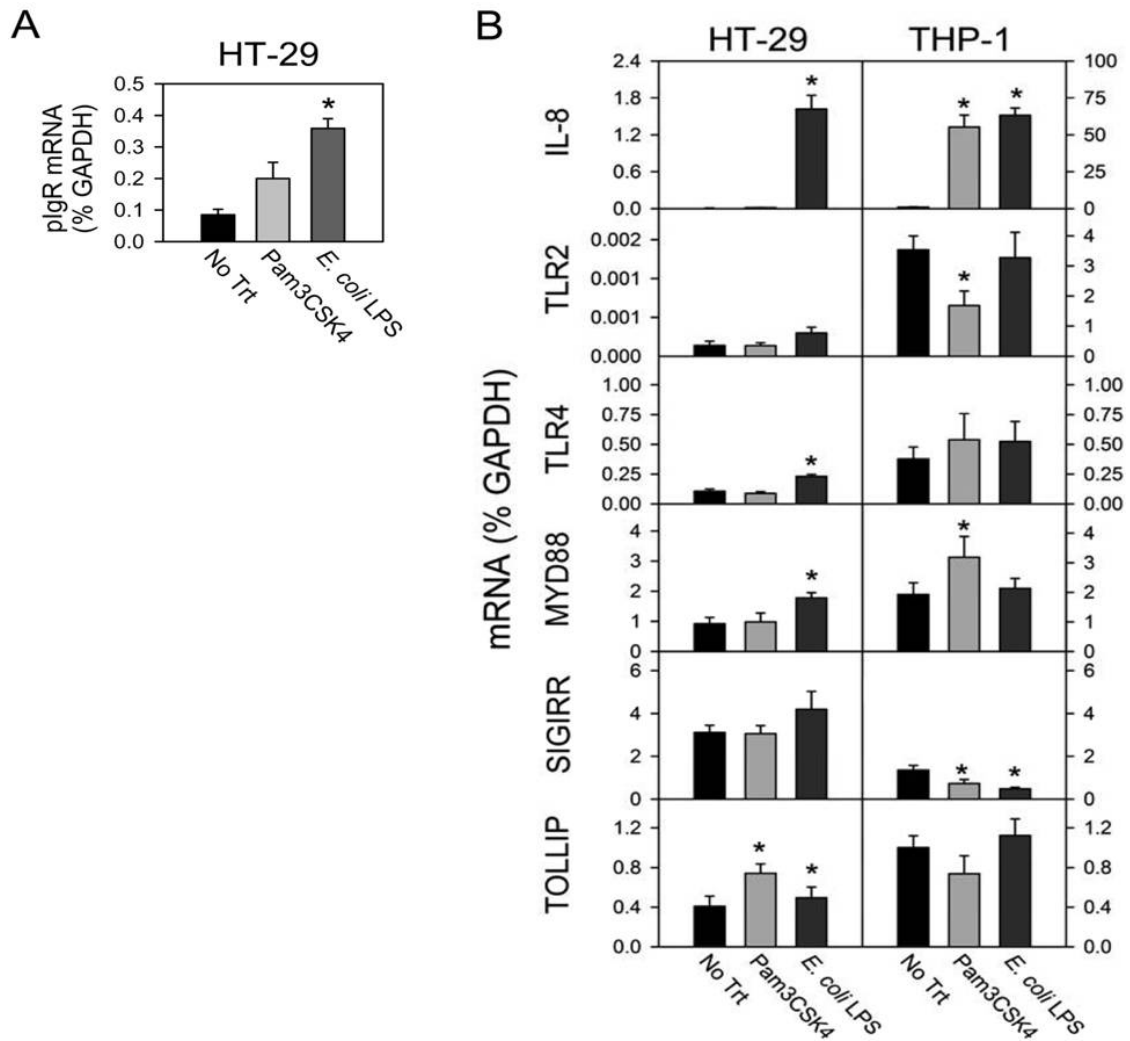


Figure 4-5: Regulation of gene expression by purified ligands for TLR2 and TLR4.

A. HT-29 cells were cultured for 24 h in the absence or presence of Pam3CSK4 (TLR2 ligand) or highly purified LPS from *E. coli* O26:B6 (TLR4 ligand), each at a concentration of 1 µg/ml. mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA. B. HT-29 and THP-1 cells were cultured for 3 h in the absence or presence of Pam3CSK4 or LPS, and mRNA levels were analyzed as described for panel A. Data from 2 independent experiments were combined and expressed as mean ± SEM

Figure 4-5 (continued) (n = 8). Asterisks indicate that the mean is significantly different from the corresponding mean for untreated cells from the same cell-line ($p < 0.05$).

(Bruno, M.E. *et al.* Regulation of the polymeric immunoglobulin receptor in intestinal epithelial cells by Enterobacteriaceae: implications for mucosal homeostasis. *Immunol Invest* **39**, 356-382 (2010)) *Reprinted with permission.*

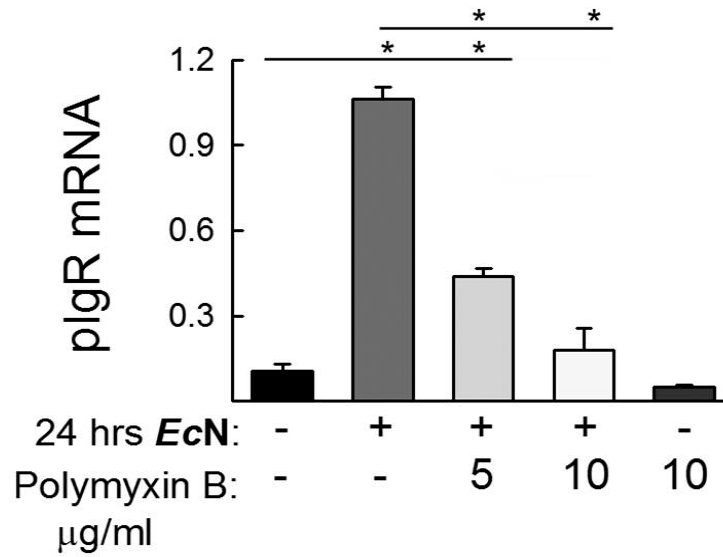


Figure 4-6 Effect of TLR4 inhibition on the induction of pIgR expression by *E. coli*

Nissle. HT-29 cells were stimulated with *EcN* in the presence or absence of varying concentrations of Polymyxin B, an inhibitor of LPS. mRNA levels were quantified by qRT-PCR, normalized to b2-M mRNA, and expressed as mean \pm SEM (n = 6). Asterisks indicate that the mean is significantly different for the indicated treatment groups (p < 0.01); N.S., not significant. Data from 2 independent experiments were combined and expressed as mean \pm SEM (n = 8)

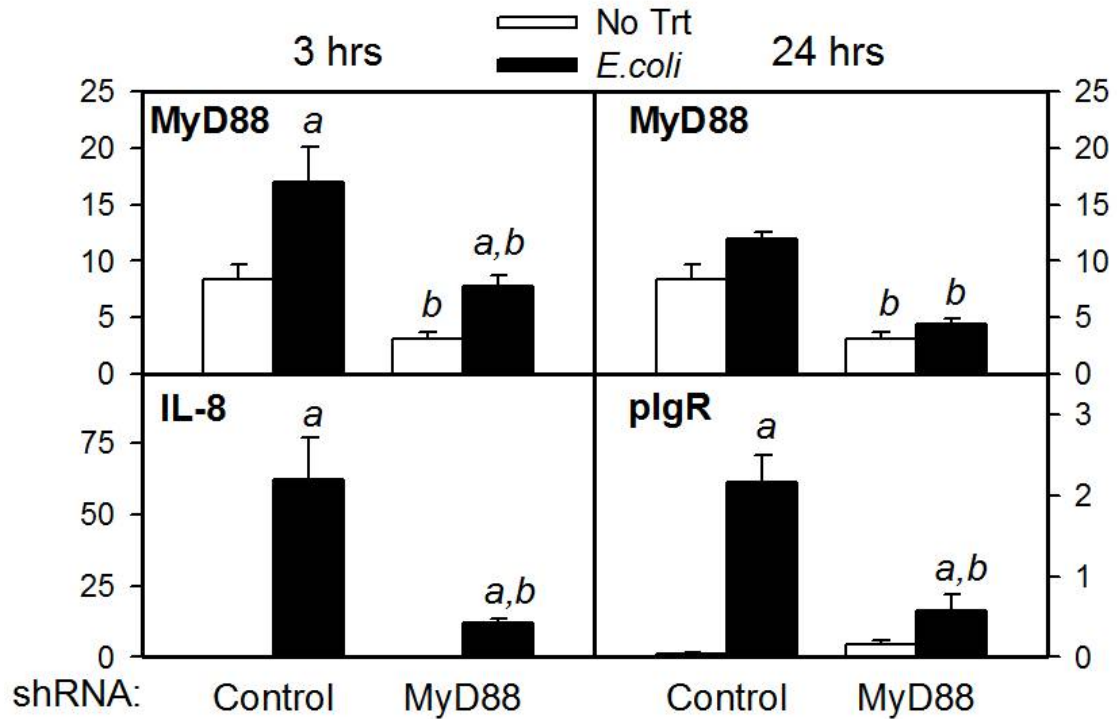


Figure 4-7. shRNA-mediated knockdown of MyD88 mRNA in a human colon carcinoma cell-line HT-29. Clones of HT-29 cells stably transfected with the indicated shRNA were stimulated with 10^6 *E. coli* for 3 and 24 hours. mRNA levels were quantified by qRT-PCR, normalized to b2-microglobulin mRNA, and expressed as mean \pm SEM (n = 6): *a*, mean for cells treated with *E. coli* is significantly different from the corresponding mean for untreated cells; *b*, mean for cells expressing MyD88 shRNA is significantly different from the mean for cells expressing control siRNA ($p < 0.05$).

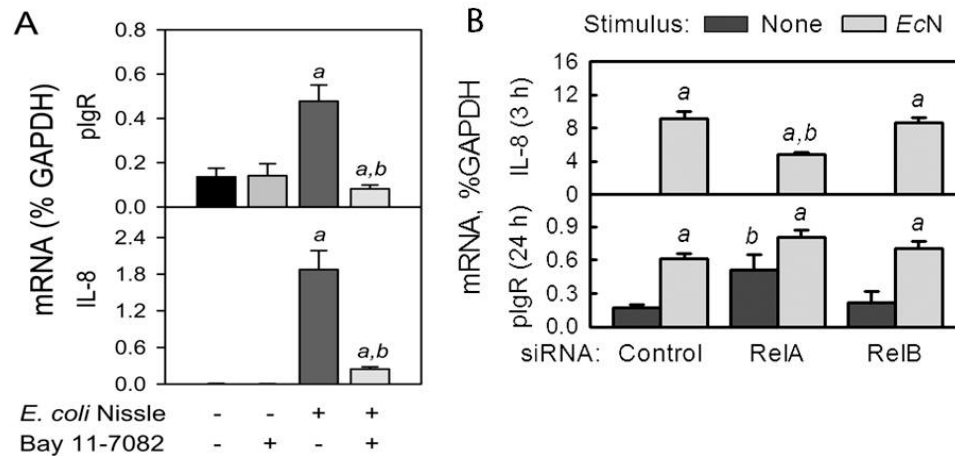


Figure 4-8: Effect of NF-κB inhibition on the response of HT-29 cells to *E. coli*

Nissle. A. HT-29 cells were stimulated for 3 or 24 h with heat-killed *EcN*, at a ratio of 10 bacterial cells per eukaryotic cell, in the presence or absence of 10 μM Bay 11 7082.

mRNA levels were quantified by qRT PCR and normalized to GAPDH mRNA. Data from 3 independent experiments were combined and expressed as mean ± SEM (n = 9): *a*, mean for cells stimulated with *EcN* is significantly different from the corresponding mean for unstimulated cells; *b*, mean for cells treated with Bay 11-7082 is significantly different from the corresponding mean for untreated cells. (Bruno, M.E. *et al.* Regulation of the polymeric immunoglobulin receptor in intestinal epithelial cells by Enterobacteriaceae: implications for mucosal homeostasis. *Immunol Invest* **39**, 356-382 (2010)) *Reprinted with permission.* **B.** HT-29 cells expressing the indicated siRNA were stimulated with *EcN*. mRNA levels were quantified by qRT-PCR, normalized to GAPDH mRNA, and expressed as mean ± SEM (n = 4): *a*, mean for cells treated with *EcN* is significantly different from the mean for untreated cells; *b*, mean for cells expressing RelA or RelB siRNA is significantly different from the mean for cells expressing control siRNA (p < 0.05).

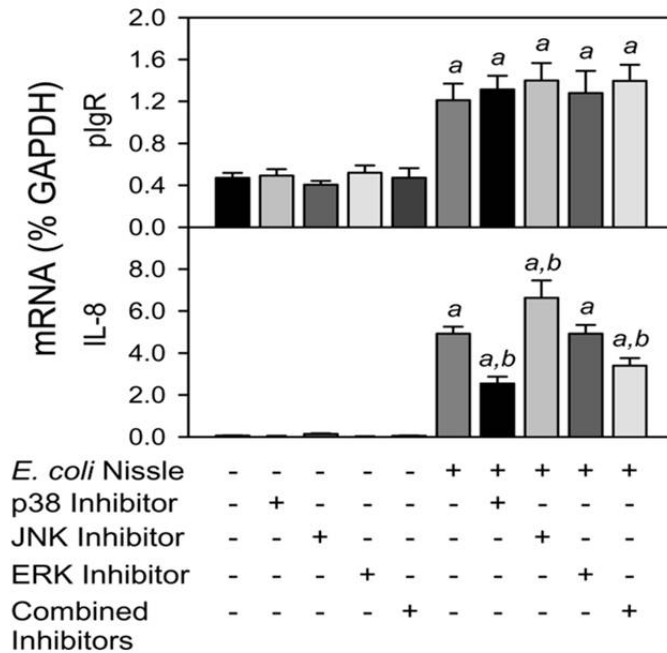


Figure 4-9: Effect of MAPK inhibition on the response of HT-29 cells to *E. coli*

Nissle. HT-29 cells were stimulated with *EcN* for 3 or 24 h in the presence or absence of MAPK inhibitors (PD98059, ERK inhibitor; SB20358, p38 inhibitor; SP600125, JNK inhibitor), individually or combined, each at a concentration of 10 μ M. mRNA levels were quantified by qRT PCR and normalized to GAPDH mRNA. Data from 2 independent experiments were combined and expressed as mean \pm SEM (n = 8): *a*, mean for cells stimulated with *EcN* is significantly different from the corresponding mean for unstimulated cells; *b*, mean for cells treated with MAPK inhibitors is significantly different from the corresponding mean for untreated cells. (Bruno, M.E. *et al.* Regulation of the polymeric immunoglobulin receptor in intestinal epithelial cells by Enterobacteriaceae: implications for mucosal homeostasis. *Immunol Invest* **39**, 356-382 (2010)). Reprinted with permission.

Chapter Five: Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides

Introduction

The mammalian intestine harbors an estimated 100 trillion microorganisms, which normally maintain a mutually beneficial relationship with the host.⁴⁰ The intestinal epithelium provides a physical barrier as well as innate immune defense, preventing this vast community of microbes from entering host tissues.¹⁵³ Defects in epithelial barrier and immune functions can lead to infections with opportunistic and pathogenic microbes¹⁵⁴ and contribute to the etiology of inflammatory bowel disease (IBD).¹⁵⁵ Cross-talk between the gut microbiota and intestinal epithelial cells (IECs) is mediated by pattern recognition receptors, including the Toll-like receptor (TLR) family,¹⁵⁶ leading to expression of gene products that enhance epithelial barrier function and innate immunity. TLR signaling in the gut is a double-edged sword; whereas physiological TLR signaling promotes intestinal homeostasis in healthy individuals, excessive TLR signaling in response to invasive microbes can exacerbate intestinal inflammation.²⁴ The cytoplasmic adaptor protein MyD88 transduces signals from TLRs that recognize bacterial products.⁷⁸ The finding that *Myd88*-deficient mice exhibited increased susceptibility to chemically-induced colitis highlighted the importance of physiological TLR signaling for protection of the epithelial barrier.¹⁵⁷ Studies using bone marrow chimeras of *Myd88*-deficient and wild-type mice in several mouse models of colitis demonstrated that MyD88 signaling in hematopoietic cells was required for development of intestinal inflammation, whereas

MyD88 signaling in non-hematopoietic cells was required for host survival.^{158,159} Here we used cell type-specific deletion of the *Myd88* gene to investigate the role of epithelial-specific MyD88 signaling in antibacterial immunity and protection against intestinal inflammation.

Secretory immunoglobulin A (SIgA) acts as the first line of antigen-specific immunity at the interface between the gut microbiota and the intestinal epithelium.⁴⁷ Polymeric IgA secreted by plasma cells in the intestinal *lamina propria* is transported across IECs by the polymeric immunoglobulin receptor (pIgR).¹⁶⁰ Importantly, we found that reduced expression of pIgR was correlated with greater disease severity in patients with IBD⁶⁰ and in mouse models of experimental colitis.¹⁶¹ Our finding that expression of pIgR is regulated in IECs by Toll-like receptor signaling⁶² suggests that microbial-epithelial cross-talk is crucial for optimal production of SIgA. Consistent with this concept, we found that MyD88-deficient mice had reduced colonic expression of pIgR and deficient transport of SIgA.¹⁶⁰ However, in that study it was not determined whether pIgR was regulated directly by MyD88 signaling in IECs or indirectly by immune cells in the intestinal *lamina propria*. In the present study we used targeted deletion of the *Myd88* gene to demonstrate an essential role for epithelial-specific MyD88 signaling in maintaining optimal expression of pIgR and transport of SIgA.

The thick layer of intestinal mucus acts to separate luminal bacteria from the epithelial surface.¹⁶² This mucus is rich in antimicrobial peptides¹⁶³ and associates with SIgA via glycan-mediated interactions,¹⁶⁴ thus forming an immunological as well as a physical barrier against microbial invasion. Here we report that targeted deletion of MyD88 in IECs causes down-regulated expression of pIgR, mucin-2 (the major protein

constituent of intestinal mucus) and antimicrobial peptides in IECs. We propose a model in which MyD88-dependent bacterial-epithelial cross-talk is crucial for maintenance of physical and immunological barrier function in the intestine.

Results

Loss of MyD88 signaling in colonic epithelial cells disturbs the segregation of microbiota and host

To investigate the role of epithelial MyD88 signaling in intestinal homeostasis, we generated mice with a targeted deletion of the *Myd88* gene in intestinal epithelial cells (MyD88^{ΔIEC}), by crossing mice carrying loxP-flanked *Myd88* alleles (MyD88^{Flox}) with mice that express *Cre* recombinase under the control of the IEC-specific *Vill* promoter.¹⁶⁵ Levels of MyD88 mRNA were significantly reduced in isolated colonic epithelial cells (ECs) from MyD88^{ΔIEC} mice at 9 weeks of age (**Figure 5-1a**). Immunostaining revealed expression of MyD88 in colonic epithelial cells (ECs) and *lamina propria* mononuclear cells (LPMCs) of MyD88^{Flox} mice, whereas MyD88^{ΔIEC} mice lacked MyD88 expression in ECs but retained expression in LPMCs (**Figure 5-1b**). Compared to MyD88^{Flox} littermates, MyD88^{ΔIEC} mice showed no histological signs of intestinal inflammation at 9 weeks of age (**Figure 5-1b**), or when followed up to 12 months (**Figure 5-2**). No pathological changes in colon morphology were observed. Although there were no differences in numbers of culturable bacteria in feces from MyD88^{Flox} and MyD88^{ΔIEC} mice, we observed increased mucus-associated CFU in MyD88^{ΔIEC} mice (**Figure 5-1c**). These differences were not restricted to culturable bacteria, since similar trends were observed when using qRT-PCR of 16s rDNA to

quantify bacterial numbers (**Figure 5-3**). Furthermore, increased bacterial attachment to the epithelial surface in MyD88^{ΔIEC} mice was correlated with increased translocation of bacteria to the draining mesenteric lymph nodes (MLNs) (**Figure 5-1d, e**). Interestingly, about 25% of bacterial colonies cultured from MLNs of MyD88^{ΔIEC} mice had a large, mucoid morphology and were identified as *Klebsiella pneumoniae* by sequencing of 16S rRNA genes and biochemical tests (**Figure 5-4**). No bacteria could be cultured from the spleens of MyD88^{ΔIEC} mice, suggesting that infection with gut-derived bacteria was confined to the mucosal immune system. These findings indicate that epithelial-specific MyD88 signaling is required for normal segregation of gut commensals from host tissues.

Loss of MyD88 signaling in colonic epithelial cells compromises epithelial barrier function

We hypothesized that increased epithelial permeability in MyD88^{ΔIEC} mice, coupled with increased numbers of mucus-adherent bacteria, could result in leakage of bacteria into the *lamina propria* and travel via the lymphatics to MLNs. Measurement of transmucosal electrical resistance (TER) in isolated colonic tissues revealed significant decreases in MyD88^{ΔIEC} mice, indicative of decreased epithelial barrier function (**Figure 5-5a**). This *ex vivo* analysis was supported by the *in vivo* observation of elevated levels of serum albumin in the feces of MyD88^{ΔIEC} mice, reflecting leakage of serum proteins into the intestinal lumen (**Figure 5-6**). To determine whether increased mucosal adherence and epithelial translocation of bacteria in MyD88^{ΔIEC} mice was selective for opportunistic pathogens such as *K. pneumoniae*, we treated mice orally for 7 days with an environmental, nonpathogenic strain of *E. coli* (Nissle 1917),¹⁶⁶ which had been

transformed to kanamycin resistance. We observed similar numbers of Kan^R *E. coli* in feces and luminal washes from MyD88^{Flox} and MyD88^{ΔIEC} mice, but significantly increased numbers in the colonic mucus of MyD88^{ΔIEC} mice (**Figure 5-5b**), consistent with the finding that mucus extracted from MyD88^{ΔIEC} mice had reduced antibacterial activity compared to MyD88^{Flox} mice (**Figure 5-5c**). However, these Kan^R *E. coli* did not translocate to the MLNs of MyD88^{ΔIEC} mice (data not shown). These results suggested that increased mucosal adherence of bacteria in MyD88^{ΔIEC} mice may be non-selective, whereas translocation to the draining lymph nodes may be selective for more aggressive bacteria such as *K. pneumoniae*.

We hypothesized that loss of epithelial MyD88 signaling could result in increased bacterial invasion of IECs. To assess epithelial invasion independent of the confounding effects of local immune cells, we performed *in vitro* bacterial invasion assays in the human HT-29 colon carcinoma cell-line. We tested the effects of shRNA-mediated knockdown of MyD88 (**Figure 4-7** for shRNA knockdown of MyD88) on attachment and invasion of three species of Gram-negative bacteria of the family *Enterobacteriaceae*: the non-invasive *E. coli* strain Nissle 1917, the opportunistic pathogen *K. pneumoniae* (a strain isolated from the MLN of a MyD88^{ΔIEC} mouse), and the invasive pathogen *Salmonella typhimurium* (strain SL1344) (**Figure 5-5d**). Knockdown of MyD88 expression in HT-29 cells resulted in significantly increased adherence of all 3 bacterial species to the epithelial surface. As expected, the magnitude of epithelial invasion by *E. coli* and *K. pneumoniae* was dramatically lower than was observed for *S. typhimurium*, but invasion of all 3 species was significantly increased when expression of MyD88 was reduced. Taken together, our findings suggest that

MyD88 signaling in ECs plays a crucial role in intestinal immunity by increasing antibacterial activity of epithelial mucus, reducing bacterial adherence to the epithelial surface, maintaining epithelial barrier function, and preventing bacterial invasion of epithelial cells.

Loss of MyD88 signaling in intestinal epithelial cells alters the composition of the gut microbiota

We hypothesized that compromised innate immunity resulting from loss of epithelial MyD88 signaling would affect the balance of the gut bacterial community, resulting in an altered composition of the gut microbiota in MyD88^{ΔIEC} mice. The richness and diversity of fecal bacteria was analyzed by the PhyloChipTM assay, a microarray-based method that measures the relative abundance of more than 59,000 individual microbial taxa in fecal DNA by analysis of the entire 16S ribosomal RNA gene sequence,¹⁰⁸ in MyD88^{Flox} and MyD88^{ΔIEC} mice. While the overall richness of the fecal microbiota of MyD88^{Flox} and MyD88^{ΔIEC} mice was similar (**Figures 5-7 and 5-8**), significant differences were observed in the relative abundance of operational taxonomic units (OTUs, defined as a group of highly similar 16S rRNA gene sequences, in most cases with similarity > 99%) within several bacterial families (**Figure 5-9**). A large proportion of families in the phylum *Bacteroidetes* were decreased in abundance in MyD88^{ΔIEC} mice, whereas a large proportion of families in the phylum *Proteobacteria* were increased. Within the phylum *Firmicutes*, the abundance of some families were increased while others were decreased in MyD88^{ΔIEC} mice. Principal component analysis (PCA), which was used to visualize complex relationships among multiple bacterial

families in individual mice, revealed significant differences between MyD88^{Flox} and MyD88^{ΔIEC} mice (**Figure 5-10a**, left panel). Interestingly, the composition of the microbiota was much more variable among individual MyD88^{Flox} mice than among individual MyD88^{ΔIEC} mice, as indicated by the tight clustering of the five MyD88^{ΔIEC} mice in the 2-dimensional PCA plot. This result suggests that loss of epithelial MyD88 signaling may cause specific and reproducible changes in the bacterial community, consistent with the finding that the abundance of 2647 OTUs differed significantly between MyD88^{Flox} and MyD88^{ΔIEC} mice (**Figure 5-10a**, right panel). The Prediction Analysis for Microarrays (PAM) method¹¹¹ was used to identify “distinctive” OTUs whose relative abundance characterizes the unique microbiota of MyD88^{Flox} and MyD88^{ΔIEC} mice (**Figure 5-10b**). This analysis identified 4 distinctive OTUs in the TM7 candidate division that were characteristic of MyD88^{ΔIEC} mice, and 7 OTUs within the genus *Lactobacillus* (phylum *Firmicutes*) that were characteristic of MyD88^{Flox} mice. Interestingly, the relative abundance of five bacterial species that have been associated with altered innate and adaptive immunity in the gut, including *Candidatus arthromitus* (SFB),¹⁶⁷ *Escherichia coli*,⁷⁷ *Pseudomonas fluorescens*,⁷⁷ *Klebsiella pneumoniae*¹⁶⁸ and *Proteus mirabilis*,¹⁶⁸ did not differ significantly in the fecal microbiota of MyD88^{Flox} and MyD88^{ΔIEC} mice (**Figure 5-10c**). This finding suggests that translocation of *K. pneumoniae* to the MLNs of MyD88^{ΔIEC} mice (**Figure 5-1e**) was due to a specific defect in the host response to this bacterium, and not simply to an increase in the abundance of *K. pneumoniae* in the gut microbiota.

Loss of MyD88 signaling in intestinal epithelial cells results in altered epithelial gene expression and reduced transcytosis of IgA

To determine whether loss of epithelial MyD88 signaling resulted in altered patterns of gene expression, we isolated colonic epithelial cells from MyD88^{Flox} and MyD88^{ΔIEC} mice and analyzed mRNA levels. 36 genes were chosen for mRNA analysis based on our previous studies and other reports implicating these genes as targets of MyD88 signaling, or having roles in epithelial cell function, innate immune signaling and/or colonic inflammation (**Table 5-2**). These genes included: 5 signature biomarkers that we have shown to be correlated with disease severity in human IBD and mouse models of colitis (*Pigr*, *Rela*, *Tnfaip3*, *Tnf*, *Cxcl2*)¹⁶¹; *Relb*, which encodes the RELB subunit of the alternative NF-κB signaling pathway, which we have shown to be implicated in regulation of *Pigr* gene transcription¹⁰⁵; *Muc2*, which encodes the major colonic mucin protein, the loss of which is associated with spontaneous development of colitis³⁷; genes associated with epithelial homeostasis and barrier function (*Areg*¹⁵⁸, *Bcl2l1*¹⁶⁹, *Birc2*¹⁷⁰, *Mmp3* and *Mmp9*¹⁷¹, *Tcf4*¹⁷², *Mylk*⁷¹ and *Tjp1*¹⁷³); antimicrobial peptides (*Defa6*¹⁶³, *Defa-rs1* [FE Johansen, personal communication], *Reg3g*⁴², *Tff3*¹⁷⁴); genes involved in MyD88-dependent TLR signaling and protection against experimental colitis (*Myd88*¹⁵⁹, *Tlr2*¹⁷⁵, *Tlr4*¹⁷⁶, *Tlr5*⁸⁰); genes involved in intracellular immune signaling (*Smad7*¹⁷⁷, *Stat3*¹⁷⁸); and a number of pro- and anti-inflammatory cytokines, chemokines and biological mediators that have been implicated in protection against or exacerbation of colitis (*Ccl2*¹⁷⁹, *Cxcl1*¹⁷⁹, *Il10*¹⁸⁰, *Il13*¹⁸¹, *Il18*¹⁸², *Il1b*¹⁸³, *Il6*¹⁵⁷, *Ptgs2*¹⁸⁴, *Tgfb1*¹⁷⁷, *Tnfs13b*⁴⁵, *Tslp*¹⁸⁵). Among these genes, five (including MyD88, as a positive control) were down-regulated in MyD88^{ΔIEC} mice, and none were up-regulated (**Figure**

5-11a). The most significantly down-regulated gene was the polymeric immunoglobulin receptor (*Pigr*), the epithelial transporter for polymeric IgA antibodies. Immunostaining of colon tissues revealed that expression of pIgR and co-localization with IgA were severely down-regulated in epithelial cells of MyD88^{ΔIEC} mice (**Figure 5-11b**), and fecal levels of IgA levels were significantly reduced (**Figure 5-11c**). Importantly, this reduction in luminal IgA was similar to the reduction we previously reported in MyD88^{-/-} mice¹¹, supporting a direct role for epithelial MyD88 signaling in regulating IgA transcytosis. Consistent with these results, we confirmed a direct role for epithelial MyD88 signaling in regulating pIgR expression by demonstrating that shRNA-mediated knockdown of MyD88 expression in the human HT-29 colon carcinoma cell-line significantly reduced the up-regulation of pIgR in response to bacterial stimulation (**Chapter 4, Figure 4-7**). Other down-regulated genes included mucin-2 (*Muc2*), the major protein constituent of intestinal mucus, and the antimicrobial peptides regenerating islet-derived IIIγ (*Reg3g*) and defensin-α related sequence 1 (*Defa-rs1*). Although there were no significant differences in goblet cell numbers in the colonic epithelia of MyD88^{Flox} and MyD88^{ΔIEC} mice (**Figure 5-12**), immunostaining revealed that the level of mucin-2 protein per goblet cell was reduced in MyD88^{ΔIEC} mice (**Figure 5-11d**). Down-regulation of RegIIIγ and Defa-rs1 likely contributed to the reduced antimicrobial activity of colonic mucus in MyD88^{ΔIEC} mice (**Figure 5-5c**). Interestingly, we found that expression of many epithelial genes that have been previously been reported to be regulated by MyD88 signaling, including *Areg*, *IL-6*, *TLR4*, and *Ptgs2*, was not altered in MyD88^{ΔIEC} mice. It is possible that MyD88-dependent signaling from other cell types may supply cues that regulate expression of these genes.

Loss of MyD88 signaling in colonic epithelial cells results in increased susceptibility to dextran sulfate sodium-induced colitis

Inappropriate inflammatory responses to normal components of the intestinal microbiota are thought to play a major role in the pathogenesis of IBD.^{79,155,186} Although we observed no overt inflammatory disease in MyD88^{ΔIEC} mice, they exhibited several defects that have been reported in IBD patients, including reduced expression of pIgR,⁶⁰ MUC2,¹⁸⁷ and antimicrobial peptides^{163,188} as well as perturbed spatial relationships between microbiota and host.^{42,69} To test the hypothesis that MyD88^{ΔIEC} mice are more susceptible to experimental colitis, acute epithelial damage was induced by administration of 2% dextran sulfate sodium (DSS) in the drinking water for 7 days. MyD88^{ΔIEC} mice had significantly higher disease activity than did MyD88^{Flox} mice, beginning as early 1 day after initiation of DSS treatment (**Figure 5-13a**). At the termination of the experiment, increased inflammatory cell infiltration and epithelial destruction were observed in colons of MyD88^{ΔIEC} mice (**Figure 5-13b,c**). Surprisingly, we did not detect expression of several genes that have previously been reported to be produced by intestinal epithelial cells (*Defa-6*, *Tnfsf13b*, *Il-13*, *Il-10*, *Tslp*) in epithelial cells from either MyD88^{ΔIEC} or MyD88^{Flox} mice under normal or inflammatory conditions (**Table 5-2**). Nineteen genes from a total of 36 were selected for principal component analysis (PCA), based on ranking of P values for the comparison of mRNA transcript levels in MyD88^{ΔIEC} and MyD88^{Flox} mice, with and without DSS treatment (n = 31) (**Tables 5-2, 5-3 and 5-4**). Factor analysis by the principal component method³ detected a significant relationship among the 19 transcripts ($X^2 = 816.7$, $p < 0.0001$), and reduced the data to 4 principal components (PCs) (**Figure 5-14**). PC1 and PC2 together

accounted for 68.8% of the overall variance in gene expression, whereas PC3 and PC4 only contributed minimally (variance proportion < 0.1). Scores for PC1 and PC2 for each individual mouse were calculated as the sum of normalized expression levels for each gene multiplied by the corresponding coefficient for PC1 and PC2. This principal component analysis of gene expression in colonic ECs following DSS treatment revealed significantly different patterns in MyD88^{ΔIEC} and MyD88^{Flox} mice (**Figure 5-13d**)

Expression of 10 genes was significantly down-regulated and expression of 5 genes was up-regulated in colonic ECs from MyD88^{Flox} mice following DSS treatment (**Figure 5-13e**, top panel). Interestingly, MyD88^{ΔIEC} mice were defective in their ability to up-regulate the expression of the pro-inflammatory factors TNF, CXCL2 and IL-1β in colonic ECs in response to DSS-induced damage (**Figure 5-13e**, bottom panel). Aberrant up-regulation of the antimicrobial peptide Defa-rs1 in colonic ECs of MyD88^{ΔIEC} mice may have been caused by increased microbial stimulation secondary to increased epithelial damage in these mice. These findings suggest that lack of epithelial MyD88 expression causes specific defects in innate immune gene expression, resulting in a compromised host response to epithelial injury and increased colonic inflammation.

Dhewulqp

Protection against bacterial invasion and infection in the intestine is dependent on the resident microbiota, the mucus layer containing SIgA and antimicrobial peptides, and the monolayer of IECs that provide a physical and innate immune barrier¹⁵⁴. Here we demonstrated that loss of epithelial MyD88 signaling compromises antibacterial immunity at all three layers of defense, resulting in translocation of bacteria to mesenteric lymph nodes. Loss of MyD88 in IECs did not cause spontaneous colitis, but rendered mice more susceptible to inflammation caused by the epithelial damaging agent DSS. These findings demonstrate the critical role of epithelial MyD88 signaling in maintenance of intestinal barrier function and protection against inflammation.

Unlike the inflammatory response generated by MyD88-dependent TLR signaling in immune cells, IECs respond by producing factors involved in epithelial cell proliferation, maintenance of barrier function, innate antimicrobial immunity and transport of SIgA.^{160,6} Here we report that targeted deletion of the *Myd88* gene in IECs was associated with reduced expression of pIgR and epithelial transport of IgA, providing direct evidence for the first time that epithelial MyD88 signaling regulates the production of SIgA. These SIgA antibodies in turn modulate the composition of the gut microbiota and form the first line of antigen-specific immunity that prevents invasion of IECs by members of the gut microbiota.⁴⁵ This connection between epithelial MyD88 signaling, SIgA production and host-microbial homeostasis provides a potential explanation for our previous findings that reduced pIgR expression and SIgA transport were correlated with disease severity in human inflammatory bowel disease⁶⁰ and in mouse models of experimental colitis.¹⁶¹ We previously reported¹⁸⁹ that expression of pIgR was up-

regulated by TNF and IL-1 β , the latter of which signals through a MyD88-dependent pathway.¹⁹⁰ We found here that loss of epithelial MyD88 signaling was associated with defective production of IL-1 β and TNF in colonic ECs in response to DSS-induced inflammation, suggesting that MyD88 may coordinate the response of IECs to autocrine and paracrine stimulation with IL-1 and TNF, as well as TLR signaling.

The intestinal epithelium is protected by a mucus layer that increases in thickness from the small to the large bowel in proportion to the density of the microbiota.³⁶ In addition to its role as a physical barrier, mucus serves to trap protective factors such as SIgA and antimicrobial peptides.^{154, 191} We found that colonic ECs from MyD88 ^{Δ IEC} mice produced significantly less Muc-2, antimicrobial peptides RegIII γ and Defa-rs1, and SIgA, consistent with reduced antimicrobial activity in colonic mucus. Vaishnav *et al.*⁴² recently reported that mice with a targeted deletion of MyD88 in IECs had reduced expression of the antimicrobial peptide RegIII- γ in the terminal ileum, and increased bacterial colonization of small intestinal surfaces. Gong *et al.*⁸¹ reported that mice transgenic for an IEC-targeted, dominant-negative mutant of MyD88 had reduced expression of antimicrobial peptides in the small intestine and increased bacterial translocation to MLNs. MyD88 signaling may also provide cues to immune cells for regulating the expression of antimicrobial and innate immune factors. Ismail *et al.*¹⁹² found that expression of RegIII- γ in small intestinal $\gamma\delta$ intraepithelial lymphocytes (IELs) was dependent on epithelial-specific MyD88 signaling, but not on expression of MyD88 within the IELs themselves. Thus epithelial MyD88 signaling provides direct and indirect signals to augment production of antimicrobial factors that segregate the microbiota from host tissues.

In our studies, a hallmark of epithelial-specific MyD88 deficiency was translocation of bacteria to the draining MLNs, including the opportunistic pathogen *Klebsiella pneumoniae*. This Gram-negative bacterium of the family *Enterobacteriaceae* is commonly found among the resident gut bacteria in both humans and mice, but has limited invasive potential in healthy individuals.¹⁹³ Although the defect in epithelial barrier function in MyD88^{ΔIEC} mice may have contributed to paracellular leakage of bacteria into the *lamina propria*, we did not observe translocation of commensal *E. coli* to the MLNs of MyD88^{ΔIEC} mice, even though these bacteria colonized the mucus layer of the colon. We conclude that virulence factors expressed by *K. pneumoniae*, but not by commensal *E. coli*, may be required for survival during transit from the mucosal surface to the MLNs. In contrast, Fukata *et al.*⁷⁷ reported translocation of *E. coli* to the MLNs in mice deficient in MyD88 in all cell types. MyD88-deficient mice have been shown to be highly susceptible to systemic infections with a broad range of bacteria.¹⁹⁴ Intratracheal infection of MyD88-deficient mice with *K. pneumoniae* resulted in severe pneumonia, attenuated neutrophil influx, and systemic dissemination of bacteria.¹⁹⁵ Rare cases of autosomal recessive MyD88 deficiency in humans were found to be associated with recurrent life-threatening infections with pyogenic bacteria early in life,¹⁹⁴ including an acute urinary tract infection caused by *K. pneumoniae* in a 4 month-old boy. However, we did not observe systemic bacterial infections in MyD88^{ΔIEC} mice. We conclude that epithelial MyD88 signaling is required for preventing the translocation of *K. pneumoniae* and possibly other opportunistic pathogens to the MLNs, but that MyD88-sufficient immune cells can prevent the systemic spread of resident gut bacteria that have penetrated the mucosal barrier.

Our findings have revealed for the first time a unique role for epithelial MyD88 signaling in shaping the composition of the resident gut microbiota, including increased numbers of OTUs in the phylum *Proteobacteria* and decreased numbers of OTUs within the phylum *Bacteroidetes* in MyD88^{ΔIEC} mice. Interestingly, similar changes in the composition of the gut microbiota have been observed in human IBD patients.¹⁹⁶ Altered host factors that are believed to impact commensal bacteria in IBD patients include increased intestinal permeability, decreased production of antimicrobial peptides and aberrant SIgA responses,¹⁹⁶ all of which characterize the immune defects in mice with a targeted deletion of MyD88 in IECs. In addition to changes at the level of bacterial phyla and families, the Prediction Analysis for Microarrays technique identified 4 “signature” OTUs in the candidate division TM7, whose increased abundance was characteristic of the altered microbiota of MyD88^{ΔIEC} mice. Importantly, increased abundance of TM7 bacteria have been reported in humans with oral inflammation¹⁹⁷ and IBD.¹⁹⁸ Increased abundance of TM7 bacteria has been reported in the gut microbiota of mice deficient in the NLRP6 inflammasome, accompanied by the development of spontaneous intestinal inflammation and increased susceptibility to DSS-induced colitis.¹⁸² Reduced production of IL-18, which transduces signals through MyD88, was characteristic of NLRP6-deficient mice. Thus impaired MyD88 signaling in the intestine may alter the local microenvironment to favor expansion of TM7. In contrast, we did not observe changes in the gut microbiota of MyD88^{ΔIEC} mice that have been associated with other mouse models of intestinal inflammation caused by defects in immune cells, such as increased abundance of Gram-positive segmented filamentous bacteria (*Candidatus arthromitus*), which have been implicated in the expansion of Th17 cells and inflammation of the gut

and extraintestinal tissues¹⁶⁷. Neither did we observe expansion of *Proteus mirabilis* nor *Klebsiella pneumoniae*, two Gram-negative species of the family *Enterobacteriaceae*, the abundance of which was increased in the gut microbiota of mice with defective immunity in dendritic cells in the intestinal *lamina propria*.¹⁶⁸ Increased colonization with *Enterobacteriaceae* and elevated titers of Enterobacteriaceal antibodies have been associated with IBD in humans,¹⁹⁹ and species of the genus *Klebsiella* are observed more frequently in the stool of ulcerative colitis patients than healthy controls.²⁰⁰ Our finding of unique changes in the gut microbiota of MyD88^{ΔIEC} mice suggests that epithelial MyD88 signaling is required to protect against abnormal expansion of specific members of the gut microbial community.

Although loss of epithelial MyD88 signaling was not sufficient to cause spontaneous intestinal inflammation for at least one year of life, it was associated with increased severity of colitis following oral administration of the epithelial damaging agent, DSS. A potential mechanism for the increased susceptibility to DSS-induced colitis was suggested by our observation that MyD88^{ΔIEC} mice failed to up-regulate expression of TNF, CXCL2 and IL-1 β in colonic ECs in response to epithelial damage. This finding was somewhat surprising, considering that these factors are generally considered to be pro-inflammatory and their increased expression has been linked to increased disease severity in experimental colitis.¹⁵⁷ Our data are consistent with the notion that “physiological” inflammatory responses by IECs are a normal response to the continuous presence of the gut microbiota, and are necessary for repair of damaged epithelial cells. On the other hand, massive infiltration of gut microbes into the intestinal

DISCUSSION

Protection against bacterial invasion and infection in the intestine is dependent on the resident microbiota, the mucus layer containing SIgA and antimicrobial peptides, and the monolayer of IECs that provide a physical and innate immune barrier¹⁵⁴. Here we demonstrated that loss of epithelial MyD88 signaling compromises antibacterial immunity at all three layers of defense, resulting in translocation of bacteria to mesenteric lymph nodes. Loss of MyD88 in IECs did not cause spontaneous colitis, but rendered mice more susceptible to inflammation caused by the epithelial damaging agent DSS. These findings demonstrate the critical role of epithelial MyD88 signaling in maintenance of intestinal barrier function and protection against inflammation.

Unlike the inflammatory response generated by MyD88-dependent TLR signaling in immune cells, IECs respond by producing factors involved in epithelial cell proliferation, maintenance of barrier function, innate antimicrobial immunity and transport of SIgA.^{160,6} Here we report that targeted deletion of the *Myd88* gene in IECs was associated with reduced expression of pIgR and epithelial transport of IgA, providing direct evidence for the first time that epithelial MyD88 signaling regulates the production of SIgA. These SIgA antibodies in turn modulate the composition of the gut microbiota and form the first line of antigen-specific immunity that prevents invasion of IECs by members of the gut microbiota.⁴⁵ This connection between epithelial MyD88 signaling, SIgA production and host-microbial homeostasis provides a potential explanation for our previous findings that reduced pIgR expression and SIgA transport were correlated with disease severity in human inflammatory bowel disease⁶⁰ and in mouse models of experimental colitis.¹⁶¹ We previously reported¹⁸⁹ that expression of pIgR was up-

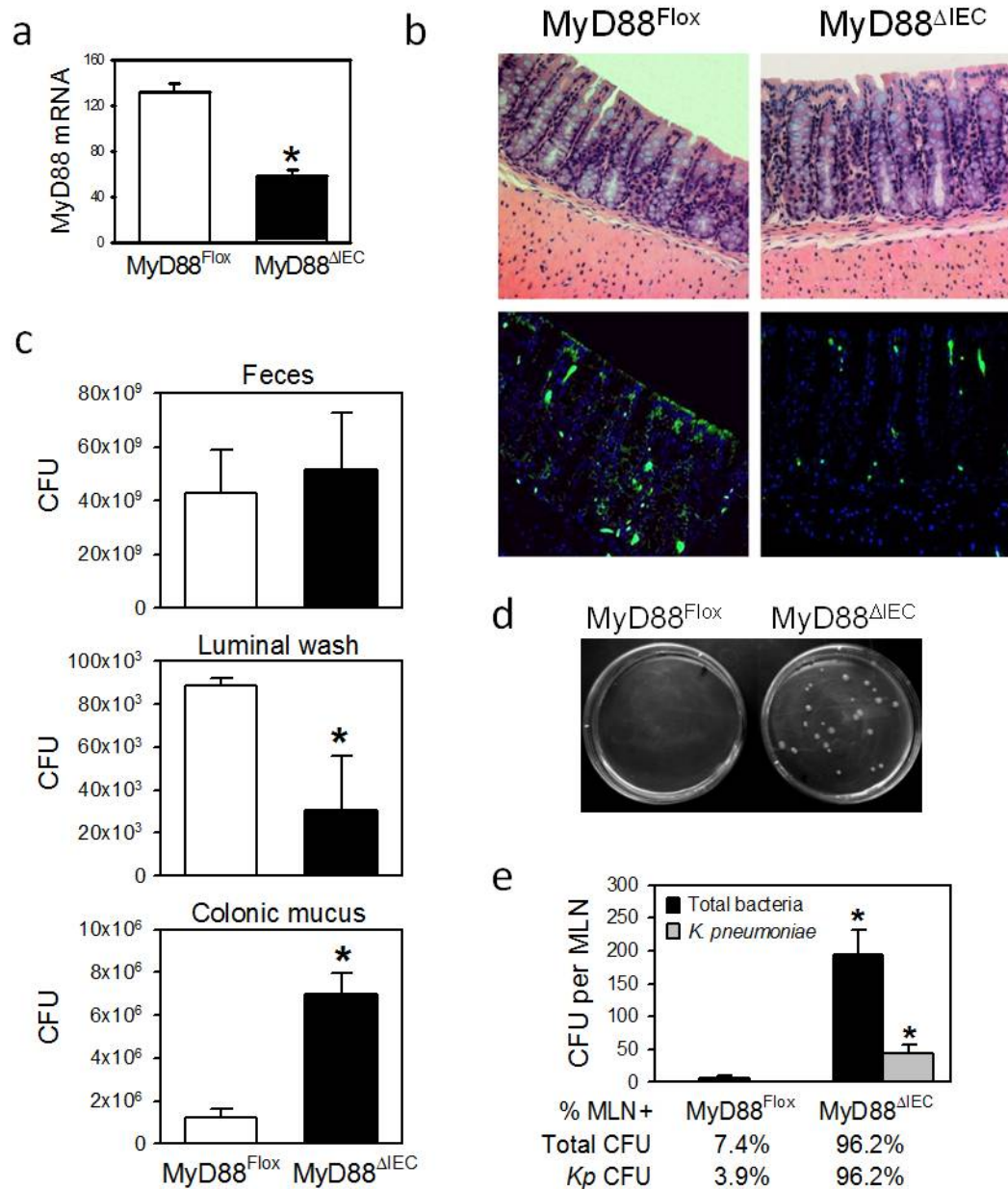


Figure 5-1 Targeted deletion of MyD88 in intestinal epithelial cells results in increased numbers of mucus-associated bacteria and bacterial translocation to mesenteric lymph nodes. (a) Mice with a targeted deletion of the *Myd88* gene in intestinal epithelial cells (IEC) (MyD88^{ΔIEC}) were created by crossing mice in which both copies of the *MyD88* gene were flanked with *loxP* sites (MyD88^{Fllox}) with mice expressing *Cre* recombinase under the control of the IEC-specific *Vill* promoter.

Figure 5-1 (continued) Levels of MyD88 mRNA were analyzed in colonic epithelial cells isolated from 9 week-old littermate MyD88^{Flox} and MyD88^{ΔIEC} mice by Nanostring nCounterTM hybridization. mRNA transcript abundance was normalized as described in Materials and Methods, and expressed as mean ± SEM (n=15). **(b)** Colon tissue sections from MyD88^{Flox} and MyD88^{ΔIEC} mice were stained with hematoxylin and eosin (upper panels) or FITC-labeled antibodies to MyD88 (green) and the nuclear stain DAPI (blue) (lower panels). **(c)** Bacterial colony-forming units (CFU) were enumerated following anaerobic culture of fecal homogenates (CFU/g feces), luminal washes (CFU/ml PBS), and surface mucus (CFU/100μl mucus) from colons of MyD88^{Flox} and MyD88^{ΔIEC} mice. Data are expressed as mean ± SEM (n = 6). **(d)** Bacterial colonies cultured under anaerobic conditions from mesenteric lymph nodes (MLNs) of representative MyD88^{Flox} and MyD88^{ΔIEC} mice. Large mucoid colonies from MyD88^{ΔIEC} mice were identified as *Klebsiella pneumoniae* by sequence analysis of 16S rRNA genes and biochemical tests **(Figure 5-3)**. **(e)** CFU of total anaerobic bacteria and *K. pneumoniae* cultured from MLNs of MyD88^{Flox} and MyD88^{ΔIEC} mice. Data in the bar graph are expressed as CFU per MLN (mean ± SEM, n = 27). The numbers below the bar graph indicate the percentage of mice of each genotype from which bacteria (total and *K. pneumoniae*) could be cultured from MLNs. In all panels, asterisks indicate that the mean for MyD88^{ΔIEC} mice is significantly different from the mean for MyD88^{Flox} mice (p < 0.05). (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

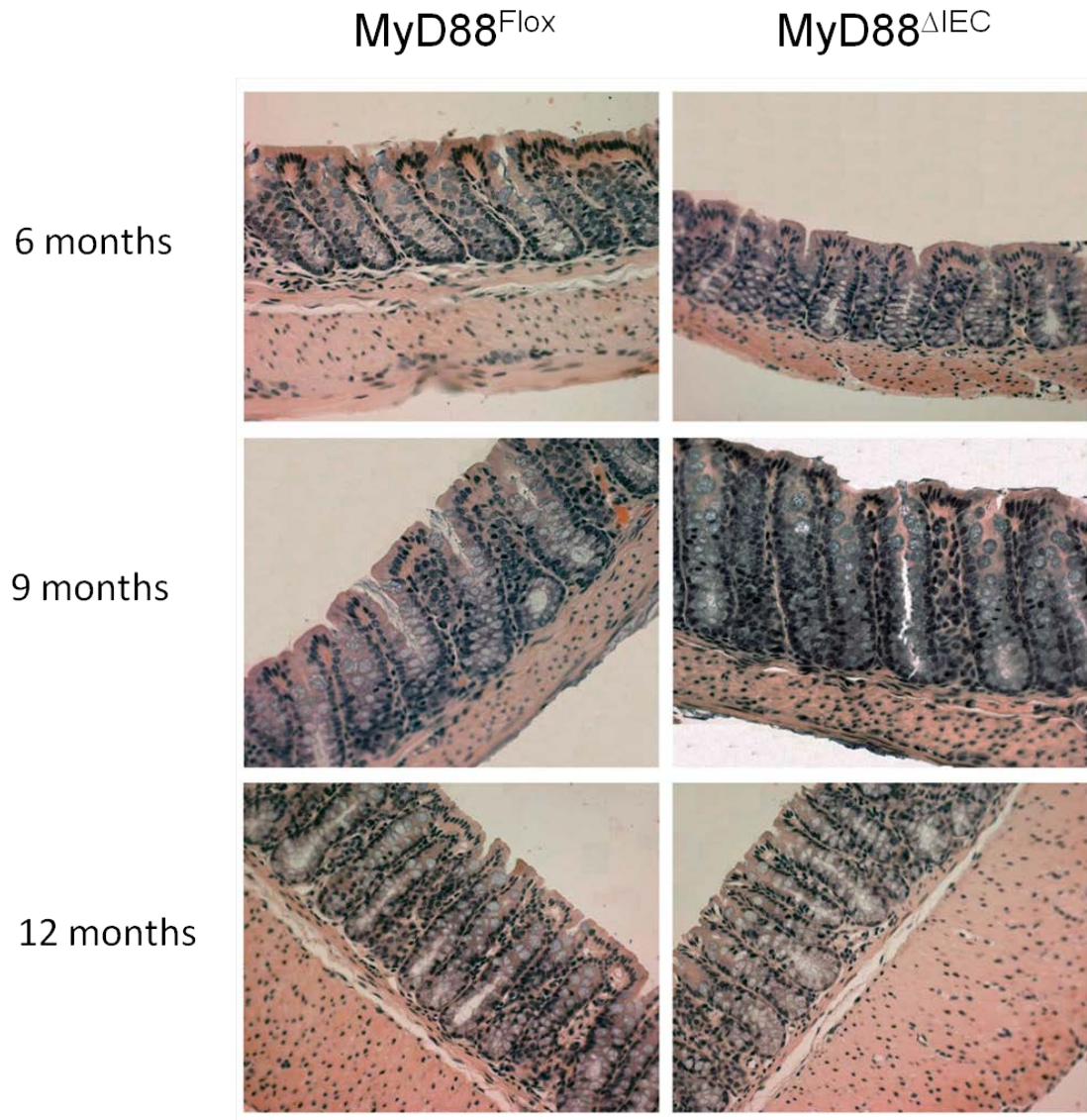


Figure 5-2 MyD88^{ΔIEC} mice do not develop spontaneous colitis with age. Colon histology from representative mice at 6, 9 and 12 months of age. Images of formalin-fixed colon tissues stained with hematoxylin and eosin were captured with a 20X objective. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission

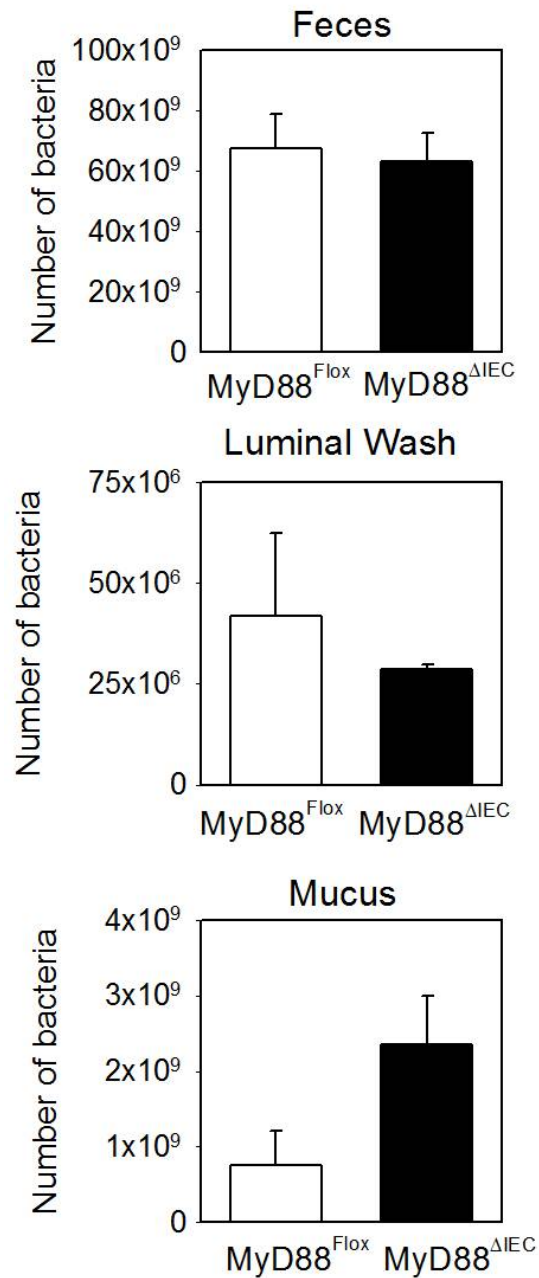


Figure 5-3. Quantification of bacteria in colonic niches of MyD88^{Flox} and MyD88^{ΔIEC} mice. Bacterial 16sRNA was quantified by qRT-PCR from fecal homogenates (CFU/g feces), luminal washes (CFU/ml PBS), and surface mucus (CFU/100 ml mucus) from

Figure 5-3 (continued) MyD88^{Flox} and MyD88^{ΔIEC} mice. Data are expressed as mean ± SEM (n = 5). (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

a Sequence of 16S rRNA gene amplified from mucoid colonies isolated from MLN of MyD88^{ΔIEC} mice

TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGACAAGGTTATTAAC
CTCACCGCCTTCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGC
ATCAGGCTTGCGCCCATTTGTGCAATATTCGCCACTGCTGCCTCCCGTAGGAGTAAT

b BLAST alignments of unknown 16S rRNA gene sequence with known bacterial genomes

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AFXH01000115.1	Klebsiella pneumoniae 1191100241 Contig115, whole genome shotgun sequence	339	339	98%	6e-93	97%
ACZD01000038.1	Klebsiella pneumoniae subsp. rhinoscleromatis ATCC 13884 contig00038, whole genome shotgun sequence	339	339	98%	6e-93	97%
NC_011283.1	Klebsiella pneumoniae 342, complete genome	339	2690	98%	6e-93	97%
NC_012731.1	Klebsiella pneumoniae NTUH-K2044 chromosome, complete genome	339	2695	98%	6e-93	97%
NC_009648.1	Klebsiella pneumoniae subsp. pneumoniae MGH 78578 chromosome, complete genome	339	2684	98%	6e-93	97%
NC_011601.1	Escherichia coli O127:H6 str. E2348/69, complete genome	322	2223	98%	1e-86	96%
CADZ01000104.1	Escherichia coli HM605, WGS project CADZ00000000 data, strain HM605, contig00104, whole genome shotgun sequence	316	316	98%	7e-85	95%
AFWP01000003.1	Escherichia coli O104:H4 str. GOS2, whole genome shotgun sequence	316	316	98%	7e-85	95%

Alignments

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>gb|AFXH01000115.1| Klebsiella pneumoniae 1191100241 Contig115, whole genome shotgun
sequence
Length=36321

Score = 339 bits (183), Expect = 6e-93
Identities = 191/195 (98%), Gaps = 0/195 (0%)
Strand=Plus/Minus

Query 1      TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGACA 60
Sbjct 27689   TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGACA 27630

Query 61     AGGTTATTAACTCACCGCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC 120
Sbjct 27629   AGGTTATTAACTTATCGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTC 27570

Query 121    TTCATACACGCGGCATGGCTGCATCAGGCTTGGCGCCCATTTGTGCAATATTCGCCACTGCT 180
Sbjct 27569   TTCATACACGCGGCATGGCTGCATCAGGCTTGGCGCCCATTTGTGCAATATTCGCCACTGCT 27510

Query 181    GCCTCCCGTAGGAGT 195
Sbjct 27509    GCCTCCCGTAGGAGT 27495
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>gb|ACZD01000038.1| Klebsiella pneumoniae subsp. rhinoscleromatis ATCC 13884 contig00038,
whole genome shotgun sequence
Length=1742

Score = 339 bits (183), Expect = 6e-93
Identities = 191/195 (98%), Gaps = 0/195 (0%)
Strand=Plus/Plus

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Query 1      TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGACA 60
Sbjct 1058   TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGACA 1117

Query 61     AGGTTATTAACTCACC GCCTTCCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC 120
Sbjct 1118   AGGTTATTAACTTATCGCCTTCCCTCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTC 1177

Query 121    TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCT 180
Sbjct 1178   TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCT 1237

Query 181    GCCTCCCGTAGGAGT 195
Sbjct 1238   GCCTCCCGTAGGAGT 1252
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Features in this part of subject sequence:
rRNA-16S ribosomal RNA

Score = 339 bits (183), Expect = 6e-93
Identities = 191/195 (98%), Gaps = 0/195 (0%)
Strand=Plus/Minus

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Query 61     AGGTTATTAACTCACC GCCTTCCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC 120
Sbjct 455147  AGGTTATTAACTCACC GCCTTCCCTCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTC 455088

Query 121    TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCT 180
Sbjct 455087  TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCT 455028

Query 181    GCCTCCCGTAGGAGT 195
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Features in this part of subject sequence:
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Score = 339 bits (183), Expect = 6e-93
Identities = 191/195 (98%), Gaps = 0/195 (0%)
Strand=Plus/Minus

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Query 61     AGGTTATTAACTCACC GCCTTCCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC 120
Sbjct 1223505  AGGTTATTAACTCACC GCCTTCCCTCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTC 1223446

Query 121    TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCT 180
Sbjct 1223445  TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCT 1223386

Query 181    GCCTCCCGTAGGAGT 195
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```

Features in this part of subject sequence:
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Score = 339 bits (183), Expect = 6e-93
Identities = 191/195 (98%), Gaps = 0/195 (0%)
Strand=Plus/Plus

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Query 61     AGGTTATTAACTCACC GCCTTCCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC 120
Sbjct 4546151  AGGTTATTAACTCACC GCCTTCCCTCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTC 4546210

Query 121    TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCT 180
Sbjct 4546211  TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCT 4546270

Query 181    GCCTCCCGTAGGAGT 195
Sbjct 4546271  GCCTCCCGTAGGAGT 4546285
```

>ref|NC_011601.1| **E** Escherichia coli O127:H6 str. E2348/69, complete genome
Length=496553

Features in this part of subject sequence:
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Score = 322 bits (174), Expect = 1e-86
Identities = 190/197 (96%), Gaps = 4/197 (2%)
Strand=Plus/Minus

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Query 1      TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGA-C 59
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Query 60     -AAGGTATTAACTTCACCGCCTTCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCCT 118
Sbjct 4238897 AAAGG-TATTAACCTTACCCCTTCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCCT 4238839

Query 119    TCTTCATACACGCGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCTG 178
Sbjct 4238838 TCTTCATACACGCGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCTG 4238779

Query 179    CTGCCTCCCGTAGGAGT 195
Sbjct 4238778 CTGCCTCCCGTAGGAGT 4238762

```

Features in this part of subject sequence:
rRNA-16S ribosomal RNA

Score = 316 bits (171), Expect = 7e-85
Identities = 190/198 (96%), Gaps = 6/198 (3%)
Strand=Plus/Minus

```

Query 1      TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGA-C 59
Sbjct 234257  TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAAT-GAGC 234199

Query 60     -AAGGTATTAACTTCAC-CGCCTTCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCC 117
Sbjct 234198  AAAGG-TATTAACCTTACTC-CCTTCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCC 234141

Query 118    TTCTTCATACACGCGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCT 177
Sbjct 234140  TTCTTCATACACGCGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCT 234081

Query 178    GCTGCCTCCCGTAGGAGT 195
Sbjct 234080  GCTGCCTCCCGTAGGAGT 234063

```

Features in this part of subject sequence:
rRNA-16S ribosomal RNA

Score = 316 bits (171), Expect = 7e-85
Identities = 190/198 (96%), Gaps = 6/198 (3%)
Strand=Plus/Plus

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Query 1      TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGA-C 59
Sbjct 2961541 TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAAT-GAGC 2961599

Query 60     -AAGGTATTAACTTCAC-CGCCTTCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCC 117
Sbjct 2961600 AAAGG-TATTAACCTTACTC-CCTTCCTCCCGCTGAAAGTACTTTACAACCCGAAGGCC 2961657

Query 118    TTCTTCATACACGCGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCT 177
Sbjct 2961658 TTCTTCATACACGCGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCT 2961717

Query 178    GCTGCCTCCCGTAGGAGT 195
Sbjct 2961718 GCTGCCTCCCGTAGGAGT 2961735

```

Features in this part of subject sequence:
rRNA-16S ribosomal RNA

C Biochemical tests

Differentiation media	Clone #1 isolated from MLN of MyD88 ^{ΔIEC}	Clone #2 isolated from MLN of MyD88 ^{ΔIEC}	<i>E. coli</i> strain Nissle 1917	Expected results for <i>K.</i> <i>pneumoniae</i>	Expected results for <i>K. pneumoniae</i> subsp. <i>ozaenae</i>	Expected results for <i>K. pneumoniae</i> subsp. <i>rhinoscleromatis</i>	Expected results for <i>K.</i> <i>oxytoca</i>	Expected results for <i>E. coli</i>
Indole	-	-	+	-	-	-	+	+
Simmons citrate	+	+	-	+	-	-	+	-
Christensen's urea	+	+	-	+	-	-	+	-

Figure 5-4 Identification of *Klebsiella pneumoniae* in the mesenteric lymph nodes

(MLN) of MyD88^{ΔIEC} mice. (a) Sequence of the 16S rRNA gene amplified from mucoid

Figure 5-4 (continued) bacterial colonies isolated from MLNs of MyD88^{ΔIEC} mice.

Identical sequences were obtained from colonies isolated from 2 different MyD88^{ΔIEC}

mice. **(b)** BLAST alignments of the unknown 16S rRNA sequence with known bacterial genome sequences in the National Center for Biotechnology Information database.

Alignments are shown for 5 *Klebsiella pneumoniae* strains and 3 *Escherichia coli* strains with at least 98% query coverage, ranked by maximum alignment score. **(c)** Biochemical tests used to differentiate *Klebsiella pneumoniae* from other *Klebsiella* species and *Escherichia coli*. Two different clones isolated from MLNs of different MyD88^{ΔIEC} mice and *Escherichia coli* strain Nissle1917 were tested and compared to expected results.

(Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

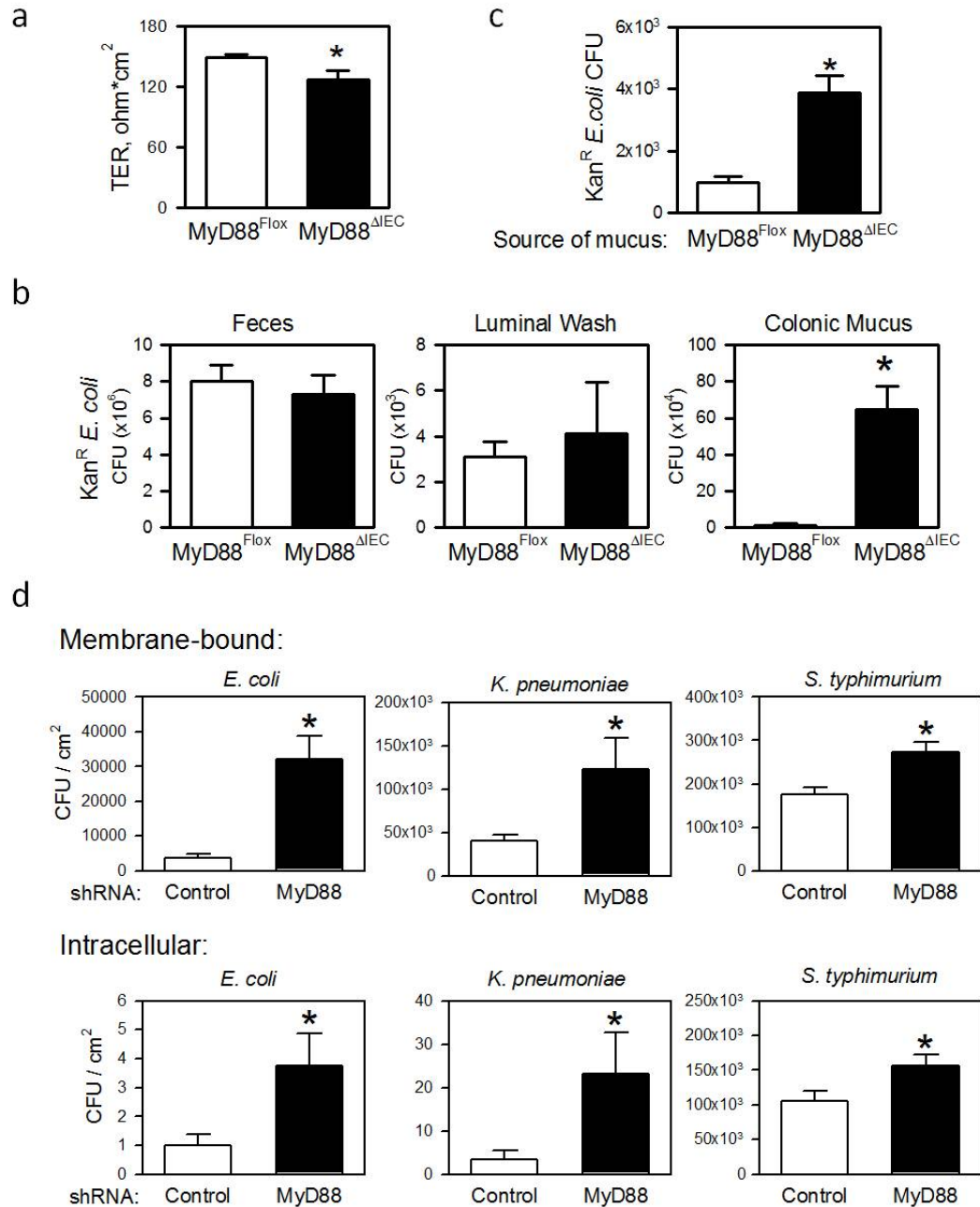


Figure 5-5 Loss of MyD88 expression in intestinal epithelial cells compromises

epithelial barrier integrity. (a) Epithelial barrier function. Transmucosal electrical

resistance (TER) was analyzed *ex vivo* in isolated mouse colon tissues placed in Ussing

chambers. Data are expressed as a mean ± SEM (n = 8). **(b)** Association of exogenous,

noninvasive bacteria with colonic mucus. MyD88^{Flox} and MyD88^{ΔIEC} mice were

Figure 5-5 (continued) administered *E. coli* strain Nissle 1917 that had been transformed to kanamycin resistance (Kan^R *E. coli*) at a dose of 2.5×10^7 CFU/ml in the drinking water for 7 days. Kanamycin-resistant colonies were enumerated after anaerobic culture of fecal homogenates (CFU/g feces), luminal washes (CFU/ml PBS), and epithelial-associated mucus (CFU/100 μ l mucus). Data are expressed as mean \pm SEM (n = 6).

(c) Antibacterial activity of colonic mucus. Aliquots of mucus harvested from the colons of MyD88^{Flox} and MyD88 ^{Δ IEC} mice were incubated for 15 min with 10^6 CFU of Kan^R *E. coli*, then cultured in the presence of kanamycin to enumerate surviving *E. coli*. Data are expressed as mean \pm SEM (n = 6). (d) *In vitro* bacterial invasion assays. Clones of the HT-29 human colon carcinoma cell-line were stably transfected with shRNA specific for MyD88 or a random control sequence. Cell monolayers (2 cm²) were incubated with 10^6 CFU (MOI of 10:1) of *E. coli* strain Nissle 1917, a strain of *K. pneumoniae* isolated from MLNs of MyD88 ^{Δ IEC} mice, or *Salmonella typhimurium* strain SL1344. Membrane-bound CFU were enumerated by anaerobic culture of cell homogenates after washing to remove unbound bacteria, and intracellular CFU were enumerated in parallel plates after 2 hours of treatment of cell monolayers with gentamicin to kill all extracellular bacteria. Data are expressed as mean \pm SEM (n = 6). Asterisks indicate that the mean for MyD88 ^{Δ IEC} mice is significantly different from the mean for MyD88^{Flox} mice (panels **a-c**) or that the mean for cells expressing MyD88 shRNA is significantly different from the mean for cells expressing control shRNA (panel **d**) ($p < 0.05$). (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

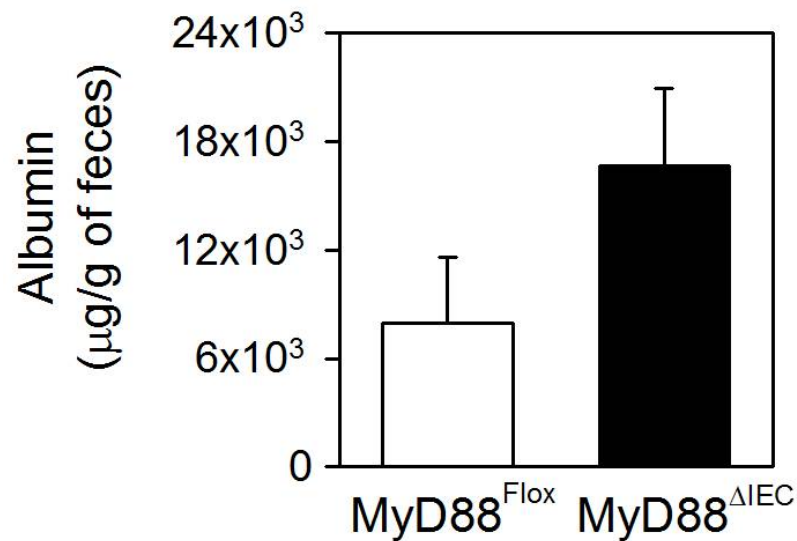


Figure 5-6 Fecal Albumin levels in MyD88^{ΔIEC}. Feces were collected from 10 week old MyD88^{Flox} and MyD88^{ΔIEC} mice and serum albumin levels were determined by ELISA. Data are expressed as mean ± SEM (n = 5). (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

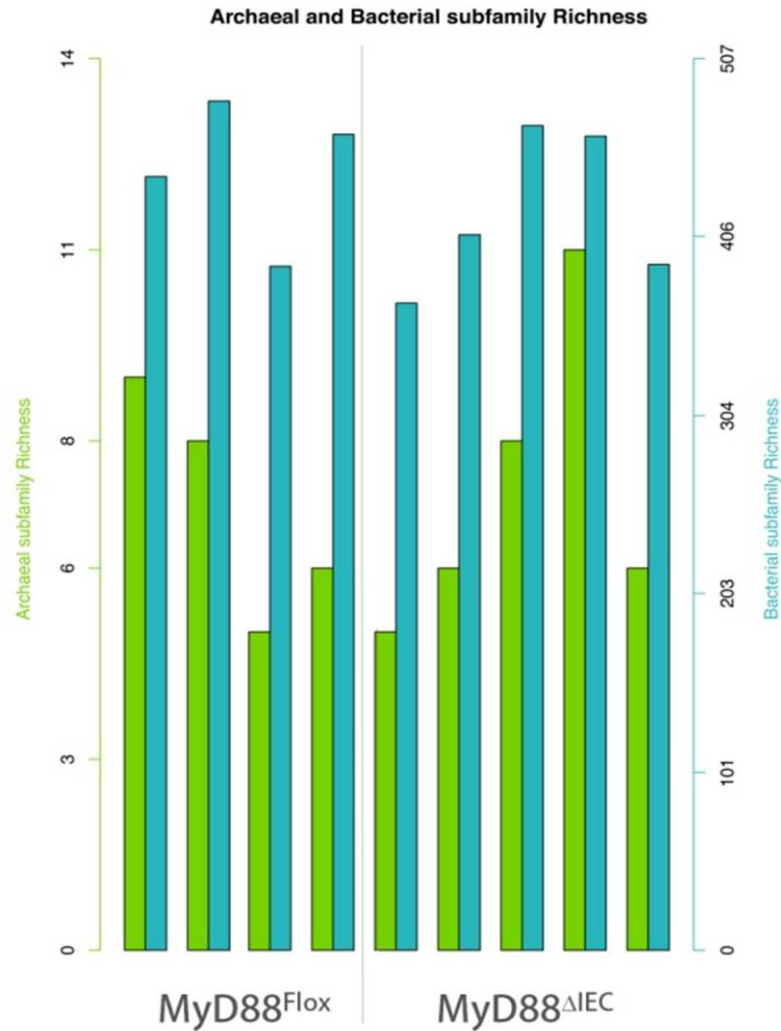


Figure 5-7 Archaeal (green) and bacterial (blue) subfamily richness in individual *MyD88^{Flox}* and *MyD88^{ΔIEC}* mice. Each bar represents an individual mouse. The Y axes indicate the number of distinct bacterial or archaeal subfamilies identified in fecal DNA from each mouse, based on taxonomic classification of 16S rRNA sequences. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

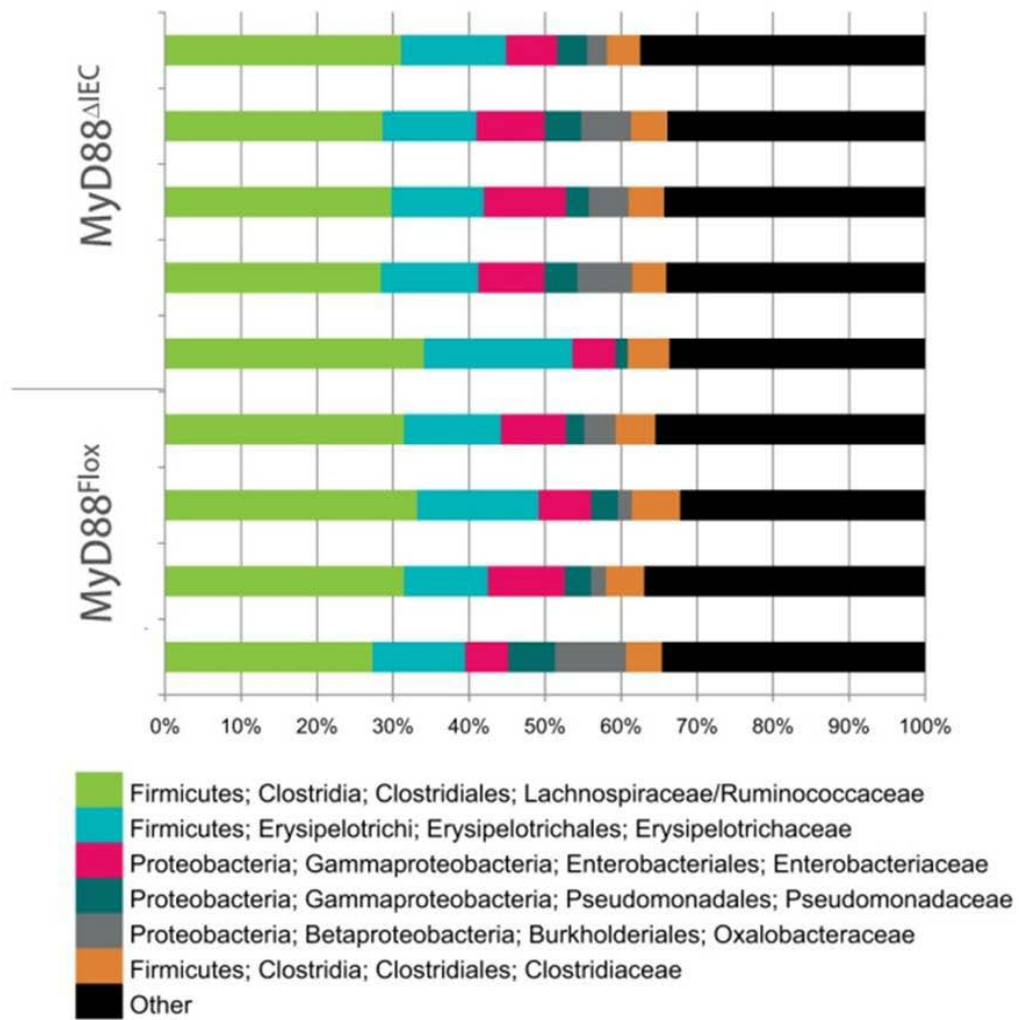


Figure 5-8 Proportions of operational taxonomic units (OTUs) classified at the family rank in fecal DNA from *MyD88^{ΔIEC}* and *MyD88^{Flox}* mice. Each bar represents an individual mouse. The size of each color block represents the number of detected OTUs in that family relative to the total number of OTUs detected in fecal DNA from that mouse. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

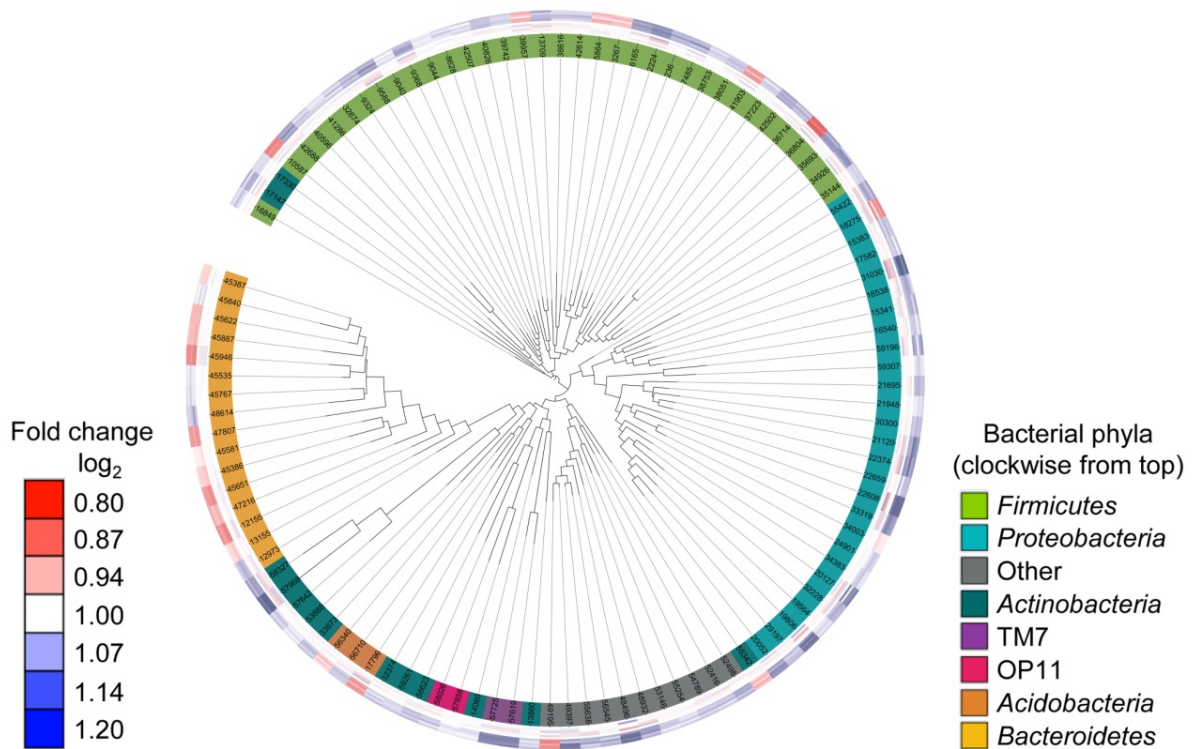


Figure 5-9 Differences in the composition of the fecal microbiota of MyD88^{Flox} and MyD88^{ΔIEC} mice. The relative abundance of operational taxonomic units (OTUs) was quantified by PhylochipTM microarray analysis of 16S rRNA gene sequences in fecal DNA from 4 MyD88^{Flox} and 5 MyD88^{ΔIEC} littermate mice (see Methods for details of data analysis). A representative 16S rRNA gene from each of 109 differentially expressed OTUs was aligned and used to infer the phylogenetic tree shown in this figure. The rings around the tree comprise a heatmap where the inner ring includes the samples from MyD88^{Flox} mice, and the outer ring includes the samples from MyD88^{ΔIEC} mice. Blue lines indicate that the OTU was more abundant in that sample than in the mean of MyD88^{Flox} mice samples, and red lines indicate that the OTU was less abundant. The color saturation indicates the fold difference in OTU abundance for each mouse compared to the mean for MyD88^{Flox} mice. (Frantz *et al.* Targeted deletion of MyD88 in

Figure 5-9 (continued) intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*,in press 2012) Reprinted with permission.

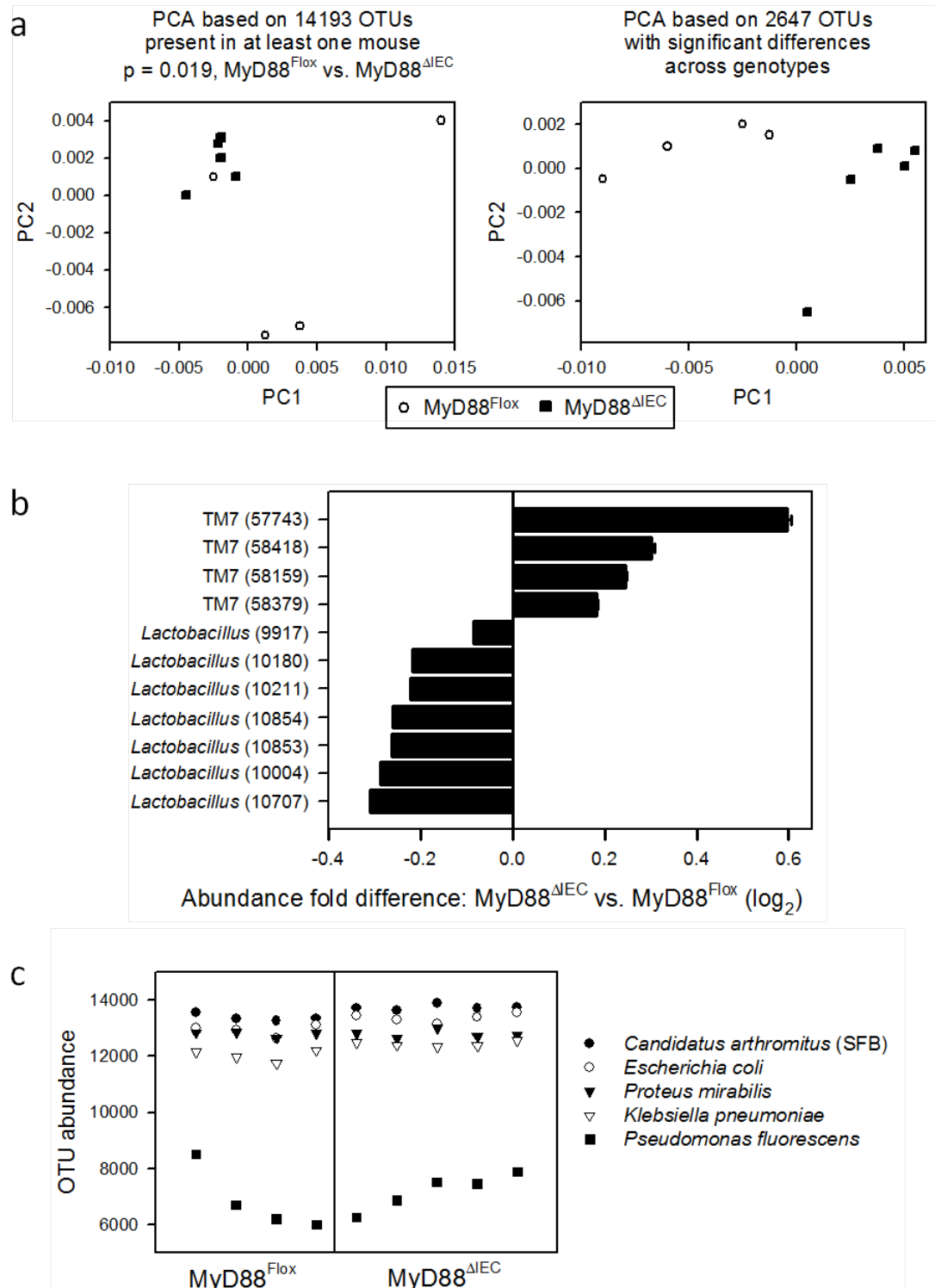


Figure 5-10 Loss of MyD88 expression in intestinal epithelial cells is associated with changes in the composition of the fecal microbiota. (a) Principal component analysis

Figure 5-10 (continued) (PCA) was used to generate two-dimensional ordination plots that visualize complex relationships between the fecal microbiota of individual MyD88^{Flox} and MyD88^{ΔIEC} mice, based on weighted Unifrac distances between OTUs detected in fecal samples. The graph on the left is based on 14193 OTUs present in at least one mouse, and the graph on the right is based on 2647 OTUs with significant differences in abundance between MyD88^{Flox} and MyD88^{ΔIEC} mice. **(c)** Significant OTUs that characterize the distinctive microbiota of MyD88^{Flox} and MyD88^{ΔIEC} mice were identified using the Prediction Analysis for Microarrays (PAM) method. Data in the bar graph are expressed as fold difference (\log_2 , mean \pm SEM) in abundance between MyD88^{ΔIEC} (n = 5) and MyD88^{Flox} mice (n = 4). Bars to the right of the zero line represent distinctive OTUs that were more abundant in MyD88^{ΔIEC} mice, all of which were classified in the candidate phylum TM7. Bars to the left represent distinctive OTUs that were more abundant in MyD88^{Flox} mice, all of which were classified in the genus *Lactobacillus* (phylum *Firmicutes*). Numbers in parentheses on the Y-axis are the IDs for individual OTUs (see **Table 5-1** for complete taxonomy of the distinctive OTUs). **(d)** Relative abundance of 5 species of fecal bacteria in individual MyD88^{Flox} and MyD88^{ΔIEC} mice. Because each of these species (except *Candidatus arthromitus*) comprised multiple OTUs, the OTU which represented the biggest difference in abundance between MyD88^{Flox} and MyD88^{ΔIEC} mice is displayed. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

OTU ID	Phylum	Class	Order	Family	Genus	Representative Sequence		
						GenBank Accession ID	Green Genes ID	Annotation
57743	TM7	TM7-3	CW040	F16	unclassified	AY990056	135089	Mouse cecum clone C12_N15
58418	TM7	TM7-3	CW040	F16	unclassified	EU474524	290190	Sheep feces clone BHSD_aaa04f03
58159	TM7	TM7-3	CW040	F16	unclassified	EU461530	292119	Rhinoceros feces clone RH_aaj01g08
58379	TM7	TM7-3	CW040	F16	unclassified	EU474432	296978	Sheep feces clone BHSD_aaa04g04
9917	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	EF460497	215674	Lactobacillus vaginalis strain SP8
10180	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	EF412975	214229	Lactobacillus reuteri strain C1
10211	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	DQ057469	137125	Chicken cecum clone ii1348
10854	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	AF371487	49835	Swine intestine clone p-3083-SwA-3
10853	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	AY993443	131279	Mouse cecum clone C19_B05
10004	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	EU510009	275831	Mouse cecum clone WD1_aaf15f04
10707	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	AY993406	135404	Mouse cecum clone C19_I03

Table 5-1 Taxonomic annotations of operational taxonomic units (OTUs) with significant differences in abundance between MyD88^{Flox} and MyD88^{ΔIEC} mice.

Distinctive OTUs were identified by predictive analysis microarrays (see **Figure 5-10c**).

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Gene		mRNA, Number of Normalized Transcripts					
Symbol	Alias	Mean MyD88 ^{FLOX} n = 12	Mean MyD88 ^{ΔIEC} n = 12	Fold Change	Fold Change Log ₂	P-Value	P-Value -log ₁₀
<i>Areg</i>	AREG	3234.4	3513.1	1.09	0.12	0.6358000	0.1967
<i>Bcl2l1</i>	Bcl-XL	57.4	56.2	0.98	-0.03	0.8838000	0.0536
<i>Birc2</i>	cIAP2	112.8	119.0	1.05	0.08	0.6842000	0.1648
<i>Ccl2</i>	MCP-1	B.D.	B.D.				
<i>Cxcl1</i>	KC	267.9	271.4	1.01	0.02	0.9058000	0.0430
<i>Cxcl2</i>	MIP-2	25.3	28.1	1.11	0.15	0.5988000	0.2227
<i>Defa6</i>	Defcr6	B.D.	B.D.				
<i>Defa-rs1</i>	Defcr-rs1	66.0	43.5	0.66	-0.60	0.0313000	1.5045
<i>Il10</i>	IL-10	B.D.	B.D.				
<i>Il13</i>	IL-13	B.D.	B.D.				
<i>Il18</i>	IL-18	1483.4	1638.6	1.10	0.14	0.5409000	0.2669
<i>Il1b</i>	IL-1β	B.D.	B.D.				
<i>Il6</i>	IL-6	B.D.	B.D.				
<i>Mmp3</i>	MMP-3	B.D.	B.D.				
<i>Mmp9</i>	MMP-9	B.D.	B.D.				
<i>Muc2</i>	MUC-2	11303.0	9014.0	0.80	-0.33	0.0232000	1.6345
<i>Myd88</i>	MyD88	131.3	68.6	0.52	-0.94	0.0000001	7.0000
<i>Mylk</i>	MLCK	193.8	211.2	1.09	0.12	0.5253000	0.2796
<i>Pigr</i>	plgR	16200.7	12022.9	0.74	-0.43	0.0186000	1.7305
<i>Ptgs2</i>	Cox-2	B.D.	B.D.				
<i>Reg3g</i>	RegIIIγ	430.9	124.3	0.29	-1.79	0.0415000	1.3820
<i>Rela</i>	RelA/p65	520.2	589.5	1.13	0.18	0.1650000	0.7825
<i>Relb</i>	RelB	610.4	651.9	1.07	0.09	0.4783000	0.3203
<i>Smad7</i>	Smad7	101.9	109.1	1.07	0.10	0.5980000	0.2233
<i>Stat3</i>	STAT3	708.1	792.2	1.12	0.16	0.1458000	0.8362
<i>Tcf4</i>	Tcf4	105.8	111.7	1.06	0.08	0.5811000	0.2357
<i>Tff3</i>	ITF	2732.4	2482.2	0.91	-0.14	0.7114000	0.1479
<i>Tgfb1</i>	TGFβ1	24.8	21.5	0.87	-0.21	0.4145000	0.3825
<i>Tjp1</i>	ZO-1	347.5	407.7	1.17	0.23	0.0915000	1.0386
<i>Tlr2</i>	TLR2	69.7	61.3	0.88	-0.19	0.4706000	0.3273
<i>Tlr4</i>	TLR4	219.8	264.7	1.20	0.27	0.1282000	0.8921
<i>Tlr5</i>	TLR5	20.1	13.2	0.66	-0.61	0.1692000	0.7716
<i>Tnf</i>	TNF	167.6	183.7	1.10	0.13	0.5062000	0.2957
<i>Tnfaip3</i>	A20	1063.9	1225.2	1.15	0.20	0.0927000	1.0329
<i>Tnfsf13b</i>	BAFF	B.D.	B.D.				
<i>Tslp</i>	TSLP	B.D.	B.D.				

Table 5-2 Gene expression in colonic epithelial cells from MyD88^{Flox} and MyD88^{ΔIEC} mice. Colonic epithelial cells were isolated from 9 week-old mice . Relative levels of 36 individual mRNA transcripts were analyzed by Nanostring nCounterTM hybridization and normalized to 4 housekeeping genes. B.D., below detection. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

Gene		mRNA, Number of Normalized Transcripts					
Symbol	Alias	Mean MyD88 ^{FLOX} n = 8	Mean MyD88 ^{FLOX} + DSS n = 7	Fold Change	Fold Change Log ₂	P-Value	P-Value -log ₁₀
Areg	AREG	2258.9	1619.9	0.72	-0.48	0.006967	2.1570
Bcl2l1	Bcl-XL	47.6	24.8	0.52	-0.94	0.003141	2.5029
Birc2	cIAP2	92.1	65.6	0.71	-0.49	0.002997	2.5233
Ccl2	MCP-1	9.2	10.5	1.14	0.19	0.915884	0.0382
Cxcl1	KC	236.7	192.7	0.81	-0.30	0.226830	0.6443
Cxcl2	MIP-2	21.1	62.5	2.96	1.57	0.000060	4.2218
Defa6	Defcr6	B.D.	B.D.				
Defa-rs1	Defcr-rs1	60.7	24.4	0.40	-1.32	0.067800	1.1688
Il10	IL-10	B.D.	B.D.				
Il13	IL-13	B.D.	B.D.				
Il18	IL-18	1148.8	989.6	0.86	-0.22	0.128209	0.8921
Il1b	IL-1β	-2.5	21.2	8.55	3.10	0.000003	5.5229
Il6	IL-6	B.D.	B.D.				
Mmp3	MMP-3	B.D.	B.D.				
Mmp9	MMP-9	B.D.	B.D.				
Muc2	MUC-2	10867.3	6928.3	0.64	-0.65	0.001059	2.9751
Myd88	MyD88	126.1	82.4	0.65	-0.61	0.000006	5.2218
Mylk	MLCK	154.8	125.2	0.81	-0.31	0.161632	0.7915
Pigr	plgR	13339.3	10355.3	0.78	-0.37	0.000509	3.2933
Ptgs2	Cox-2	-2.8	9.4	3.39	1.76	0.001612	2.7926
Reg3g	RegIIIγ	231.8	2511.1	10.83	3.44	0.000007	5.1549
Rela	RelA/p65	464.9	408.7	0.88	-0.19	0.150123	0.8236
Relb	RelB	542.6	364.6	0.67	-0.57	0.000751	3.1244
Smad7	Smad7	89.8	41.3	0.46	-1.12	0.000135	3.8697
Stat3	STAT3	644.3	712.7	1.11	0.15	0.239217	0.6212
Tcf4	Tcf4	88.4	81.9	0.93	-0.11	0.387354	0.4119
Tff3	ITF	1603.1	1191.1	0.74	-0.43	0.008034	2.0951
Tgfb1	TGFβ1	18.8	17.8	0.94	-0.08	0.753089	0.1232
Tjp1	ZO-1	311.1	225.6	0.73	-0.46	0.008192	2.0866
Tlr2	TLR2	53.7	51.9	0.97	-0.05	0.830451	0.0807
Tlr4	TLR4	191.1	188.8	0.99	-0.02	0.923129	0.0347
Tlr5	TLR5	11.5	6.6	0.57	-0.81	0.220282	0.6570
Tnf	TNF	155.2	248.1	1.60	0.68	0.000074	4.1308
Tnfaip3	A20	1066.1	1000.8	0.94	-0.09	0.576085	0.2395
Tnfsf13b	BAFF	B.D.	B.D.				
Tslp	TSLP	B.D.	B.D.				

Table 5-3 DSS-induced changes in gene expression in colonic epithelial cells from MyD88^{Flox} mice. Mice (8 weeks of age) were given 2% DSS or regular drinking water for 7 days prior to isolation of colonic epithelial cells. Relative levels of 36 individual mRNA transcripts were analyzed by Nanostring nCounterTM hybridization and normalized to 4 housekeeping genes. B.D., below detection. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

Gene		mRNA, Number of Normalized Transcripts					
Symbol	Alias	Mean MyD88 ^{Flox} + DSS n = 7	Mean MyD88 ^{ΔIEC} + DSS n = 9	Fold Change	Fold Change Log ₂	P-Value	P-Value -log ₁₀
<i>Areg</i>	AREG	1619.9	1841.5	1.14	0.19	0.308406	0.5109
<i>Bcl2l1</i>	Bcl-XL	24.8	32.3	1.31	0.38	0.280251	0.5525
<i>Birc2</i>	clAP2	65.6	70.3	1.07	0.10	0.560356	0.2515
<i>Ccl2</i>	MCP-1	10.5	7.8			0.821752	
<i>Cxcl1</i>	KC	192.7	175.0	0.91	-0.14	0.611531	0.2136
<i>Cxcl2</i>	MIP-2	62.5	39.1	0.63	-0.68	0.010570	1.9759
<i>Defa6</i>	Defcr6	B.D.	B.D.				
<i>Defa-rs1</i>	Defcr-rs1	24.4	55.6	2.28	1.19	0.025500	1.5935
<i>Il10</i>	IL-10	B.D.	B.D.				
<i>Il13</i>	IL-13	B.D.	B.D.				
<i>Il18</i>	IL-18	989.6	965.2	0.98	-0.04	0.807478	0.0929
<i>Il1b</i>	IL-1β	21.2	12.4	0.59	-0.77	0.036702	1.4353
<i>Il6</i>	IL-6	B.D.	B.D.				
<i>Mmp3</i>	MMP-3	B.D.	B.D.				
<i>Mmp9</i>	MMP-9	B.D.	B.D.				
<i>Muc2</i>	MUC-2	6928.3	5807.4	0.84	-0.25	0.294908	0.5303
<i>Myd88</i>	MyD88	82.4	41.4	0.50	-0.99	0.000011	4.9586
<i>Mylk</i>	MLCK	125.2	147.6	1.18	0.24	0.272931	0.5639
<i>PiGr</i>	plgR	10355.3	10476.4	1.01	0.02	0.871202	0.0599
<i>Ptgs2</i>	Cox-2	9.4	3.7	0.40	-1.34	0.105633	0.9762
<i>Reg3g</i>	RegIIIγ	2511.1	1892.4	0.75	-0.41	0.136005	0.8664
<i>Rela</i>	RelA/p65	408.7	394.7	0.97	-0.05	0.707231	0.1504
<i>Relb</i>	RelB	364.6	403.1	1.11	0.15	0.407225	0.3902
<i>Smad7</i>	Smad7	41.3	51.5	1.25	0.32	0.347107	0.4595
<i>Stat3</i>	STAT3	712.7	656.9	0.92	-0.12	0.322349	0.4917
<i>Tcf4</i>	Tcf4	81.9	83.2	1.02	0.02	0.853857	0.0686
<i>Tff3</i>	ITF	1191.1	1226.2	1.03	0.04	0.804705	0.0944
<i>Tgfb1</i>	TGFβ1	17.8	21.7	1.22	0.29	0.236940	0.6254
<i>Tjp1</i>	ZO-1	225.6	258.3	1.14	0.20	0.273328	0.5633
<i>Tlr2</i>	TLR2	51.9	52.8	1.02	0.03	0.910508	0.0407
<i>Tlr4</i>	TLR4	188.8	175.3	0.93	-0.11	0.559386	0.2523
<i>Tlr5</i>	TLR5	6.6	11.5	1.74	0.80	0.213818	0.6700
<i>Tnf</i>	TNF	248.1	192.1	0.77	-0.37	0.007741	2.1112
<i>Tnfaip3</i>	A20	1000.8	940.5	0.94	-0.09	0.595508	0.2251
<i>Tnfsf13b</i>	BAFF	B.D.	B.D.				
<i>Tslp</i>	TSLP	B.D.	B.D.				

Table 5-4 Differences in DSS-induced changes in gene expression in colonic epithelial cells from MyD88^{Flox} and MyD88^{ΔIEC} mice. Mice (8 weeks of age) were given 2% DSS in the drinking water for 7 days prior to isolation of colonic epithelial cells. Relative levels of 36 individual mRNA transcripts were analyzed by Nanostring nCounterTM hybridization and normalized to 4 housekeeping genes. B.D., below detection. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

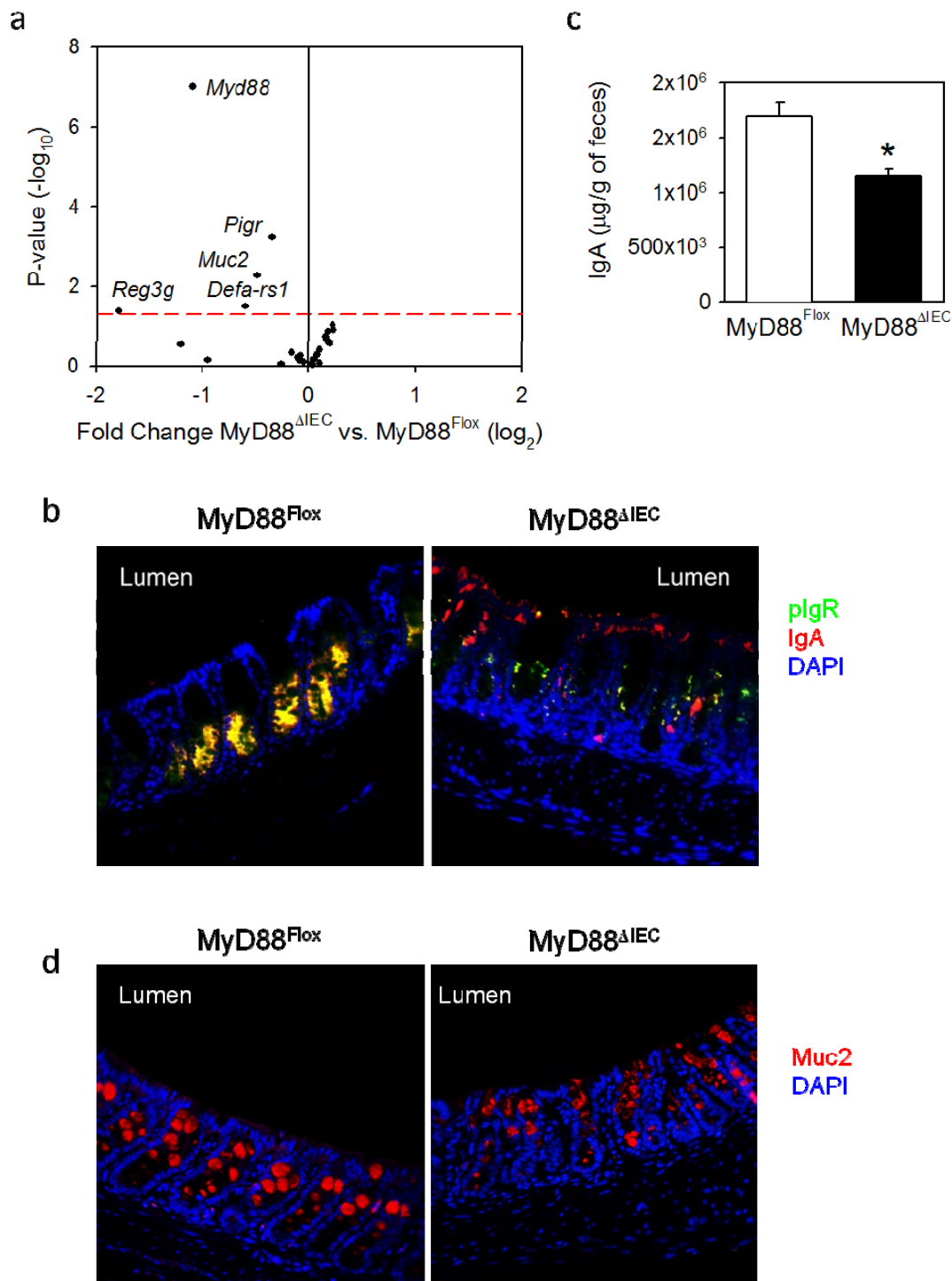


Figure 5-11. Loss of MyD88 expression alters epithelial gene expression and compromises pIgR-mediated IgA transport. (a) Colonic epithelial cells were isolated

Figure 5-11 (continued) from 8 week-old MyD88^{ΔIEC} and MyD88^{Flox} mice (n = 12). Levels of 36 individual mRNA transcripts were analyzed by Nanostring nCounterTM hybridization and plotted as mean fold difference (log₂) between MyD88^{ΔIEC} and MyD88^{Flox} mice vs. P-value (-log₁₀). mRNA transcript levels for genes above the red dashed line were significantly different in MyD88^{ΔIEC} mice compared to MyD88^{Flox} mice (p < 0.05). Genes to the left of the zero line were down-regulated, while genes to the right were up-regulated. **(b)** Immunofluorescence staining of colon tissues from 9 week-old MyD88^{Flox} and MyD88^{ΔIEC} mice (pIgR = red, IgA = green, DAPI-stained nuclei = blue). **(c)** Feces were collected from 10 week old MyD88^{Flox} and MyD88^{ΔIEC} mice and IgA levels were determined by ELISA. The asterisk indicate that the mean for MyD88^{ΔIEC} mice is significantly different from the mean for MyD88^{Flox} mice (p<0.05). Data are expressed as mean ± SEM (n = 5). **(d)** Immunofluorescence staining of colon tissues from 9 week-old MyD88^{Flox} and MyD88^{ΔIEC} mice (Muc2 = red, DAPI-stained nuclei = blue). (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

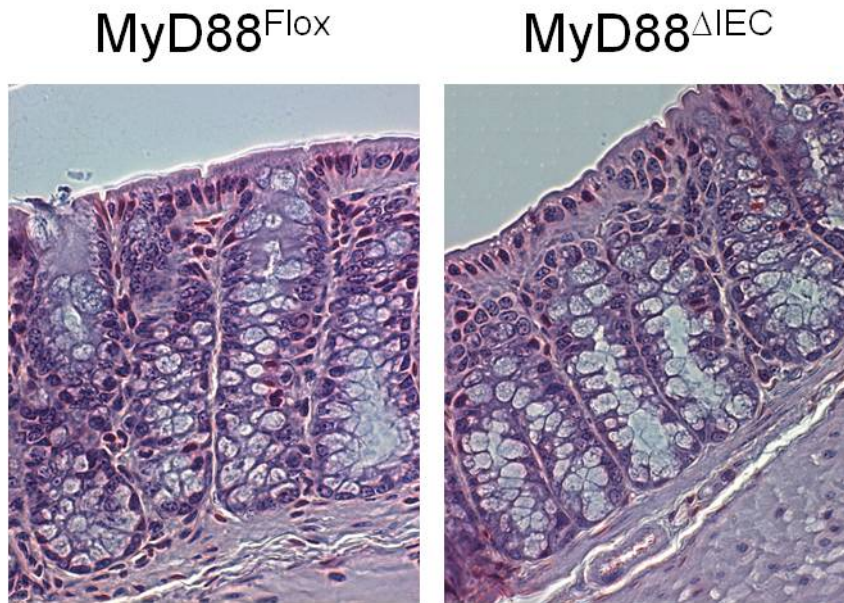


Figure 5-12. Loss of epithelial MyD88 expression does not affect goblet cell numbers in the colon. Images of formalin-fixed colon tissues from representative 10 week old mice, stained with hematoxylin and eosin, were captured with a 40X objective. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

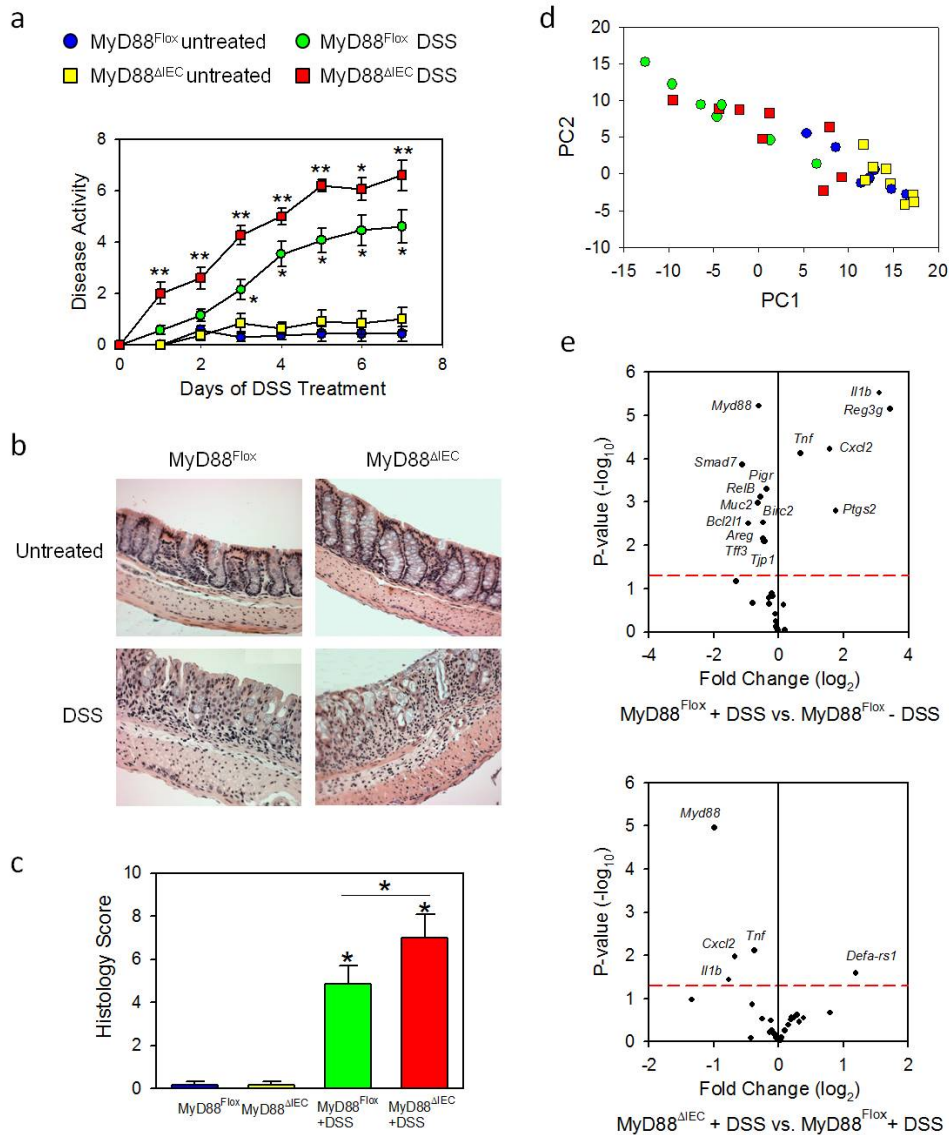


Figure 5-13. Loss of MyD88 expression in intestinal epithelial cells increases the severity of DSS-induced colitis. (a) Disease activity in MyD88^{Flox} and MyD88^{ΔIEC} mice given regular drinking water or 2% DSS for 7 days. Data are expressed as mean ± SEM (n = 8). One asterisk indicates that the mean for DSS-treated mice is significantly greater than the corresponding mean for untreated mice, and two asterisks indicate that the mean for DSS-treated MyD88^{ΔIEC} mice is greater than the mean for DSS-treated MyD88^{Flox} mice. (b) Colon histology from representative mice at day 7. Images of

Figure 5-13 (continued) formalin-fixed colon tissues stained with hematoxylin and eosin were captured with a 20X objective. **(c)** Histological inflammatory scores for MyD88^{Flox} and MyD88^{ΔIEC} mice on day 7 days. The asterisk indicate that the means for DSS-treated mice are significantly different from the means for untreated mice of the same genotype, and that the mean for MyD88^{ΔIEC} mice is significantly different from the mean for MyD88^{Flox} mice ($p < 0.05$). Data are expressed as mean \pm SEM ($n = 8$). **(d)** Principal component analysis was used to generate two-dimensional ordination plots that visualize complex relationships in expression of multiple genes in colonic EC of individual MyD88^{Flox} and MyD88^{ΔIEC} mice, with or without DSS treatment. Normalized mRNA transcript levels for 19 genes were reduced to 2 PCs (see **Figure 5-15** for details), which are plotted for individual mice. **(e)** Levels of 36 individual mRNA transcripts were measured by Nanostring nCounterTM hybridization. Data are plotted as mean fold difference (\log_2) between MyD88^{Flox} mice with or without DSS treatment vs. P-value ($-\log_{10}$) (upper panel), and mean fold difference (\log_2) between DSS-treated MyD88^{ΔIEC} and MyD88^{Flox} mice vs. P-value ($-\log_{10}$) (lower panel). Individual dots represent the mean of treatment groups ($n = 8$). mRNA transcript levels for genes above the red dashed line were significantly different for each comparison ($p < 0.05$). Genes above the red dashed line were significantly different in DSS-treated mice compared to untreated mice ($p < 0.05$). Genes to the left of the zero line were down-regulated, and genes to the right were up-regulated. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of **Figure 5-13 (continued)** polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

a

Factor Analysis Summary	
Number of Variables	19
Number of Factors	4
Number of Cases	31
Degrees of Freedom	189
Bartlett's Chi Square	816.7
P-value	<0.0001
Factor Extraction Method:	Principal Component Analysis

Eigenvalues		
Factors	Magnitude	Variance Proportion
PC1	9.424	0.496
PC2	3.449	0.182
PC3	1.334	0.070
PC4	1.069	0.056

Gene	Coefficient	
	PC1	PC2
<i>Areg</i>	0.886	0.317
<i>Bcl2l1</i>	0.683	-0.173
<i>Birc2</i>	0.850	-0.042
<i>Cxcl2</i>	-0.651	0.471
<i>Defars1</i>	0.177	0.204
<i>Il-18</i>	0.723	0.327
<i>Il-1b</i>	-0.725	0.407
<i>Muc2</i>	0.439	-0.616
<i>Mylk</i>	0.709	0.361
<i>Pigr</i>	0.552	-0.478
<i>Ptgs2</i>	-0.621	0.466
<i>Reg3g</i>	-0.857	0.144
<i>RelA</i>	0.811	0.418
<i>RelB</i>	0.918	0.277
<i>Smad7</i>	0.902	0.136
<i>Tff3</i>	0.324	-0.715
<i>Tjp1</i>	0.874	0.391
<i>TNF</i>	-0.485	0.689
<i>Tnfaip3</i>	0.639	0.628

b

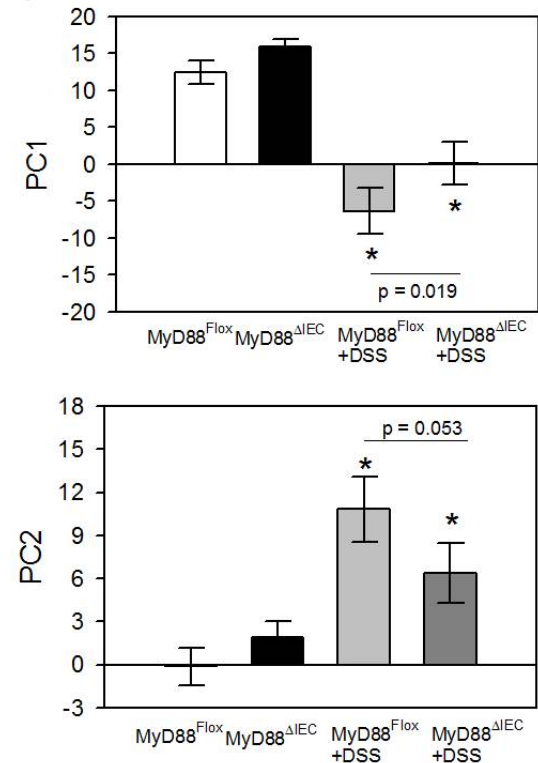


Figure 5-14. Principal component analysis (PCA) of gene expression in colonic epithelial cells from MyD88^{ΔIEC} and MyD88^{Flox} mice in the presence and absence of DSS treatment (a) Nineteen genes from a total of 36 were selected for PCA, based on ranking of P values for the comparison of mRNA transcript levels in MyD88^{ΔIEC} and MyD88^{Flox} mice, with and without DSS treatment (n = 31) (see **Tables 5-2, 5-3 and 5-4**). Data for mRNA transcript levels from individual mice were reduced to two principal components that accounted for 68.8% of the overall variance in gene expression (PC1

Figure 5-14 (continued) and PC2). Weighted coefficients are listed for each variable comprising PC1 and PC2. **(b)** PC1 and PC2 scores were calculated for each individual mouse as the sum of normalized expression levels for each gene multiplied by the corresponding coefficient for PC1 and PC2. Bar graphs indicate the mean \pm SEM for each treatment group (n = 7-9). Asterisks indicate that the mean for DSS-treated mice is significantly different from the mean for untreated mice of the same genotype ($p < 0.05$). P-values for the comparisons between DSS-treated MyD88^{Flox} and MyD88 ^{Δ IEC} mice are noted on the graph. (Frantz *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, in press 2012) Reprinted with permission.

Chapter Six – Discussion and Future Directions

Maintenance of pIgR expression is required for intestinal homeostasis

An important finding in this work is that the downregulation of pIgR expression that is observed in patients with IBD, is also a common feature in mouse models of acute and chronic experimental colitis. Notably, this downregulation in epithelial gene expression is correlated with disease severity. These results raise the question of whether dysregulation of pIgR expression is a cause or consequence of intestinal inflammation. It is possible that inflammation-induced damage to the intestinal epithelium may disrupt intracellular signaling pathways and alter protein trafficking, leading to the dysregulation of pIgR expression. For example, chronic inflammation has been shown to reduce the activity of critical chaperone proteins required for proper protein trafficking, resulting in loss of epithelial polarity²⁰¹. Loss of epithelial polarity leads to dysregulation of pIgR expression and transport of IgA⁴⁴. Thus, independent of the mechanism by which intestinal inflammation develops, pIgR expression is significantly impaired. We found that this defect is associated with dysregulated epithelial transcytosis of IgA, reduced levels of luminal SIgA, accumulation of IgA in the intestinal *lamina propria*, as well as increased levels of circulating IgA. Multiple studies suggest that reduced transport of SIgA exacerbates intestinal inflammation. pIgR deficient mice, which have significantly reduced SIgA in external secretions, were found to be more susceptible than wild-type mice to acute DSS-induced colitis⁵⁸. Furthermore, pIgR deficient mice are more susceptible to a variety of bacterial infections²⁰²⁻²⁰⁴. Taken together, it can be concluded that maintaining high levels of pIgR expression is necessary to maintain intestinal homeostasis.

Not only does maintaining high levels of pIgR expression maintain sufficient transcytosis of SIgA, but it also provides sufficient levels of free SC in the intestinal lumen. The SC moiety of SIgA has also been shown to contribute to innate defense mechanisms, including prevention of bacterial adherence to the intestinal mucus layer and neutralization of potential pro-inflammatory factors^{54,134}. However, the work presented here suggests that expression of pIgR and transport of free SC has a role in protection against intestinal inflammation. Since Rag^{-/-} mice do not produce IgA, any protective effects of T regulatory cells in this model do not involve maintaining IgA function. Interestingly, we found that in mice that received T effector and T regulatory cells, pIgR expression was restored to a level that was not significantly different than control mice. Accordingly, weight loss, colon weight to length ratio and PC values for mice who received T effectors and T regulatory cells were no different than control mice. Thus, restoration of pIgR expression, independent of IgA, is associated with colitis prevention. It has recently been reported that anti-TNF therapy not only neutralizes TNF, but promotes the activation and expansion of T regulatory cells in CD patients¹³⁷. Accordingly, maintaining an environment that promotes pIgR expression in epithelial cells could be an additional mechanism by which T regulatory cells protect against T cell dependent intestinal inflammation and restore intestinal homeostasis.

Overall these results suggest that therapeutic strategies aimed to maintain pIgR expression may be beneficial. Recently, probiotics, defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host, are receiving increasing attention as potential treatment options for intestinal inflammation. However, the mechanisms by which probiotics achieve their beneficial effects remain unclear.

Interestingly, *E. coli* strain Nissle 1917 (*EcN*) is the best characterized probiotic strain of *E. coli*. *EcN* has been tested as a treatment for IBD in several clinical trials, and has been shown to be as effective as mesalazine in maintaining remission in ulcerative colitis patients¹⁶⁶. *In vitro* experiments with cultured IEC have revealed several mechanisms by which *EcN* may act, including induction of tight junction proteins, maintenance of barrier function, inhibition of invasion by invasive bacteria, induction of antimicrobial β -defensins and chemokine synthesis, and inhibition of TNF induced pro-inflammatory response²⁰⁵⁻²⁰⁷. Our lab has shown that *EcN* was unique among representative species of the four major phyla of human colonic bacteria in its ability to upregulate expression of pIgR in HT-29 cells⁶². Here we show that the induction of pIgR by *EcN* is mediated by the TLR4-MyD88 dependent activation of NF- κ B. In particular, this response is primarily mediated by the classical RelA-dependent pathway, but the alternative RelB-dependent pathway can compensate if necessary to maintain pIgR expression. Previous work has demonstrated that bacterial-dependent MyD88 signaling by IECs promotes B cell recruitment and IgA class-switching in the *lamina propria*^{4,19}. Accordingly, the induction and maintenance of high levels of pIgR expression, as well as enhanced IgA production, resulting in increased transport of SIgA and SC, may be an additional novel mechanism by which *EcN* achieves its probiotic effects. These results provide evidence for the continued investigation and the therapeutic use of *EcN* as a probiotic treatment.

Epithelial MyD88 signaling in the colon

A primary goal of this work was to understand the role of intestinal epithelial MyD88 signaling. The generation of mice with an epithelial-specific deletion of the

Myd88 gene is a relatively new model. Vaishnava *et al.*⁴² recently reported that *MyD88*^{ΔIEC} mice had reduced expression of the antimicrobial peptide RegIII-γ in the terminal ileum and increased bacterial colonization of small intestinal surfaces. These authors concluded that epithelial *MyD88* regulation of RegIII-γ, which has specificity for Gram-positive bacteria, is a mechanism to restrict numbers of mucus-associated Gram-positive bacteria in specific niches of the intestine. Consistent with these results, we show that RegIII-γ expression is reduced in the colon of *MyD88*^{ΔIEC} mice and extend these findings to show that the antimicrobial peptide Defa-rs1, which is constitutively expressed by IECs and upregulated in response to bacterial stimulation²⁰⁸, is also reduced in the colons of *MyD88*^{ΔIEC} mice. These defects are associated with reduced antimicrobial activity of colonic mucus, resulting in increased mucus-associated bacteria in the colon, similar to published results in the small intestine⁴². However, we show that *MyD88*^{ΔIEC} mice also have reduced expression of Muc-2 and pIgR (associated with reduced transcytosis of IgA), which was not observed by Vaishnava *et al.* in the small intestine of *MyD88*^{ΔIEC} mice. These discrepancies could be due to the structural and functional differences of these organs. It is logical to assume that the colon, which houses exponentially greater numbers of commensal bacteria than the small intestine, has evolved mechanisms to efficiently regulate expression of antimicrobial and innate immune genes to effectively deal with this huge bacterial load. Accordingly, our results support the idea that in the colon, antimicrobial peptides, with specificities for both Gram-positive and Gram-negative bacteria, as well as critical genes associated with epithelial barrier function and immunity (Muc-2 and pIgR) are regulated by commensal bacteria themselves. Thus, a feed-back system is established in which commensal

bacteria induce the expression, via epithelial MyD88 signaling, of epithelial genes required to maintain epithelial barrier function and homeostasis in the colon. In this situation, the function of colonic epithelial MyD88 signaling is unique compared to other cell types and locations.

Novel role of epithelial MyD88 signaling in regulating pIgR expression

Interestingly, pIgR was the most significantly downregulated gene in isolated epithelial cells from MyD88^{ΔIEC} mice. Furthermore, this downregulation of pIgR expression was associated with reduced transcytosis of SIgA and reduced levels of IgA in the feces of MyD88^{ΔIEC} mice. While it has been suggested that MyD88 signaling in epithelial cells regulates the expression of several genes necessary to maintain homeostasis^{6,78}, this is the first study to demonstrate that epithelial MyD88 signaling is required to maintain sufficient pIgR expression and SIgA transcytosis *in vivo*. Our lab previously showed that pIgR expression and IgA transport was reduced in MyD88 deficient mice¹⁶⁰, but that study did not determine whether pIgR was regulated directly by MyD88 signaling in IECs or indirectly by immune cells in the intestinal *lamina propria*. While these new findings do not exclude the possibility that epithelial MyD88 signaling provides immune cells with signals that regulate the production of SIgA, these results do indicate that epithelial MyD88 signaling is necessary to regulate the expression of pIgR and transport of IgA. Accordingly, it would be interesting to examine pIgR expression and IgA transport in mice with myeloid-specific deletion of MyD88. Several studies had revealed an essential role for dendritic cell MyD88 signaling in luminal sampling and the induction of B cell differentiation in to IgA⁺ plasma cells^{6,209,210}. Using bone-marrow

chimeras it has been demonstrated that MyD88 signaling in myeloid cells mediates susceptibility to chronic intestinal inflammation⁶. It would be hypothesized that while pIgR expression may be slightly reduced in mice with MyD88-sufficient epithelial cells and MyD88-deficient myeloid cells, the production of IgA would be significantly altered; thus, dysregulated SIgA responses may also contribute to disease in this model.

This work also reveals a novel role of epithelial NF- κ B signaling, downstream of MyD88, in maintaining pIgR expression in response to commensal bacteria. While pro-inflammatory responses to commensal bacteria are dependent on the classical RelA pathway, both the classical and alternative NF- κ B pathways can induce pIgR expression. By utilizing signaling pathways that are distinct from those involved in regulating pro-inflammatory responses, it is possible that IECs are able to upregulate pIgR expression in response to microbial and host stimuli while suppressing a potentially damaging pro-inflammatory response. Taken together, these results suggest that the regulation of pIgR expression in IECs may be an important mechanism by which MyD88-dependent activation of NF- κ B maintains intestinal homeostasis. Consistent with this idea, we found that loss of intestinal homeostasis associated with experimental colitis and human IBD is generally accompanied by reduced expression of epithelial RelA. Likewise, expression of RelA and pIgR were significant contributors to homeostatic PC1 in models of acute and chronic experimental colitis, as well as human CD.

An important observation in this work is that loss of epithelial MyD88 signaling was associated with alterations in the composition of the gut microbiota. Interestingly, similar compositional changes have been observed in human IBD patients¹⁹⁶. Altered host factors that are believed to impact commensal bacteria in IBD patients include

increased intestinal permeability, decreased production of antimicrobial peptides and aberrant SIgA responses¹⁹⁶, all of which were observed in MyD88^{ΔIEC} mice. Specifically, IgA is known to have a role in shaping the composition of the microbiota by preferentially promoting the maintenance of beneficial and symbiotic bacterial communities^{19,211}. In contrast, the composition of the microbiota has been shown to alter the production and transcytosis of IgA^{46,212}. Taken together, our findings identify a novel role for epithelial MyD88 signaling in shaping the composition of the gut microbiota, at least in part by regulating pIgR expression and IgA transport. This idea highlights the complexity and importance of epithelial cells in integrating bacterial signals, as well as immune cell interactions, to promote intestinal homeostasis.

MyD88 and TLR independent signaling pathways in the regulation of pIgR expression

It should be noted that pIgR expression was significantly reduced in epithelial cells from MyD88^{ΔIEC} mice, not abolished. Inhibiting the binding of *E.coli* LPS to TLR4 did appear to completely abolish the induction of pIgR, implying TLR4 signaling is required in response to commensal bacteria. However, TLR4 utilizes both MyD88 and Trif to initiate target gene transcription. Likewise, while shRNA mediated knockdown of MyD88 in HT-29 cells resulted in reduced expression of pIgR in unstimulated cells and reduced upregulation of pIgR expression in response to *E.coli* stimulation, it did not completely abolish this response. Furthermore, work presented here, as well as previous studies^{44,141}, clearly demonstrates that TLR3 signaling, which is Trif-dependent and MyD88-independent, induces pIgR expression *in vitro*; however the specific contribution

of the Trif-dependent signaling pathway was not examined in these studies.

Interestingly, this work, as well as previous work from the lab¹⁴¹, has shown that stimulation of TLR3 in HT-29 cells elicits a much stronger pro-inflammatory response than stimulation of TLR4, even though the induction of pIgR mRNA is similar. Thus, it is probable that the Trif-dependent pathway, as well as additional signaling pathways, is responsible for maintaining the reduced level of pIgR expression observed in MyD88^{ΔIEC} mice, while the levels of most pro-inflammatory chemokines and cytokines remained unchanged. However, it has been demonstrated that Trif^{-/-} bone marrow chimeras display no detectable differences in response to DSS treatment¹⁵⁸, indicating TRIF signaling does not play a significant role in the setting of acute experimental colitis. Therefore, while TRIF-dependent signaling can induce pIgR expression, it probably plays a minor role during intestinal inflammation.

Importantly, as demonstrated here, one common feature of the MyD88, TRIF and TNF signaling pathways in the induction of pIgR expression is the activation of NF-κB. While the MAPK signaling pathway contributed to the induction of the pro-inflammatory response, NF-κB was solely required for the induction of pIgR expression. Accordingly, it would be interesting to examine pIgR expression in NEMO^{ΔIEC} mice. Similar to MyD88^{ΔIEC} mice, reduced expression of antimicrobial peptides and increased bacterial translocation was observed in NEMO^{ΔIEC} mice³⁴. However, unlike MyD88^{ΔIEC} mice, NEMO^{ΔIEC} mice develop severe spontaneous intestinal inflammation. Interestingly, crossing NEMO^{ΔIEC} mice with MyD88 deficient mice prevented the development of intestinal inflammation, demonstrating that bacterial-dependent TLR signaling is essential for the development of intestinal inflammation in this model³⁴. We have made

the observation that downregulation of pIgR expression is correlated with disease severity in IBD patients and mouse models of colitis; therefore it would be hypothesized that pIgR expression and IgA transport would be greatly diminished in NEMO^{ΔIEC} mice, and that this defect could contribute to disease severity.

Additional studies have demonstrated that host cytokines, including IL-4, IL-1, IFN- γ , TNF, and IL-17 can also induce pIgR expression *in vitro*^{44,160,213} in a TLR-independent manner. IL-4, IFN- γ , and TNF have been shown to work synergistically to induce pIgR expression⁴⁴, suggesting cooperativity between host factors to maintain high pIgR expression. It is likely that TLR-independent pathways are also affected in MyD88^{ΔIEC} mice. In particular, IL-1, whose receptor signals through MyD88 and is a MyD88 target gene, is a prominent pro-inflammatory cytokine released by macrophages that are recruited to the *lamina propria* in response to bacterial sampling and epithelial penetration²¹⁴. IECs secrete IL-1 in an autocrine and paracrine manner in response to bacterial stimulation²¹⁵. Here we observed that MyD88^{ΔIEC} mice were unable to upregulate epithelial IL-1 expression in response to DSS colitis. Accordingly, it is possible that the reduction in pIgR expression and IgA transport in MyD88^{ΔIEC} mice is, in part, due to an indirect effect of the loss of bacteria-induced MyD88 signaling, resulting in the inability to upregulate and respond to IL-1. Consistent with this line of thinking, MyD88^{ΔIEC} mice were unable to upregulate epithelial TNF expression in response to DSS colitis, which was associated with increased disease severity. As we have shown here, TNF-dependent activation of NF- κ B is a strong inducer of pIgR expression in IECs. Interestingly, it has previously been shown that chronic exposure of IECs to TNF in a cell culture model of chronic inflammation significantly increases pIgR mRNA stability¹⁴³.

Therefore, the inability of MyD88^{ΔIEC} to upregulate epithelial TLR-induced TNF expression would also impair autocrine TNF signaling in IECs, further disabling the induction of pIgR expression and altering mRNA stability. Alternatively, MyD88^{ΔIEC} mice had increased histological inflammatory scores, including an increase in inflammatory immune cell infiltration into the *lamina propria* in response to DSS colitis. While gene expression was not analyzed in *lamina propria* immune cells, these inflammatory conditions are generally associated with increased production of inflammatory cytokines, including TNF, by macrophages and activated T cells. Thus, production of TNF, as well as other inflammatory stimuli, by MyD88-sufficient immune cells could signal in a paracrine fashion to induce pIgR expression in IECs, independent of epithelial MyD88 signaling, which could partially account for the pIgR expression in MyD88^{ΔIEC} mice. Nonetheless, the capacity of such diverse factors, as well as the utilization of several different signaling pathways, to induce pIgR expression suggests that maintaining high levels of pIgR and transport of SIgA and SC is of significant evolutionary importance for host fitness.

Communication between all neighbors is required to prevent translocation of opportunistic bacteria.

As previously mentioned, a significant finding in this work is that loss of epithelial-MyD88 signaling caused increased translocation of bacteria to the MLN. It has been proposed that low numbers of commensal bacteria continuously translocate the epithelial barrier in healthy immunocompetent hosts; however, these bacteria are generally killed en route or within the lymphoid organ, rarely ever establishing a

persistent presence²¹⁶. This is not the case in MyD88^{ΔIEC} mice. Three primary mechanisms that promote the translocation of enteric bacteria from the intestine have been identified: Overgrowth of intestinal bacteria, deficiencies in host immune system, and increased epithelial permeability²¹⁶. While an overgrowth of *K. pneumoniae* was not observed in MyD88 mice, multiple defects in innate immunity as well as increased epithelial permeability were observed. While these primary defects in epithelial defenses are central to the translocation of intestinal bacteria, cell-mediated immunity, including macrophages, neutrophils and T cells in the *lamina propria* are involved in reducing bacterial translocation²¹⁶. Previous studies have demonstrated that the depletion of these cells types in the *lamina propria* promoted the translocation of *E.coli* into the MLN^{216,217}. It has been suggested that epithelial TLR signaling is responsible for releasing soluble factors that regulate the development of secondary lymphoid structures, recruitment of immune cells to the *lamina propria*, as well as antigen sampling by DCs^{1,6,218,219}. It is likely that a loss of MyD88 signaling in epithelial cells affects the development, recruitment and function of MyD88-sufficient immune cells. This idea is supported by the results from Ismail *et al.*¹⁹² who found that expression of RegIII- γ in small intestinal $\gamma\delta$ intraepithelial lymphocytes (IELs) was dependent on epithelial-specific MyD88 signaling, but was not dependent on expression of MyD88 within the IELs themselves. Thus, not only does loss of epithelial MyD88 signaling cause defects in epithelial defenses, it may also lead to *lamina propria* immune cell dysfunction, further promoting bacterial translocation and susceptibility to intestinal inflammation.

Importantly, a dramatic increase in the translocation of *Klebsiella pneumoniae* was observed in MLN from MyD88^{ΔIEC} mice. This bacterial species is the most clinically

important member of the *Klebsiella* genus of the family *Enterobacteriaceae*, due to its role in nosocomial lung and intestinal infections in pediatric patients^{220,221}. It has recently been shown that oral infection with *K. pneumoniae* exacerbated intestinal inflammation in DSS colitis²²². While the technique used to identify bacteria in the MLN only allows the identification of live culturable bacteria, the fact that translocation of *K. pneumoniae* was observed in >95% of MyD88^{ΔIEC} mice and very rarely in MyD88-sufficient mice indicates that epithelial MyD88 signaling may have general and specific roles in antibacterial immunity. *K. pneumoniae* is an encapsulated bacterium. This capsule is considered a virulence factor because it prevents desiccation and phagocytosis by host immune phagocytes and thus enhances the ability of the bacteria to cause disease. Accordingly, it is possible that loss of epithelial MyD88 signaling specifically increases susceptibility to resilient opportunistic bacteria. While facultative, encapsulated anaerobic bacteria in the *Enterobacteriaceae* family are considered to translocate at high efficiency, both *E. coli* and *Proteus mirabilis* are thought to be the most efficient at translocation^{216,223}. However, increased translocation of *E. coli*, and/or an overgrowth of either *E. coli* or *Proteus mirabilis*, was not observed in MyD88^{ΔIEC} mice. Taken together, these results support the idea that epithelial MyD88 signaling is required to defend against *K. pneumoniae* adherence and penetration of the epithelial layer, as well as indirectly required to efficiently kill bacteria that have penetrated the epithelial barrier.

Bacterial translocation is considered an important early step in the pathogenesis of opportunistic infections²¹⁶. Although there have been recent reports of MyD88-dependent immune responses to lung infection with *K. pneumoniae*^{195,224}, the role of MyD88 signaling in enteric infections with this opportunistic pathogen has not been studied.

While we detected significant numbers of *K. pneumoniae* in the MLNs of MyD88^{ΔIEC} mice, we were unable to detect these bacteria in the spleen, suggesting that the antibacterial activity of MyD88-sufficient immune cells was sufficient to prevent systemic infection. Therefore while this suboptimal immunity of MyD88^{ΔIEC} mice may be sufficient to control the numbers of *K. pneumoniae* normally found in the commensal gut microbiota, it may be insufficient to control larger numbers of *K. pneumoniae*. Accordingly, it would be hypothesized that MyD88^{ΔIEC} mice would be more susceptible than MyD88^{Flox} mice to enteric infection with *K. pneumoniae*. The results could offer the potential to increase our understanding of the pathogenesis of opportunistic enteric infections in immunocompromised individuals.

Role of MyD88-sufficient *lamina propria* immune cells in the regulation of intestinal homeostasis.

One important aspect that was not addressed in these studies is the effect of loss of epithelial MyD88 signaling on MyD88-sufficient immune cells of the *lamina propria*. As previously mentioned in this chapter, it is possibly that loss of epithelial MyD88 signaling could have indirect effects on the production of SIgA. However, expression of B cell activating factor, BAFF (Tnsf13b), as well as the lymphocyte growth factor thymic stromal lymphopoietin (TSLP), in isolated ECs from MyD88^{Flox} and MyD88^{ΔIEC} mice were below the detectable limit. It would be worthwhile to compare the expression of other epithelial-derived factors that influence B cell differentiation, such as a proliferating inducing ligand (APRIL), in MyD88^{Flox} and MyD88^{ΔIEC} mice. APRIL has been shown to be released by IECs in response to TLR signaling and can directly induce B cell

differentiation²²⁵. Furthermore, while immunofluorescence analysis of IgA expression in colonic sections did not reveal differences in the presence of IgA⁺ plasma cells in the *lamina propria*, the presence of additional immune cell types was not investigated. It would be valuable to analyze the cellular composition of immune cells isolated from the *lamina propria* of MyD88^{Flox} and MyD88^{ΔIEC} mice. As previously discussed, while the translocation of bacteria, in particular *K. pneumoniae*, observed in MyD88^{ΔIEC} mice is a result of defective epithelial barrier function, the ability of these bacteria to survive transit to the MLN suggests an impairment of *lamina propria* immune cells to efficiently contain these bacteria that have penetrated the epithelium. For example, impairment in the recruitment of DCs and T cell into the *lamina propria* could also indirectly effect the production of SIgA. Analyzing the cellular composition of the *lamina propria* immune cells may provide insight into the cell-types responsible for these antibacterial and immune defects.

It would also be important to analyze pro-inflammatory gene expression in isolated *lamina propria* immune cells from MyD88^{Flox} and MyD88^{ΔIEC} mice. By using a model in which MyD88 expression is restricted to myeloid cells, Malvin *et al.* have recently shown that myeloid cells, macrophages in particular, in untreated mice are in an activated state displaying enhanced NF-κB activity²²⁶. These authors conclude that MyD88 signaling in non-myeloid cells can inhibit myeloid cell activation. Considering the increase in bacterial penetration of the intestinal epithelium that was observed in MyD88^{ΔIEC} mice, it would be hypothesized that the *lamina propria* of MyD88^{ΔIEC} would also contain hyperactivated-immune cells. Here we have shown that THP-1 monocytes are hyper-responsive to TLR stimulation, as compared to HT-29 epithelial cells.

Furthermore, it is possible that while this hyper-activated state may offer some level of immune protection against the normal microbiota in MyD88^{ΔIEC} mice, it may be damaging in response to the high bacterial stimulation that occurs with DSS treatment. Accordingly, Asquith *et al.* demonstrated that MyD88 signaling in hematopoietic cells were responsible for the pro-inflammatory responses that increased colitis severity scores in these bone marrow chimeric mice¹⁵⁹. It is possible that these effects could contribute to the enhanced susceptibility of MyD88^{ΔIEC} mice to DSS.

Bacterial-dependent signaling by IECs regulates intestinal homeostasis and inflammation

Taken together, the results presented here suggest a model in which maintenance of intestinal homeostasis requires active communication among the microbiota, epithelial cells and immune cells. However, while communication must occur, physical and immunological barriers must be maintained. Protection against bacterial invasion and the development of inflammation in the intestine is dependent on the resident microbiota, the mucus layer containing SIgA and antimicrobial peptides, and the monolayer of IECs that provide a physical and innate immune barrier. Here we demonstrate that MyD88 signaling in IECs is a main regulator of these processes in the colon (**Figure 6-1A**). Epithelial MyD88 signaling regulates the expression of Muc-2, the main structural component of colonic mucus, as well as the expression of antimicrobial peptides. Importantly, MyD88 signaling in epithelial cells regulates the expression of pIgR and the transport of SIgA. In response to *E. coli*, the regulation of pIgR induction is dependent on the TLR4-activation of NF-κB. However, when dysregulation of these defenses occur,

bacterial translocation and intestinal inflammation can result (**Figure 6-1B**). Accordingly, the loss of epithelial MyD88 signaling leads to reduced mucin secretion, reduced mucus-associated antimicrobial activity, reduced transcytosis of SIgA and increased epithelial permeability. These impairments in antibacterial and innate immunity result in alterations in the composition of the resident microbiota, increased numbers of mucus-associated bacteria and increased bacterial translocation to the mesenteric lymph nodes. The inability to maintain homeostasis with the intestinal microbiota increases the host's susceptibility to intestinal inflammation and systemic infection. Notably, sustained reduction of pIgR expression and altered transport of SIgA are characteristic of intestinal inflammation and further exacerbates disease. Taken together, these results highlight the critical role of intestinal epithelial cells in maintaining intestinal homeostasis and suggest that changes in IEC gene expression and function may be central to understanding the underlying mechanisms of intestinal inflammation and disease. As a result, a multifactorial approach to examine epithelial gene expression may be the most informative for the diagnosis of intestinal disease and for evaluating potential therapeutic options. These results provide insight into the importance of the complex relationships between bacteria and IECs, as well as IECs and immune cells, and offer evidence for the continued investigations into potential therapeutic strategies. Thus in the intestine, good epithelial fences do make good neighbors.

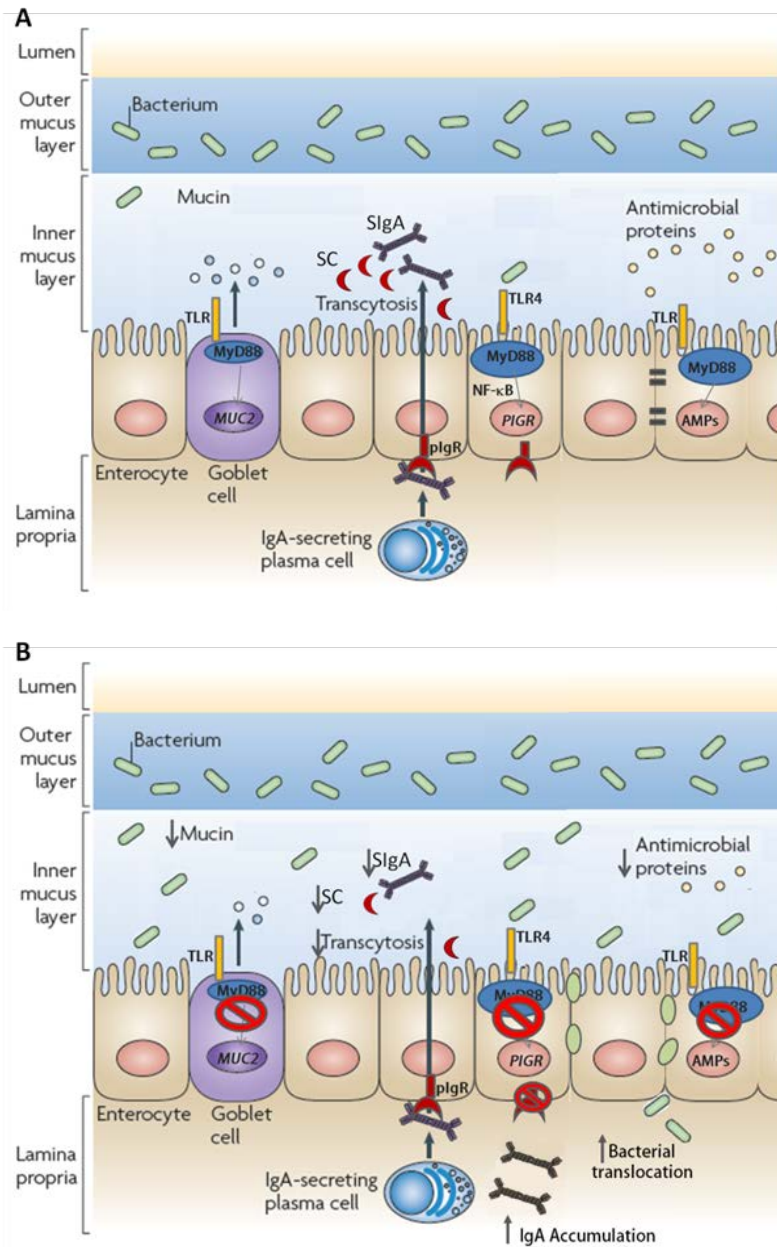


Figure 6-1 Bacterial-dependent signaling by IEC is required to maintain intestinal homeostasis. (A) Under homeostatic conditions, colonic epithelial cells maintain a physical and immunological barrier. Bacterial-dependent MyD88 signaling regulates muc-2 production in goblet cells; regulates expression of antimicrobial peptides; regulates the expression of pIgR and the transport of SIgA via activation of NF-κB; and maintains an impermeable epithelial barrier. **(B)** Loss of colonic epithelial MyD88

Figure 6-1 (continued) expression results in dysregulation of Muc-2, AMPs, and pIgR expression as well as impairs SIgA transport and increases epithelial permeability. These defects cause increased bacterial adherence and translocation, increasing the host's susceptibility to intestinal inflammation. (Adapted with permission from Hooper *et al.* Nat. Rev. Immunol. 10(3):159-69 2010)

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EDUCATION

University of Kentucky, Lexington KY

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HONORS and AWARDS

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- University of Kentucky Opportunity Fellowship, August 2010 – August 2011
- University of Kentucky Opportunity Fellowship, August 2009 – August 2010
- International Congress of Mucosal Immunology Travel Award , July 2009
- University of Kentucky Graduate Student Travel Award, June 2009
- University of Kentucky Opportunity Fellowship, August 2008 – August 2009
- University of Kentucky Graduate School Academic Fellowship, August 2007 – August 2008

PROFESSIONAL POSITIONS

- Graduate Research Assistant, University of Kentucky, Department of Microbiology, Immunology and Molecular Genetics, August 2007 – Present
- Teaching Assistant for Medical Microbiology, University of Kentucky College of Medicine August 2009 - December 2009
- Laboratory Technician , University of Kentucky, Department of Microbiology, Immunology and Molecular Genetics, August 2006 – August 2007
- Undergraduate Researcher, University of Kentucky, Department of Biology, May 2005-June 2006

PUBLICATIONS

Aubrey L. Frantz, Eric W. Rogier, Christopher Weber, Le Shen, Donald A. Cohen, Maria E.C. Bruno, and Charlotte S. Kaetzel. Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with the down-regulation of polymeric immunoglobulin receptor, mucin-2 and antibacterial peptides. *Mucosal Immunology*, 2012 *In press*.

Aubrey L. Frantz, Maria E.C. Bruno, Eric W. Rogier, Halide Tuna, Donald A. Cohen, Subbarao Bondada, Ralph L. Chelvarajan, J. Anthony Brandon, C. Darrell Jennings, and Charlotte S. Kaetzel. Multifactorial Patterns of Gene Expression in Colonic Epithelial Cells Predict Disease Phenotypes in Experimental Colitis. *Inflammatory Bowel Disease*, March 2012 *In press*.

Weikang Cai, Jennifer L. Rudolph, Ling Jin, Susan M.W. Harrison, Aubrey L. Frantz, Douglas A. Harrison, and Douglas A. Andres. An evolutionarily conserved Rit GTPase-p38 MAPK signaling pathway mediates oxidative stress resistance. *Mol Biol Cell*. 2011 Jul 7. E11-05-0400

Bruno, M.E.C., Frantz, A. L., Rogier, E.W., Johansen, F.E. and Kaetzel, C.S. Differential roles of the RelA and RelB subunits of NF- κ B in regulation of the polymeric immunoglobulin receptor and pro-inflammatory genes by TNF and TLR signaling in intestinal epithelial cells. *Mucosal Immunol*. 2011; 4(4):468-78.

Bruno, M.E.C., Rogier, E.W., Frantz, A.L., Stefka, A. Thompson, S. & Kaetzel, C.S. Regulation of the polymeric immunoglobulin receptor in intestinal epithelial cells by *Enterobacteriaceae*: Implications for mucosal homeostasis. *Immunological Investigations* 2010; 39:356.

PRESENTATIONS

July, 2011, International Congress of Mucosal Immunology, Paris, France. Aubrey Frantz, Eric Rogier, Maria E. C. Bruno and Charlotte S. Kaetzel. Intestinal Epithelial Cell-Specific MyD88 Signaling Promotes Homeostasis by Regulating Expression of the Polymeric Immunoglobulin Receptor. Oral presentation.

November, 2010, Autumn Immunology Conference, Chicago, IL. Aubrey Frantz, Maria Bruno, Subbarao Bondada, Donald Cohen & Charlotte Kaetzel. Signature biomarkers in Crohn's disease predict phenotypes in mouse models of colitis. Poster presentation and short talk.

November, 2009, Autumn Immunology Conference, Chicago, IL. Aubrey Frantz, Eric Rogier, Maria E. C. Bruno and Charlotte S. Kaetzel. Induction of the Polymeric Immunoglobulin Receptor by Colonic Bacteria through TLR-Dependent Activation of NF- κ B. Poster presentation and short talk.

July, 2009, International Congress of Mucosal Immunology, Boston, MA Aubrey Frantz, Eric Rogier, Maria E. C. Bruno and Charlotte S. Kaetzel. Induction of the Polymeric Immunoglobulin Receptor by Colonic Bacteria through TLR-Dependent Activation of NF- κ B. Poster presentation.

November, 2008, Autumn Immunology Conference, Chicago, IL Aubrey Frantz, Eric Rogier, Maria E. C. Bruno and Charlotte S. Kaetzel. Differential responses of intestinal epithelial cells and monocytes to commensal bacteria. Poster presentation and short talk.

Intramural Presentations - University of Kentucky:

October, 2008, University of Kentucky Cancer Research Day. Aubrey Frantz, Eric Rogier, Maria E.C. Bruno and Charlotte S. Kaetzel. The Role of MyD88-Dependent Toll-like Receptor Signaling in Intestinal Inflammation. Poster presentation.

October, 2008, Department of Microbiology, Immunology and Molecular Genetics Retreat. Aubrey Frantz, Maria E. C. Bruno and Charlotte S. Kaetzel. The Role of MyD88-Dependent TLR Signaling in Intestinal Homeostasis. Poster presentation.

August, 2009, IBS Orientation for the Department of Microbiology, Immunology and Molecular Genetics Retreat. Aubrey Frantz, Eric Rogier, Maria E. C. Bruno and Charlotte S. Kaetzel. Induction of the Polymeric Immunoglobulin Receptor by Colonic Bacteria through TLR-Dependent Activation of NF- κ B. Poster presentation.

October, 2009, Department of Microbiology, Immunology and Molecular Genetics Retreat. Aubrey Frantz, Eric Rogier, Maria E. C. Bruno and Charlotte S. Kaetzel. Induction of the Polymeric Immunoglobulin Receptor by Colonic Bacteria through TLR-Dependent Activation of NF- κ B. Poster presentation.

October, 2009, Department of Microbiology, Immunology and Molecular Genetics Retreat. Aubrey Frantz, Eric Rogier, Maria E. C. Bruno and Charlotte S. Kaetzel. Induction of the Polymeric Immunoglobulin Receptor by Colonic Bacteria through TLR-Dependent Activation of NF- κ B. Poster presentation.

August, 2010, University of Kentucky Cancer Research Day. Aubrey Frantz, Maria Bruno, Subbarao Bondada, Donald Cohen & Charlotte Kaetzel. Signature biomarkers in Crohn's disease predict phenotypes in mouse models of colitis. Poster presentation.

October, 2010, Department of Microbiology, Immunology and Molecular Genetics Retreat. Aubrey Frantz, Maria Bruno, Subbarao Bondada, Donald Cohen & Charlotte Kaetzel. Signature biomarkers in Crohn's disease predict phenotypes in mouse models of colitis. Poster presentation and short talk.

March, 2011, University of Kentucky Cancer Research Day. Aubrey Frantz, Eric Rogier, Gavin Ellis, Cathryn Kurkijun, Razvan Arsenescu and Charlotte Kaetzel. Down-regulation of the polymeric immunoglobulin receptor in ulcerative colitis and in experimental murine colitis-associated cancer. Poster presentation.

October, 2011, Department of Microbiology, Immunology and Molecular Genetics Retreat. Aubrey Frantz, Maria Bruno, Subbarao Bondada, Donald Cohen & Charlotte Kaetzel. . Intestinal Epithelial Cell-Specific MyD88 Signaling Promotes Homeostasis by Regulating Expression of the Polymeric Immunoglobulin Receptor. Poster presentation.

Seminars:

April 20, 2012 “The role of intestinal epithelial cells and the regulation of the polymeric immunoglobulin receptor in homeostasis and inflammation”

January 28, 2011 “The role of intestinal epithelial cells in homeostasis, inflammation and cancer”

April 30, 2010, “Regulation of the polymeric immunoglobulin receptor in intestinal homeostasis and inflammation”

September 12, 2008, “Allele-specific regulation of the imprinted Igf2/H19 locus”