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CROSSTALK BETWEEN GASTROINTESTINAL EPITHELIAL CELLS AND RESIDENT MICROBIOTA PROMOTES IMMUNE HOMEOSTASIS

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CROSSTALK BETWEEN GASTROINTESTINAL EPITHELIAL CELLS AND RESIDENT MICROBIOTA PROMOTES IMMUNE HOMEOSTASIS

ABSTRACT OF 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

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2012

ABSTRACT OF DISSERTATION

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The gastrointestinal tract houses one of the most dense and diverse communities of bacteria on the planet. The mutualistic relationship between the host and commensal microbe permits the microbe an ideal environment to grow and provides the host with increased caloric intake, maturation of the adaptive immune system, and resistance against invading pathogens. To maintain a system in which both parties benefit, the epithelium has evolved numerous strategies to ensure epithelial cells respond to microbes appropriately and that potentially hazardous commensals remain distanced from the soma proper. Breakdown of these propitiating mechanisms elicits unchecked inflammation and can lead to pathology and reduction of host fitness. We show that oral and intestinal epithelial cells respond to the circulating hormone adiponectin in the presence of bacterial constituents, and that adiponectin has the potential to downregulate NF-κB signaling. We also show many commensal bacteria have no effect on TNF or IL-8 proinflammatory gene expression in intestinal cells. Commensals within the family *Enterobacteriaceae* can increase TNF and IL-8 expression, but also expression of the NF-κB regulator A20 and MAPK phosphatase MKP-1. Importantly, *Enterobacteriaceae* also increased expression of the IgA transporter pIgR. In the mouse model, we show pIgR expression along the intestinal epithelium is necessary for SIgA accumulation in the outer mucus layer where commensal bacteria reside. Loss of the mucus layer, but not pIgR is sufficient to allow direct bacterial-epithelial cell contact and induce spontaneous inflammation along the colon. Secretory IgA is supplied maternally through breast milk early in life to compensate for the neonate's inability to produce sufficient endogenous amounts. By utilizing a breeding scheme in which mouse dams were unable to provide their offspring with SIgA, we show the necessity of maternally-supplied SIgA to control bacterial invasion to mesenteric lymph nodes before weaning. In addition, 8-10 week old adult offspring not receiving SIgA as neonates showed both a unique intestinal microbiota and different patterns of intestinal epithelial cell gene expression with and without chemically-induced acute colitis. In summary, we reveal new mechanisms the mammalian host utilizes in order to maintain peace between the commensal microbe and host immune system.

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July 30, 2012

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This is dedicated to Dr. Robert Guse, who always tried to see the whole picture

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Abbreviations used in this document

AMP – antimicrobial peptide

BLAST – Basic Local Alignment Search Tool

CBC cell – crypt base columnar cell

CD – Crohn's disease

CEC – colonic epithelial cell

CFU – colony forming unit

DAI – disease activity index

DC- dendritic cell

DNA – deoxyribonucleic acid

DSS – dextran sodium sulfate

ELISA – enzyme-linked immunosorbant assay

EPS – exopolysaccharide

FAE – follicle associated epithelium

FISH – fluorescence *in situ* hybridization

FITC – fluorescein isothiocyanate

GALT – gut associated lymphoid tissue

GI tract – gastrointestinal tract

GWAS – genome-wide association study

H&E stain – Hematoxylin and eosin stain

IBD – inflammatory bowel disease

IEC – intestinal epithelial cell

IFNγ - interferon gamma

IL – interleukin

ILF – isolated lymphoid follicle

ISC – intestinal stem cell

J chain – joining chain

LPS – lipopolysaccharide

LRC – label-retaining cell

M cell – microfold cell

MAPK - mitogen-activated protein kinase

MAMP – microbial associated molecular pattern

MLN – mesenteric lymph node

NALT – nasal associated lymphoid tissue

NEC – necrotizing enterocolitis

NF-κB – nuclear factor kappa beta

NLR – Nod-like receptor

OTU- operational taxanomic unit

PC – plasma cell

PCA/PCoA – principal component analysis

PCR – polymerase chain reaction

pIgR – polymeric immunoglobulin receptor

PP – Peyer's patch

PPARγ - Peroxisome proliferator-activated receptor gamma

PRR – pattern recognition receptor

RLH – Rig-like helicase

SC – secretory component

SCFA – short chain fatty acid

SIgA – secretory immunoglobulin A

TLR – Toll-like receptor

TNF – tumor necrosis factor

UC – ulcerative colitis

Chapter 1

INTRODUCTION

mu'tu·al (mūˈchoo-əl) *adj*. **1,** preferred, exerted, or performed by each other with respect to each other; reciprocal. **2,** denoting a company whose members share the expenses and the profits

-The New American Webster Dictionary

Friendships are sometimes difficult to maintain. Occasionally, one party will attempt to benefit at the expense of the other, and the resulting conflict may require long amounts of time to resolve and repair the relationship. Humans deal with these interpersonal issues on a daily basis, but rarely give thought to the interchange between two entities that has been happening within their own bodies since the minute they were born. During birth, infants must endure the monumental transition from an environment in which they are the sole organism to an environment where other organisms not only exist, but share intimate cohabitation. Most of these organisms we cannot see, and these microorganisms colonize infants at the moment of birth with the vast majority of the microbes belonging to the domain Bacteria (1). This colonization has deep evolutionary roots within the kingdom Anamalia, and is seen among many branches of the evolutionary tree, including: insects (2), fish (3), and higher vertebrates (4, 5). In humans, the highest degree of bacterial colonization in or on the body is found along the length of the gastrointestinal (GI) tract (6). At its essence, the GI tract is simply an elongated tube beginning at the mouth and extending to the anus, but this tube isn't simply an inert barrier. Throughout evolutionary history, humans and other mammals have evolved complex systems at each of the unique sites along the GI tract to allow for not only tolerance of the bacteria which reside at the GI surface, but also the formation of a mutualistic relationship with the resident bacterial population – also frequently referred to as the microbiota (7, 8). The surface of the GI tract which is exposed to the outside environment is composed of the subunit of the epithelial cell, which, collectively, is denoted as the GI epithelium. At this interface, bacteria and their human host are in constant communication with each other by various molecular signals. Since disruption of this inter-Domain dialogue can be grave for both the bacterium and the host, the epithelium employs multiple redundant systems that are in place to ensure the peace is kept. The oral cavity and intestine are the two sites along the GI tract which are in contact with the highest concentrations of bacteria (9, 10), so our focus will be the mechanisms used by these two sites to maintain a cordial relationship with the resident microbiota.

A. VARIABLE STRUCTURE OF THE GI EPITHELIUM AND RESIDENT BACTERIA

The oral epithelium: Friends by association

The epithelium within the oral cavity (oral mucosa) is characterized as stratified squamous epithelium, and is further sub-classified depending on the presence or absence of keratin (11, 12). Non-keratinized epithelia of the mouth are classified at lining mucosa and constitute the cheek and other soft epithelium. The hard palate on the mouth's roof and gingiva surrounding the teeth are both examples of masticatory mucosa. Figure **1.1a** illustrates the amorphous, stratified nature of the oral epithelium and the cell types located directly beneath. Fibroblasts underneath the epithelium quickly replace constantly shedding epithelial cells, while macrophages (among other local myeloid and lymphoid cells) allow for localized immune responses (12). Figure **1.1b** shows a section of human oral tissue which has been stained with hematoxylin and eosin (H&E stain) to emphasize different cell types present in the tissue. The stratified nature of the epithelium and delineation between epithelial cells and other underlying cell types is also clearly apparent.

As profiling of bacterial communities has moved away from culture-dependent and microscopic methods to high-throughput genetic sequencing, the definifition of a unique bacterial 'species' has been blurred due to obvious genetic differences between bacteria, but no discernable morphological, metabolic, or other biological variance. More recently, the idiom 'phylotype' or 'operational taxanomic unit' (OTU), which relies on sequence similarity of 16S rRNA, is commonly used to segregate bacteria (8). A common standard for stating two bacteria are both of the same species/OTU is a rRNA sequence similarity of >98% (13), and will be used in this document to designate unique bacteria.

The human mouth is home to approximately 100-500 distinct bacterial species (11) which frequently colonize the gingiva at the border of tooth and tissue. The current paradigm for bacterial organization within the oral cavity is shown as a multi-species biofilm where bacteria and occasional single-celled eukaryotes share a common biological scaffold (10, 14). This framework is known as the exopolysaccharide matrix (EPS matrix, or ECM) and is composed from an amalgam of secreted bacterial sugar derivatives (15). In a clinical setting, prolonged ECM accumulation leads to dental plaque, which hardens to calculus if not removed regularly. Currently, the spatiotemporal model of bacterial colonization helps to explain community dynamics by dividing the oral microbiota into early and late colonizers (10). Early colonizers include multiple species within the genera *Streptococcus* and *Actinomyces* which are able to associate with host salivary pellicle proteins including: mucins, agglutinins, and amylases (16,

17). These early colonizers stimulate biofilm formation by coaggregation with later successive colonizers and secretion of EPS substrate. Late colonizers include *Fusobacterium nucleatum*, *Treponema denticola*, and many others which allow further enhancement of the multi-species biofilm by continued coaggregation with neighboring bacteria and secretion of quorum sensing molecules (10). While the aforementioned bacteria are regular inhabitants of the oral mucosa, determining consistent relative percentages of constituents of the oral microbiota by phylum or any lower taxanomic order is difficult due to the dynamic nature of the location. However, current efforts are underway through the Human Microbiome Project to try to identify a 'core microbiome' in the mouth which is unanimous for all humans. The bacterial species which do inhabit the mouth are rarely found outside this environment, showing the specifically-evolved dynamic of the residents for this particular site (10).

The small intestinal epithelium: Mountains beyond mountains

The organ in the human body with the greatest surface area exposed to the outside environment is the small intestine, which has a surface area approximately the size of a squash court (18). All three sections of the small intestine: duodenum, jejunum, and ileum, are all lined by a specialized simple epithelium, which allows digestion of water and nutrient absorption (19). The morphology of this specialized epithelium is arranged into units of crypts, which invaginate into the epithelium, and villi, which extend like pillars above the epithelium. These structures greatly increase the surface area of the organ and allow for greater nutrient uptake, but also allowmore exposure to the outside environment and all possible threats associated with it.

As with the oral cavity, the cells constituting the small intestine epithelium are constantly being renewed, and it is estimated the entire epithelial surface is replaced within a span of 4-5 days (19). After much early debate, this process has been better illuminated by the discovery of the existence bona fide intestinal stem cells which display characteristics of stem cells in other compartments in the body (20). However, controversy still remains about the exact characteristics of this cell type since multiple cells with both unique gene expression patterns and displaying different cell surface markers have been shown to have stem cell-like properties of self-regeneration and differentiation into less-potent cell types (21). After the initial introduction of intestinal stem cells (ISCs), two hypotheses preside with one based on the quiescent feature of adult stem cells (called the +4 Label-Retaining Cell, LRC) and the other based on functional and genetic evidence (Lrg5+ Crypt Base Columnar, CBC, cell) (22, 23). Both of these cells reside near the bottom of the intestinal crypt with +4 LRCs at four cell widths from the lowest point of the crypt and Lrg5+ CBCs interspersed along the crypt base. More recently, this paradigm has

been modified with the finding that +4 LRCs express the hematopoietic and neural stem cell gene *Bmi1*, and the concept that two distinct types of ISC populations exist (24), which may even be able to replenish each other's populations in times of stress or injury (25). The stem cells in the intestine differentiate into two classes: absorptive and secretory. Cells in the absorptive lineage (absorptive enterocytes) are dependent upon expression of the transcription factor Hes1 which arises from Notch signaling in the ISC early in differentiation (19, 22). Conversely, intestinal secretory cells (goblet, enteroendocrine, and Paneth cells) all express the Math1 transcription factor, which is a result of ISC signaling through the Wnt pathway. Both of these transcription factors antagonize the expression of the other in the same cell, so cells maintain a committed lineage once beginning differentiation.

Absorptive enterocytes constitute the majority of epithelial cells (~75%) on the small intestinal epithelium and serve to take in dietary nutrients, absorb water, and regulate ion exchange. Along the apical surface of this cell type are structures known as microvilli (which, collectively, is termed the brush border) which allow for further augmentation of available surface area to complete these biological processes (21). Located deep within the crypts and interspersed within ISCs, Paneth cells function as innate immune cells by secreting antimicrobial peptides and lysozyme (19, 26). Enteroendocrine cells make up a very small percentage of the epithelium (~5%) and secrete hormones into the intestinal lumen. The most prevalent cell type from the secretory lineage is the goblet cell (10-15%), which is responsible for excretion of mucus into the intestinal lumen. Figure **1.1c** shows the organization of the crypt-villus axis with the perpetual requirement for intestinal epithelial cell (27) renewal due to constant cells shedding from the villus apex. Shown to the right is the basic schematic for ISC migration and differentiation into the for epithelial cell types. The left side of Figure **1.1d** shows an H&E stained section of a mouse small intestine with both the villus and crypt displayed. To the right are the four epithelial cell types shown *in situ*: b) goblet cells stained positive for mucus with periodic acid-Schiff,c) an enteroendocrine cell stained positive for synaptophysin, d) paneth cells stained positive for lysozyme, e) and absorptive enterocytes stained positive with alkaline phosphatase.

Another cell type found along the distal section of the small intestine is the microfold cell (M cell). Constituting less than 3% of epithelial cells in the distal jejunum and length of ileum in humans, these cells play a unique role in the sampling of bacteria and other particles from the intestinal lumen and assisting in the development of the host-driven adaptive immune response (17, 28). These cells are present mainly in conjuncture with the follicle-associated epithelium (FAE), which manifests as isolated lymphoid follicles (ILFs) and Peyer's patches (PPs) in the

distal small intestine (29-31). M cells line the surface of these structures to allow for antigen sampling of luminal contents and the subsequent genesis of appropriate immune responses by dendritic cells (DCs), T cells, and B cells directly underneath the epithelial surface. Afferent lymphatic drainage from the small and large intestine to neighboring mesenteric lymph nodes (MLNs) also provides a site for adaptive immune cell maturation by way of bacterial and other antigens present in the intestinal lumen (31).

An immense pH gradient exists from the duodenum to the distal ileum which has large implications for the bacteria trying to colonize the different sites. Bacteria able to withstand the high concentrations of hydrogen ions from the duodenum through the jejunum predominantly include species within the genera *Streptococcus* and *Lactobacillus* (9, 17). However, even these microbes do not necessarily thrive under these conditions, as bacterial concentrations only creep into amounts of 10^2 bacterial/mL luminal contents. The ileum provides a more alkaline environment – exposing niche availability and allowing greater bacterial diversity and quantity (32). In the ileum, bacteria within the phylum *Firmucutes* and *Bacteroidetes* begin to establish successful colonization, and concentrations reach upwards of 10^8 bacteria/mL luminal contents (9). For these reasons, it is no coincidence that FAE structures begin to appear at the terminal jejunum, and increase in prevalence as one moves toward the distal ileum. Bacterial communities in the small intestine are loosely arranged due to the absence of an organized mucus layer, and often are found in adherence with transient food particles or detached mucus globules (33).

The large intestinal epithelium: Divide and colonize

With respect to morphology, the most profound difference between the small and large intestine is the absence of villi in the latter. Crypts extend deep into the lamina propria underneath the surface, but retain their small diameter, giving the large intestinal epithelium a smooth appearance. The cell types of this epithelium remain the same as in the small intestine, but in dissimilar quantities. Absorptive enterocytes still constitute the majority of epithelial cells, but the enteroendocrine and Paneth cells of the secretory lineage are almost non-existent (19). With the exception of low numbers in the caecum, Peyer's patches are mostly absent from the large intestine, so M cells are only found when in association with ILFs (31). Even with the deficit of other secretory cells, goblet cells still show a strong presence in the large intestine, constituting around 20% of all cells on the epithelium. The combination of a relatively smooth surface and increased presence of mucus-secreting goblet cells allows for the development of a structured mucus layer which increases in thickness from the cecum to the end of the GI tract at the rectum (34). This mucus layer has two distinct divisions: a tightly-packed inner mucus layer

and less-dense outer mucus layer (35, 36). Proteins within the Mucin family provide the scaffolding necessary to form these layers (37).

Immediately following the distal ileum, bacterial density and community diversity explodes as aided by more neutral pH and structure of large intestine (9). Concentrations of bacteria start around 10^9 microbes/mL in the cecal contents and rise to within 10^{12} bacteria as one nears the rectum. Throughout the length of the large intestine in humans, members of the bacterial phylum *Firmicutes* preside as the dominant inhabitants, composing 50-60% of all unique bacteria (38). Within this phylum, it is estimated upwards of 95% of these bacteria belong to the class *Clostridia*. The phylum *Bacteroidetes* includes the next highest abundance of large intestinal bacteria with 30-40% of bacteria included under this taxonomy. Within *Bacteroidetes*, species within genus *Bacteroides* have been the closest studied intestinal bacteria, and have been found to contain a plethora of adaptations that adjusted for life within the intestine (39, 40). The phyla *Proteobacteria* and *Actinobacteria* each compose around 5% of the large intestinal microbiome, while other phyla of lesser abundance commonly present in humans include *Verrucomicrobia*, *Fusobacterium*, and the candidate phylum TM7 (38, 41). It is thought that biofilms also play an important role in community dynamics for bacteria residing within the intestine (42, 43), but this has not been assessed as thoroughly as in the oral cavity.

Figure **1e** illustrates the basic structure of the large intestinal epithelium with mucin secretion from goblet cells providing overlying mucus layers and the majority of bacterial habitation in the outer mucus layer. Immunoglobulin A (IgA), as well as antimicrobial proteins are also found within the mucus layers, and will be discussed in depth in Section C. Figure **1.1f** shows an H&E-stained section of a mouse colon showing deep penetration of crypts into the lamina propria and formation of the luminal mucus layer by goblet cells exudates.

B. COMMUNICATION BETWEEN HOST AND MICROBE

Pattern recognition receptors and intracellular signaling pathways

The innate immune system provides multiple opportunities for any bacterium or bacterial product to be recognized by the host through a diverse array of pattern recognition receptors (PRRs) on the plasma membrane surface, within the endosome, and throughout the cytoplasm of epithelial cells (see Fig. **1.1g**). The biological ligands for these receptors are known as microbial-associated molecular patterns (MAMPs). For the purposes of focusing on epithelial cells , the C-type lectin PRRs, which are primarily expressed on the surface of myeloid-derived cells, will not be discussed (44). Likewise, the viral PRRs including Rig-like helicases (RLHs) and Toll-like

receptors (TLRs) 3,7,8,9 will also not be discussed (45). Currently, the plasma membrane-bound TLRs are separated by numerical classification according to which microbial pattern they recognize. All TLRs share a common structure as a Type 1 transmembrane glycoprotein with an ectodomain containing a series of leucine-rich repeats which allow for recognition of microbial molecular patterns (17) The heterodimer of TLR1 and 2 recognizes triacylated lipopeptides from Gram (-) bacteria, and heterodimer TLR2/6 recognizes diacylated lipopeptides from Gram (+) bacteria and mycoplasma (46). The flagellin subunit of bacterial flagella is recognized through TLR5 while TLR4 is activated by certain kinds of bacterial lipopolysaccharide (LPS). Importantly, certain bacteria within the intestinal microbiota have been shown to inhibit TLR4 responses through production of LPS derivatives with different acylation and phosphorylation patterns on the Lipid A motif (47, 48). Mutualistic bacteria within the phylum *Bacteroidetes* have a 'monotonously similar' Lipid A composition with two phosphate and four primary acyl chains which is virtually unrecognized by the Cd14-MD2-TLR4 signaling complex (47). Lipid A molecules with 6 acyl chains and multiple phosphate groups provide the most potent stimulus for TLR4 activation, and are found on many human pathogens within the phylum *Proteobacteria* (48).

Even if bacteria or their products are able to bypass plasma membrane TLRs, the family of cytoplasmic Nucleotide Oligomerization Domain-like receptors (NOD-like receptors, NLRs) allows for further detection by the host cell (49). The NOD receptors NOD1 and 2 both recognize peptidoglycan from Gram (+) bacteria, but only NOD 2 is able to identify peptidoglycans from Gram (-) microbes (50, 51). More NLRs such as IPAF, CIITA, NAIP, and the pyrin domain-containing NALPs can also initiate signaling pathways in the presence of MAMPs (49). Just by observation of sheer variety and quantity, it is clearly apparent that it is to the benefit of the host to be able to sense and respond to bacteria on the epithelial surface.

All plasma-membrane bound TLRs utilize the adaptor protein myeloid differentiation primary response gene 88 (MyD88) to initiate downstream signaling cascades in response to microbial products (46). In turn, MyD88-mediated signaling is able to activate both the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways. Cytoplasmic NOD1 and 2 are also able to perpetuate NF-κB signaling, although through a different adaptor protein, RIP-like interacting CLARP kinase (also, RICK)(52). Signaling through the canonical NF-κB cascade requires translocation of the p50/p65 NF-κB heterodimer to the nucleus of the epithelial cell, where it acts as a transcription factor to induce transcription of both pro- and anti-inflammatory genes (53). Proteins involved in the promotion of a mucosal inflammatory response include chemokines such as interleukin 8 (IL-

8), cytokines assisting in leukocyte extravasation such as tumor necrosis factor (TNF) and IL-1, and lymphocyte and leukocyte activators such as IL-12, IL-13, and interferon gamma (IFNγ)(54). Other proteins are secreted by the epithelium in order to diminish inflammatory signaling, and include the apoptosis-inducing transforming growth factor beta (TGFβ) and NF-κB-inhibiting IL-10. In addition, epithelial cells produce proteins which remain intracellular and inhibit NF-κB signaling. Peroxisome proliferator-activated receptor gamma (PPARγ) shuttles the NF-κB heterodimer out of the nucleus, while A20 prevents NF-κB translocation from taking place (55, 56). Since nuclear translocation is one of the terminal events in the NF-κB cascade, determining amount of NF-κB in a cell's nucleus is a common way to measure the impact microbial products have had on cell stimulation through PRR signaling (57, 58). The intestinal mutualist *Bacteroides thetaiotaomicron* has been shown to directly attenuate pathogen-induced inflammatory responses through inhibition of NF-κB signaling *in vivo* and *in vitro* by PPARγ (59). Figure **1.1g** identifies many of the PRRs and their respective ligands as well as the intracellular signaling pathways used by cells along the epithelium to sense pathogens and the resident microbiota.

Short chain fatty acids and their receptors

Pattern recognition receptors are directly involved in the detection of MAMPs that are shed from living or dead bacteria, but other metabolic products are recognized by the epithelium that convey multiple benefits to the host. Many ingested complex polysaccharides not utilized by the host due to lack of appropriate digestive enzymes are readily used as the primary food source for bacteria along the GI tract, with the highest areas of utilization at the cecum and proximal colon (60). From the metabolism of these complex molecules, bacteria produce numerous short chain fatty acids (SCFAs) with the three of highest intestinal concentration being acetate, propionate, and butyrate. These SCFAs are easy energy sources and readily absorbed by the intestinal epithelium and can be found in systemic circulation. Current estimates state that a healthy individual gains upwards of 15% of their daily caloric intake from bacterial metabolic products, and this hypothesis is well-supported by the fact that mice lacking an intestinal microbiota are much leaner than their microbe-exposed counterparts (61, 62). On a more immunological basis, SCFAs are widely thought to potentiate anti-inflammatory responses through activation of host G-protein coupled receptors Gpr41 and 43 (60, 63). Signaling through the type II interferon receptor and canonical NF-κB pathway have both been shown to be attenuated after treatment with SCFAs (64, 65). Furthermore, experimental models of

inflammation in animals deficient for SCFA receptors have shown worse disease in sites far removed from the GI tract (61, 63), implicating microbial-derived SCFAs in the aid of global inflammation control.

C. HOST AND BACTERIAL MECHANISMS FOR MAINTAINING HARMONY

Early establishment of an advantageous microbiota

Upon entry into the world, the newborn infant has unknowingly just created a new niche for microbes to thrive on and within its body (6). These bacteria will populate this human's skin and mucosal surfaces perpetually over tens of thousands of binary fission events, and will remain until the human body expires. As with many things in a newborn's life, the first exposure to bacteria is out of the child's control, and recent evidence suggests early microbial composition in the infant is mostly dependent upon mode of delivery. Children born via vaginal delivery tend to possess a more diverse microbiota in both the oral cavity (66) and along the intestinal tract (67, 68). Very strong correlations exist between the vaginal microbiota of the mother and intestinal microbiota of the naturally-born infant (69). Conversely, the microbiota of a cesarean infant is a mirror-image of the mother's skin bacterial community.

In humans, the first year of life is known to be a particularly dynamic period of shifts in bacterial density and composition (1, 70), but these perturbations tend to wane as the child moves beyond the first few years (71). In addition to providing the initial inoculum to germinate the neonate's microbiota, the mother is also able to continually cultivate this developing biome through products in the breastmilk. Some oligosaccharides in human milk are resistant to human digestive enzymes (72), and their only perceived purpose is to provide a food source for expanding microbial communities – specifically bacteria within the genera *Bifidobacterium* and *Bacteroides* whose genomes are well-adapted for scavenging these molecules. (73, 74). Infants on formula-only diets, which would lack many of these human oligosaccharides, tend to have higher amounts of *Firmicutes* and *Proteobacteria* along their intestinal mucosa when compared to their breast fed peers (70).

Another abundant milk product that has not received as much attention are the mucosal antibodies that are directly transferred to the GI tract of the nursing infant (Chapter 5). As opposed to immunoglobulins the infant would be able to produce (termed active antibodies), the maternal supplementation of immunoglobulins to the infant is referred to as transfer of passive antibodies. Immunoglobulin A (IgA), IgG, and IgM are all present in human breast milk, but IgA far exceeds the other immunoglobulins in quantity by constituting an impressive 4% by weight of

colostrum and 0.1% of mature milk (17). As IgA and IgM both require the polymeric immunoglobulin receptor (pIgR) to traverse epithelial barriers (75), the release of these antibodies from pIgR in the mammary epithelium creates a new molecule of secretory IgA (SIgA) or SIgM. Multiple studies have shown SIgA in the promotion of biofilm formation on the oral epithelium (76) and in the gut (77, 78), but never by SIgA received through maternal transmission. It is tempting to speculate that maternal milk may promote a benevolent microbiota by a two-pronged approach: a) providing prebiotics to stimulate growth of mutualistic bacteria, and b) supplementation with SIgA to frame the mutualists into an enduring biofilm. Proper community establishment by IgA extends far beyond the Bacteria domain, as SIgA is able to inhibit invasion of enteric viruses such as rotavirus and norovirus (79, 80), which the infant is particularly susceptible to.

Mucin proteins and the mucus layer

Mucins are secreted onto the epithelium throughout the GI tract. The function of these proteins is site-specific, and the relative mucin protein concentrations vary widely between sites. Mucin proteins can broadly be classified into two types which are similar in biological function: secreted and membrane-bound. The secreted mucins (muc2,5AB,5B,6) are all located within the 11p15 locus and serve to form mucus layers on top of epithelial surfaces (37). The membrane bound mucins (muc1,3A,3B,4,12,13), which are still tethered to the epithelial cell, are known to serve as steric hindrances to pathogen receptors on the epithelium (33). The common bond between all mucin proteins is the presence of repeating contiguous proline-threonine-serine residues (PTS-regions) in which proline flattens the molecule into β-sheets and serine and threonine allow for heavy O-glycosylation (37). Only secreted mucins contain cysteine-rich domains, which permit crosslinking of mucin proteins and structure a mucus layer with considerable depth.

Limited amounts of mucins are secreted into the oral cavity by salivary glands and produced by oral epithelial cells. Mucin5B appears to be the only secreted mucin of appreciable quantities, while both muc1 and 4 can be found on the surface of the epithelium (81). Low molecular weight muc5B has been shown to have direct inhibitory affects against agglutination of oral pathogens *Streptococcus mutans* and *S. sanguis* (82). In addition, the oral mutualists *S. oralis* and *S. gordonii* were able to use Muc5B as a nutrient source, but only within the structure of a biofilm, and not in the planktonic state (83).

Small and large intestinal goblet cells secrete copious amounts of gel-forming muc2, which is heavily glycosylated and present in high-molecular weight oligomers as it leaves the

cells (84). Though considerable amount of protein and polysaccharide are present in these mucus granules, as a true gel, the main component of the mucus is water. The buffer zone the mucus provides intestinal epithelial cells aids in separating host from bacteria, but mucus layers are not simply sedentary barriers. Bacterial products capable of stimulating TLRs and SCFA receptors have been shown to increase mucus production in goblet cells (85-87), implicating a primary host response to bacterial recognition is to reinforce the mucus layer. Further discovery has found that germ-free mice possess an intestinal mucus layer of 3-fold decreased thickness when compared to conventionally-raised mice (36). The necessity to form a mucus layer in the intestine is shown by the presence of spontaneous colitis in mice deficient for the muc2 protein (88, 89). After extrusion from goblet cells, mucus in the lumen organizes into two distinct layers. The organization and proteins within the two layers are also distinct (Chapter 4). The outer loose mucus layer is less dense and thicker than the inner layer, and is thought to arise from proteolytic cleavage of mucin proteins that were initially part of the inner layer (90). One of the striking findings of the outer mucus layer was the presence of bacteria in only this layer in immune-competent animals (36, 91). This outer layer actually provides a very suitable niche for many mutualists within the intestinal microbiota, and multiple bacteria express proteins that allow consumption and metabolism of carbon-rich mucus (92, 93). Specifically, bacteria within the genus *Lactobacillus* express extracellular proteins with mucus-binding domains (94), while inhabitants of the *Bacteroides* genus produce the Sus family of proteins which allow mucus binding, transport across the periplasm, and enzymatic degradation within the microbe (39, 40). Along with offering a dense barrier between the intestinal lumen and host epithelium, the inner mucus layer provides a sink for antimicrobial peptides to be deposited after secretion from host cells, and will further be discussed in Section 1.9.

The polymeric immunoglobulin receptor and secretory immunoglobulins

Though both IgM and IgG can be found in external secretions, IgA secretion along the GI mucosa far exceeds amounts of the other two immunoglobulins (17). Most B cell maturation into IgA-producing plasma cells takes place within the PPs and ILFs of the gut-associated lymphoid tissue (GALT) (95). Other appreciable B cell maturation occurs in other mucosal lymphoid regions, such as along the nasal (NALT) and bronchial (BALT) epithelia, but the tremendous amount of bacterial antigen within the intestinal tract allows the GALT to be a robust site of immune cell development. The importance of a microbiota in development of IgA plasma cells (IgA^+ PCs) is shown by the germ-free mouse model in which mice never exposed to bacteria and

have extensive defects in GALT and MLN development as well as severely reduced IgA^+ PC numbers and systemic IgA levels (96).

B cells and plasma cells both produce the 'J chain' polypeptide which is able to increase immune activity of polymerized IgA and IgM. Though not required for antibody multimerization, the J chain is thought to provide an additional adhesive to the connecting heavy chains through disulfide bridging (97). For the purposes of antibody secretion into the GI lumen, the J chain is crucial since it allows binding of polymerized IgA and IgM to the polymeric immunoglobulin receptor (pIgR) on the basal surface of epithelial cells. Due to the large size of antibody multimers, antibody movement across the GI epithelium requires active transport (98). Once bound to pIgR, the antibody-J chain-pIgR complex traverses the epithelial cell through a series of endosomal sorting pathways until the apical surface of the epithelial cell is reached (99). Once at the apical surface, the pIgR molecule is proteolytically cleaved to release the complex into the luminal space which is now referred to as a secretory (S) antibody (e.g.SIgA). Apical cleavage of pIgR can also be achieved when the receptor is not bound, releasing the secretory component (SC) fragment into the lumen (100) (Figure **1.1h**).

In the oral cavity, SIgA is found in salivary secretions with higher amounts found in whole versus parotid saliva (95). Localized IgA^+ PCs are thought to arise from the distant GALT, the neighboring NALT, and surrounding salivary glands. Plasma cells matured outside the oral mucosa are able to secrete a more polyclonal antibody which has possible functions in agglutination of the resident microbiota and promotion of biofilm development (101). However, infection with the oral pathogens *Streptococcus mutans* or *Actinobacillus actinomycetemcomitans* both elicit a pathogen-specific antibody response (102, 103) , which is similar to what is observed in the GALT after infection with enteric pathogens (104).

On a daily basis, the intestinal epithelium devotes an enormous amount of metabolic energy to the production of IgA and J chain by plasma cells, creation of pIgR within epithelial cells, and endosomal trafficking of immune molecules across the epithelium. In humans, it is estimated the intestinal tract discharges around 3g SIgA daily (105), on top of an additional 82mg of free SC (106). These molecules share redundant functions for the benefit of the host, as well as unique protein-specific functions. The majority of bacteria in the intestine are bound by SIgA (107) which provides a protective mechanism to the host through immune exclusion. Both bacteria and their antigens bound by SIgA have a greatly reduced capacity for epithelial invasion and stimulation on PRRs on the intestinal surface (100). Polysaccharide residues on SIgA and free SC likely promote association between these proteins and the heavily glycosylated mucus layer, allowing for entrapment of bacterial products away from the epithelium (108). Moreover,

the mucin-binding domains of *Lactobacillus* bacteria have also been found to directly bind SIgA (109), suggesting a close association between bacteria, mucins, and SIgA. As in the oral cavity, SIgA in the intestine probably plays a role in the formation of biofilms (77, 78, 110). Even if the epithelial cell is breached by viruses or MAMPs, pIgR-bound IgA in endosomal compartments is able to adhere to viruses to thwart replication as well as prevent bacterial antigen activation of inflammatory signaling pathways (100, 105). Increased mucosal leakiness is a hallmark of a compromised epithelium, and this allows for antigens to cross the epithelial layer that would normally be segregated within the lumen. The professional immune cells residing in the lamina propria could 'overreact' to increased antigen concentration, so IgA-bound pIgR and pIgR alone shuttle deleterious antigens back into the intestinal lumen (111-113). Figure **1.1h** shows the transepithelial shuttling of pIgR and IgA into the mucus layer in addition to immune functions provided by these proteins. Section (a) displays immune exclusion to the mucus layer while (b) shows intracellular neutralization of virions and (c) intracellular neutralization of MAMPs plus prevention of NF-κB signaling; (d) illustrates antigen secretion back into the intestinal lumen.

Antimicrobial peptides

The diverse collection of antimicrobial peptides (AMPs) is used by the host in both the oral and intestinal epithelium, and many of the exact same peptides are used at both locations. Many of these molecules can be produced by all epithelial cell types, but the small intestinal Paneth cells are apparently differentiated to perform only this function (33). Many types of immune cells also have the capacity for AMP production. At the broad classification level, mucosal AMPs can be partitioned into one of seven groups: defensins, cathlecidins, angiogenins, lectins, lysozymes, collectins, and histatins (33). This is not an exhaustive list, as novel peptides with antibacterial activity are being discovered at a consistent rate (114). Most of these small molecules are amphipathic and carry a positive charge at physiological pH, which allows them to integrate into and disrupt bacterial membranes (115). Within the defensin family, the β-defensins are found to be produced by epithelial surfaces and are especially prevalent within both the oral and intestinal mucosa (116). The α-defensins are produced by neutrophils and Paneth cells, so these proteins are at considerably greater expression in the small intestine. Cathlecidins, lysozymes, and histatins are readily found in both the oral cavity and intestinal lumen (33). Due to the greater bacterial challenge faced along the intestinal tract, additional AMPs within the clusters of lectins (RegIIIα,β,γ), collectins, and angiogenins are also found in the small and large intestines. Where an established mucus layer exists, AMPs are arranged much like fruit pieces in a Jell-O salad. The inner mucus layer holds the greatest density of these peptides which aid to

inhibit bacterial survival and growth in this region and, ultimately, keep the microbiota separated from the epithelium (117).

Hormones and their receptors

Though numerous hormones are produced by the human host, research considering hormone activity in the relationship between host and microbe has generally been limited to metabolic proprieties. It is well known bacterial products such as SCFAs and TLR ligands are able to impact production of and efficacy of appetite hormones such as leptin and ghrelin (118, 119). The adipokine adiponectin modulates inflammatory signaling pathways in numerous cell types, including: monocytes/macrophages (120, 121), endothelial cells (122-124), myocytes (125-127), synovial fibroblasts (128), and intestinal epithelial cells (129). Various cell models have shown that adiponectin has the capacity to modulate intracellular signaling through the NF-κB pathway (120, 127, 128) which is also utilized by all plasma membrane-bound TLRs (130).

The receptors for adiponectin, AdipoR1 and 2, have been found on the epithelium of the oral (131), gastric (132, 133), and intestinal (134-136) surfaces. In particular, the oral and intestinal epithelial surfaces are in constant interaction with adiponectin as supplied through saliva (137, 138) and intestinal cells (139, 140), respectively. Furthermore, it is possible that adiponectin reaches epithelial cells through serum outflow from the surrounding microvasculature. Mice unable to produce adiponectin show worse response to colitogenic (141, 142) and carcinogenic (136, 143, 144) conditions, leading to the paradigm that adiponectin helps the epithelium mitigate excessive inflammatory signaling that may be caused by the local microbiota (Chapter 3).

D. CAUSES AND EFFECTS OF GI DYSBIOSIS AND INFLAMMATORY DISORDERS

Dysbiosis overview

With the discovery of microbes inhabiting all human mucosal surfaces and providing services the host genome is unable to generate, the fusion between the host and its microbiota is currently referred to as a superorganism (8). In addition, the body's microbial inhabitants contain genomes that collectively are thought to contain between 10 and 60 million unique genes (145, 146), at least a 1000-fold greater amount than the human genome. Integration of functions performed by the host and microbial genomes creates a 'metagenome' which is the synthesis of the host's mutualistic relationship with resident microbes. The overarching goal of the Human Microbiome Project is to "understand the range of human genetic and physiological diversity"

(8), which extends well beyond host genome. Currently, the idea of what it means for the microbiota to be 'normal' is subjective based upon the defining criteria of what a normal or healthy human is. One strategy the Human Microbiome Project has employed to reduce variability is the characterization of microbiome communities at specific locations (e.g. subgingival plaque, nasal airway, stool) and then relating microbiotas to clinical data (145). The goal is an elucidation of a 'core microbiota' at each of these sites which is common among all humans regardless of geographical location, dietary habits, or any other variable (8). For our purposes, two types of dysbiosis will be discussed in this document: 1) the development and persistence of an abnormal microbiota, 2) the perturbation of the microbial community associated with a host action or disease state.

The evolutionary journey arriving at *Homo sapiens* has experienced the host-microbe relationship for hundreds of millions of years, and both participants are well-evolved to tolerate the other. Appropriate maturation of the mammalian immune system is dependent upon presence of the GI microbiota (147-149), and studies in germ-free mice (150) and pigs (151) have shown a plethora of immune abnormalities during the animals' development. Two of the most striking features germ-free animals display is a stark reduction in number of B cells which have matured to immunoglobulin-secreting plasma cells and the increased susceptibility to challenges with bacteria and viruses, most of which would be innocuous to animals reared under microbial exposure. Though humanity has been unsuccessful creating the germ-free person, the widespread adoption of the Westernized lifestyle has decreased both microbial exposure and the diversity of the individual microbiota (152, 153). Concurrently, the prevalence of many allergic and autoimmune diseases is dramatically increasing in the Western world (154). The first to receive credit for attempting to associate these two trends was David Strachan who, in 1989, proposed the pronounced increase in hay fever cases among wealthy children in Great Britain was due to "declining family size, improvements in household amenities, and higher standards of personal cleanliness" (155). This now-termed Hygiene Hypothesis of a reduced antigenic environment during immune system development helps explain the rise of many allergic, asthmatic, autoimmune , and metabolic diseases (152).

In most inflammatory disorders along the GI tract, no single bacterium can fulfill all of Koch's postulates. While *Porphyromonas gingivalis* inoculation has certainly been implicated in the progression of periodontitis (156), most, but not all, humans with the disease carry the germ in their oral cavity (157). Likewise, the search for the microbial agent causing Crohn's Disease (also, CD) was initially thought to be over with the finding of *Mycobacterium avium* subspecies *paratuberculosis* in CD patients (158). However, since the initial finding, high-throughput

genetic analysis of thousands of intestinal bacterial communities has shown not all CD patients harbor *M. avium* (159, 160), and more broad groups of bacteria are now thought to play an etiological role (161). With the aid of sequencing and microarray tools which allow a complete survey of the microbiome at a certain mucosal site, broad community dysbiosis is the contemporary paradigm for many human inflammatory diseases with known microbial components (162). A diverse array of human diseases are known to be associated with dysboises: periodontitis, cystic fibrosis, celiac disease, inflammatory bowel disease, Type I diabetes, metabolic syndrome, psoriasis, esophageal disease, vaginosis, and irritable bowel syndrome (159). The etiological significance of these dysbiotic states is hard to interpret, since the change in bacterial community may be inciting the disease or simply a result of the disease state. Instead of drawing a straight line between a microbiota and a disease, more integrative approaches are being adopted to help explain the significance of a dysbiosis in the context of host phenotype and disease. Figure **1.1i** illustrates an example of a structural equations model which accounts for host genotype and covariates, gene and protein expression, physiological measurements, and microbiome composition as all contributors to host phenotype.

Periodontitis

Damage and loss of the tissue surrounding the tooth are the hallmarks of periodontal disease, and clear microbial components have been identified. The bacteria *P. gingivalis*, *A. actinomycetemcomitans*, *Tannerella forsythia*, and *Treponema denticola* are all associated with patients with progressive periodontal disease (157). At a community level, gingivitis and periodontitis show a strong dysbiosis with decreases in Gram (+) bacteria such as the mutualists *Streptococcus* and *Actinomyces* but increases in Gram (-) rods (163). Increases in the amounts of Gram (-) bacteria provide higher concentrations of LPS capable of stimulating TLR4 on the gingival epithelium and on immune cells directly beneath the squamous epithelium. The pathogen *P. gingivalis* is known to modulate its own gene expression in order to more easily integrate into oral biofilms and persist in the mouth (164). By its own association into dental biofilms, *P. gingivalis* likely evicts mutualists which are unable to compete with the pathogen.

Obesity

With the rise of obesity and closely-associated metabolic syndrome in developed countries, the elucidation of the intestinal microbiota from affected individuals has shown pronounced insights about the disease. Obesity-induced dysbiosis appears to be a causative and resulting factor linked to the disease. Studies from the Jeffery Gordon lab have shown a clear

positive correlation between an individual's body mass index (BMI) and percentage of intestinal bacteria within the phylum *Firmicutes*. The group went on to show that as a person's BMI decreases over time, the relative percentage of *Firmicutes* decreases while *Bacteroidetes* rises closer to that seen in lean individuals (165). Other studies show overall decreased phylogenetic diversity of the intestinal microbiota in conjecture with obesity and increased bacterial gene expression of proteins involved in carbohydrate, lipid, and amino-acid metabolism (166). This obese microbiota is transmissible in the mouse model, as shown when germ-free mice that received stool pellets from obese, but not lean, mice also displayed symptoms of obesity (167). Persons with hypertrophied adipose tissue are thought to be in a state of chronic low-grade inflammation, and are over-represented in IBD and colorectal cancer patient populations (168). In addition, circulating levels of the hormone adiponectin negatively correlating with BMI may play a role in promoting a more inflammatory environment (169).

Inflammatory bowel diseases

The two classical IBDs are designated as Crohn's Disease (also, CD) and ulcerative colitis (UC). The presentation of CD is generally found in the distal ileum and along multiple sites in the colon, showing a characteristic 'skip lesion' pattern of multiple sites of transmural inflammation (170). More superficial inflammation is seen in UC, with disease severity increasing towards the distal colon in parallel with increasing amounts of intestinal bacteria (9). Both diseases show a skewing of T cell populations toward a Th1 and Th17 pro-inflammatory phenotype with reductions in the prevalence of T regulatory (Treg) cells (171). With the advent of genome-wide association studies (GWAS), high-throughput profiling of intestinal microbiota, and elucidation of patient covariates which increase IBD susceptibility, the realm of IBD etiology is thought to be extremely multifactorial with multiple factors likely synergizing to cause a disease state (171). Figure **1.1j** illustrates the multifactorial nature of IBDs with many of the factors which are currently thought to play a role in patient susceptibility to the disease.

An individual's genome or patterns of gene expression may increase IBD vulnerability through excessive responses to intestinal bacteria, inability to detect intestinal bacteria, breakdown of intestinal epithelial cell physiology and barrier function, or disruption of many other biological roles which maintain intestinal homeostasis (172). Currently, GWAS have shown CD susceptibility loci to be generally within the categories of cellular innate immunity and other immune-mediated functions (173). Likewise, UC susceptibility loci show many hits for immune-mediated functions, but gene mutations leading to defects in the epithelial barrier are also enriched in UC patients. This notion is logical that disruption of any number of diverse

biological functions at the intestinal epithelium may predispose the host to reoccurring excessive inflammation. With the incessant microbial challenge the ileum and colon must continually endure, the absence of any of the numerous homeostatic mechanisms employed by the intestine may tip the balance in favor of war with the intestinal microbiota as opposed to mutualistic peace (174).

As with dysbiosis in obesity, evidence exists for intestinal microbiota dysbiosis both playing a causative role in IBD and a result of IBD activity. Transmissible UC mediated by the microbiota was found in studies using genetically susceptible mice (175), and animals infected with adherent-invasive bacteria within the family *Enterobacteriaceae* progress to a disease state mimicking IBDs (176, 177). However, adherent-invasive bacteria are not always found in humans with IBDs (178), and host-mediated inflammation can also incite a state of dysbiosis (179). At the perspective of the bacterial community, IBD presence shows a reliable decrease in the diversity and amount of microbes residing in the intestine (180, 181). Bacteria within the phyla *Firmicutes* and *Bacteroidetes* show strong reductions while the *Enterobacteriaceae* family consistently increases in relative percentage of the total microbiota (41, 171). These changes in composition also point to another advantage given to the host by the mutualistic microbiota: colonization resistance. Resident microbes are able to fill ecological niches and secrete metabolic products that make colonization of new bacteria extremely difficult (32). The enteric pathogen *Salmonella enterica* decreases colonization resistance through promotion of host inflammatory responses which will kill numerous mutualists (182). This may be a strategy also used by bacteria associated with IBD in order to give themselves a colonization advantage.

The mouse as a model to study human intestinal diseases

Both morphological and immunological differences exist between man and the laboratory mouse (183), but the mouse has nevertheless become an important model in the pursuit of information about human intestinal diseases. Advantages include reduction of genetic variability through inbreeding and the fast generation of experimental subjects. Laboratory mice also have a very similar intestinal microbta to humans with major variations only arising at the family and lower taxanomic levels (184). However, many types of mouse immune and epithelial cells produce different cytokines and express different cell surface proteins as well as mouse B cells repertoires having different IgG and IgA subtypes (183). Also, to ensure mice are not unintentionally exposed to pathogens, animals are housed under sterile conditions where water, food, and bedding are all disinfected which is not representative of our microbial-painted world. One of the most powerful tools at the researcher's disposal is the knockout (KO) mouse, which

cannot produce protein(s) of interest due to genetic alterations. Many transgenic mice develop spontaneous inflammation in the intestine, and include mice unable to produce immunoregulatory proteins such as IL-10 and TGF-β, barrier regulation proteins WASP and N-cadherin, mucus proteins muc2 and trefoil factor, and inhibitors of NF-κB signaling A20 and IKKγ (185). Intestinal inflammation in almost all of these transgenic mice is microbial-mediated and shown to be ameliorated when the mice are raised under germ-free conditions (150). Mice can also be subjected to chemically-induced colitis, the most common form being supplementation of dextran sulfate sodium (DSS) in animal's drinking water which mimics the increased inflammation in the distal colon as seen in UC (186). Overall, though clear differences exists between mouse and human immunity, the mouse model provides researchers with an effective tool to study causes and effects of inflammation in the intestine its resident microbiota.

E. SCOPE AND SIGNIFICANCE OF THIS DISSERTATION

This dissertation further illuminates many of the sophisticated languages used by host and bacterium in order to maintain an environment which is beneficial for both parties. For all studies, the oral or intestinal epithelium is the central focus of the work. In Chapter 3, the first language discussed is how the hormone adiponectin modulates oral microbial TLR ligand and host TNF signaling to and cause dramatic changes in oral and colonic epithelial cell gene expression and cytokine production as well as changes in the NF-κB signaling cascade. Chapter 4 demonstrates how intestinal bacteria only within certain phyla are able to induce colonic epithelial cell (also, CEC) gene expression of pIgR and other microbial response genes. This study further shows the polarized nature of CEC stimulation gene expression dynamics over periods of extended stimulation. In addition, this chapter shows the localization of SIgA and SC in the outer mucus layer of the colon in both mice and humans and that intestinal bacteria localize with these proteins in the outer mucus layer. Chapter 5 shows the impact of SIgA within maternal milk on the establishment of the intestinal microbiota and CEC gene expression in adult mice. Interestingly, very specific genotypic and phenotypic differences were seen between mice receiving maternal IgA, but not SIgA, and mice receiving only IgA were in an immunocompromised state early and later in life, well after dissociation from their mothers. Collectively, these studies help shed further light on the complex relationship between the host and its microbiome and the necessity for multiple mechanisms to ensure mutualism.

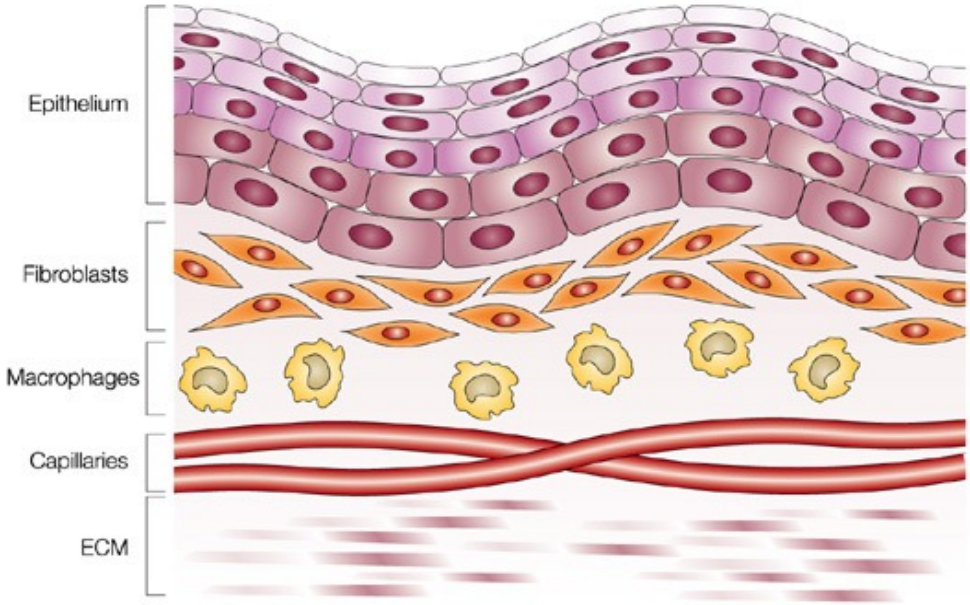


Figure 1.1a [Adapted from Ref. (12) with permission]. Structure of stratified squamous oral epithelium showing multi-layered epithelial cells overlying fibroblasts and macrophages.

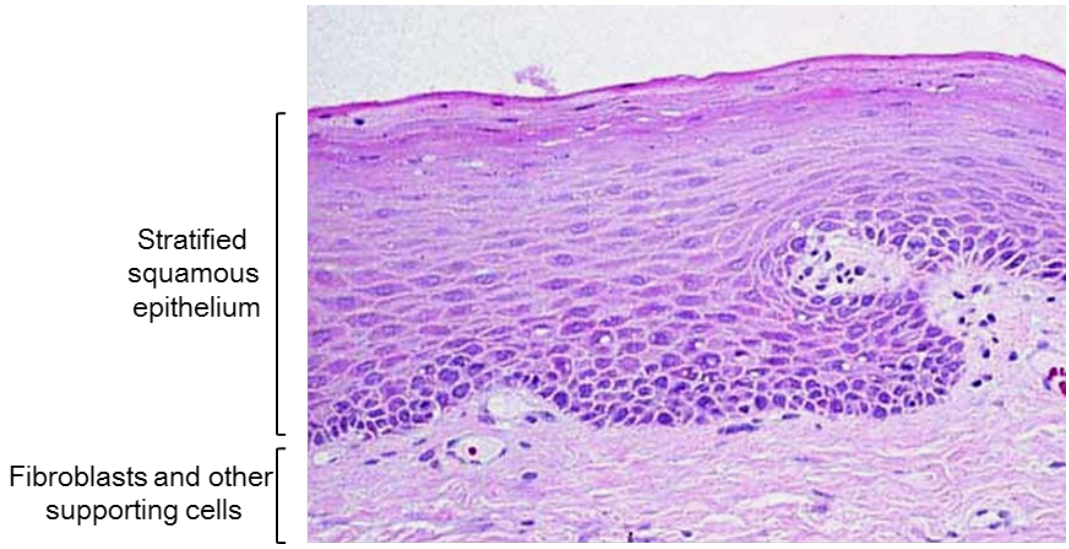


Figure 1.1b [Adapted from Ref. (187)]. Hematoxylin and eosin stain of oral gingival biopsy showing clear stratified nature of the epithelium and distinct transition from epithelial cells to fibroblasts.

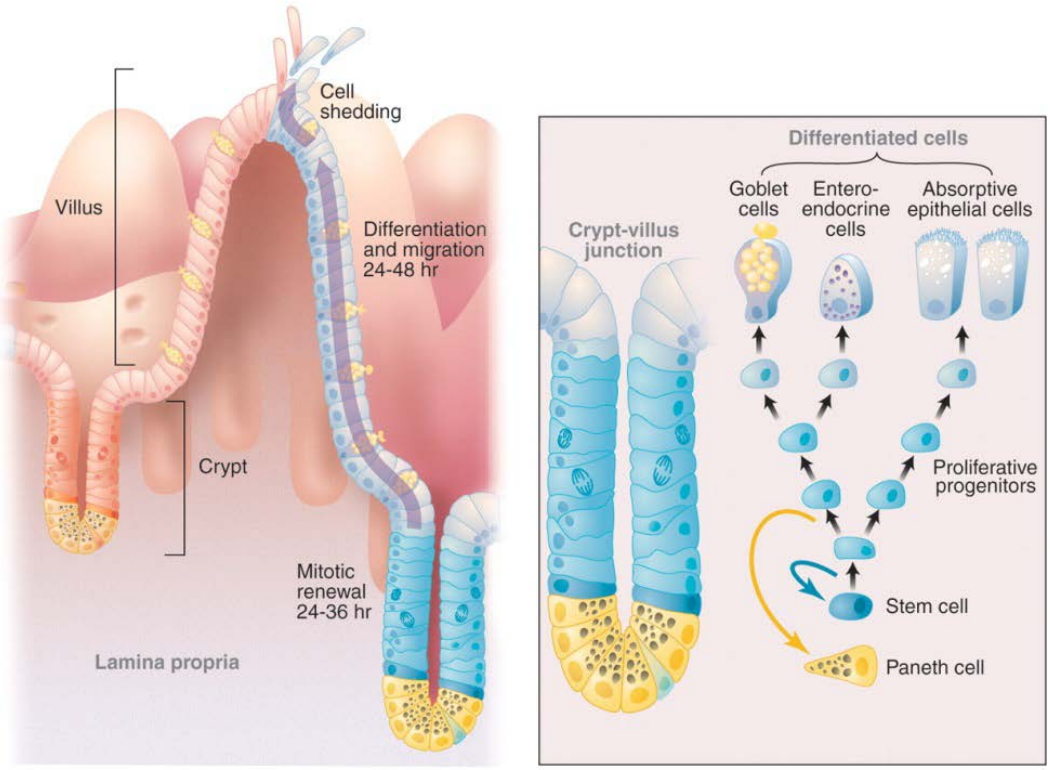


Figure 1.1c [Adapted from Ref. (18) reprinted with permission from AAAS]. Architecture of small intestinal epithelium showing villus/crypt axis and stem cell maturation into one of four intestinal epithelial cells (IECs). IECs life span is thought to be less than five days from nascent differentiation to shedding into the intestinal lumen, so consistent replenishment of these cells is required by the host.

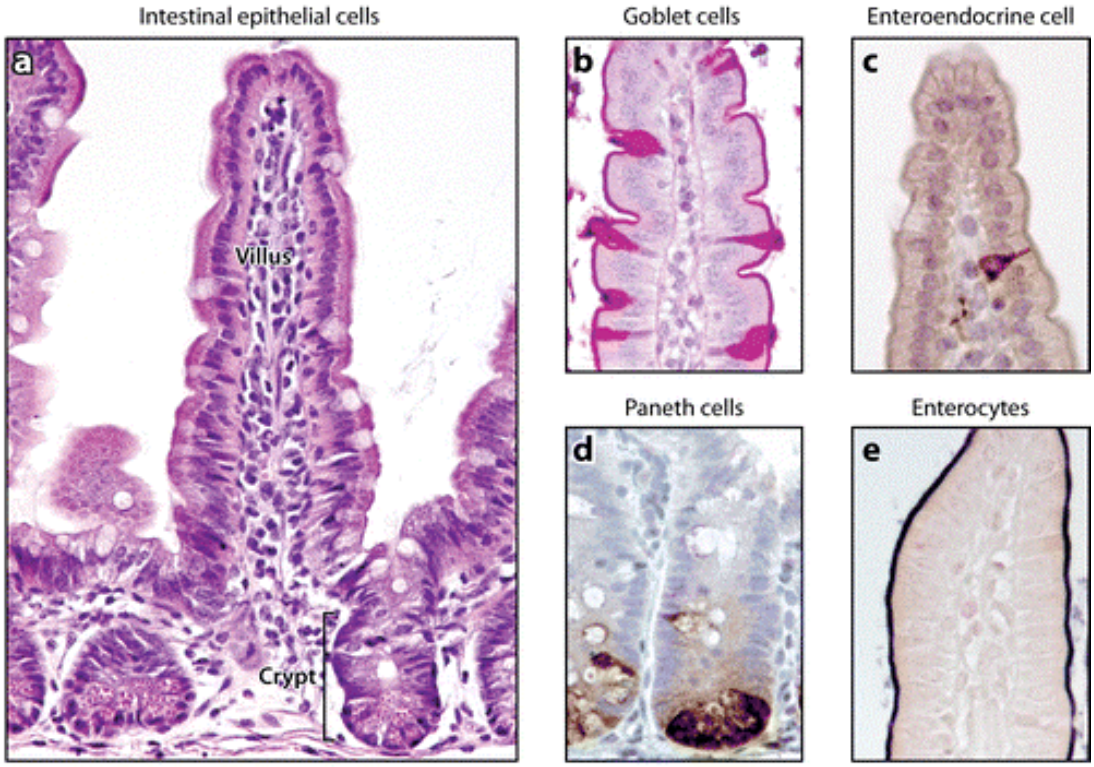


Figure 1.1d [Adapted from Ref. (19) reprinted with permission]. Small intestinal architecture and cell types. a) Hematoxylin and eosin stain of mouse small intestinal biopsy showing stricture of villus and crypt. b) goblet cells along the vilus stained positive for mucus with periodic acid-Schiff, c) an enteroendocrine cell stained positive for synaptophysin, d) paneth cells stained positive for lysozyme, e) and absorptive enterocytes stained positive with alkaline phosphatase.

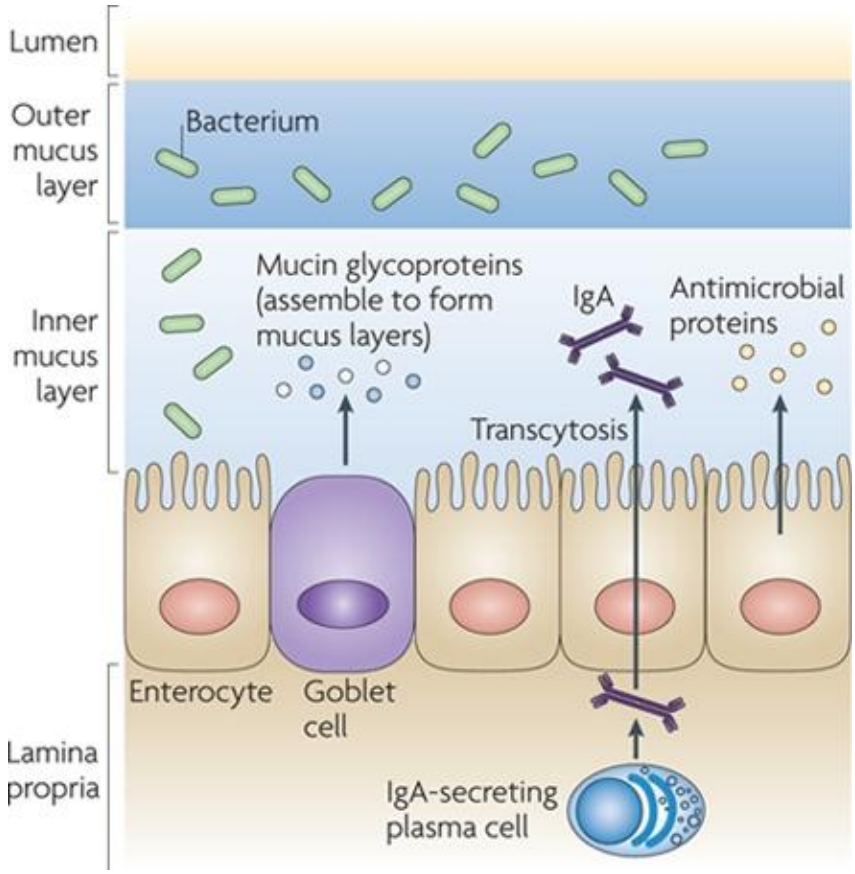


Figure 1.1e [Adapted from Ref. (149) reprinted with permission]. Structure of large intestinal epithelium and organized mucus layers. Goblet cells along large intestinal epithelium secrete mucus into intestinal lumen, forming two distinct layers. Bacteria are segregated largely to outer mucus layer while host antimicrobial proteins from epithelial cells and IgA from lamina propria plasma cells are found throughout mucus layer.

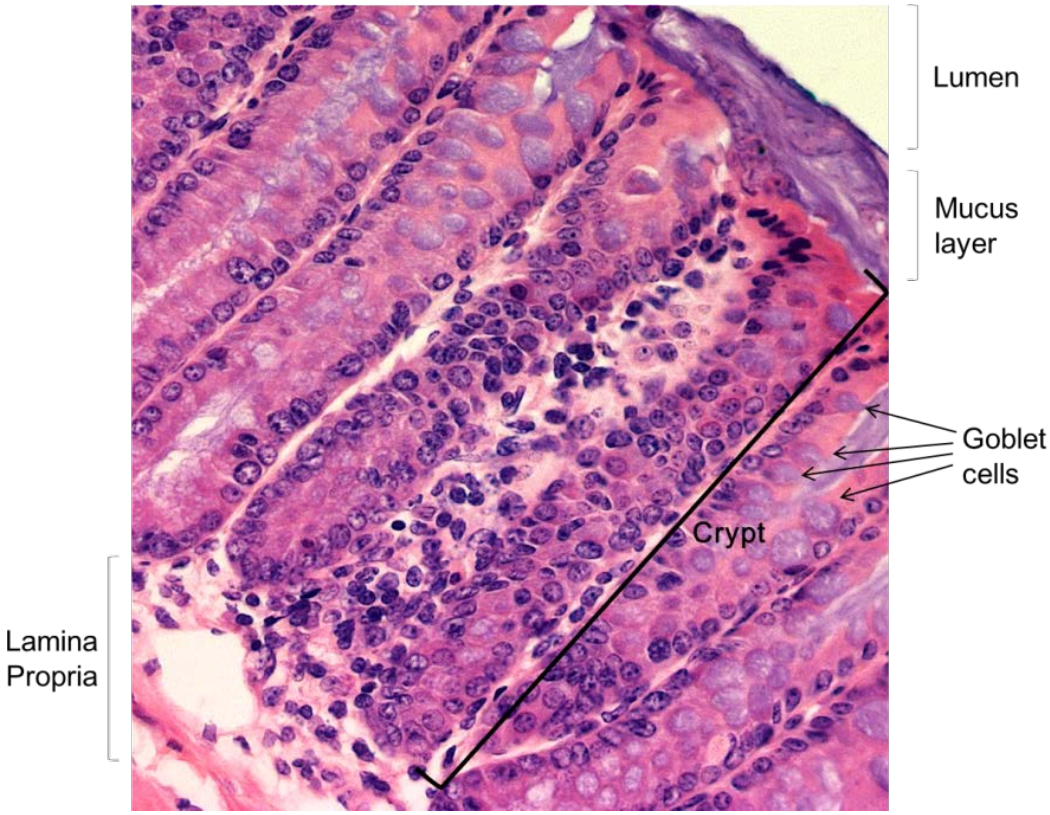


Figure 1.1f [Adapted from Ref. (188)]. H&E-stain section of mouse colon showing deep crypt penetration into the lamina propria, goblet cell size and localization *in situ*, and secreted mucus filling crypt crevasses and migrating to luminal space. Once in the luminal space, mucus remains adhered to colonic epithelium and forms an organized layer.

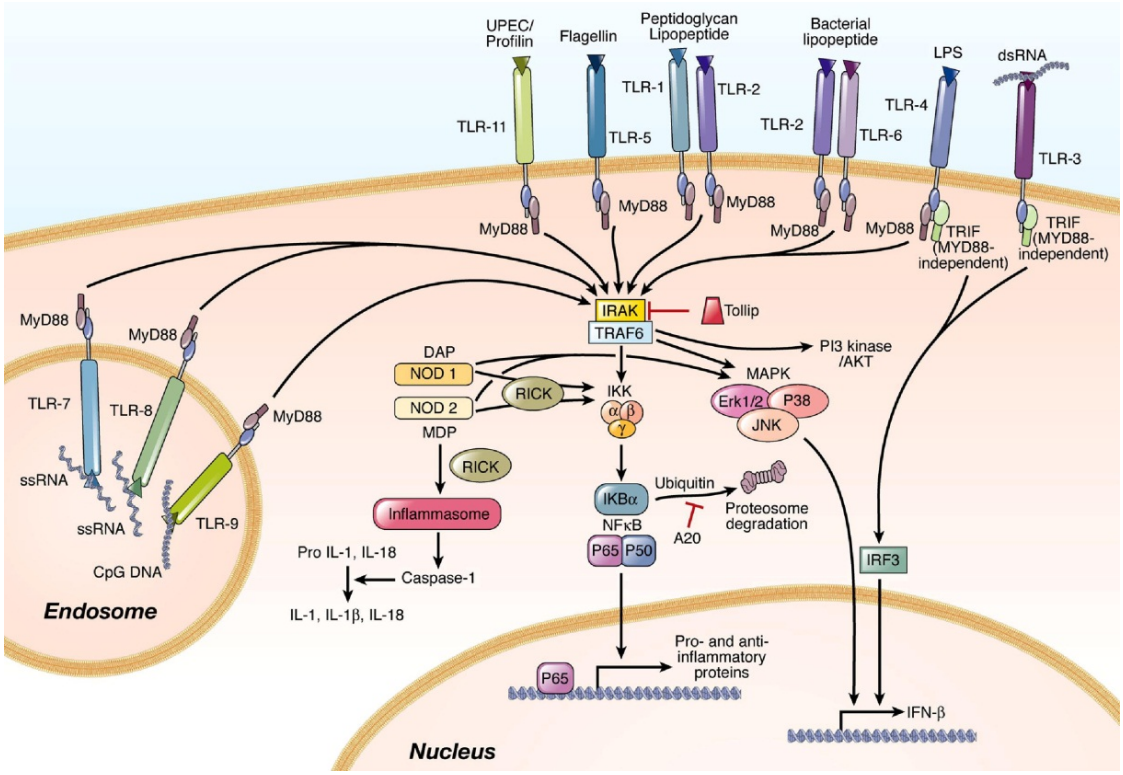


Figure 1.1g [Adapted from Ref. (9) reprinted with permission]. Microbial recognition by epithelial cells. Membrane bound and intracellular Toll-like receptors (TLRs) as well as the Nod-like receptors NOD 1 and 2 are shown *in situ* with their respective microbial ligands. All TLRs (except TLR3) signal through the MyD88 adaptor protein to activate the mitogen-activated protein kinase (MAPK) and NF-κB cascades and induce host response and gene expression. NOD receptors utilize the adaptor protein RICK to signal through NF-κB, but are also able to signal through the MAPK pathway. The inhibitors of signal transduction, Tollip and A20, are shown at which steps in the pathway they disrupt.

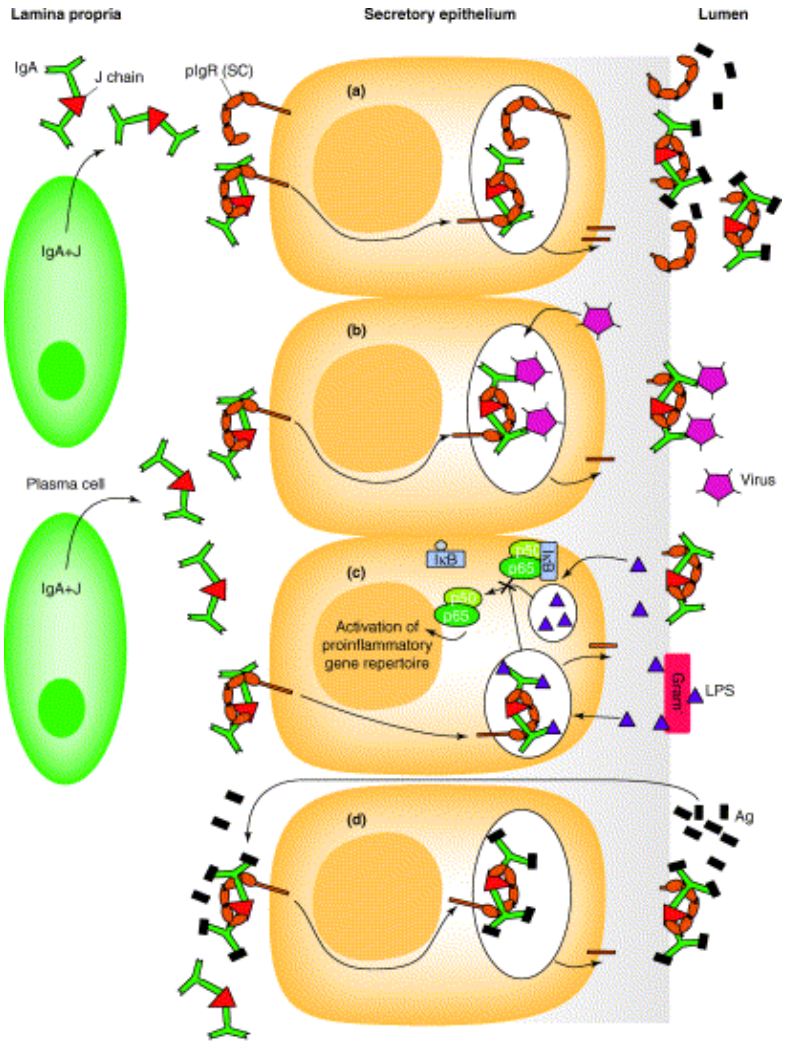


Figure 1.1h [Adapted from Ref. (105) reprinted with permission]. Mechanism of transport and functions for polymeric IgA and polymeric immunoglobulin receptor (pIgR/SC) at the epithelial surface. Production of IgA and J chain by IgA^+ plasma cells allows the efficient multimerization of IgA proteins with connecting J chain. Polymerized IgA then binds pIgR on the basolateral surface of the epithelial cell and is transported through endosomal vesicles to the apical surface of the cell where it is cleaved and released (now SIgA) into the mucus layer. Multiple immune functions are mediated by the SIgA and SC proteins. Section (a) displays immune exclusion of antigens (Ag) to the mucus layer while (b) shows intracellular neutralization of virions and (c) intracellular neutralization of microbial products plus prevention of NF-κB signaling; (d) illustrates antigen secretion back into the intestinal lumen.

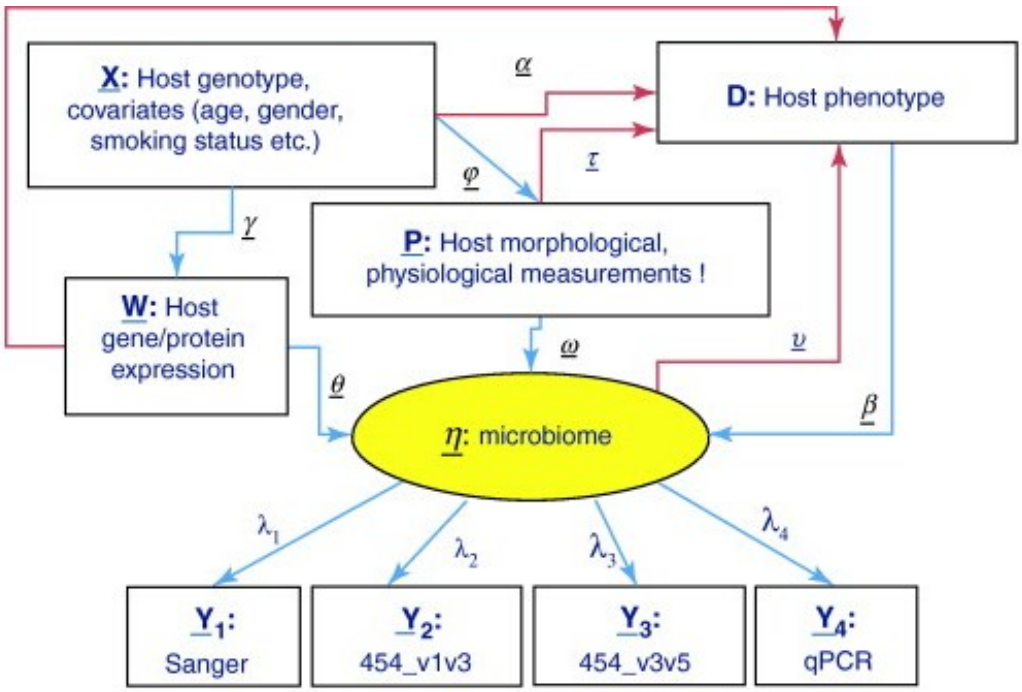


Figure 1.1i [Adapted from Ref (159) reprinted with permission]. A comprehensive structural equations modeling (also, SEM) approach to link host variables (with certain emphasis on host microbiome) with host phenotype. Collectively, the red and blue connectors (nodes) forms a set of interrelated regression equations with random independent as well as dependent variables that allow formulation and testing of directional causal pathway hypotheses (189). The path coefficients α,β,γ,etc. are regression coefficients of each nodal regression equation which can be analyzed individually. However, the optimal resolution for the host in a clinical setting is the simultaneous analysis of the entire equations system. The host microbiome can be sampled by various sequencing techniques, some of which include: Sanger sequencing (Y_1), 454 pyrosequencing of V1 and V3 regions of bacterial 16S rRNA (Y_2), pyrosequencing of V3 and V5 regions (Y_3), and quantitative PCR (Y_4).

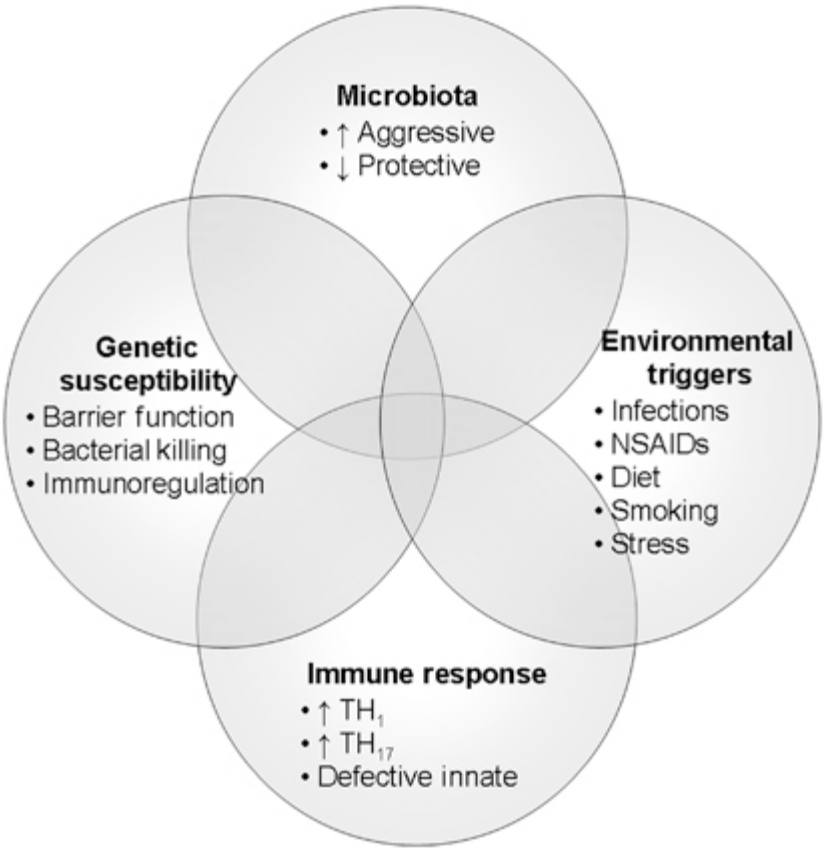


Figure 1.1j [Adapted from Ref (190) reprinted with permission]. The multifactorial nature of chronic inflammatory bowel disease (IBD). IBDs may present and persist by the overlap of any or all of these susceptibility factors. Mutations in genes allowing for control of intestinal epithelial barrier integrity, killing of bacteria in close contact with the epithelium, or regulation of host immune response increase likelihood of IBD, but are probably not enough in themselves to incite disease. A common finding among IBD patients is the increased skewing of T cell subsets towards the Th1 and Th17 pro-inflammatory lineages which overact to innocuous intestinal microbes. Multiple environmental factors have been implicated in IBD occurrence, and include many practices associated with the Westernized lifestyle. Consistent trends are seen in IBD patients with or without active disease by dysbiosis of intestinal microbiota with increases in relative percentage of facultative aerobic species within the family *Enterobacteriaceae* and broad decreases in amounts and percentages of the *Firmicutes* and *Bacteroidetes* phyla.

Chapter 2

MATERIALS AND METHODS

Collection of tissue biopsies from human subjects (Chapter 3,4). Adult volunteers (age 18-80) for these studies were recruited from patients undergoing colonoscopy at the University of Kentucky Medical Center, after institutional review board approval and written informed consent. For patients with inflammatory bowel disease (IBD), including Crohn's disease (191) or ulcerative colitis (UC), the indication for colonoscopy was either to evaluate disease exacerbation or to screen for dysplasia and colorectal cancer. Diagnosis of IBD was based on clinical, radiological and endoscopic criteria and supported by histopathological findings. Control subjects age 50 or older underwent screening colonoscopies for colon cancer in accordance with current guidelines. Control subjects age 18-49 underwent colonoscopy for evaluation of constipation or chronic abdominal pain. Individuals were classified as "normal" when endoscopic, radiologic and pathologic evaluation of randomly obtained biopsies revealed no disease of the small or large bowel. 'Healthy' (control) subjects defined by the absence of CD, UC, indeterminate colitis, or celiac disease at any time in their life. Mucosal biopsies were collected from the terminal ileum and pooled from multiple locations in the ascending, transverse and descending colon. Subjects who consented to participate in the study subsequently underwent periodontal examination at the University of Kentucky College of Dentistry, and biopsies were obtained from regions of the gingiva without macroscopic inflammation. Biopsied tissue was immediately immersed in an RNA stabilizing solution (RNAlater) (Qiagen, Valencia, CA), and stored at -80°C. Gene expression analysis of gingival biopsies only preformed on patients without intestinal inflammation.

Mice (Chapter 4,5). Male and female wild-type C57BL/6 mice were obtained from ongoing mouse colony under the supervision of Dr. Charlotte Kaetzel at the University of Kentucky which had originally been purchased from The Jackson Laboratory (Bar Harbor, ME). Transgenic mice with Neo^R cassette inserted into exon 3 of mouse *PIGR* locus and unable to produce functional Pigr protein were initially created as described previously (75) and were a kind gift from Dr. Finn-Eirik Johansen, University of Oslo, Norway. Mucin 2 (*Muc2*) deficient mice which had initially been created by replacing of exons 2-4 in *Muc2* gene with *PKG-Neo* cassette (88) were a kind gift from Dr. Anna Velcich, Albert Einstein College of Medicine, New York, NY. All mice were bred in the animal facility at the University of Kentucky College of Medicine. All mice were kept in microisolator cages with sterile bedding and were fed sterile food and water. For

experiments utilizing the maternal milk breeding scheme (Figure 5.1A), mice were separated by gender and *Pigr* genotype at the time of weaning for the duration of their lives. Mice were maintained and used in accordance with the policies and guidelines set forth by the University of Kentucky Institutional Animal Care and Use Committee.

Eukaryotic cell culture (Chapter 3,4). The HT-29v20 subclone of the human colon adenocarcinoma cell-line HT-29 (192) and the human acute myeloid leukemia cell line THP-1 (193) (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 μg/ml streptomycin and 250 ng/ml fungizone) – collectively, 'Complete Medium'. The immortalized human oral keratinocyte OKF6/hTERT-2 (194) cell-line, which has normal growth and differentiation characteristics that represent a model of oral epithelium (195), was cultured in serum-free keratinocyte medium as previously described (196). All cell lines were grown at at 37°C and 5% CO_2. All cell culture reagents were from Lonza Walkersville (Walkersville, MD) or Invitrogen Life Technologies (Carlsbad, CA). For experiments with bacterial stimulation (Chapter 4), HT-29 cells were plated in 24-well dishes or Transwell inserts and grown for 24-48 h to a density of approximately 10^6 cells/well prior to initiation of experiments. THP-1 cells were maintained in suspension culture, and were diluted to a density of $1x10^6$ cells/well in 24-well dishes immediately before exposure to bacteria. Heat-killed or live bacteria were diluted to a final concentration of approximately 10^7 cells/well. For adiponectin stimulation experiments (Chapter 3), OKF6 and HT-29 cells were grown to approximately 70% confluence, then treated as described in the figure legends. Recombinant high molecular weight human adiponectin (HMWAd) produced in HEK293 cells, which mimics serum adiponectin by forming high molecular weight (HMW) and hexameric species, was obtained from Enzo Life Sciences, Farmingdale, NY. Recombinant human TNF, produced in *E. coli*, was obtained from eBioscience, San Diego, CA. The LPS purified from *E. coli* O26:B6 was obtained from Sigma-Aldrich, St. Louis, MO, and recombinant flagellin from *Salmonella typhimurium* was obtained from InvivoGen, San Diego, CA. At the termination of all cell culture experiments, culture supernatants were collected and stored at -20°C for cytokine analyses, and cells were harvested in buffer RLT and stored at -20°C for RNA purification.

Bacterial cell culture (Chapter 4). *Escherichia coli* Nissle 1917 was the generous gift of Dr. Ulrich Sonnenborn, Ardeypharm GmbH, Herdecke, Germany. *Salmonella typhimurium* SL1344 was the generous gift of Dr. Sarah D'Orazio, University of Kentucky. All other bacteria were

obtained from the American Type Culture Collection (Manassas, VA). Prior to incubation with eukaryotic cells, bacteria were grown to mid-log phase in Schaedler broth (Oxoid, LTD, Basingstoke, UK) under anaerobic conditions at 37^{o}C. For quality control, the identity of each bacterial species was confirmed by PCR amplification of the 16S rRNA gene with species-specific primers. Heat-killed bacteria were prepared by diluting liquid cultures to approximately 10^8 colony-forming units (CFU)/ml in eukaryotic tissue culture medium and heating at 65^{o}C for 30 min. Aliquots of bacterial cultures prior to heat treatment were plated on Schaedler agar to quantify CFU. For experiments with live bacteria, aliquots of bacterial suspensions before and after co-culture with eukaryotic cells were plated on Schaedler agar to quantify CFU.

In vitro bacterial invasion assay and FITC dextran flux (Chapter 4). The ability of live *E. coli* Nissle or *S. typhimurium* SL1344 to invade monolayers of HT-29 cells was analyzed as described (197). Approximately 10^7 bacteria were added to 24-well dishes containing approximately 10^6 HT-29 cells. After 1 h, triplicate cultures of HT-29 cells were washed extensively with Hank's Balanced Salt Solution (HBSS), lysed with 1% Triton X-100, and plated on Schaedler agar to enumerate total cell-associated CFU. Separate triplicate cultures of HT-29 cells were treated with gentamicin (480 μg/ml in HBSS) for 90 min at 37^{o}C to kill bacteria bound to the external cell surface, washed extensively, lysed, and plated on Schaedler agar to enumerate internalized CFU. Membrane-associated bacteria were calculated by subtracting internalized CFU from total cell-associated CFU. Permeability across epithelial monolayers measured by adding 5mg/mL 3kD FITC-dextran (Sigma) to Transwell apical chamber and collecting well basal chamber volume after 3h and reading flow-through FITC concentration by spectrophotometer. Flux across HT-29 monolayer measured by: [ug FITC-dextran in basal chamber / time (in seconds)].

NF-κB reporter assay (Chapter 3). HT-29 cells incubated in serum-free medium 19h after transfection for 6h. Cells pretreated with HMW adiponectin for 18h and subsequent 6h TLR ligand or TNF stimulation. Cell lysates were analyzed for firefly and *Renilla* luciferase activities using the Dual Luciferase Reporter Assay System (Promega). NF-κB enhancer activity was calculated by subtracting the normalized luciferase activity of cells treated with pTK-luc from the normalized luciferase activity of cells treated with pNF-κB-TK-luc.

DSS colitis regimen (Chapter 5). Acute colitis induced in mice by feeding of 2% dextran sodium sulfate (DSS, MP Biomedicals, Aurora, OH) in drinking water for 8 days. Mice were

weighed every day of treatment and surveyed for stool consistency and occult or visible blood for calculation of disease activity index (also, DAI) by summation of scores from all three parameters. On day 9 of the treatment regimen mice were sacrificed, colons were extracted and measured, and tissue fixed for histology or processed for epithelial cell isolation.

Isolation of bacteria from mesenteric lymph nodes and identification of prominent aerobic species (Chapter 5). Mesenteric lymph node (MLN) sections surrounding the cecum were dissected from 21d old mice under sterile conditions, homogenized in sterile PBS, and cultured on Schaedler agar plates under both aerobic and anaerobic conditions. To identify the prominent aerobic bacteria found in weanlings not receiving SIgA, 2 colonies derived from two different mouse MLNs were harvested and cultured overnight in Schaedler broth at 37^{o} under aerobic conditions. Bacterial DNA was extracted by QIAamp DNA Stool Isolation Kit (Qiagen, Germantown, MD), and the 16S rRNA gene was amplified by real-time PCR using the universal Eub primers (198). 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), and shipped to ACGT, Inc. (Wheeling, IL) for sequence analysis. Identical sequences of the unknown 16S rRNA 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).

Fixation and rehydration of tissues and histological scoring of colons (Chapter 4,5). Colons were dissected from euthanized mice and fixed in formalin, Carnoy's fixative (all figures of colons), or prepared for epithelial cell isolation. Fixed tissues were embedded in paraffin and sectioned onto glass slides. Tissue sections were dewaxed in xylene and rehydrated by ethanol gradient by previous protocol (186). For histological scoring, rehydrated colonic sections were stained with H&E to visualize colonic attributes and morphology and allow scoring. Scoring was performed in a blinded fashion with possible score from 0-4 for each of four sites along colonic length scored (total possible score of 16 indicating most severe disease).

Epithelial cell isolation from mouse colons (Chapter 5). Epithelial cells from colonic tissue were isolated as previously described (186, 199). Colons were opened longitudinally, cut into small 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 Complete Medium. The supernatants were discarded, and the epithelium was stripped from the tissue pieces by incubation for 1 h at 37°C in a solution of 0.5 M EDTA in Complete Medium. The suspension was vortexed, supernatant containing epithelial cells was poured off. Isolation of epithelial cells 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 epithelial cells was removed and transferred into a 15 ml conical tube. The volume was brought to 10 ml with supplemented tissue culture medium, and epithelial cells pelleted by centrifugation at 2000 rpm for 10 min at 4°C and resuspended in buffer RLT.

Immunofluorescence of in vitro cell lines (Chapter 3). For p65 (RelA) staining on cell lines, cells were grown on glass 8-Chamber Slide system (Lab-Tek, Naperville, IL) and fixed with 4% paraformaldehyde for 10min and subjected to additional permeabilization in methanol for 15min at -20^{o} after fixing. Slides were mounted with mounting medium containing DAPI (Vectashield, Burlingame, CA). Permeabilized cells stained for p65 localization to the nucleus as described previously (58) with rabbit anti-p65 (1:100, Santa Cruz) overnight at 4^{o} and sheep anti-rabbit IgG (1:200, Sigma-Aldrich) 1h at room temperature. Images taken using a Zeiss Axiophoto confocal microscope with Axiovision image software.

Immunofluorescence staining and tissue microscopy (Chapter 4,5). After dewaxing and rehydration of tissue slides, antigen retrieval was performed by incubating slides for 20 min at 95°C in 25 ml of 10mM sodium citrate buffer, pH 6. For only slides undergoing pIgR/SC staining, endogenous peroxidase activity was quenched by incubation for 10 min at room temperature in 25 ml of 3% (v/v) H_20_2. Rehydrated slides blocked for 30min in PBS containing 0.1% TritonX-100 and 10% serum from secondary antibody species. Primary antibodies kept on slides overnight after dilution in blocking buffer at concentrations: pIgR/SC 1:250 (produced in goat, R&D Systems), muc2 1:100 (produced in rabbit, Santa Cruz). Secondary antibodies diluted in blocking buffer and kept on slides for 2h at room temperature at concentrations: pIgR/SC 1:500 (HRP-rabbitαgoat IgG, Invitrogen), muc2 1:200 (Rhodamine bovineαrabbit IgG, Santa Cruz), IgA 1:200 (FITC, goatαmouse IgA, Santa Cruz) . To visualize pIgR/SC protein, bound peroxidase was revealed with TSA Plus-TMR Reagent (Perkin-Elmer, Waltham MA). To visualize nuclei, sections were counterstained with VectaShield containing DAPI. Imaging was performed on a Zeiss Axiophoto confocal microscope with Axiovision image software.

Fluorescence in situ hybridization (Chapter 4,5). After rehydration, tissue slides were probed for bacteria as previously described (36). Briefly, the Eub probe recognizing all bacteria (198) conjugated to Alexafluor647 fluorophore (Invitrogen) was diluted to 25ng/mL in hybridization buffer (20mM TrisHCl, 0.9M NaCl, 0.1%SDS, pH 7.4) and kept at 50^{o}C overnight. Slides were washed in 50^{o}C wash buffer (hybridization buffer without SDS) for 20min, washed once more briefly, and mounted with VectaShield containing DAPI.

mRNA analysis of cell culture lines for bacterial studies (Chapter 4). Total cellular RNA was extracted from cell lines using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). The quality of purified RNA was analyzed by agarose gel electrophoresis. cDNA was synthesized using 200-300 ng of total cellular RNA and the TaqMan Gold RT-PCR kit with random hexamers (Applied Biosystems, Foster City, CA). Specific mRNA levels were quantified by real-time reverse transcriptase PCR (qRT-PCR), using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems). The sequences of primers and fluorescent probes for the human genes pIgR, interleukin (IL)-8, tumor necrosis factor (TNF) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have previously been reported (200). The proprietary software PrimerExpress™ (Applied Biosystems) was used to design the primers and probe for mouse pIgR as follows (5'-3'): forward primer: CTATTGGTGTCTTACCAATGGTGACT; reverse primer: CTGTTGCGTTCTGTGGCGT; TaqMan probe: ACAATAGAACTCCAGGTTGCCGAAGCT ACAAGG. Pre-designed primers and probes for A20 and MAPK phosphatase (MKP)-1 were purchased from Applied Biosystems. PCR was performed using 40ng reverse-transcribed RNA and TaqMan Universal PCR Master Mix, no UNG (Applied Biosystems). Amplification of the cDNA template was measured at every cycle by fluorescence resonance energy transfer (ΔRn). The threshold cycle (145), which is inversely proportional to the level of a given mRNA transcript, is defined as the cycle at which the ΔRn exceeds the threshold of fluorescence detection. To determine the relative level of target mRNA in individual samples, the C_T values for each message were normalized to the C_T for the control genes GAPDH (HT-29 and THP-1 cells) by the formula $(2^{-(C_T \text{target} - C_T \text{control})}) \times 100\%$.

mRNA analysis for adiponectin studies (Chapter 3). Biopsies or cells in buffer RLT were lysed by MagNA Lyser (Roche, Basel Switzerland) and total RNA purified by MagNA Pure Compact RNA Isolation Kit (Roche) protocol with elution volume of 50μL. RNA quality and quantity were determined with a spectrophotometer (NanoDrop Technologices, Wilmington, DE). cDNA of purified RNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen).

Real-time quantitative RT-PCR of cDNA products performed by BioRad CFX96 Real-time System (BioRad, Hercules, CA) in Microseal low profile plates (BioRad). Primers (nucleotide sequence not provided) for β_2 microglobulin (β2m), IL-8, TNF, A20, p65 (RelA), adiponectin receptor 1 (AdipoR1), AdipoR2, PPARγ purchased from SA Biosciences (Frederick, MD) and real-time cycles ran according to their protocol with RT^2 Fast SYBR Green qPCR Master Mix (SA Biosciences). PCR specificities were confirmed by melting curve analysis of amplified gene products displaying single peak. mRNA levels normalized to MHC molecule β_2 microglobulin present on all nucleated cells and quantified by ΔCt method: $[(2^{-(Ct\ test - Ct\ \beta 2m)}) \times 100\%]$.

mRNA analysis by microarray and Nanostring nCounterTM Hybridization (Chapter 5). Total RNA was purified from isolated CEC cells in buffer RLT by lysing on MagNA Lyser (Roche, Basel Switzerland) and total RNA purification by MagNA Pure Compact RNA Isolation Kit (Roche) according to their protocol with elution volume of 50μL. RNA quality and quantity were determined with a spectrophotometer (NanoDrop Technologices, Wilmington, DE). Microarray analysis was done using Affymetrix Mouse Gene 1.0 ST Array GeneChip according to their protocol (www.affymetrix.com). For mRNA analysis using the Nanostring nCounter™ hybridization technique, colonic epithelial cells were isolated from freshly dissected colons as previously described (199). Abundance of mRNA transcripts was analyzed by Nanostring nCounter™ hybridization (201), and normalized using an algorithm developed by NanoString, Inc. (www.nanostring.com).

Biological pathway overrepresentation analysis of microarray gene expression data (Chapter 5). Gene lists found to be significantly ($p < 0.01$) up- or downregulated from three experimental factors or combination of factors by microarray analysis (see Venn diagram, Fig. 5.5A) were queried for biological pathway overrepresentation by Reactome database (www.reactome.org). Reactome is an open source database and website for the exploration and analysis of human biological pathways (202, 203). Reactome contains core datasets for systems biology. Pathways are built from biological reactions that are connected as steps in the pathway. Each reaction describes a biological event (e.g. binding, phosphorylation, transport, or enzymatic process) based on information provided by expert biologists, peer-reviewed to ensure the resulting pathways represent the biological consensus. Every reaction is supported by published experimental data stored in bioinformatics databases, including: NCBI Entrez Gene, Ensembl, UniProt, UCSC and HapMap genome browsers, KEGG compound, ChEBI small molecule, PubMed, and Gene Ontology. Pathways are anthrocentric, but may incorporate data from model

organisms in the case of conserved functions, although these reactions are clearly differentiated from those that were experimentally determined in humans. Each topic is represented as a hierarchy of pathway diagrams. Gene list overrepresentation of biological event at $p < 0.01$ determined to be significant for this study.

Cytokine quantification by electrochemical luminescence (ECL) (Chapter 3). Cell supernatants were analyzed for pg/ml concentrations of IFNγ, IL-10, total IL-12, IL-13, IL-17, IL-1β, IL-8 (CXCL8), and TNF by Custom Human 10-spot ECL plate (Meso Scale Descovery, Gaithersburg, MD) according to their protocol.

Analysis of IL8 from cell supernatants and fecal IgA by enzyme-linked immunosorbant assay (ELISA) (Chapter 4,5). Cell culture supernatants analyzed for IL8 protein levels as described by manufacture's protocol (R&D systems, Minneapolis, MN). In the same way, mouse feces were weighed, liquefied in ELISA dilution buffer, and analyzed by ELISA for total IgA (Bethyl Laboratories, Montgomery, TX). For both ELISA kits, HRP substrate was bought from R&D systems. Optical density (OD) measured by absorbance at 450nm on Spectramax M5 (Molecular Devices, Sunnyvale, CA) and fitted to standard curve of known dilutions by SoftMax Pro software.

Analysis of mouse fecal microbiota (Chapter 5). Bacterial DNA from mouse feces purified using QIAamp DNA Stool Isolation Kit according to manufacturer's protocol (Qiagen). For qRT-PCR analysis of bacteria, primers for 16S rRNA regions of intestinal bacteria were used as published previously (198) with iQ SYBR Green Supermix (Bio-Rad) after optimization by our lab. Cycle threshold levels were determined on ABI Prism® 7700 Sequence Detection System. For phyochip analysis of fecal microbiota, samples were sent to Second Genome, Inc. (San Bruno, CA) for analysis by the PhyloChip™ microarray (204). Bacterial 16S rRNA genes were amplified by PCR using the forward primer 5'-AGRGTTTGATCMTGGCTCAG-3' and reverse primer 5'-GGTTACCTTGTTACGACTT-3', fragmented, biotin labeled, and hybridized to the PhyloChip™ 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 communities (205). For weighted UniFrac, (WuniFrac), both the abundance and dissimilarities among OTUs were considered. Phylogenic trees was generated using the "Interactive Tree of

Life" software tool (206). Principal Component Analysis (PCA) was used to position points on two-dimensional ordination plots based on dissimilarity values. Significant OTUs whose abundance characterizes each mouse genotype were identified by Prediction Analysis for Microarrays (PAM analysis), which utilizes a nearest shrunken centroid method (207).

Statistical Analysis (Chapter 3). Statistical significance of differences in gene or protein expression among treatment groups was assessed by ANOVA and Fisher's protected least significant difference test. Statistical significance of differences in gene expression between paired samples of non-inflamed and inflamed tissues from individual IBD patients was determined by non-parametric paired sign test.

Statistical Analysis (Chapter 4). Statistical differences among treatment groups were determined by ANOVA and Fisher's protected least significant difference test.

Statistical Analysis (Chapter 5). Statistical differences among treatment groups for protein, IgA, bacterial abundance, and bacterial CFU amounts determined by ANOVA and Fisher's protected least significant difference test. Analysis of fecal microbiota at the population level included both statistical analysis of abundance and binary metrics at each taxa-sample intersection. Sample-to-sample distance functions comparing dissimilarity between communities determined by the Unifrac distance metric (13) using unweighted (absence/presence) or weighted (+ abundance) considerations. Two-dimensional ordinations and hierarchical clustering maps of the samples in the form of dendrograms were created to graphically summarize the inter-sample relationships. To create dendrograms, the samples from the distance matrix are clustered hierarchically using the average-neighbor (HC-AN) method. Principal Coordinate Analysis (PCoA) by two-dimensional ordination plotting used to visualize dissimilarity values to position sample points relative to each other. Lists of significant taxa whose abundance characterizes each class performed using Prediction Analysis for Microarrays (PAM) which utilizes a nearest shrunken centroid method (208). The Adonis test utilized for finding significant whole microbiome differences among discrete categorical or continuous variables. In this randomization/monte carlo permutation test, the samples randomly reassigned to the various sample categories, and the mean normalized cross-category differences from each permutation are compared to the true cross-category differences. The fraction of permutations with greater distinction among categories (larger cross-category differences) than that observed with the non-permuted data reported as the p value for the adonis test. For significance testing of paired sample

sets a paired Student's t-tests was performed on abundance values of each OTU per paired sample (p-value threshold of 0.05 and 0.01). For analysis of mRNA abundance, PCoA utilized to determine sample distribution and population clustering by two-dimensional plotting. Analysis of Variance measurements calculated differences between sample populations with statistical significance determined at $p < 0.05$.

Chapter 3

HIGH MOLECULAR WEIGHT ADIPONECTIN MODULATES IMMUNE RESPONSES OF ORAL AND INTESTINAL EPITHELIAL CELLS THROUGH NF-κB-DEPENDENT TNF AND TOLL-LIKE RECEPTOR SIGNALING

INTRODUCTION

Adiponectin is the primary adipokine secreted by white adipose tissue in humans (209). Serum levels are variable, but tend to average in low μg/ml quantities with a range of 2-17μg/ml in a healthy individual (169) which inversely correlates with BMI (210-212). By itself, full-length adiponectin has been shown to be capable of signaling through various cell types *in vitro* and *in vivo* including: monocytes/macrophages (120, 121), endothelial cells (122-124), myocytes (125-127), synovial fibroblasts (128), and intestinal epithelial cells (129). Globular adiponectin, which is a cleavage product of the full-length protein, appears to have high affinity for adiponectin receptor 1 (AdipoR1) and low affinity for adiponectin receptor 2 (AdipoR2) whereas full-length adiponectin employs the opposite scheme with high AdipoR2 and low AdipoR1 affinity (213, 214).

Both AdipoR1 and 2 have been detected on the epithelium of the oral (131), gastric (132, 133), and intestinal (134-136) surfaces. In particular, the oral and intestinal epithelial surfaces are in constant interaction with adiponectin as supplied through saliva (137, 138) and intestinal cells (139, 140), respectively. Furthermore, it is possible that adiponectin reaches epithelial cells through serum outflow from the surrounding microvasculature. Data is limited, but AdipoR1 and 2 expression appears to be downregulated in the gingiva during periodontal disease (131). Full-length adiponectin and the adiponectin receptors are expressed at lower levels in patients with gastric cancers (132, 133) and in mice subjected to DSS colitis (140), leading to the view that adiponectin acts in a protective, anti-inflammatory manner. Furthermore in the mouse model, knockout of the adiponectin gene has been shown to worsen host response to colitogenic (141, 142) and carcinogenic (136, 143, 144) conditions. Conflicting reports have shown that adiponectin has no protective effect in preventing colitis or colorectal cancer, and may even worsen the condition (139, 215).

In addition to the presence of adiponectin, the oral and intestinal epithelium are persistently exposed to exogenous antigens from the resident microbiota that signal to epithelial cells through pattern recognition receptors (PRRs) on the cell surface (216, 217). Due to the continuous presence of bacterial products at the oral and intestinal interface, epithelial cells at these locations are required to respond in a subdued manner or the host will suffer acute

inflammation. Toll-like receptors (TLRs) constitute the primary bacterial surveillance tool epithelial cells employ in order to respond to bacterial presence and pathogen invasion. Endogenous stimuli is also provided to the epithelium by tumor necrosis factor alpha (TNFα) that is produced by local immune and epithelial cells and is able to signal to epithelial cells through TNF receptor 1 (TNFR1) on the epithelial surface (218). Various cell models have shown that high molecular weight adiponectin (HMWAd) has the capacity to modulate intracellular signaling through the NF-κB pathway (120, 127, 128) which is also utilized by all plasma membrane-bound TLRs (130) as well as TNFR1 (218). However, studies are lacking which investigate the combined effects on epithelial cells of these exogenous and endogenous stimulants in the presence of HMW adiponectin. Here, we test the hypothesis that physiological concentrations HMWAd will dampen the capacity of bacterial products and TNFα to activate the NF-κB signaling cascade in oral and intestinal epithelial cells and subsequently modulate gene expression and cytokine secretion. In addition, we show that AdipoR1 is expressed at higher levels than AdipoR2 at multiple sites along the gastrointestinal epithelium and that expression of AdipoR2, but not AdipoR1, is reduced in inflamed colonic tissue from patients with inflammatory bowel diseases.

RESULTS

Expression of adiponectin receptors along gastrointestinal epithelium. With the discoveries of adiponectin outside of systemic circulation in the oral and intestinal cavities, we investigated whether the adiponectin receptors were expressed on the epithelium of these sites along the gastrointestinal tract. We found substantial ADIPOR1 and 2 expression in oral, ileal, and colonic epithelial biopsies from healthy volunteers (Fig. 3.1). The expression level of both receptors on the intestinal epithelium was relatively equivalent, but ADIPOR1 expression was significantly higher in the oral epithelium.

HMWAd modulates cytokine secretion by epithelial cells. Full-length adiponectin assembles into multiples of three during post-translational processing within the adipocyte (169). Multimers of 12-18 proteins connected have been classified as the high molecular weight form of the protein and are capable of affecting numerous cell types throughout the body. Though AdipoR1 and AdipoR2 are found at very high expression levels on epithelial cells throughout the upper and lower gastrointestinal tract, little work has been done to elucidate what effects HMWAd has on these cells which mediate the barrier between the body proper and the outside environment.

The transformed OKF6 oral epithelial (194) and colonic adenocarcinoma HT-29 (200, 219) cell lines are widely used as *in vitro* models to represent the main cell type of the oral and colonic epithelium, respectively. OKF6 and HT-29 cells were treated with or without HMWAd for 18h and then stimulated with TNFα for 6h (Fig. 3.2). Measurements of cytokine secretion in cell supernatants revealed that oral epithelial cells' IFNγ, IL-12, IL-13, IL-8, TNFα, IL-10, IL-17, and IL-1β production was significantly raised after TNFα stimulation and IL-12, IL-8, IL-10, and IL-17 further increased after combined HMWAd and TNFα presence (Fig.3.2A). Following HMWAd pretreatment, intestinal epithelial cells showed an increase in all cytokines measured, but only further increases of IFNγ over TNFα stimulation alone (Fig. 3.2B).

OKF6 and HT-29 cells were also exposed to bacterial lipopolysaccharide (LPS, ligand for TLR4) or flagellin (ligand for TLR5) after HMWAd pretreatment (Fig.3). In oral cells, significant increases in IFNγ, IL-12, IL-13, and IL-8 secretion was shown only after combined HMWAd pretreatment and flagellin stimulation and not with bacterial flagellin alone. Consistent with previous studies in other cell types (122, 123), HMWAd by itself was able to induce IL-8 expression and secretion in OKF6 cells. Intestinal epithelial cells showed a different pattern of cytokine release with increases in production of all cytokines measured after stimulation with LPS or flagellin alone. However, when cells were pretreated with HMWAd and then stimulated with flagellin, a significantly higher amount of IFNγ was produced while considerably less IL-12 was made. These findings suggest that HMWAd is important in regulating the epithelial cell response to endogenously-produced TNFα as well as perpetually present bacterial LPS and flagellin.

Negative regulators of NF-κB signaling increased in response to HMWAd, TLR, and TNFR ligands. Expression of AdipoR1 and AdipoR2 by various cell types has been shown to be slightly downregulated by cell exposure to TLR ligands (220) or cytokines (132). Consistent with previous findings, we saw a trend of slightly decreased AdipoR1 and AdipoR2 expression when stimulated with TNFα (Fig. 3.4A) or TLR ligands (Fig. 3.5A). However, expression levels of these two receptors were high on both OKF6 and HT-29 cells and were generally unaffected by provided stimuli.

Signaling through all plasma membrane-bound TLRs utilizes the adaptor protein MyD88, which is able to activate gene expression through NF-κB activation and translocation to the nucleus (130). In response to TLR 4 and 5 ligands and HMWAd, we measured changes in expression levels of the p65 (RelA) subunit of the NF-κB heterodimer, p65 reverse shuttling protein PPARγ, and IκBα deubquinating protein A20. The p65 subunit is known to be a biomarker for intestinal homeostasis (221), while PPARγ (59) and A20 (222) work to directly

impede the NF-κB cascade. OKF6 cells showed no significant changes in p65 or PPARγ expression in response to TNFα alone or combined with HMWAd, but gene expression of A20 rose very sharply after a combined exposure to these two stimulants (a 350x-fold increase over unstimulated control) (Fig. 3.4B). Significant increases in p65 and A20 expression were found in HT-29 cells stimulated with TNFα (Fig. 3.4B). When pretreated with HMWAd before TNFα exposure, p65 and PPARγ both showed significant increases in expression over TNFα stimulation alone, while A20 showed an increase, but was below statistically significant levels.

Consistent with TNFα stimulation, oral epithelial cells saw no changes in p65 or PPARγ expression, but immense upregulation of A20 expression in response to HMWAd (10x-fold increase) and even further increases with HMWAd pretreatment and LPS stimulation (Fig. 3.5B). Intestinal epithelial cells increased expression of p65 only after dual stimulation with HMWAd and a TLR ligand, suggesting synergy between the two unique signaling pathways. The expression of A20 was upregulated in response to TLR ligands, but HMWAd had no additional effect on expression levels. With dramatic increases in expression of negative regulators to NF-κB signaling after combined HMWAd pretreatment with or without TNFα or TLR ligand stimulation, HMWAd appears to be involved in the process of dampening the NF-κB signaling cascade.

TNFα-induced NF- κB activity decreased in OKF6 cells after following HMWAd treatment. Our gene expression data from both oral and intestinal epithelial cell types showed a possible role of HMWAd in the control of the NF-κB signaling cascade. To test this possibility, we transfected OKF6 and HT-29 cells with an NF-κB reporter plasmid which expresses firefly luciferase when NF-κB-mediated transcription is initiated in the cell. We then stimulated the epithelial cells in the similar fashion as previous experiments with an 18h HMWAd pretreatment and 6h stimulus with a TLR ligand or TNFα. OKF6 cells did not alter NF-κB signaling when pretreated with HMWAd alone or stimulated with TLR ligands (Fig. 3.6A). However, the oral cells did show a significant increase in NF-κB signaling when stimulated with TNFα, but this increase was brought down to basal levels when the cells were pretreated with HMWAd. No change in NF-κB activity was seen when OKF6 cells were stimulated with LPS alone, but activity was significantly decreased below basal levels when cells were pretreated with HMWAd before LPS stimulation. As shown in Fig 3.5A, combination of HMWAd and LPS was also responsible for significantly higher A20 expression when compared to HMWAd pretreatment alone. This data is consistent with Figures 2 through 5 showing OKF6 cells' hyporesponsiveness to TLR ligands alone and the profound ability of TNFα to stimulate oral cells. HT-29 cells also showed a lack of increased NF-κB signaling in response to the TLR ligands LPS and flagellin,

but significantly higher signaling in response to TNFα. Pretreatment with HMWAd did not affect the HT-29 cells' ability to induce NF-κB mediated transcription for all stimuli provided.

Translocation of p65 subunit of NF-κB to the cell's nucleus is necessary to activate transcription through the canonical NF-κB signaling pathway (223). To visualize this, we stimulated OKF6 and HT-29 cells for 2h with TNFα following pretreatment with or without HMWAd and then stained cells for intracellular localization of p65 (Fig. 3.6B). In OKF6 cells, p65 was found almost exclusively in the nucleus after TNFα stimulation. Pretreatment with HMWAd before TNFα stimulation showed the intracellular distribution of p65 to be much more similar to cells not treated with TNFα, indicating one possible mechanism employed by HMWAd to subdue NF-κB-mediated signaling. HT-29 cells did not show a visible difference in p65 translocation to the nucleus after TNFα stimulation. OKF6 and HT-29 cells were also stimulated in a similar fashion for 1 and 4h with TNFα and showed no visible differences in p65 localization with respect to HMWAd pretreatment (Fig. 3.7). Both cell types were also stimulated with LPS and flagellin in the same manner at various time points, but no differences were visible in the intracellular localization of p65 (data not shown).

AdipoR1 and AdipoR2 expression in noninflamed and inflamed human gingival and colonic biopsies. Inflammatory bowel diseases (IBDs) are characterized by uncontrolled pathological inflammation along the intestinal tract and the main subdivisions classified as Crohn's Disease (CD) and Ulcerative Colitis (UC) (171). Skip lesions of inflammation along the ileum and colon are characteristic of CD whereas UC generally presents as superficial ulceration along the distal colon. Gene expression levels of AdipoR1 and AdipoR2 have been shown to be decreased in inflamed gingival tissue (131) and lower receptor expression is found to be associated with colorectal cancers (135) and worse prognosis with gastric cancer (133). We investigated whether inflamed colonic tissue in CD and UC patients showed a difference in AdipoR1 and 2 expression when compared to non-inflamed colonic tissue within the same patient. Paired biopsies were taken from patients with active flares that had been previously diagnosed with CD or UC: one from a region of inflammation in the colon and one from an area without inflammation. Measurement of AdipoR1 and 2 gene expression from the paired biopsies showed no significant differences in AdipoR1 expression but a clear decrease in AdipoR2 expression in inflamed biopsies for 82% (27 of 33) of paired biopsies (Fig. 3.8). Interestingly, AdipoR2 has been identified as the receptor for the HMW multimer of the full-length adiponectin protein (214). These data point to the possibility of loss of HMWAd recognition in the colon as a possible contributing factor in the exacerbation of inflammation in IBDs.

SUMMARY

The results of this present study demonstrate the high-molecular weight form of the full-length adiponectin multimer (HMWAd) acting through the NF-κB pathway in an "appropriate inflammatory" manner with both pro- and anti-inflammatory effects on oral and intestinal epithelial cells. Through our observations, HMWAd itself had little impact on epithelial cell stimulation, but when combined with the bacterial products LPS and flagellin or the cytokine TNFα, epithelial cells showed increased secretion of pro-inflammatory cytokines as well as increased expression of negative regulators of the NF-κB signaling cascade.

TNFα is continuously produced in small amounts along the epithelium by local immune cells and epithelial cells in response to microbial and environmental cues. Consistent with previous reports, our oral and intestinal epithelial cell lines were very sensitive to stimulation with TNFα (200, 224), showing increased secretion of all cytokines measured (Fig. 3.2). When cells were first pretreated with HMWAd, further increased production of multiple cytokines such as IL-12, IL-8, IL-10, and IL-17 in OKF6 cells and IFNγ in HT-29 cells shows the potential capacity of HMWAd to facilitate a pro-inflammatory response. However, pretreatment with HMWAd and subsequent TNFα stimulation also induced expression of multiple negative regulators of NF-κB signaling, namely, A20 in OKF6 cells and both PPARγ and A20 in HT-29 cells (Fig. 3.4B). Following HMWAd pretreatment, NF-κB-induced transcriptional activity and p65 nuclear translocation were both reduced in OKF6 cells when compared to TNFα stimulation alone, showing how the HMW multimer can also act to dampen the NF-κB cascade and prevent an excessive inflammatory response (Fig. 3.6). These findings add another piece to the body of evidence that NF-κB signaling in the epithelium is necessary for both the promotion and control of the local inflammatory response (225, 226).

We found that OKF6 cells were generally unresponsive to LPS with only increased secretion of IL-8 at the experimental concentration used (Fig. 3.3). However, we did notice an interesting finding in that combined LPS stimulation and HMWAd pretreatment was able to significantly increase A20 expression over HMWAd pretreatment alone (Fig. 3.5B). This revealed further consequences as shown by decreased NF-κB activity below basal levels when OKF6 cells were exposed to both HMWAd and LPS (Fig. 3.6A). In contrast with oral cells, HT-29 cells responded robustly to LPS and flagellin with increased secretion of IFNγ, IL-12, IL-13 IL-8, and TNFα (Fig. 3.3). In a similar fashion to TNFα stimulation, HT-29 cells also increased PPARγ expression with HMWAd pretreatment and LPS stimulation (Fig. 3.5). In addition, adiponectin has been shown to bind the LPS of *γ-Proteobacteria* (227), suggesting another novel mechanism in which adiponectin inhibits PRR signaling. Multiple flagellated bacteria found on

the oral and intestinal epithelium continuously shed the flagellin subunit which is recognized by another PRR, TLR5. Much like LPS, OKF6 cells were generally unresponsive to flagellin alone. However, unlike LPS, when HMWAd pretreatment was combined with flagellin stimulation, IFNγ, IL-12, and IL-13 were all increased in these cells (Fig. 3.3). Though HT-29 cells were able to increase multiple cytokines with flagellin stimulation alone, a similar phenomenon was found with IFNγ production significantly higher and IL-12 production lower when combining HMWAd pretreatment and flagellin stimulation. For the HT-29 cells, this further extended into gene expression of NF-κB regulators, with only HMWAd and flagellin able to increase p65 and PPARγ expression (Fig. 3.5). The modulation of cytokine secretion and gene expression patterns not by HMWAd alone or bacterial product alone but only in unison leads to the notion of intersecting intracellular signaling pathways between AdipoR2 and the TLRs.

We found that epithelial cells in the human mouth, ileum, and colon express both of the putative adiponectin receptors, AdipoR1 and AdipoR2 (Fig. 3.1). Constant exogenous insults from the resident microbiota require these cells to act in an appropriate manner, or potentially reduce the fitness of the host through oral or intestinal inflammation. With the discovery of adiponectin in the intestinal lumen (139, 140) and high expression of AdipoR1 and 2 on IECs (134-136), this conjuncture hints to adiponectin as providing stimuli to IECs to possibly help deal with the extreme microbial burden. We found that in IBD patients, expression of AdipoR1 in the colon was not different between non-inflamed and inflamed areas. However, the expression of AdipoR2, which is the high-affinity receptor for HMWAd, was consistently reduced in the inflamed areas of the colon for almost all IBD patients (27/33, 82%) (Fig. 3.8). Data from other groups has shown mesenteric fat as actually producing more adiponectin in Crohn's disease (228) and during experimental colitis in rats (229). However, without recognition by the epithelium through AdipoR2, no modification of inflammatory signaling pathways within IECs would take place. This data provides rational to further study HMWAd modulation of inflammatory signaling pathways within the gastrointestinal epithelium, and points to the possibility of loss of "appropriate inflammatory" HMWAd signaling in the pathogenesis of IBDs.

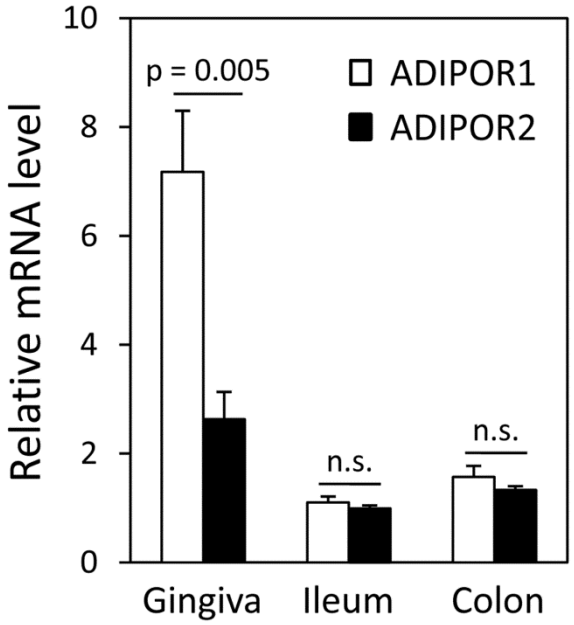


Figure 3.1. Expression of mRNA for adiponectin receptors in different regions of the gastrointestinal tract. Biopsies of oral gingiva (n = 12), ileal mucosa (n = 15), and colonic mucosa (n = 15) biopsies were collected from healthy volunteers. Levels of ADIPOR1 and ADIPOR2 mRNA were analyzed by qRT-PCR and normalized to β2-microglobulin mRNA. Data are expressed as mean ± SEM, and significance of differences between ADIPOR1 and ADIPOR2 expression are noted for each tissue; n.s. = not significant.

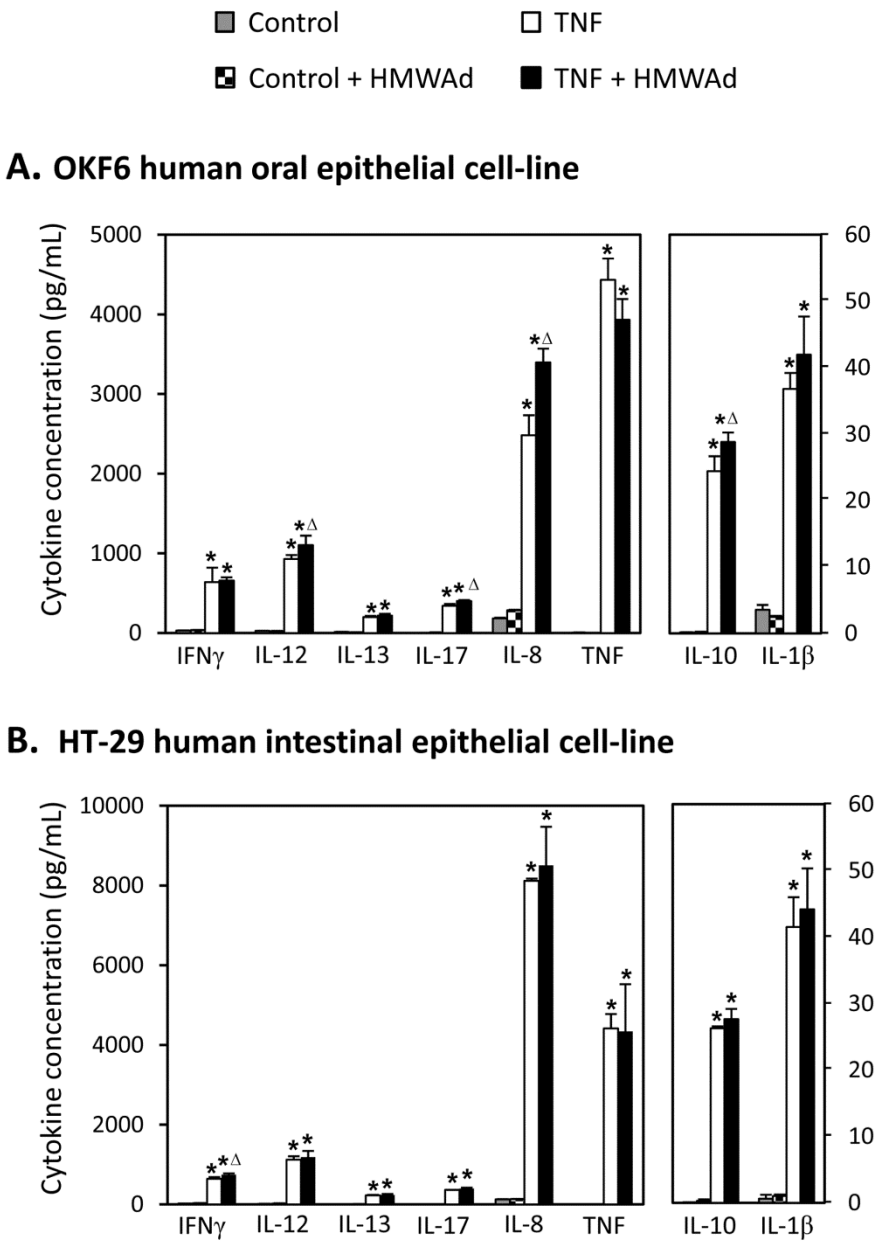


Figure 3.2. Effect of high molecular weight adiponectin (HMWAd) pretreatment on TNF-stimulated cytokine secretion by cultured human oral and intestinal epithelial cells. A, OKF6 oral epithelial and B, HT-29 intestinal epithelial cell-lines were cultured for 18 h in the absence or presence of HMWAd (1 μg/ml), then cultured for an additional 6 h without further treatment (control) or with the addition of TNF (10 ng/ml). Cell supernatants were collected at the end of the 24 h culture period, and cytokine levels were analyzed by ELISA. Data are expressed as mean ± SEM (n = 3). Asterisks denote a significant effect of TNF treatment, and triangles denote a significant effect of HMWAd pretreatment ($p < 0.05$).

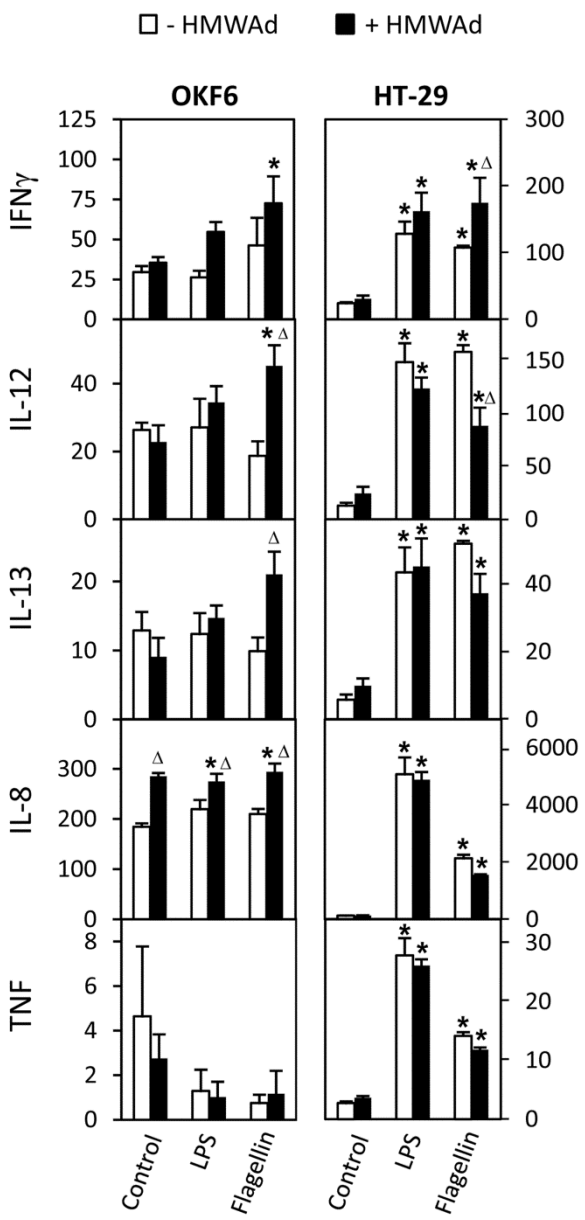


Figure 3.3. Effect of high molecular weight adiponectin (HMWAd) pretreatment on Toll-like receptor (TLR) ligand-stimulated cytokine secretion by cultured human oral and intestinal epithelial cells. A, OKF6 and B, HT-29 cells were cultured for 18 h in the absence or presence of HMWAd (1 μg/ml), then cultured for an additional 6 h without further treatment (control) or with the addition of the TLR4 ligand LPS (100 ng/ml) or the TLR5 ligand flagellin (70 ng/ml). Cell supernatants were collected at the end of the 24 h culture period, and cytokine levels were analyzed by ELISA. Data are expressed as mean ± SEM (n = 3). Asterisks denote a significant effect of LPS or flagellin treatment, and triangles denote a significant effect of HMWAd pretreatment ($p < 0.05$).

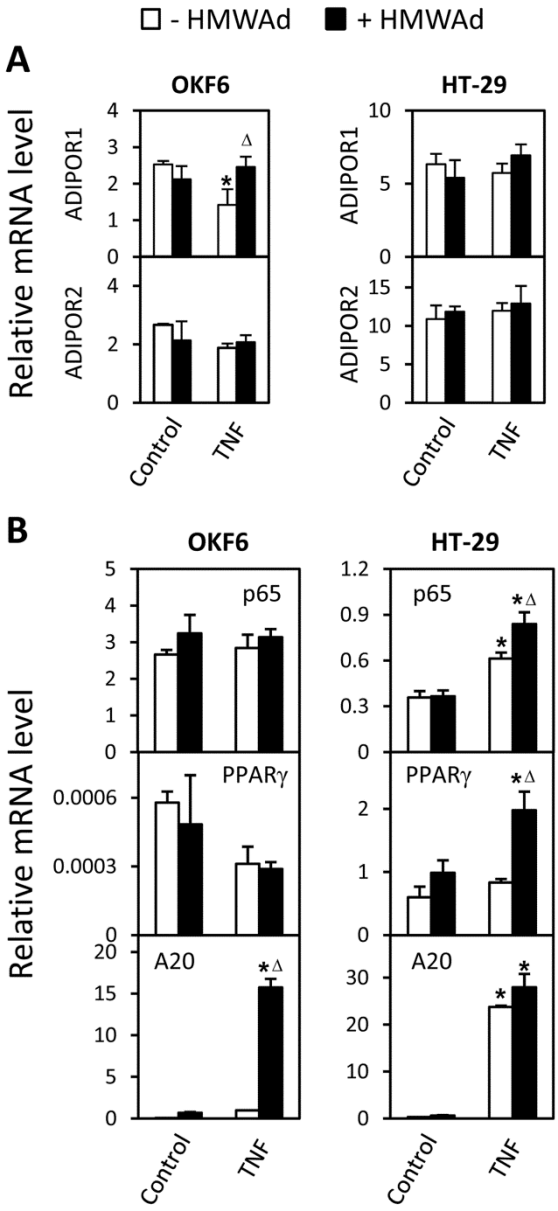


Figure 3.4. Effect of high molecular weight adiponectin (HMWAd) pretreatment and TNF stimulation on expression of A) adiponectin receptors and, B) downstream signaling components. OKF6 and HT-29 cells were cultured for 18 h in the absence or presence of HMWAd (1 μg/ml), then cultured for an additional 6 h without further treatment (control) or with the addition of TNF (10 ng/ml). Levels of mRNA encoding ADIPOR1, ADIPOR2, the p65 subunit of nuclear factor (NF)-κB, peroxisome proliferator-activated receptor (PPAR)γ, and the ubiquitin modifying enzyme A20 were analyzed by qRT-PCR and normalized to β_2-microglobulin. Data are expressed as mean ± SEM (n = 3). Asterisks denote a significant effect of TNF treatment, and triangles denote a significant effect of HMWAd pretreatment ($p < 0.05$).

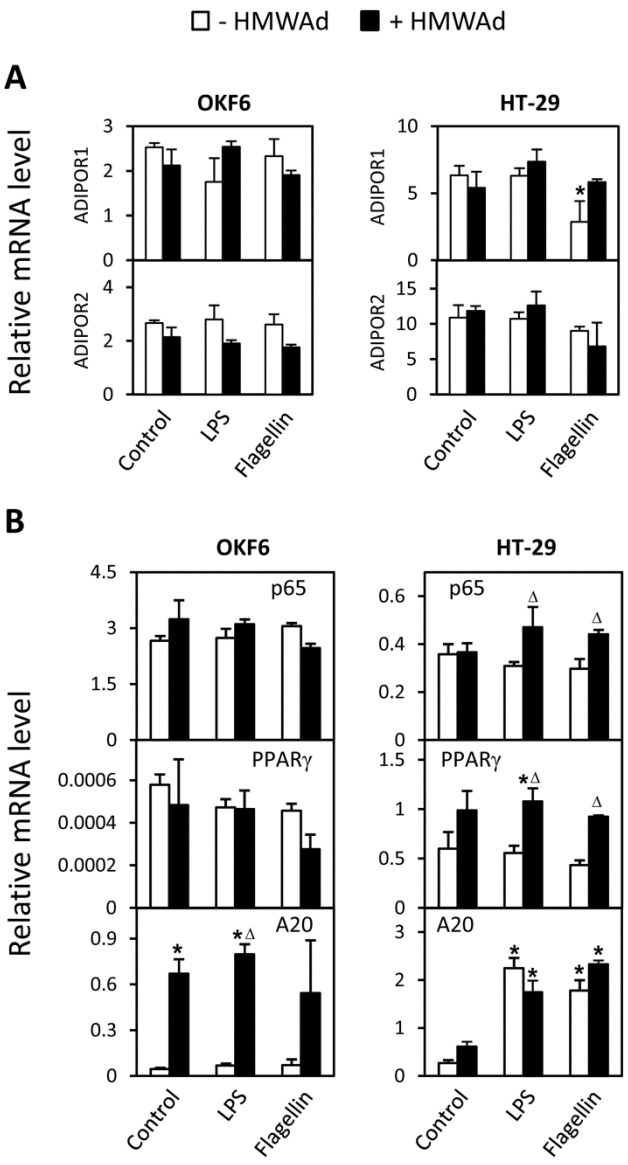


Figure 3.5. Effect of high molecular weight adiponectin (HMWAd) pretreatment and TLR ligand stimulation on expression of A) adiponectin receptors and, B) downstream signaling components. OKF6 oral epithelial and HT-29 intestinal epithelial cell-lines were cultured for 18 h in the absence or presence of HMWAd (1 μg/ml), then cultured for an additional 6 h without further treatment (control) or with the addition of the LPS (100 ng/ml) or flagellin (70 ng/ml). Levels of mRNA encoding ADIPOR1, ADIPOR2, p65, PPARγ and A20 were analyzed by qRT-PCR and normalized to β_2-microglobulin. Data are expressed as mean ± SEM (n = 3). Asterisks denote a significant effect of TLR ligand treatment, and triangles denote a significant effect of HMWAd pretreatment ($p < 0.05$).

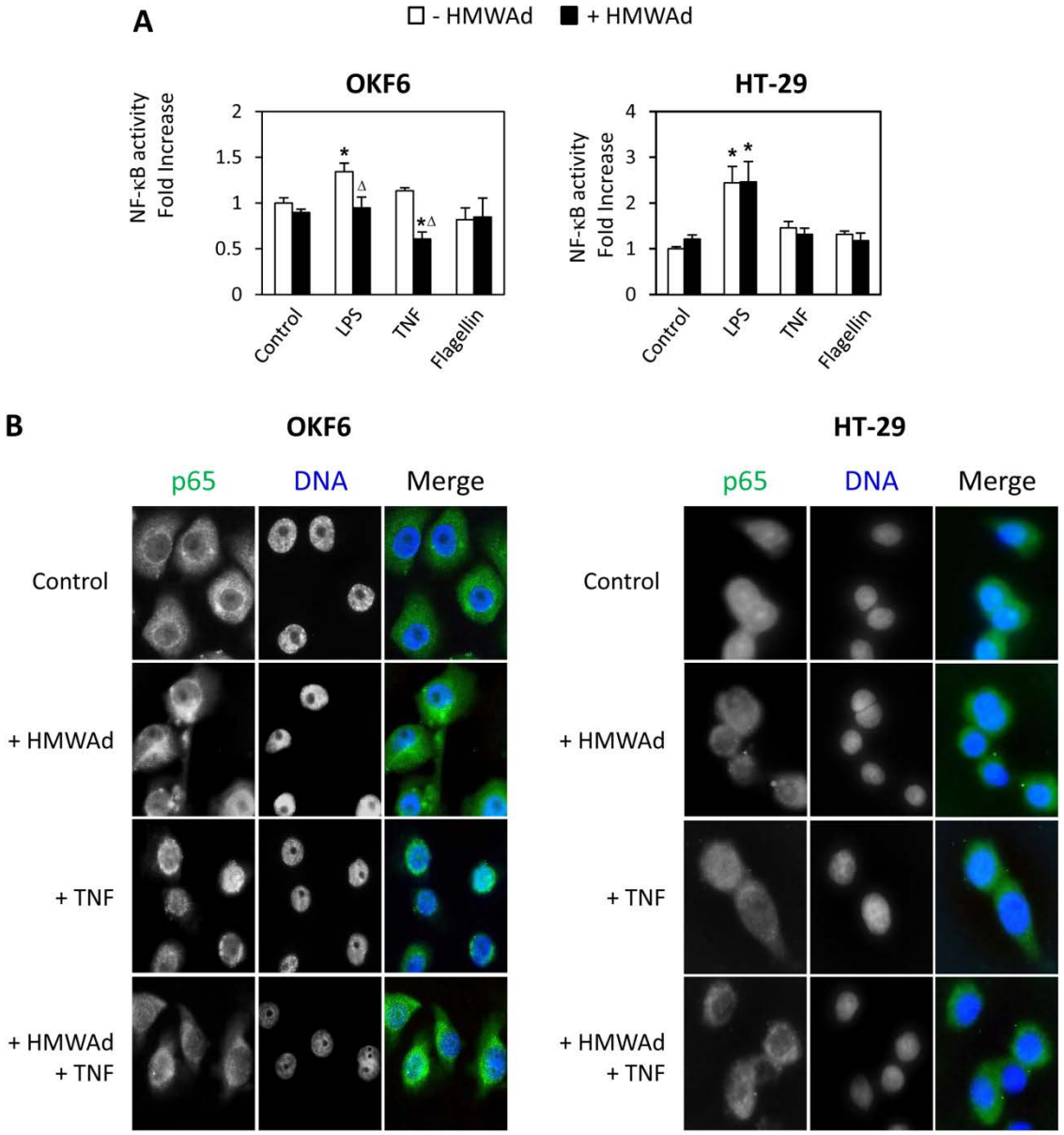


Figure 3.6. Modulation of NF-κB activation by HMWAd. A, NF-κB reporter assay. OKF6 and HT-29 cells were transiently transfected with a reporter plasmid, in which expression of firefly luciferase is regulated by NF-κB-binding elements, and a control *Renilla* luciferase plasmid. Transfected cells were cultured for 18 h in the absence or presence of HMWAd (1 μg/ml), then cultured for an additional 6 h without further treatment (control) or with the addition of LPS (100 ng/ml), TNF (10 ng/ml) or flagellin (70 ng/ml). NF-κB activity was calculated as the ratio of firefly to *Renilla* luciferase, and expressed as fold increase compared to the mean of untreated cells (mean ± SEM, n = 4 for OKF6 cells and n = 7 for HT-29 cells. Asterisks denote a significant

effect of TLR ligand treatment, and triangles denote a significant effect of HMWAd pretreatment ($p < 0.05$). B, Intracellular localization of the p65 subunit of NF-κB. OKF6 HT-29 were cultured for 18 h in the absence or presence of HMWAd (1 μg/ml), then cultured for an additional 2 h without further treatment (control) or with the addition TNF (10 ng/ml). Cytoplasmic and nuclear p65 were detected by immunofluorescence (p65 = green, DAPI-stained nuclei = blue).

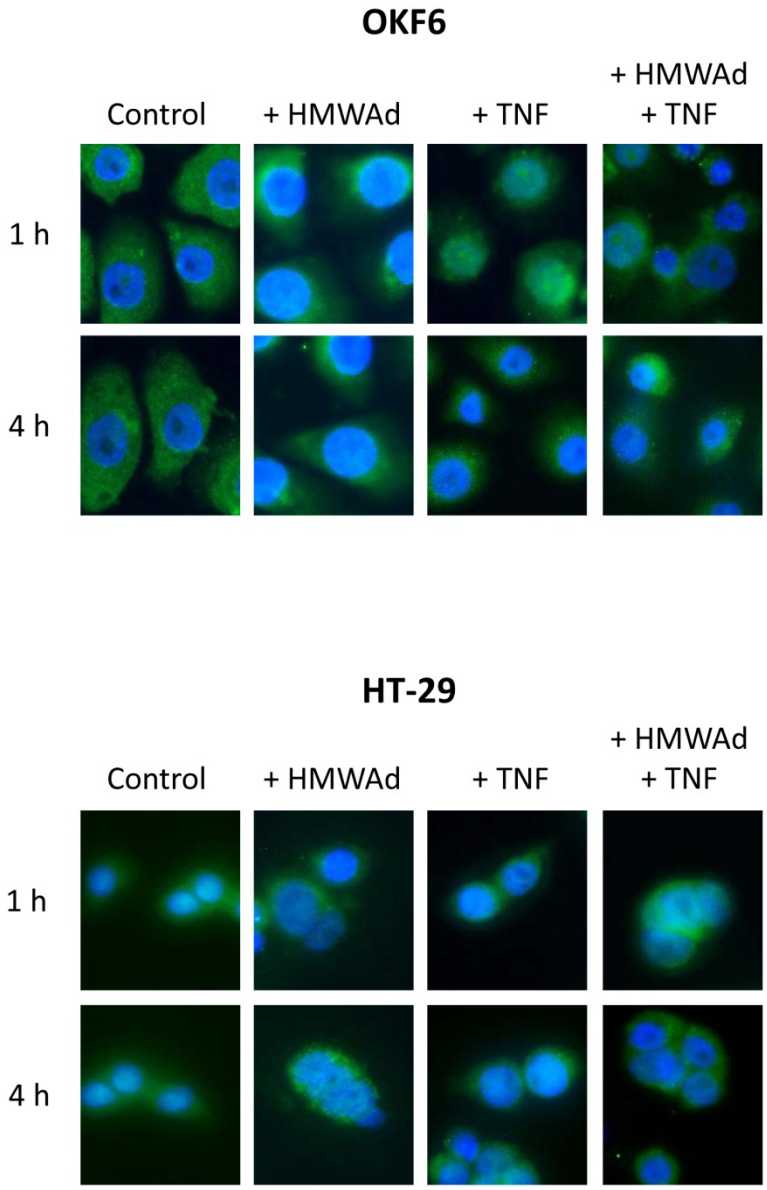


Figure 3.7. Intracellular localization of the p65 subunit of NF-κB. OKF6 and HT-29 cells were cultured for 18 h in the absence or presence of HMWAd (1 μg/ml), then cultured for an additional 1 or 4h without further treatment (control) or with the addition TNF (10 ng/ml). Cytoplasmic and nuclear p65 were detected by immunofluorescence (p65 = green, DAPI-stained nuclei = blue).

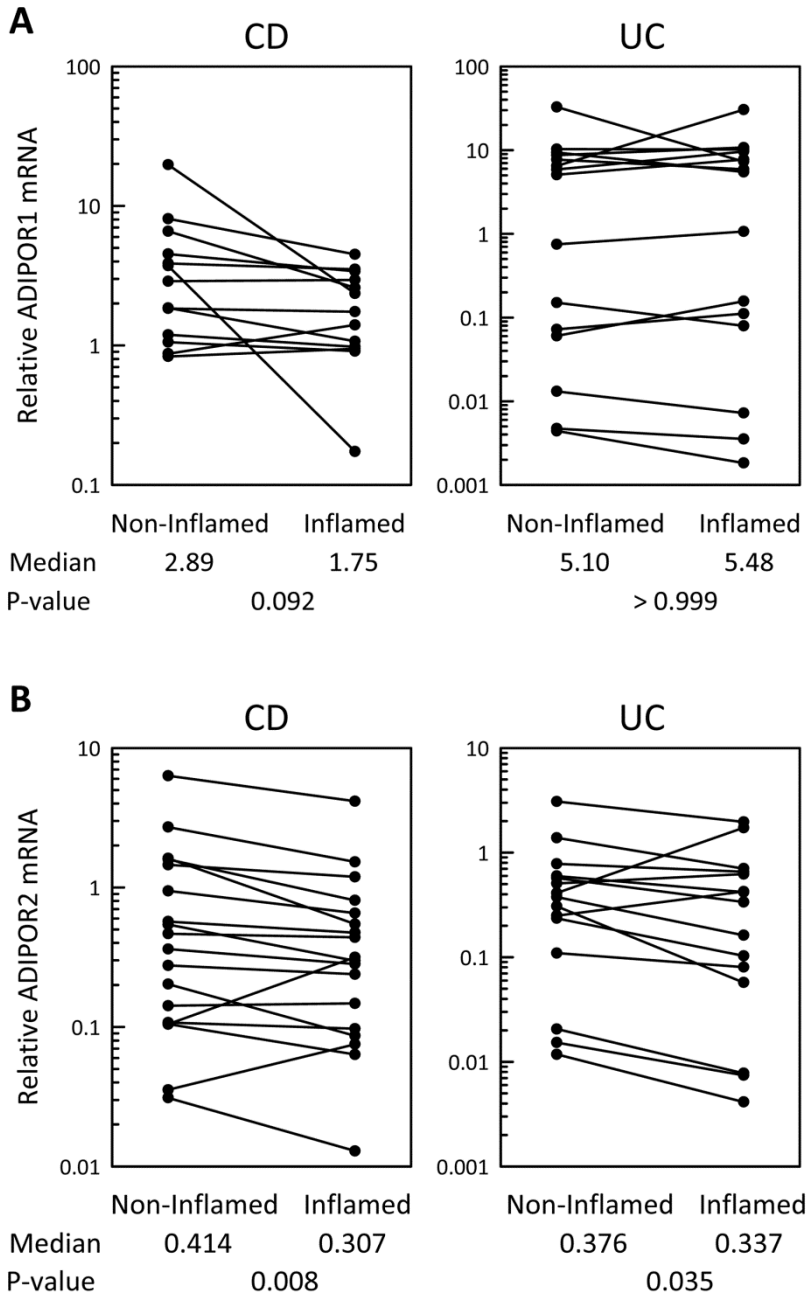


Figure 3.8. Expression of adiponectin receptors in paired biopsies of non-inflamed and inflamed colonic mucosa of patients with Crohn's disease (CD) or ulcerative colitis (UC). Levels of mRNA for A, ADIPOR1 and B, ADIPOR2 were analyzed by qRT-PCR and normalized to β2-microglobulin mRNA. Symbols represent individual patients, with lines connecting the values for non-inflamed and inflamed colonic mucosa for each patient. Statistical significance of differences in mRNA levels between paired biopsies was determined by non-parametric paired sign test.

Chapter 4

STIMULATION OF COLONIC EPITHELIAL CELL GENE EXPRESSION BY SPECIFIC COMMENSAL BACTERIA AND LOCALIZATION OF SECRETORY IgA AND SECRETORY COMPONENT IN THE COLONIC MUCUS LAYER

INTRODUCTION

The intestine must constantly contend with a huge microbial burden which increases from around ten million in the ileum to over a trillion in the large intestine (9). Secretory IgA (SIgA) antibodies play a critical role in shaping the composition of this gut microbiota and in maintenance of intestinal homeostasis (230-232). The polymeric immunoglobulin receptor (pIgR) transports polymeric IgA antibodies from the basolateral to the apical surface of intestinal epithelial cells (also, IECs)(233), where proteolytic cleavage of the extracellular domain of pIgR (also known as secretory component, SC) releases SIgA into the intestinal mucus layer (99, 233). SC, either free or complexed to SIgA, participates in a variety of innate defense mechanisms, including prevention of bacterial adherence to the intestinal mucous layer and neutralization of potential pro-inflammatory factors (29, 112). The significance of pIgR in intestinal homeostasis is highlighted by observations that *Pigr*$^{-/-}$ mice are more susceptible than are wild-type mice to infection with *Salmonella typhimurium* (234) and to development of chemically-induced colitis (235).

Colonic bacteria promote intestinal homeostasis by enhancing the physical integrity of the epithelium and preventing bacteria from translocating into the lamina propria and draining lymphoid tissues (9, 236). A recent analysis of combined data from 3 independent metagenomic studies indicated that the majority of colonic bacteria in healthy individuals belonged to one of 4 phyla: *Firmicutes* (about 65% of total species); *Bacteroidetes* (about 23%); *Proteobacteria* (about 8%); and *Actinobacteria* (about 3%) (237). Alterations in the composition of the colonic microbiota, characterized by relative increases in *Proteobacteria* and *Actinobacteria* and relative decreases in *Firmicutes* and *Bacteroidetes*, have been associated with increased risk for inflammatory bowel disease (IBD) (41). Probiotic strains of colonic bacteria have shown promise in the prevention and treatment of IBD, but further clinical and fundamental studies are needed to delineate the mechanisms by which different probiotics exert their beneficial effects (238-240).

Cross-talk between commensal bacteria and IEC is mediated by several families of pattern recognition receptors (PRRs), including the Toll-like (TLR) and NOD-like receptor families (51, 241, 242). In previous studies, we demonstrated that signaling through TLR3 and

TLR4 up-regulates pIgR expression in the human IEC line HT-29 (243). It is not known whether changes in the composition of the colonic microbiota affect pIgR expression in IEC, since the effect of different species of colonic bacteria on pIgR regulation has not been compared systematically. This present work assesses whether bacterial species representing the four major phyla of the colonic microbiota differentially regulate the expression of pIgR and other epithelial genes involved in intestinal immunity. We found that pIgR expression in IECs along the colonic crypt was more towards the apical side of the crypt as the bacterial-dense distal colon is reached. *In vitro* studies showed that pIgR expression was selectively up-regulated in HT-29 cells in response to bacteria of the family *Enterobacteriaceae* of the phylum *Proteobacteria*, including the probiotic *E. coli* strain Nissle 1917 (*Ec*N). In a similar fashion, another intestinal epithelial cells line (CaCo-2 cells) also showed selective increases in both pIgR and Il-8 gene expression with only *Ec*N able to upregulate both of these genes. Both live and dead and *Ec*N and *Salmonella typhimurium* bacteria were able to increase gene expression of pIgR and the pro-inflammatory chemokine IL-8, indicating bacterial products are the driving force behind modulation of IEC gene expression. Since epithelial cells arrange in a polarized fashion *in vivo*, we also investigated the stimulatory capacity of *Ec*N to signal by both apical and basolateral membranes in HT-29 cells. Our findings suggest that bacteria of the family *Enterobacteriaceae* regulate transport of SIgA antibodies into intestinal secretions by inducing pIgR expression in IECs by equal stimulation from both surfaces of the cell. Furthermore, other bacterial species may play a role in the maintenance of barrier integrity in the colon.

Once SIgA is cleaved from the epithelial surface, its release into the colonic mucus layer allows direct binding of the complex to bacteria residing in the host intestinal lumen. Multiple *in vitro* studies have shown SIgA in the promotion of biofilm formation of intestinal mutualists (77, 78, 110), but this concept does not have much *in vivo* backing. The colonic lumen is separated from the epithelium by the colonic mucus layer, which is organized into two distinct layers, a dense inner layer directly above the epithelium and a loose outer layer overlaying the inner (35). The mucin protein, muc2, is the gel-forming mucin secreted from host goblet cells that allows formation of the intestinal mucus layers. Proteomic analysis of these two layers have shown high levels of the IgG-binding Fc gamma binding protein (Fcγbp), but no putative host-derived proteins with IgA-binding capacity (36, 244). The current assumption is that IgA binds glycan residues on the muc2 protein and primarily resides in the inner mucus layer (110, 149, 245) and aids the host by immune exclusion of bacterial antigens from the epithelial surface.

Absence of the intestinal mucus layer is very detrimental to the host, and is a hallmark of multiple enteric diseases, including: bacterial infection (91, 246), parasitic infection (247), and

IBDs (248). Usually found only in the outer mucus layer, bacteria and fecal material are in direct contact with IECs without a mucus barrier, and mice lacking the muc2 protein develop spontaneous colitis (88, 89). Imaging of the mucus layer has historically been troublesome due to the high amount of water that is lost by tissue fixation with the conventional aldehyde- or hydrocarbon-based fixatives. With the discovery of intestinal mucus preservation by the use of Carnoy's fixative (35), a wealth of information regarding *in situ* mucus contents and bacterial interactions has been unearthed. This study utilizes mucus-fixing techniques to show intermucal localization of the SIgA and SC proteins. We found that, in mice, both of these proteins are concentrated in the outer mucus layer in the direct vicinity as the intestinal bacteria. Outer mucus layer-localization of SIgA was also confirmed in the human colon. These findings suggest one of the most important roles of SIgA and SC is immune exclusion in the outer, not inner, mucus layer and that these proteins and concentrate in areas of highest microbial product density.

RESULTS

Apical localization of pIgR-producing IECs along length of colon is directly correlated with concentration of bacteria. The colon is made of repeating subunits of the crypts of Lieberkühn (or, crypts) (Fig. 4.1A). Directly beneath and between the crypts lie the lamina propria containing immune cells. The innervated and vascularized muscularis mucosae is directly beneath the laminia propria, and the foundational submucosa provides structural support to the colon. In the colon, SIgA directly works to bind bacteria in the lumen and prevent migration into the colonic epithelium. Efflux of IgA across colonic epithelial cells requires the pIgR protein, so we investigated the crypt-specific localization of pIgR at different sites along the colon. To our surprise, seemingly specific sets of colonic epithelial cells produced pIgR depending upon which location of the colon was surveyed (Fig. 4.1B). Apical crypt localization became more prominent as the distal colon was approached with most of the pIgR-positive cells in the rectum being on the apical tip of colonic crypts. The pIgR produced in the lower portion of the crypt is likely cleaved with IgA and carried to the intestinal lumen by the mucal current, but pIgR produced at the apical tips of the crypts is probably cleaved and directly released with IgA into the inner mucus layer. This differential expression pattern could have an effect on the way SC and SIgA interacts with the microbiota in the intestinal lumen. Interestingly, pIgR localization became more apical in parallel with increasing bacterial concentration in the colon, possibly as an effect of higher MAMP-induced expression of pIgR in the distal colon.

Regulation of gene expression in human intestinal epithelial cells by commensal bacteria representing the four major phyla of the colonic microbiota. We and others have

demonstrated that pIgR expression is regulated in HT-29 cells by cytokines known to modulate intestinal immunity in humans, including IFN-γ, TNF, IL-1 and IL-4 (192, 200, 249-262). The HT-29 cell-line has subsequently been used as a model system to identify basal and cytokine-inducible regulatory elements in the human *PIGR* gene (262-270). A potential role for commensal bacteria in pIgR regulation was first suggested by the finding that butyrate, a by-product of bacterial fermentation, up-regulated pIgR expression in HT-29 cells (271). We subsequently demonstrated that bacterial LPS up-regulated pIgR expression in HT-29 cells, suggesting that bacterial-epithelial cross-talk via TLR signaling may regulate transport of secretory immunoglobulins (243). To compare the ability of different species of colonic bacteria to regulate pIgR expression, we analyzed pIgR mRNA levels in HT-29 cells stimulated with heat-killed bacteria of representative species from the four major bacterial phyla of the human colonic microbiota (Table 4.1)(Fig. 4.2A). Preliminary studies with selected species demonstrated that induction of pIgR expression in response to heat-killed or live bacteria were similar (data not shown). Table 4.1 describes the phylogeny of the bacteria used in this study, all of which were environmental strains originally isolated from human fecal material. Individual species were chosen based on their relevance to the pathogenesis or prevention of IBD in humans. A recent study reported that relative numbers of *Clostridium nexile* (also, *Cn*), *Bacteroides thetaiotaomicron* (also, *Bt*) and *Alistipes onderdonkii* (*Ao*) were reduced in the colonic microbiota of patients with IBD, whereas relative numbers of *Pseudomonas straminea* (*Pstr*) , *Escherichia coli* (*Ec*) and *Pimelobacter simplex* (*Psim*) were increased (41). The probiotic species *Lactobacillus acidophilus* (*La*) and *Bifidobacterium longum* (*Bl*) were included in this experiment because of their widespread use in fermented milk products and other probiotic formulations (239, 272). Interestingly, *Ec*N was unique among these 8 commensal species in its ability to induce expression of pIgR. Levels of pIgR mRNA increased slowly in response to *Ec*N stimulation, with a small but significant increase at 3h and a much larger increase at 24h. The delayed increase in pIgR expression in response to *Ec*N is consistent with our previous observations with LPS and other TLR ligands (243). We and others have observed delayed responses of pIgR to cytokine stimulation, due to a requirement for *de novo* synthesis of one or more transcription factors (243, 254, 255, 263, 265-267, 273). The delayed response of pIgR to bacterial stimulation suggests that there may be a similar requirement for a newly synthesized transcription factor. In our previous studies involving stimulation of HT-29 cells with cytokines and TLR ligands, we found that levels of membrane and secreted pIgR protein were directly correlated with levels of pIgR mRNA (243, 255). We found that *Ec*N caused a rapid induction in mRNA for the pro-inflammatory factors IL-8 and TNF, which declined significantly by 24 h (Fig.

4.2B). The observed down-regulation of IL-8 and TNF could be attributed in part to the induction of A20, a ubiquitin-editing enzyme that down-regulates NF-κB signaling (55), and mitogen-activated protein kinase phosphatase (MKP)-1, a negative regulator of MAP kinase signaling (274)(Fig. 4.2B). We also tested an additional colonic epithelial cell line (CaCo-2 cells) to see if the stimulatory specificity of *Ec*N was applicable to other CEC models. Using a single representative from the four prominent intestinal phyla, we found only *Ec*N was able to induce gene expression of Il-8 after 3h stimulation whereas both *Ec*N and *La* significantly increased pIgR mRNA after 24h with higher levels seen after *Ec*N treatment (Fig.4.3). Though slightly different from HT-29 cells, the results of CaCo-2 stimulation showed the same trend of the *Proteobacteria* species *Ec*N as having the most profound effect on CECs.

As a control to assess the potential activity of bacteria that did not induce gene expression in HT-29 cells, the THP-1 human monocyte cell-line was stimulated with heat-killed bacteria of the same species (Fig. 4.2C). Levels of pIgR mRNA were negligible in THP-1 cells even after bacterial stimulation (data not shown), consistent with the epithelial-specific expression of pIgR (99). Every commensal species induced expression of IL-8 and TNF in THP-1 cells to levels 100-fold higher than those seen in HT-29 cells. Only selected species, including *Ec*N, induced expression of the negative regulators A20 and MKP-1 in THP-1 cells. These results suggest that the potential to induce a pro-inflammatory response in innate immune cells is widespread among commensal bacteria, but that IEC may respond only to a subset of bacteria.

The epithelium is a polarized layer of epithelial cells with an apical side that faces the intestinal lumen and a basolateral side that faces the lamina propria. Tight junctions between epithelial cells ensure minimal diffusion of luminal contents across the epithelial barrier, but bacterial products and other antigens do occasionally traverse this barrier, especially under periods of inflammation (185). To investigate the consequences of *Ec*N stimulation from both of these surfaces, we grew HT-29 cells to confluent single-cell monolayers on Transwell inserts, which contain a porous membrane to allow diffusion of liquid to the basolateral surface of the cell monolayer. Induction of pIgR expression was equally attainable by both apical and basolateral stimulation with heat-killed *Ec*N after 3,6,12, and 24h (Fig. 4.4A). Upregulation of Il-8 was significantly higher at 6h when HT-29 cells were stimulated apically, but not different at 3,12, and 24h timepoints (Fig. 4.4A). Directional secretion of the Il-8 protein was also assayed in apical and basal supernatants (Fig. 4.4B). With the exception of the 24h timepoint, HT-29 cells consistently secreted higher amounts of Il-8 from the apical side of the cells. This chemotactic protein is well known for its ability to recruit neutrophils to the site of infection, so it is interesting that more Il-8 would be excreted towards the direction of the intestinal lumen as

opposed to the lamina propria. However, since pathogenic bacteria may reside at the epithelial surface or in colonic crypts (275), perhaps the best place for the phagocytic/reactive oxygen-producing immune cells is in the midst of the harmful microbes.

Regulation of pIgR expression in HT-29 cells by commensal and pathogenic bacteria of the family Enterobacteriaceae. We found that *Ec*N, a commensal bacterium of the family *Enterobacteriaceae* (phylum *Proteobacteria*) was unique in its ability to induce gene expression in HT-29 cells (Fig. 4.2). The failure of other commensal species to induce gene expression could be due to a lack of recognition of those species by cellular PRRs or active inhibition of PRR signaling. If the latter mechanism was operative, the presence of "inhibitory" bacteria might down-regulate the response to *Ec*N. To test this hypothesis, HT-29 cells were exposed to heat-killed *Ec*N in the presence or absence of equivalent doses of *Lactobacillus acidophilus* (*La,* phylum *Firmicutes), Bacteroides thetaiotaomicron* (*Bt*, phylum *Bacteroidetes*) or *Bifidobacterium longum* (*Bl,* phylum *Actinobacteria*) (Fig. 4.5). As expected, *Ec*N stimulation resulted in early induction of IL-8 mRNA and delayed induction of pIgR mRNA. None of the bacteria from other phyla, alone or in combination with *Ec*N, significantly affected expression of pIgR or IL-8. These findings suggest that concurrent exposure to other types of commensal bacteria does not significantly dampen the response of intestinal epithelial cells to *Ec*N. However, the possibility remains that a complex mixture of commensal bacteria could modify the response to *Ec*N or other bacteria of the family *Enterobacteriaceae.*

To examine the ability of IECs to discriminate between closely related commensal and pathogenic species of the family *Enterobacteriaceae*, HT-29 cells were co-cultured with *Ec*N or *Salmonella typhimurium* (*St*) strain SL1344. Following 1h of culture, relative numbers of *Ec*N or *St* associated with the plasma membrane of HT-29 cells were found to be similar, whereas only *St* was capable of cellular invasion (Fig. 4.6A). This result is consistent with reports demonstrating higher invasiveness of *St* in HT-29 cells compared to several nonpathogenic *E. coli* strains, including *Ec*N (276, 277). To compare the responses to living vs. dead bacteria, HT-29 cells were exposed to live or heat-killed *Ec*N or *St* for 3h (Fig. 4.6B). Interestingly, early induction of IL-8 expression was greater in response to live *Ec*N than live *St*, whereas no differences were observed in the responses to heat-killed *Ec*N vs. *St*. Expression of pIgR was not induced following 3h of stimulation with either live or dead bacteria. Comparison of the responses at 24h to *Ec*N and *St* was complicated by the fact that the viability of HT-29 cells declined after exposure to live bacteria for more than 3h (data not shown). To determine whether changes in gene expression persisted after removal of bacterial stimuli, HT-29 cells were treated for 3 h with live or heat-killed *Ec*N or *St,* washed with antibiotic-containing culture medium, and cultured for an

additional 21h (24h harvest). Under these conditions, pIgR mRNA levels were not significantly different from those in untreated HT-29 cells, suggesting that continuous exposure to bacterial components is required to induce pIgR expression (compare Fig. 4.6B to Fig. 4.2A and 4.4A). The lack of significant IL-8 expression at 24 h was likely due to a combination of withdrawal of bacterial stimulation and induction of negative regulatory pathways.

Stimulation of CEC monolayers with pathogenic or commensal bacteria reduces fluid diffusion across monolayer. Signaling of bacterial products through TLRs and other PRRs is known to improve intestinal barrier function *in vivo*, and one of the most striking features of the germ-free mouse is the ability of interstitial fluid to seep into the intestinal lumen (150). To explore the capacity of pathogenic and commensal microbes to reduce CEC barrier leakiness, we grew HT-29 cells on Transwell inserts and apically exposed the cells to live pathogenic (*St*) or commensal (*Ec*N or *Bt*) bacteria for 9h then measured monolayer permeability by FITC-dextran flux across the epithelial cell monolayer (Fig. 4.7). As expected, the presence of the HT-29 monolayer significantly reduced FITC-dextran flux across the Transwell membrane. Unexpectedly, all three live bacteria used were able to further reduce diffusion across the membrane, suggesting a common response by the intestine when pathogenic and commensal bacteria are in close contact with the epithelium.

Absence of colonic mucus changes colonic crypt morphology. Mucin2 (muc2) is the primary secreted gel forming mucin protein in the colon intestine. Constant synthesis, intracellular oligomerization, and secretion of this protein allows the formation of the colonic mucus layer (33). We observed mouse goblet cell extrusion of mucus into the empty crypt space and unidirectional migration to the colonic lumen (Fig. 4.8A). Goblet cells constitute approximately 15% of colonic epithelial cells, and their intracellular mucin granules hold the majority of the goblet cell volume (84). We observed that mouse goblet cells unable to produce the muc2 protein lose a tremendous amount of volume *in vivo* and are indistinguishable from absorptive enterocytes (Fig. 4.8B). The loss of this protein has other effects on the intestinal mucosa, and clear differences are seen with respect to crypt elongation, erosion of the epithelial surface, and detachment of IECs from the apical surface of the crypts.

Intestinal IgA and pIgR secretory component (SC) both concentrated in outer colonic mucus layer. An immense amount of SIgA and SC are secreted by the intestinal epithelium daily (105), and can be found in both mucosal scrapings and stool samples. The mouse deficient for the pIgR protein is unable to actively transport IgA into the intestinal lumen, and is unable to produce SIgA or SC (75), while the muc2-deficient mouse cannot form an intestinal mucus layer (Fig. 4.8B). To investigate the *in situ* localization of SIgA, we stained Carnoy's-fixed colons

from wild-type, *Pigr* $^{-/-}$, *Muc2* $^{-/-}$, and *Pigr* $^{-/-}$*Muc2* $^{-/-}$ mice with antibodies against the muc2 and IgA proteins (Fig. 4.9). In all four genotypes of mice green IgA-positive plasma cells can be seen throughout this intestinal mucosa. Wild-type mice show a clear mucus layer with inner and outer layers, and strong colocalization of muc2 and IgA in the outer mucus layer. While both mucus layers can still be seen in the *Pigr* $^{-/-}$ mice, very little dual staining exists in the outer mucus layer. The *Muc2* $^{-/-}$ mice are still able to transport IgA into the intestinal lumen, but in the absence of a mucus layer, the antibody is simply integrated into the luminal contents and has no structured distribution. The colonic epithelial interface of *Pigr* $^{-/-}$*Muc2* $^{-/-}$ mice lacks both a mucus layer and IgA transport into the lumen. The same phenomenon of IgA localization was seen at the human colonic interface (Fig. 4.13). The human colonic mucosa contains a much higher amount of IgA-secreting plasma cells than the mouse mucosa, likely due to the non-sterile environment human immune systems contend with. As with the mouse colons, we observed goblet cells actively secreting mucus into the colonic lumen, but much higher human colonic IgA concentrations allowed visualization of threads of IgA being pushed from the epithelial surface towards the outer mucus layer where IgA was actively accumulating. These observations in mice and humans points towards a common mechanism of IgA migration to the outer mucus layer in the colon.

In the same manner as IgA, we also noticed SC accumulation in the outer mucus layer (Fig. 4.10). As with IgA, the inner mucus layer provided a clear boundary between the colonic epithelium (dotted white line) and the accumulation of SC in the colonic lumen. Moreover, this boundary is eliminated when mice are unable to produce a mucus layer, and *Muc2* $^{-/-}$ mice show SC in the colonic lumen, but in a disorganized manner.

Bacteria require mucus layers, but not SIgA to remain separated from colonic epithelium. Multiple reports have shown the utility of the intestinal mucus layer in the separation of the microbiota from the epithelial surface by isolation of bacteria into the outer mucus layer (36, 91). To investigate whether IgA was also necessary for bacterial segregation, we used fluorescence *in situ* hybridization (FISH) to visualize bacterial localization in our four genotypes of mice (Fig. 4.11). With both wild-type and *Pigr* $^{-/-}$ mice, we observed a clear separation between the microbiota and the surface of the epithelium. However, when the mucus layer is absent, bacteria are in direct contact with the epithelium, regardless if IgA is in the intestinal lumen or not. These data show that the colonic mucus layer, but not IgA actively partakes in the separation between host and bacteria.

Bacterial migration into the colonic crypts is a hallmark of severe inflammatory disease in both mice (91, 275) and humans (278). We observed deep crypt invasion of intestinal bacteria in both *Muc2* $^{-/-}$, and *Pigr* $^{-/-}$*Muc2* $^{-/-}$ mice, but never in wild-type or *Pigr* $^{-/-}$ animals (Figure 4.12).

It is important to note that the colons of *Muc2* $^{-/-}$, and *Pigr* $^{-/-}$*Muc2* $^{-/-}$ mice showed no signs of overt inflammation and never presented with blood in luminal contents. This finding further shows the important nature of the colonic mucus layer, but not IgA, in keeping a barrier in between the intestinal microbiota and the host epithelium.

SUMMARY

At the mucosal surface, SIgA antibodies carry out the multiple functions including neutralizing potentially invasive pathogens and shaping the composition of the commensal microbiota (230-232). Here, we show potential mechanisms for microbiota promotion of SIgA transport to the colonic mucus layer as well as SIgA and SC localization once deposited in the mucus. We also show that mucus, but not SIgA is necessary for the separation of host and bacteria in the colon. The pIgR protein was found to be expressed in different sets of colonic epithelial cells depending on what site was surveyed along the colon (Fig. 4.1B). Interestingly, crypt apical expression of pIgR is directly correlated to the putative bacterial burden in the colonic lumen. In stimulating *in vitro* colonic epithelial cells, we found *Ec*N to be unique among representative species from the 4 major phyla of human colonic bacteria in its ability to up-regulate expression of pIgR in the human IEC line HT-29 (Fig. 4.2A). In addition, *Ec*N was also the only commensal to increase gene expression of the immune proteins Il-8 and TNF as well as the negative regulator of NF-κB signaling, A20. This finding was repeated in CaCo-2 cells with only *Ec*N able to increase both pIgR and Il-8 expression in the cells (Fig. 4.3). We found that HT-29 cells responded to whole *Ec*N, although not as robustly as the THP-1 monocyte cell-line (Fig. 4.2). Interestingly, HT-29 cells did not respond to other Gram-negative bacteria, including *Pseudomonas straminea*, which like *Ec*N is a member of the phylum *Proteobacteria*, and two members of the phylum *Bacteroidetes*, *Bacteroides thetaiotaomicron* and *Alistipes onderdonkii*. Analyses of the structural requirements for TLR4-mediated signaling have suggested that the ability to act as a ligand for TLR4 is restricted to LPS molecules from a limited group of Gram-negative bacteria, in particular those of the family *Enterobacteriaceae* (47, 279). Both the apical and basolateral and surfaces of HT-29 cells were equally responsive to stimulation with *Ec*N (Fig. 4.4A), but Il-8 secretion from the apical side of these monolayers was consistently higher than from the basal side (Fig. 4.3B). Physiologically, this may point to a method used by the intestinal epithelium in order to recruit neutrophils to the location of the bacterial threat. We unexpectedly found both pathogenic and commensal bacteria as having the ability to decrease epithelial monolayer permeability (Fig. 4.7), pointing to a common response of the epithelium to any intimate bacterial-IEC contact. Following release from the epithelium, both SC and SIgA were

found to be concentrated in the outer mucus layer, which was completely ablated in the absence of the muc2 protein (Figs. 4.9,4.10). This occurrence was not specific to mice, as the human colonic epithelium showed the same pattern of IgA localization in the outer mucus layer (Fig. 4.13). The outer mucus layer is also the residence for the colonic microbiota (36), as shown by FISH staining for bacteria and immunofluorescence detection of IgA (Fig. 4.11). Presence of the muc2 protein is necessary for separation of bacteria from the epithelial surface, but the absence of SIgA does not affect the bacterial localization. Without an established mucus layer, bacteria are able to invade the colonic crypt space, which is also independent of presence or absence of pIgR (Fig. 12). Our data point towards a model of 'selective ignorance' with of IEC recognition of only certain commensals. Furthermore, SIgA may encourage immune exclusion of the microbiota by promotion of biofilm development and, ultimately, segregation of bacteria to the outer mucus layer.

Table 4.1: Bacteria used for the stimulation of cell lines

Phylum	Class	Order	Family	Genus	Species/strain	Gram stain
Commensal:						
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	*acidophilus*	Positive
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	*nexile*	Positive
Bacteroidetes	*Bacteroidetes*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	*thetaiotaomicron*	Negative
Bacteroidetes	*Bacteroidetes*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*	*onderdonkii*	Negative
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*straminea*	Negative
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia*	*coli* Nissle 1917	Negative
Actinobacteria	*Actinobacteria*	*Bifidobacteriales*	*Bifidobacteriaceae*	*Bifidobacterium*	*longum*	Positive
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardioidaceae*	*Pimelobacter*	*simplex*	Positive
Pathogenic:						
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Salmonella*	*typhimurium* SL1344	Negative

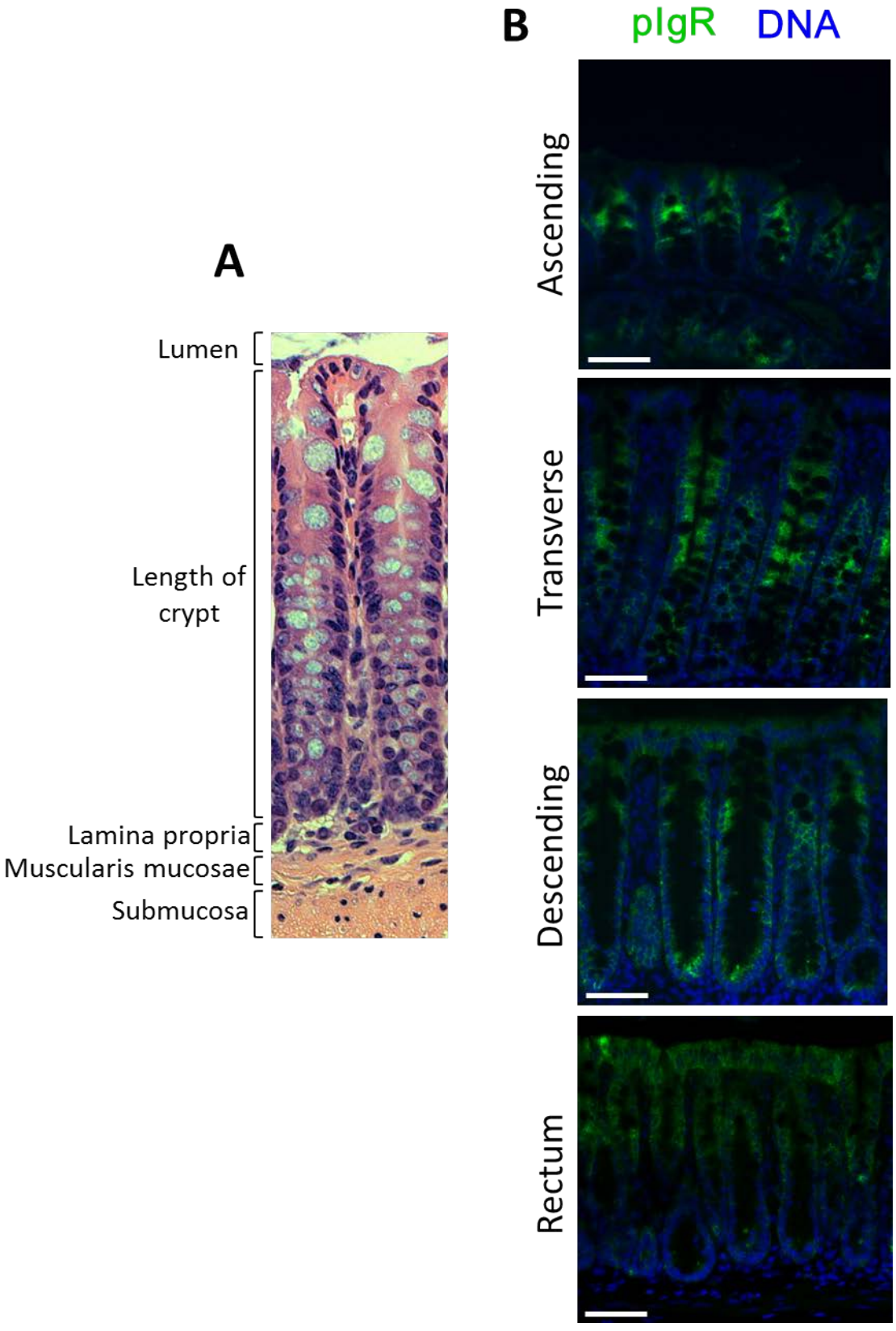

A
Lumen
Length of crypt
Lamina propria
Muscularis mucosae
Submucosa
B
pIgR
DNA
Ascending
Transverse
Descending
Rectum

Figure 4.1. Architecture of colonic crypts and localization of pIgR along mouse colon. The colon is composed of repeating units denoted as crypts. A) H&E stain of two tandem crypts in colon showing primary features as well as rotund mucus-secreting goblet cells and columnar absorptive enterocytes along length of crypt. B) Immunostaining for pIgR (FITC, green) and counterstaining for DNA (DAPI, blue) showing increased pIgR protein at crypt apical surface as the distal colon is approached. Scale bar = 50μm.

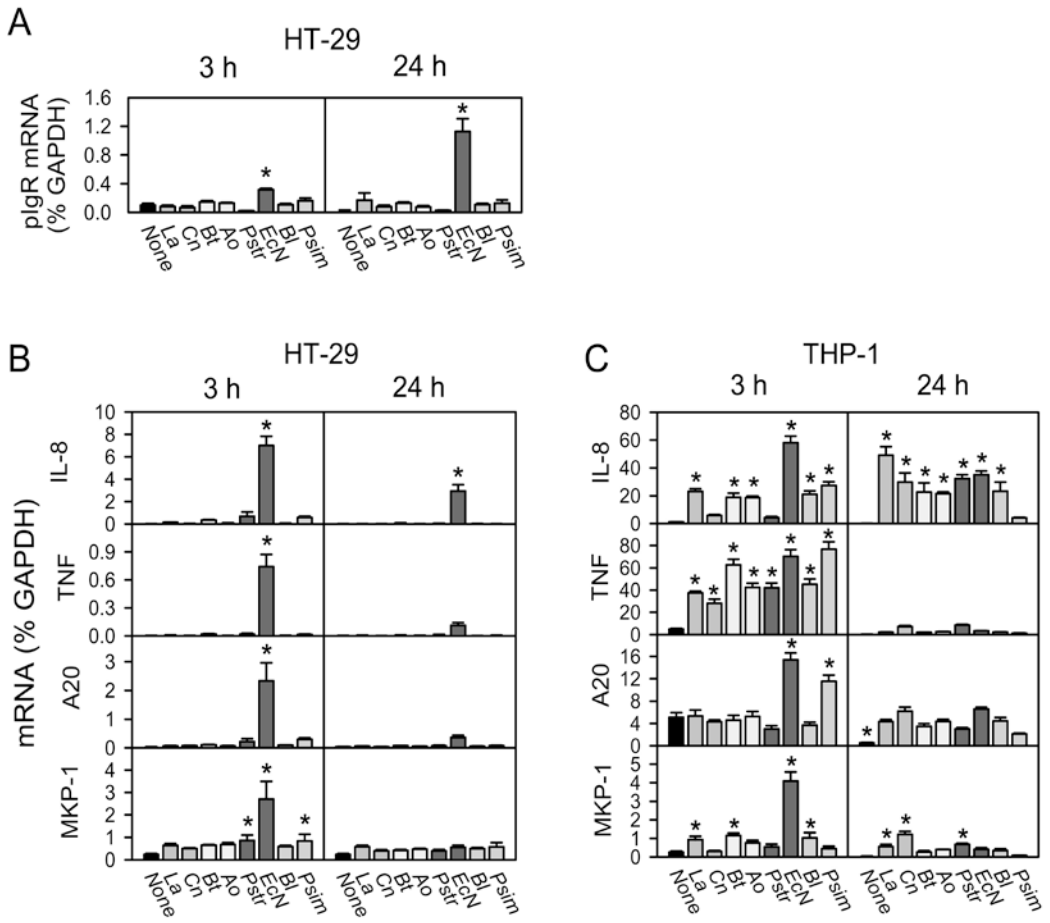


Figure 4.2. Regulation of gene expression by commensal bacteria representing the four major phyla of the human colonic microbiota. See Table 4.1 for a description of bacterial phylogeny. Abbreviations: *La, L. acidophilus; Cn, C. nexile; Bt, B. thetaiotaomicron; Ao, A. onderdonkii; Pstr, P. straminea; Ec*N, *E. coli* Nissle; *Bl, B. longum; Psim, P. simplex*. A) HT-29 cells were cultured for 3 or 24h with heat-killed bacteria at a ratio of 10-20 bacterial cells per eukaryotic cell. pIgR 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 = 8). Asterisks indicate that the mean is significantly different from the mean for untreated HT-29 cells at 3 h ($p < 0.05$). B,C) Analysis of mRNA for pro-inflammatory factors and negative regulators in HT-29 and THP-1 cells treated as described for panel A. Data from 3 independent experiments were combined and expressed as mean ± SEM (HT-29, n = 8; THP-1, n = 9). Asterisks indicate that the mean is significantly different from the mean for untreated cells from the same cell-line at 3h ($p < 0.05$).

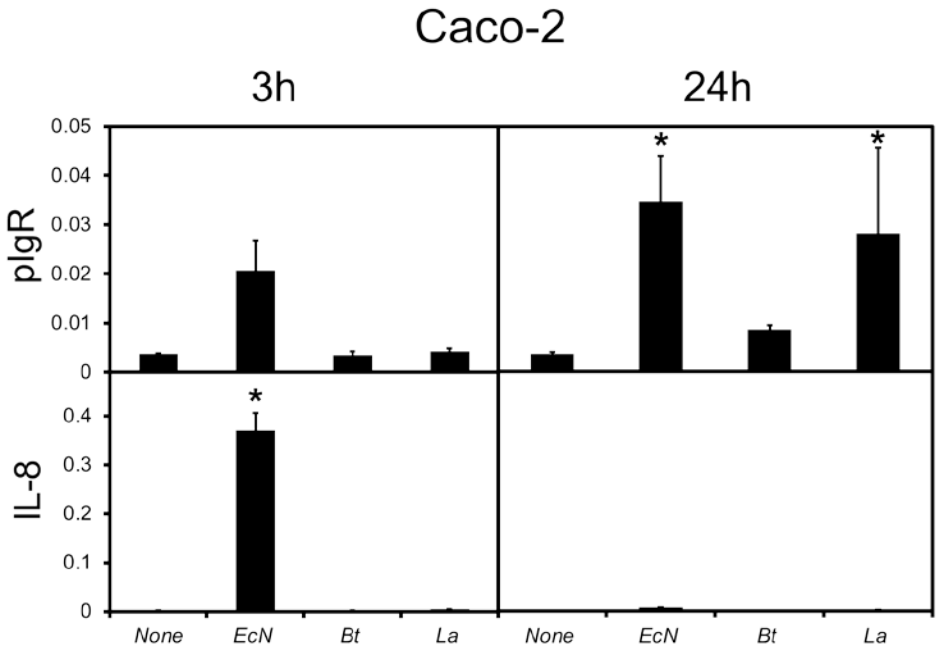


Figure 4.3. Human Caco-2 colonic adenocarcinoma cells display similar gene expression pattern as HT-29 cells when stimulated with commensal bacteria. Caco-2 cells were grown to 70% confluency and stimulated with indicated bacterium representing one of the three dominant bacterial phyla in the human intestine (abbreviations and taxonomy displayed in Table 1). As with HT-29 cells, *EcN* gave most dramatic increases in gene expression of pIgR and IL-8, but *La* was also able to significantly increase pIgR expression following 24h stimulation. Data representative of one experiment with error bars displayed as SEM (n=3). ***** -gene expression significantly ($p<0.05$) different in eukaryotic cells stimulated with bacterium versus unstimulated cells.

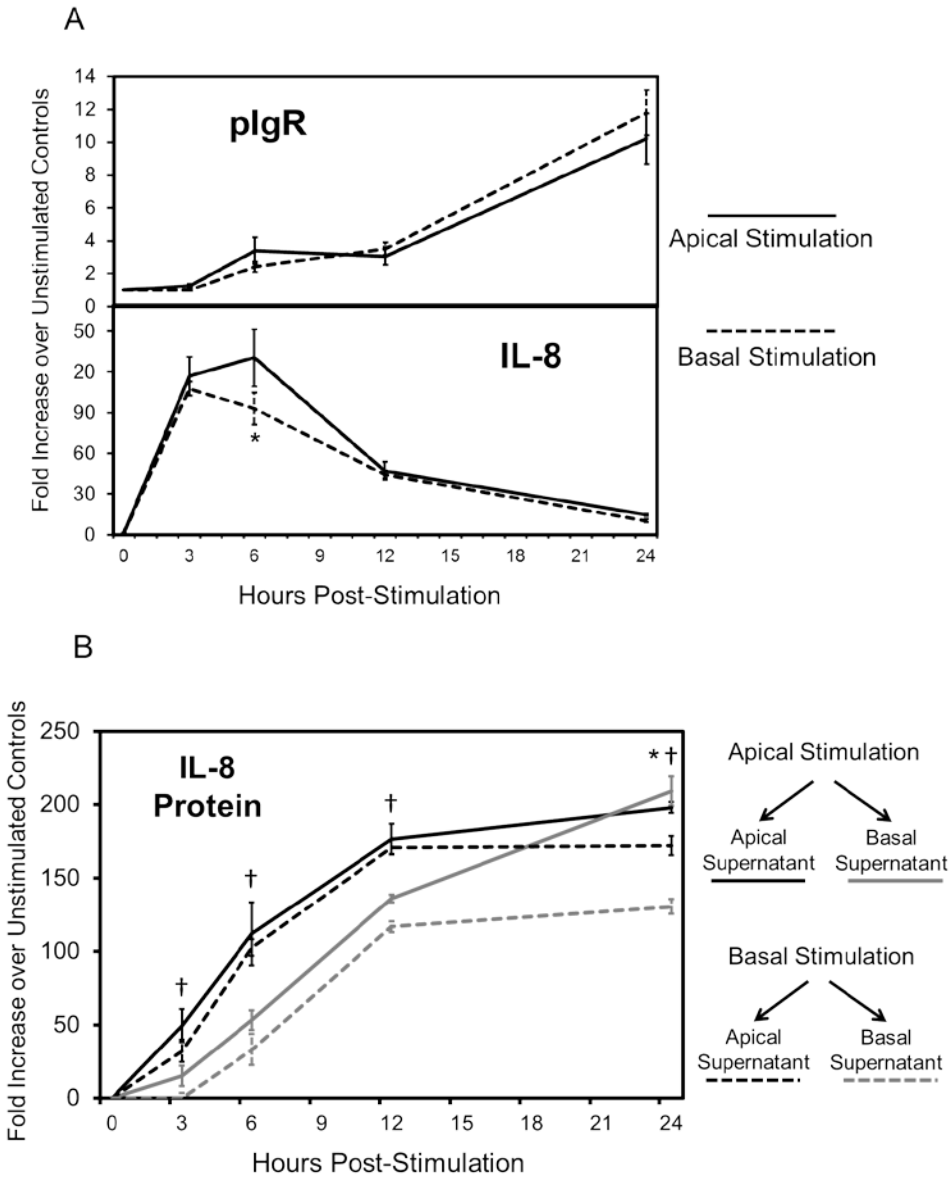
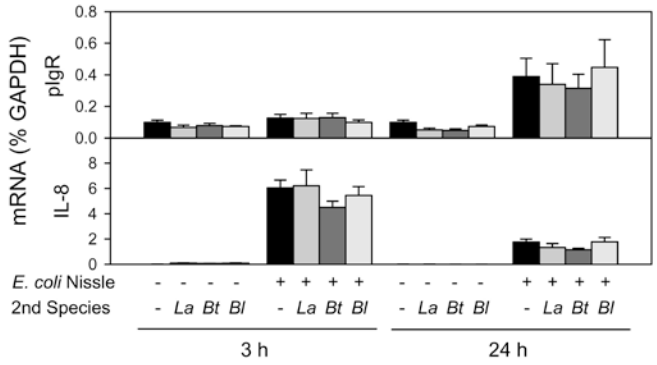


Figure 4.4. Modulation of pIgR and IL-8 gene expression and secretion of IL-8 after apical and basal stimulation of HT-29 cells with *EcN*. HT-29 cells were allowed to grow to confluent monolayer on Transwell inserts and then stimulated with 10^7CFU/mL *EcN* for 3,6,12,or 24 hours on the apical or basal side of the cells. Gene expression of pIgR and IL-8 is expressed in fold increase over unstimulated HT-29 cells. Stimulation of either apical or basal side of epithelial cells with *EcN* shows an equal capacity to induce gene expression (A). Supernatants were collected at various time points from either apical or basal side of cell monolayer to determine IL-8 secretion through ELISA. IL-8 protein shows preferential secretion from apical surface after

stimulation with *EcN* (B). Only the 24h time point of *EcN* stimulation causes that specified time point to have significant differences in apical vs. basal stimulation, however, apical stimulation showed significantly higher IL-8 secretion for the cumulative experiment of all time points when compared to basal stimulation. Data are averages±SEM of two independent experiments with $n \geq 5$ for gene expression and n=6 for ELISA data. ***** -gene expression or IL-8 secretion significantly ($p<0.05$) different in apical vs. basal stimulation at indicated time point. †- IL-8 secretion significantly ($p<0.05$) higher in apical supernatants compared to basal supernatants at indicated time point.



Analysis of Variance

	p	
	pIgR	IL-8
Time (3 vs. 24 h)	< 0.0001	< 0.0001
E. coli Nissle	< 0.0001	< 0.0001
L. acidophilus	0.4881	0.9214
B. thetaiotaomicron	0.4914	0.0950
B. longum	0.6547	0.6895

Figure 4.5. Effect of addition of bacteria from different phyla on the response of HT-29 cells to *E. coli* Nissle. HT-29 cells were cultured for 3 or 24 h with heat-killed *E. coli* Nissle, *L. acidophilus* (*La*), *B. thetaiotaomicron* (*Bt*), or *B. longum* (*Bl*), as indicated. Each bacterial species was added at a ratio of 10-20 bacterial cells per eukaryotic cell. mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA. Data from 2 independent experiments were combined and expressed as mean ± SEM (n = 7). Statistical comparisons of the overall effects of time and bacterial stimulation on pIgR and IL-8 mRNA are noted in the analysis of variance table.

A

Species	*E. coli* Nissle	*S. typhimurium*
	(CFU)	(CFU)
Initial concentration	6.1×10^6	9.8×10^6
Final concentration	6.3×10^7	1.6×10^7
Membrane-associated[a]	$1.6 \pm 0.28 \times 10^4$	$2.8 \pm 0.32 \times 10^4$
Internalized	0	$3.2 \pm 0.31 \times 10^2$

B

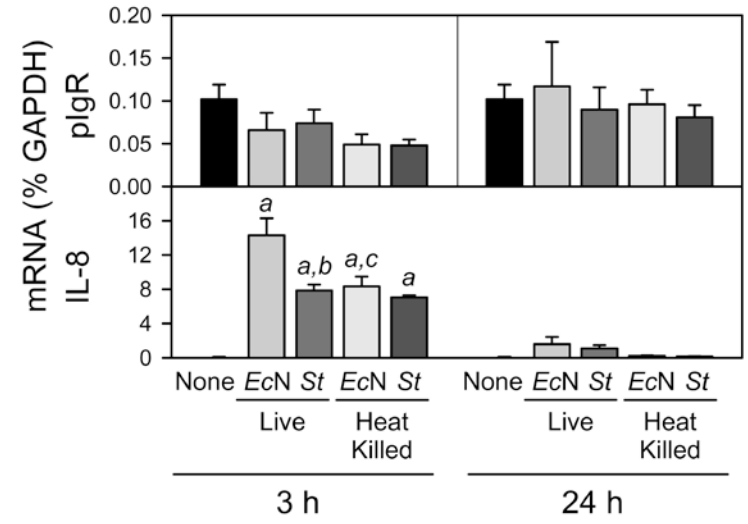


Figure 4.6. Induction of gene expression in HT-29 cells by live or heat-killed bacteria of the family *Enterobacteriaceae.* A) Bacterial invasion assay. Six replicate cultures of HT-29 cells (approximately 10^6 cells/culture) were incubated for 1h with the indicated concentration of *E. coli* Nissle or *S. typhimurium.* After removal of culture supernatants to obtain final bacterial concentrations, triplicate cultures of HT-29 cells were analyzed for either membrane-associated or internalized bacteria as described in Materials and Methods. Statistical comparison: a, numbers of membrane-associated *S. typhimurium* were significantly greater than numbers of membrane-associated *E. coli* Nissle ($p < 0.05$). B) HT-29 cells were cultured for 3h with live or heat-killed bacteria of the commensal *E. coli* Nissle (*Ec*N) or the pathogen *Salmonella typhimurium* (*St*) at a ratio of 10 bacterial cells per eukaryotic cell. HT-29 cells were either harvested immediately (3h harvest), or washed and cultured in antibiotic-containing medium for an additional 21h (24h harvest). 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 = 10). Statistical comparisons: a, mean is significantly different from the corresponding mean for untreated HT-29 cells ($p < 0.05$); b, mean for HT-29 cells treated with *St* is significantly different from the corresponding mean for cells treated with *Ec*N ($p < 0.05$); c, mean for HT-29 cells treated with heat-killed bacteria is significantly different from the corresponding mean for cells treated with live bacteria ($p < 0.05$).

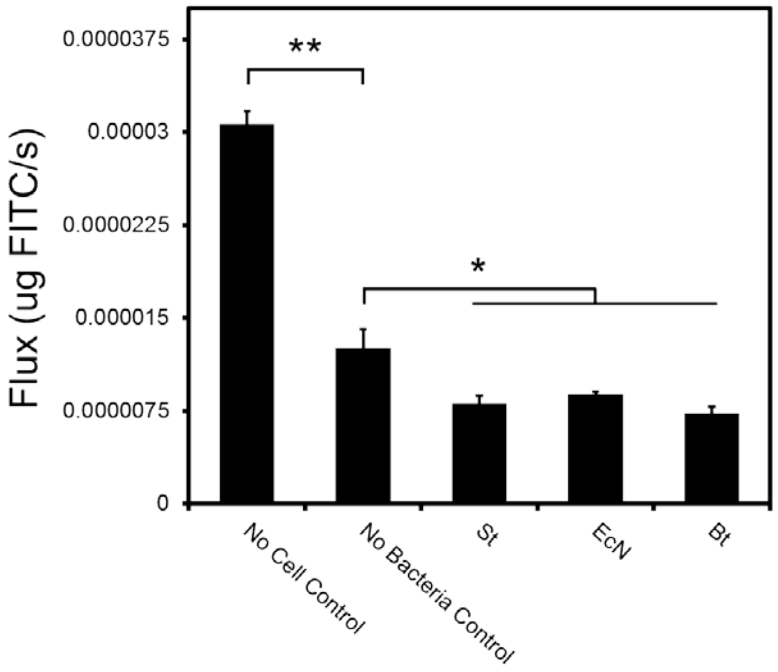


Figure 4.7. Both commensal and pathogenic bacteria decrease epithelial monolayer permeability. Epithelial cells allowed to grow to confluent monolayers on Transwell inserts and then exposed to 10^2 live bacteria for 9 hours. Monolayer permeability measured by adding 5mg/mL 3kD FITC-dextran to Transwell apical chamber and collecting well basal chamber volume after 3h and reading flow-through FITC concentration by spectrophotometer. Flux across HT-29 monolayer measured by: [μg FITC-dextran in basal chamber / time (in seconds)]. FITC-dextran ability to cross Transwell membrane significantly reduced by monolayer of epithelial cells and further reduced by pathogenic or commensal bacteria. Data are averages±SEM from 2 independent experiments with n=3. **, $p<0.0001$, *, $p<0.01$

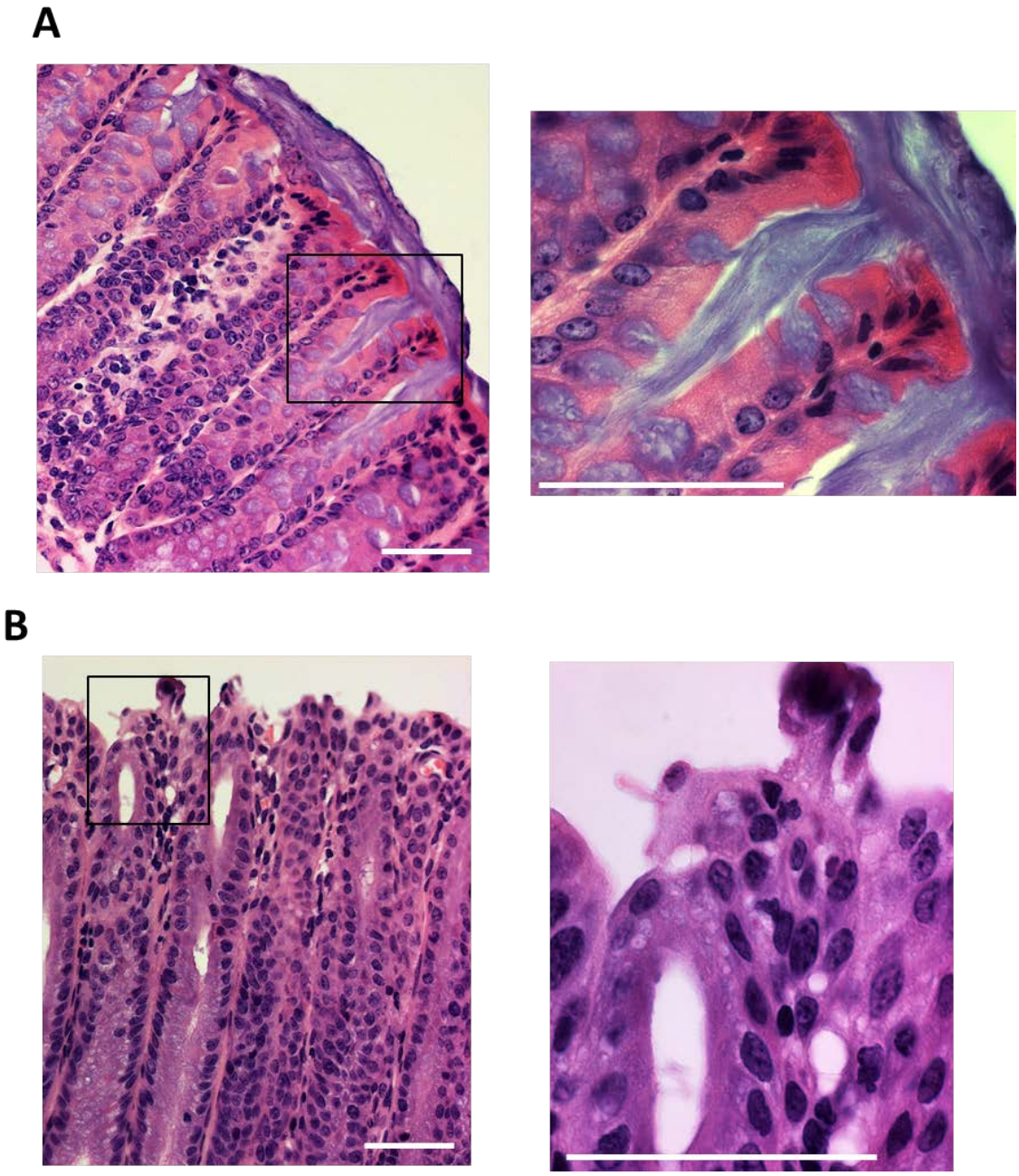


Figure 4.8. Change in colonic crypt morphology and absence of mucus layer in mice lacking Muc2 protein production. A) H&E stain of wild-type mouse colon showing crypt architecture and mucus production and excretion from goblet cells. Panel to right is magnified section of black box. B) H&E stain of *Muc2* $^{-/-}$ mouse colon showing elongated crypts, goblet cell atrophy, and erosion of the epithelium with epithelial cell detachment. Panel to right is magnified section of black box. Bar = 50μm for all panels.

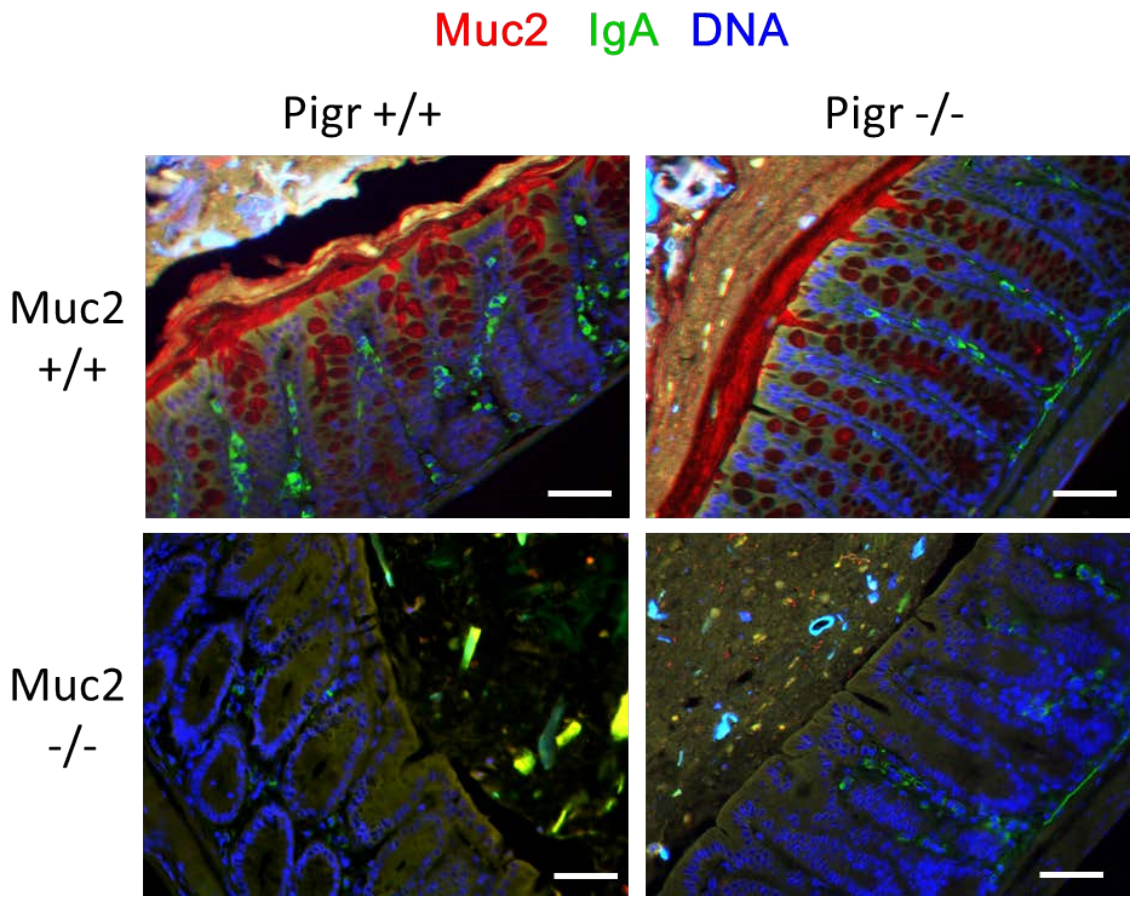


Figure 4.9. IgA localizes to outer mucus layer in colon. Immunofluorescence histochemistry of wild-type, *Pigr* $^{-/-}$, *Muc2* $^{-/-}$, and *Pigr* $^{-/-}$*Muc2* $^{-/-}$ mouse colons staining for IgA (FITC, green), Muc2 (Rhodamine, red), and counterstained for nuclei (DAPI, blue). In Muc2 +/+ mice, a clear boundry exists between the inner and outer mucus layer with high concentrations of IgA in outer layer of Pigr sufficient mice as indicated by bright yellow combination of signals. Pigr +/+ mice unable to retain IgA above colonic epithelium in absence of mucus layer. Bar =50μm.

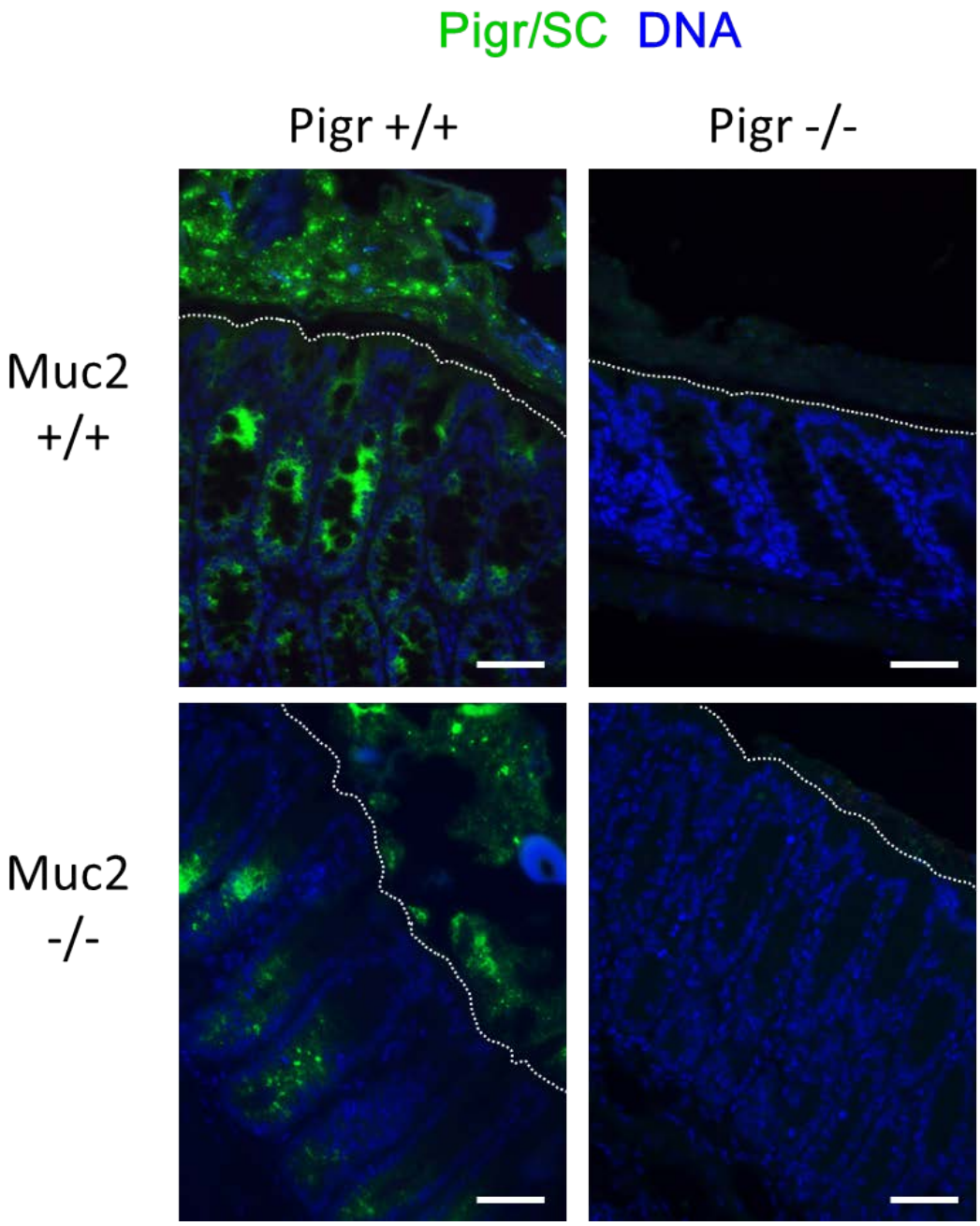


Figure 4.10. Cleavage product of Pigr, secretory component (SC), also localizes to outer mucus layer in colon. Immunofluorescence histochemistry of wild-type, *Pigr* $^{-/-}$, *Muc2* $^{-/-}$, and *Pigr* $^{-/-}$ *Muc2* $^{-/-}$ mouse colons staining for Pigr/SC (FITC, green) and counterstained for nuclei (DAPI, blue). White dotted line indicates boundary between epithelial surface and lumen. Whole pIgR protein can be seen in lower and middle crypts in the colon. Bar = 50μm.

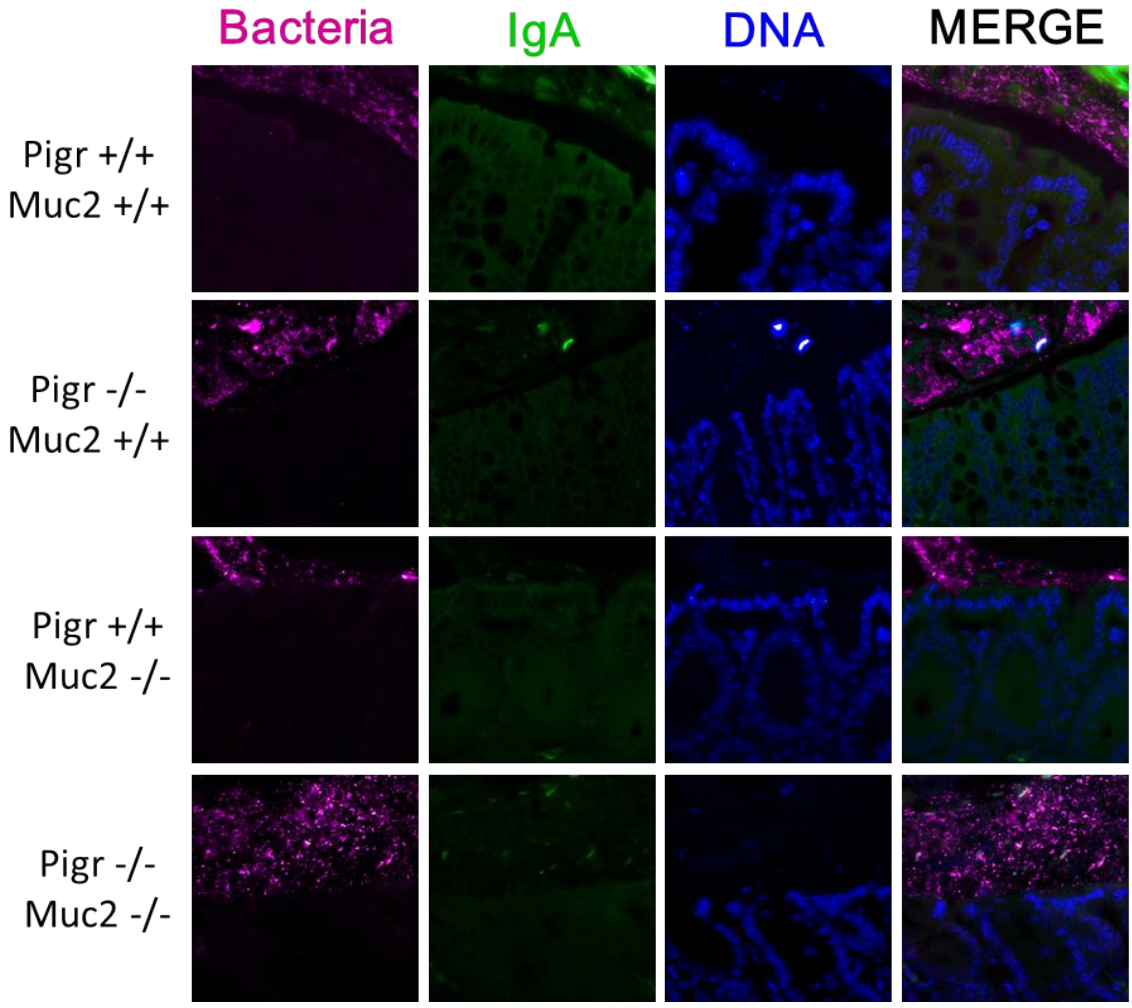


Figure 4.11. Intestinal bacteria are found in the outer mucus layer with IgA, but are in direct contact with colonic epithelium in absence of mucus layer. Also, pIgR/SC not required to keep bacteria in outer mucus layer. Wild-type, *Pigr* $^{-/-}$, *Muc2* $^{-/-}$, and *Pigr* $^{-/-}$*Muc2* $^{-/-}$ mouse colons visualized by fluorescence *in situ* hybrizidation (FISH) labeling of intestinal bacteria (Cy5-Eub probe, magenta), immunostaining for IgA (FITC, green), and counterstaining for nuclei (DAPI, blue).

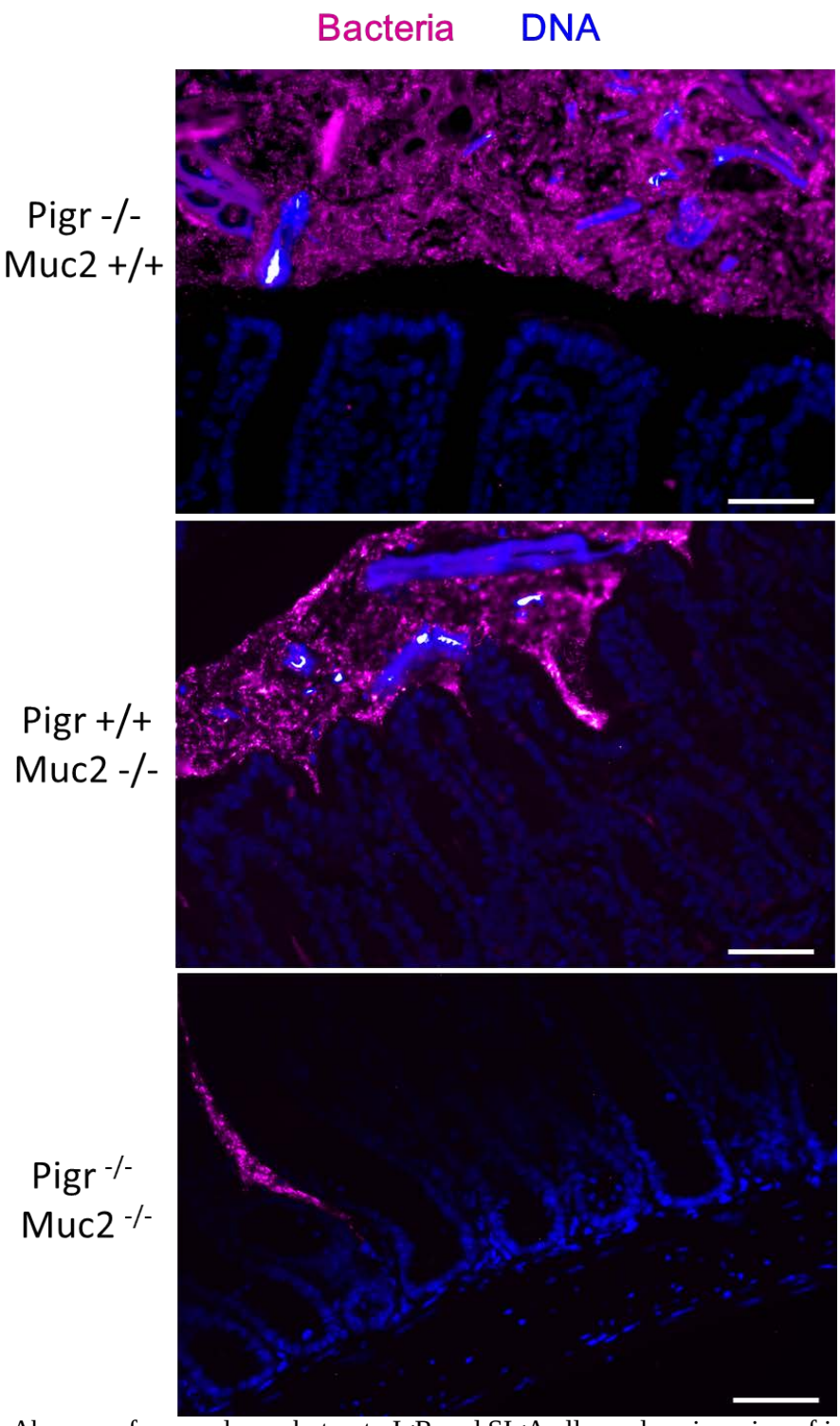


Figure 4.12. Absence of mucus layer, but not pIgR and SIgA allows deep invasion of intestinal bacteria into colonic crypts. Colons of *Pigr* $^{-/-}$, *Muc2* $^{-/-}$, and *Pigr* $^{-/-}$*Muc2* $^{-/-}$ mice labeled with FISH probe against intestinal bacteria (Cy5-Eub probe, magenta) and counterstained for nuclei (DAPI, blue). Bar = 50μm.

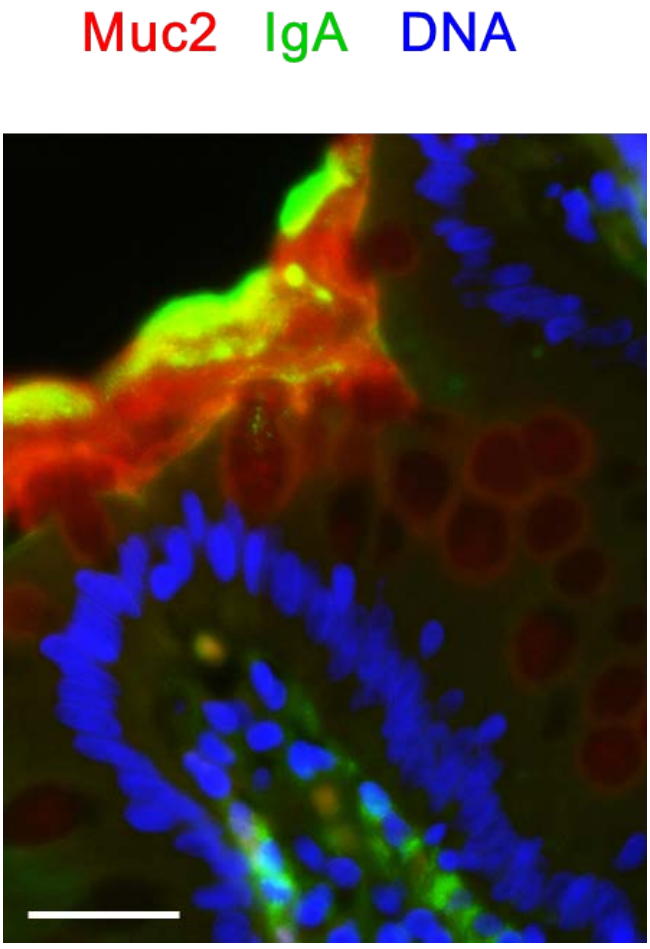


Figure 4.13. Human colon shows similar pattern of IgA migration to outer mucus layer and accumalation. A section of human colon actively secreting IgA and mucus stained for IgA (FITC, green), Muc2 (Rhodamine, red), and counterstained for nuclei (DAPI, blue). Bar = 50μm.

Chapter 5

NEONATES NOT RECEIVING MATERNAL SECRETORY IMMUNOGLOBULIN A SHOW PROFOUND SHIFTS IN INTESTINAL MICROBIOTA AND EPITHELIAL GENE EXPRESSION AS ADULTS

INTRODUCTION

Immediately following birth, infant immune systems must contend with the world of microbes that will be with them for their entire lives. Failure of the neonatal immune system to appropriately respond to these bacterial immigrants onto and in their bodies can lead to severe systemic and enteric infections, one of the most deadly being necrotizing enterocolitis (NEC) with a mortality rate upwards of 30% (280). Infant immunity is initially deficient due to lack of activating surface proteins on immune cells (281), but the child's gastrointestinal (GI) tract is bolstered by supplementation with maternal milk. Besides the standard nutritional components, human milk provides many bioactive factors to the infant, including antimicrobial proteins, prebiotics, anti-inflammatory agents, cytokines, and immunoglobulins (282). By far, the most abundant immunoglobulin in human milk is secretory IgA (SIgA), which is the product of proteolytic cleavage from the polymeric immunoglobulin receptor (pIgR) transporter at the mammary lobe epithelium. To investigate the consequences of neonates not receiving SIgA during suckling, we compared mouse neonates from pIgR-deficient dams to pIgR-sufficient dams. Using a systematic breeding scheme, we generated offspring from both maternal genotypes which were, themselves, pIgR-deficient or pIgR-sufficient. This allowed us to compare offspring phenotype as a result of maternally-supplied (passive SIgA) or endogenously-produced (active SIgA) antibodies. We found that mice not receiving passive SIgA had high amounts of the opportunistic bacteria *Ochrobactrum anthropi* in their mesenteric lymph nodes (MLNs), as well as intestinal microbiotas which were distinctive at weaning and adulthood from offspring receiving passive SIgA during suckling. In addition, passive SIgA played a much more prominent role compared to active SIgA in modulation of adult colonic epithelial cell (also, CEC) gene expression with and without chemically-induced colitis. Overall, this work supplies experimental evidence to the role of maternally-supplied SIgA in the protection against bacterial invasion, establishment of the intestinal microbiota, and influence on CEC gene expression long after the nursing period.

RESULTS

SIgA, but not IgA, travels from lactating dam to neonate colon. Our animal breeding scheme (Fig. 5.1A) allowed generation of pIgR sufficient and deficient offspring from both pIgR sufficient and deficient dams. This allowed us to compare the effects on offspring phenotype of passively-supplied versus actively-supplied SIgA. As expected, dams deficient for the pIgR protein were unable to produce SIgA in mammary lobes (Fig. 5.1B). However, mammary tissue of pIgR deficient dams still contained conglomerates of IgA-secreting plasma cells which were able to expel IgA into the lactiferous ducts. Over the course of nursing, both dam genotypes provided IgA to their offspring (Fig. 5.1C) which was found in pup stomachs (Fig. 5.1D), but only IgA with secretory component attached (SIgA) was able to traverse the entire neonatal GI tract (Fig. 5.1E) and be found in stool samples (Fig. 5.1F). These observations are supported by the findings that SIgA is more resistant than IgA to proteolytic degradation by the digestive enzymes trypsin and pepsin (283). Before and directly after weaning, mouse IgA^+ plasma cell numbers in the intestine are low (Fig. 5.1G), so the neonate's only source of SIgA is provided by maternal milk (Fig. 5.1F). These observations were completely dependent upon maternal ability to provide SIgA, and were not affected by offspring pIgR genotype. As mice age, the number of IgA^+ plasma cells increases in density along the length of the colon (Fig. 5.1G). Though no differences were seen in absolute numbers of IgA^+ cells between all offspring from breeding scheme, we found significantly less IgA in the stools of adult offspring from pIgR deficient dams (Fig 5.1H).

Neonates not receiving passive SIgA show greater bacterial translocation to MLNs of opportunistic pathogen Ochrobactrum anthropi. Infant intestines become less permeable as the child ages (284), but a constant threat during the first stage of life is the possibility of bacterial diffusion across the intestinal epithelium into the soma proper. We found that in mice, bacteria were in very intimate contact with the colonic epithelium during the first few weeks of life (Fig. 5.2A). This remained consistent regardless of dam or pup *Pigr* genotype. Likely for this reason, we occasionally found culturable anaerobic bacteria (colony-forming units, CFUs) in the MLNs of the four types of breeding scheme offspring (Fig. 5.2B). However, aerobic bacteria were only found once in pups receiving passive SIgA, and were almost always found in pups not receiving passive SIgA. Upon visual inspection of aerobic bacteria culture plates, a predominant colony morphology was seen from all mice not receiving passive SIgA. This colony was isolated and the genome region for the 16S rRNA sequenced. BLAST alignment to all known bacterial 16S rRNA genome sequences showed high sequence alignment (99%) to *Ochrobactrum anthropi* (Fig 5.2C), an opportunistic pathogen in humans. Interestingly, *O. anthropi* has only been

identified as a potential pathogen in the past two decades, has been associated with infant bacteremia and peritonitis (285, 286), and is known to carry a potent LPS (287). Total 16S rRNA signal from pup fecal samples showed no differences in the prevalence of the *Ochrobactrum* genus within the intestines of the neonates.

Infant and adult microbiota is heavily dependent upon receiving passive SIgA during nursing. To investigate the effect of passive SIgA on the intestinal microbiota, we compared neonate and adult fecal microbiotas from the offspring receiving and not receiving SIgA during nursing. Stool samples were taken at the day of weaning (21d after birth) and every following five days until the mouse was 70d old. Analysis of total bacterial DNA in stool samples showed bacterial quantities in a much more similar pattern based upon maternal *Pigr* genotype compared to offspring genotype (Fig 5.3A). Significantly higher amounts of bacterial DNA were found in stool samples from offspring receiving passive SIgA at 21 and 31 days of age. Upon real-time qPCR analysis of the community dynamics for the four dominant bacterial phyla in mammalian intestines (*Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria*), all four phyla were detected at each timepoint from the four types of mice from the breeding scheme (Fig. 5.3C). Phylochip microarray analysis was also performed on isolated bacterial DNA from selected samples. Only the fecal microbiota of offspring with the ability to produce active SIgA (*Pigr* $^{+/-}$ offspring) was surveyed. Two samples were taken from each mouse, one at 21d after birth and one at 70d. Microarray analysis further confirmed the stability of phyla presence over the 21d to 70d timecourse measured, with no significant differences in representation of the four dominant bacterial phyla regardless of the age of the mouse or the mouse's ability to receive passive SIgA (Fig. 5.3B). Though diversity of bacterial phyla did not differ throughout time or reception of passive SIgA, diversity at the species level was widely apparent, and principal component analysis of OTU presence/absence data closely clustered by maternal *Pigr* genotype early and later in the life of the mouse (Fig. 5.3 PCoA plot and adjacent dendogram). In fact, hierarchical clustering of the groups showed the greatest distance to be between the two adult groups which were only separated by receiving or not receiving passive SIgA during nursing. This data suggests a possible mechanism for long-lasting effects of initial gut microbial establishment by SIgA long after the animal has been separated from maternal contact and SIgA provision.

Human milk is the most efficient prophylactic against many neonatal intestinal inflammatory diseases, including infant enteritis and NEC (282). Many bacteria are early colonizers of the intestinal tract that have the capacity to become opportunistic pathogens, especially if the infant is pre-term or in an immunocompromised state. Several of these

opportunists have been identified from the bacterial genera *Clostridium, Klebsiella, Enterobacter, Pseudomonas,* and *Staphylococcus* (288). Using the phylochip survey of the entire 21d weanling microbiota, we saw consistent increases in many OTUs within each of these genera in the fecal microbiota of neonates not receiving passive SIgA (Fig. 5.3E). Significant increases in abundance ($p < 0.05$) were seen with specific OTUs within the genera *Staphylococcus* and *Enterococcus*. Though weanlings not receiving passive SIgA did not display overt signs of spontaneous intestinal inflammation, these increases in *Staphylococcus, Klebsiella,* and *Enterobacter* are also mirrored in human infants within days of NEC diagnosis (289). In addition, we also found greater changes in OTU abundance from weaning to adulthood of offspring not receiving passive SIgA (Fig. 5.3F). These findings promote the idea that SIgA in maternal milk positively affects the offspring microbiota by subduing abundance of opportunistic pathogens and promoting a more stable community that is less susceptible to perturbations over time.

Adult CEC gene expression patterns more dependent upon passive SIgA than active SIgA. Gene expression in colonic epithelial cells (also, CECs) is known to be directly influenced by bacterial products that can signal to the epithelial cell through pattern recognition receptors (PRRs) and short chain fatty acids (SCFAs) (46, 63). Our survey of intestinal microbiotas showed unique bacterial communities in adult mice which were dependent upon receiving passive SIgA as neonates. To further characterize changes in CEC gene expression in adult mice, we isolated CECs from adult mice generated from the breeding scheme (Fig. 5.1A) treated with or without DSS to cause acute colitis. Figure 5.4 shows the results of microarray analysis from these different cohorts of mice with segregations by factor interactions or statistical significance. As expected, DSS-induced colitis had the most profound effect on CEC gene expression, but passive SIgA showed a much more substantial effect compared to active SIgA (Fig. 5.5A,B). Using Reactome biological pathway overrepresentation analysis (www.reactome.org) for significantly up- and downregulated genes by our experimental factor(s), we again found DSS as having the greatest effect on overrepresentation of genes which map to specific biological pathways (Table 5.1). However, both by itself and in synergy with DSS treatment, passive SIgA showed profound ability compared to active SIgA in its ability to change gene expression that maps to precise biological pathways.

In humans, approximately 100 gene loci are known to be associated with the prevalence of inflammatory bowel diseases (IBDs) and 40 are known to associate with coeliac disease (290, 291). In our experiments, we found CEC gene expression of the putative genes within these known susceptibility loci to be highly dependent upon the colitic agent DSS, but also greatly

influenced by the synergy between passive SIgA and DSS (Table 5.2). While DSS treatment modulated genes within all four categories of intestinal inflammation, we found that gene expression significantly changed by the combination of DSS and passive SIgA grouped into only the categories of Crohn's and coeliac disease.

We validated many genes from our microarray results by Nanostring nCounter analysis, which quantifies the actual number of mRNA copies within a sample for a given gene. We chose 40 candidates in which to validate the gene expression trends we saw from the microarray analysis (Table 5.3). Using mRNA abundance for our different treatment groups, we applied the variable reduction power of principal component analysis (PCA) in order to plot the different treatment groups according to gene expression pattern of validated genes. We found clear separation of mean PCA values from the treatment groups (n=6 mice per group) with and without exposure to DSS (Fig. 5.6A). A more subtle, but clearly distinguishable and statistically-significant separation also occurred between groups receiving and not receiving passive SIgA. Presence or absence of active SIgA did not lead to group segregation. These data further point to the effect of receiving passive SIgA as a neonate on CEC gene expression patterns in adult animals. Blinded pathological scoring of colon sections showed a strong negative correlation between pathology score and principal component 1 (PC1), but no correlation with PC2 (Fig. 5.6B). With the exception of DSS administration, mouse treatment groups did not show any significant differences in disease activity index (DAI) (Fig. 5.6C) or colonic length (Fig. 5.6D) at time of sacrifice, suggesting that changes due to passive and active SIgA occurred more at the cellular and transcriptional levels.

SUMMARY

The human infant is born immunodeficient, and relies on the extra-uterine link the mother supplies through breastmilk (80). Here, we present data from the mouse model showing neonates not receiving SIgA during nursing as having increased translocation of the opportunistic pathogen *Ochrobactrum anthropi* to mesenteric lymph nodes (Fig. 5.2B,C). Furthermore, these mice have an intestinal microbiota that is unique from SIgA-fed neonates (Fig. 5.3D) with higher abundance of many bacteria that are opportunistic pathogens in human (Fig. 5.3E). The effects of not receiving passive SIgA during nursing extend well after mice have reached adulthood, as shown by lower fecal IgA levels (Fig. 5.1H), high degree of OTU-specific abundance shifts since weaning (Fig. 5.3F), and an intestinal microbiota dramatically different from their passive SIgA-receiving cohorts (Fig. 5.3D). In adult mice, receiving SIgA early in life had a more profound effect on CEC gene expression than the ability of the mouse to produce active SIgA by itself (Fig.

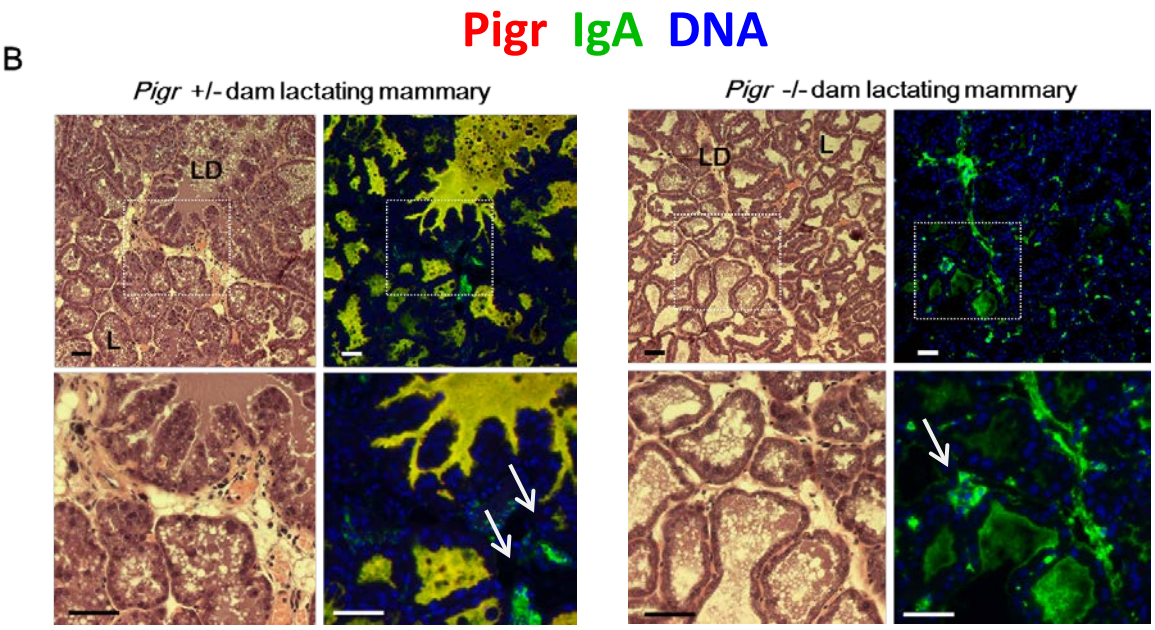
5.5A,B). Mice producing active SIgA and receiving passive SIgA during nursing had similar responses to DSS-induced acute colitis in disease presentation (Fig. 5.6), but showed unique CEC gene expression patterns with or without DSS treatment (Fig. 5.6A). By incorporation of adult CEC gene expression data into biological pathway overrepresentation analysis, we found passive SIgA to have a much greater effect over active SIgA on intracellular biological pathways affected by its presence (Table 5.1).

An interesting finding of this study was that dams deficient for the pIgR protein were still able to transmit IgA to their pups through milk (Fig. 5.1B,C). Though this IgA was found in pup stomachs (Fig. 5.1D), when lacking the secretory component, it is presumably degraded in the neonatal intestine by digestive enzymes (283) and never reaches the colonic lumen (Fig. 5.1E,F). Maternal milk is the only available source of SIgA in the neonatal mouse, as pIgR-sufficient offspring showed undetectable SIgA levels if they were being nursed by a pIgR-deficient dam (Fig. 5.1F). By mass, 4% of human colostrum and 0.1% of mature milk is composed of the protein secretory IgA (17). For the enormous amount of energy the nursing female body devotes to the production and transfer of this bioactive protein, we now show multiple mechanisms by which this metabolic effort may assist in the development and lifelong fitness of the offspring.

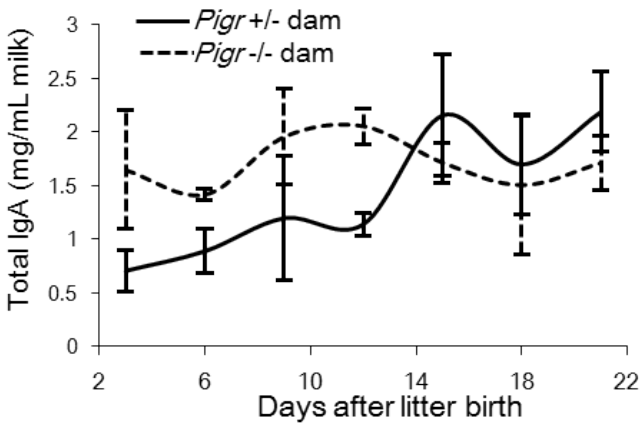
A

P1 Genotype	♀ *Pigr* $^{+/-}$ x ♂ *Pigr* $^{-/-}$	♀ *Pigr* $^{-/-}$ x ♂ *Pigr* $^{+/-}$
F1 Genotype	*Pigr* $^{+/-}$	*Pigr* $^{-/-}$	*Pigr* $^{+/-}$	*Pigr* $^{-/-}$
Passive antibody	Yes	Yes	No	No
Active antibody	Yes	No	Yes	No

B



C



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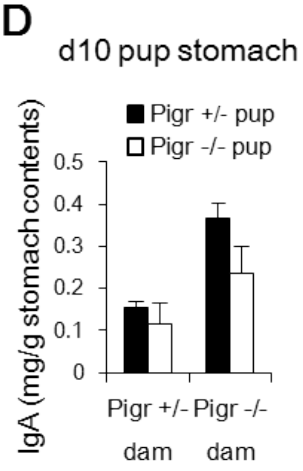
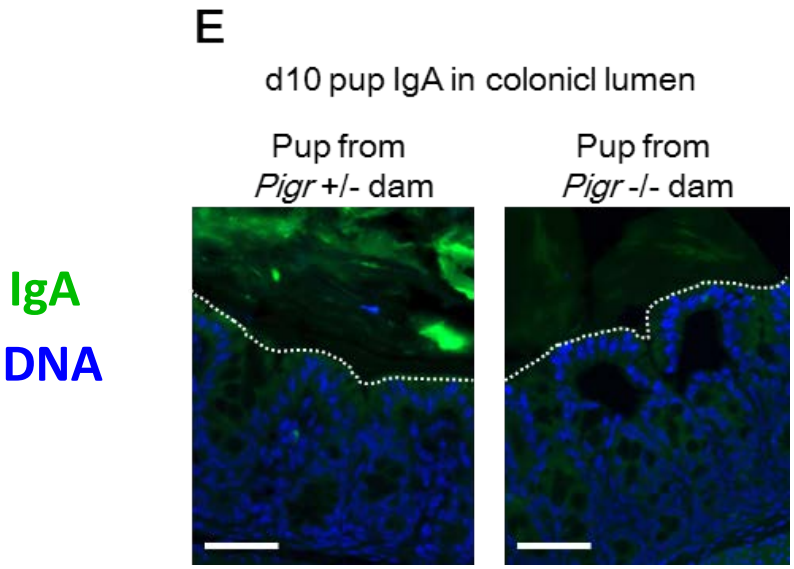
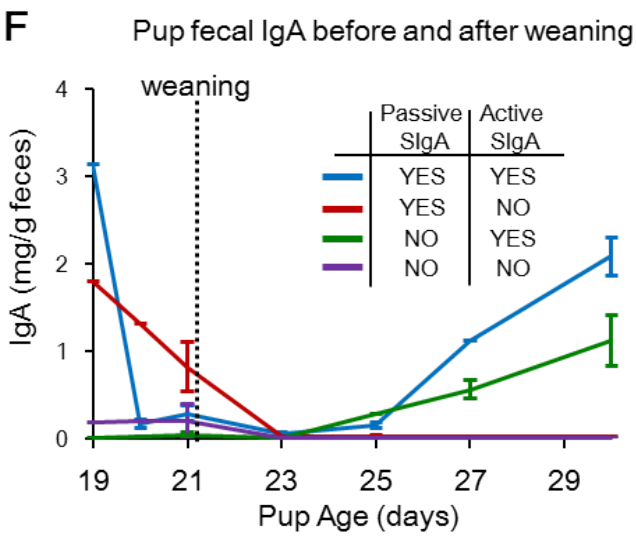
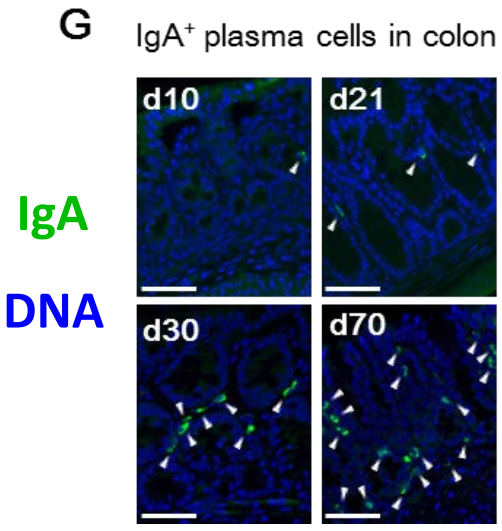



E
d10 pup IgA in colonicl lumen
Pup from
Pigr +/- dam
Pup from
Pigr -/- dam
IgA
DNA


F
Pup fecal IgA before and after weaning
weaning
IgA (mg/g feces)
Pup Age (days)
Passive SIgA
Active SIgA
YES YES
YES NO
NO YES
NO NO


G
IgA+ plasma cells in colon
IgA
DNA
d10
d21
d30
d70

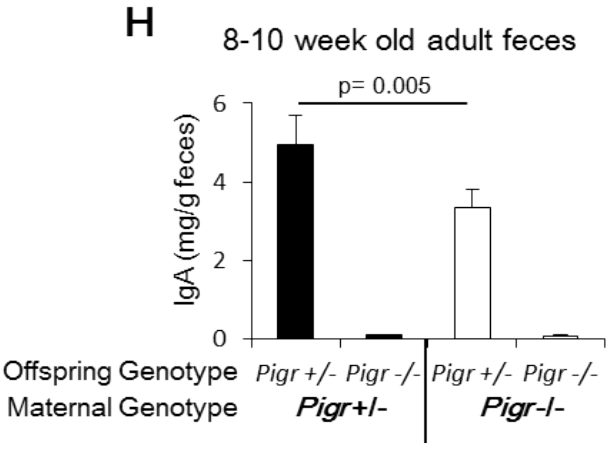

H
8-10 week old adult feces
p= 0.005
IgA (mg/g feces)
Offspring Genotype
Maternal Genotype
Pigr +/-
Pigr -/-
Pigr +/-
Pigr -/-
Pigr+/-
Pigr-/-

Figure 5.1. Neonates fed secretory IgA, but not IgA, in breastmilk have IgA in intestinal lumen. A) Breeding scheme allowing both *Pigr* $^{+/-}$ and *Pigr* $^{-/-}$ mice to be generated from a *Pigr* $^{+/-}$ or *Pigr* $^{-/-}$ mother. B) Sections of mammary tissue extracted from breeding scheme dams 10d postpartum and stained with H&E and serial section with antibody against mouse-pIgR (Rhodamine, red), mouse-IgA (FITC, green), and counterstained to visualize nuclei (DAPI, blue). Lower panels are enlarged section of hashed box in upper panel. White arrows in lower panels indicate concentrations of IgA-secreting plasma cells localized in mammary. Lactiferous ducts denoted by '**LD**' and representative lobes by '**L**'. Scale bar =50μm for all panels. C) IgA content of milk from nursing dams (mean ± SEM, n=3-6 milk samples from 2-4 dams/timepoint). D) IgA content of neonate stomach contents at 10d (mean ± SEM, n=3-15 stomach contents from multiple litters). E) Sections of 10d-old neonate colon and luminal space stained for IgA (FITC, green) and nuclei (DAPI, blue). White line denotes apical surface of colonic epithelium with colonic lumen above and epithelial cells below. Images representative of both pup genotypes for the given maternal genotype. Scale bar = 50μm. F) Fecal IgA levels of neonates around time of weaning (mean ± SEM, n=3-21 stools from multiple mice and 3-5 litters). G) IgA^+ plasma cells in colon from D10, 21, 30, and 70-old mice. White arrows indicate visible plasma cell. Images representative of both offspring genotypes from both maternal genotypes at given timepoint. Scale bar = 50μm. H) Adult fecal IgA levels from mice generated by breeding scheme (mean ± SEM, n=11-13 stools from multiple mice and multiple litters).

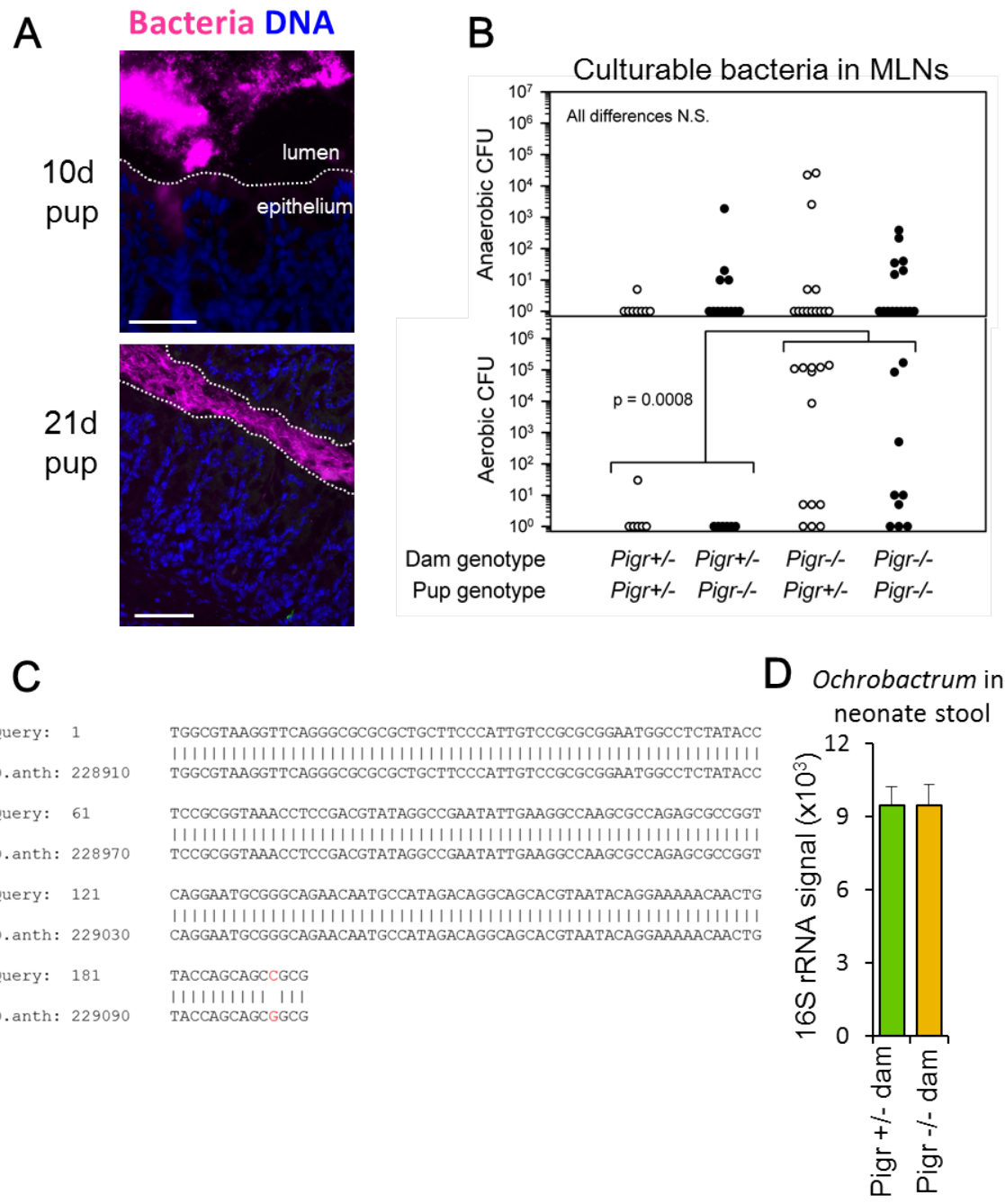
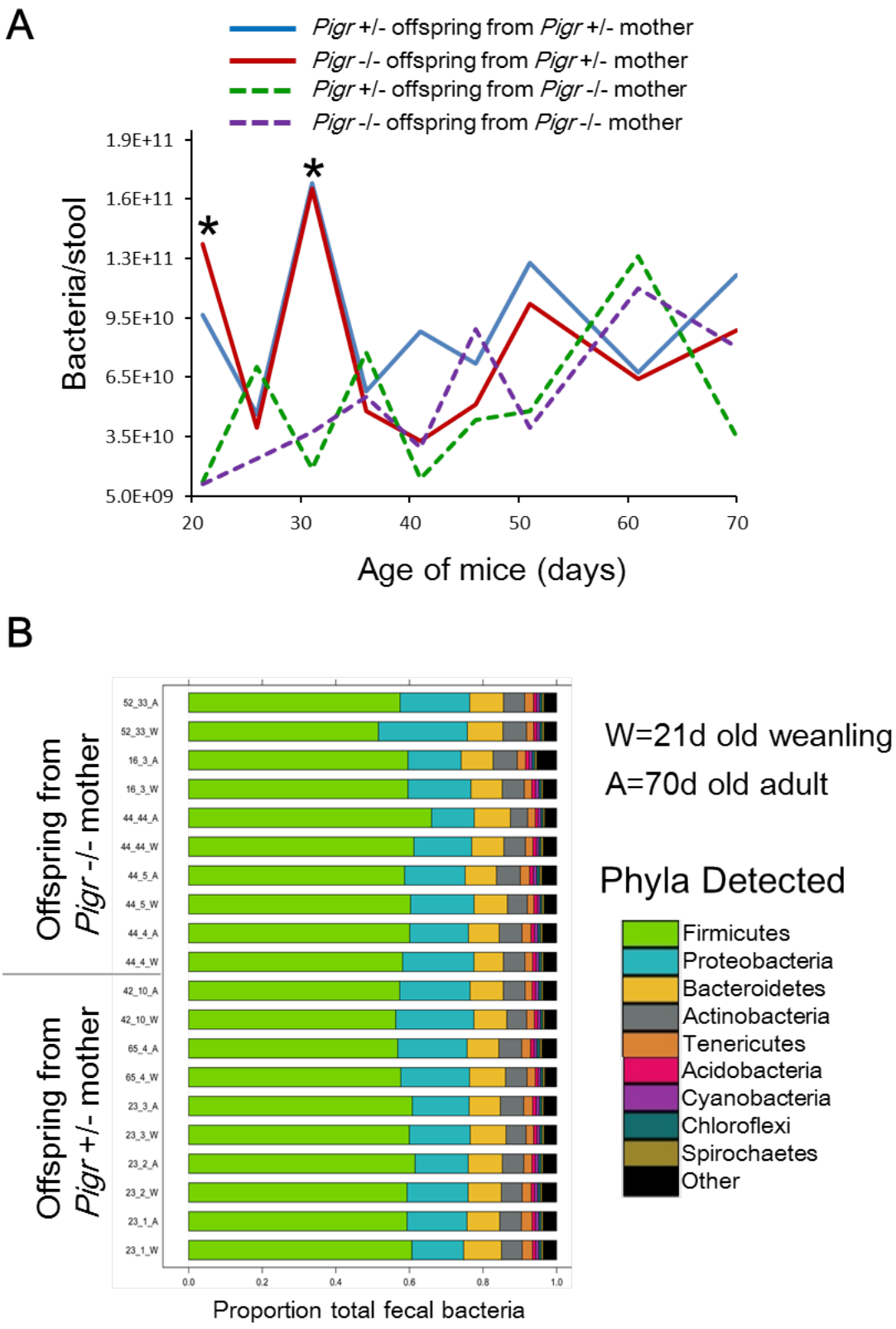
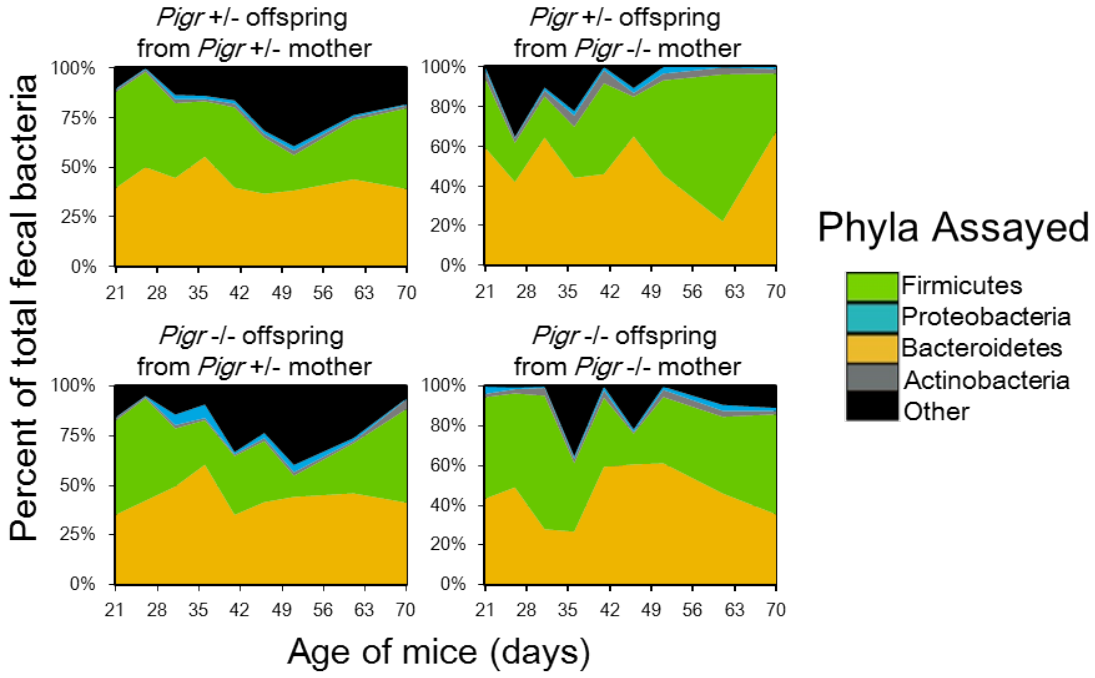


Figure 5.2. Neonates not receiving passive secretory IgA show translocation of the opportunistic pathogen *Ochrobactrum anthropi* to mesenteric lymph nodes. A) Day 10 and 21 neonate colons showing direct bacterial contact with epithelium as visualized by fluorescence *in situ* hybridization (FISH) for bacteria (Cy5, pink), immunofluorescence for IgA (FITC, green), and counterstaining to visualize nuclei (DAPI, blue). White dotted line indicates boundary between epithelium and colonic lumen. Images representative of both pup and both maternal genotypes at

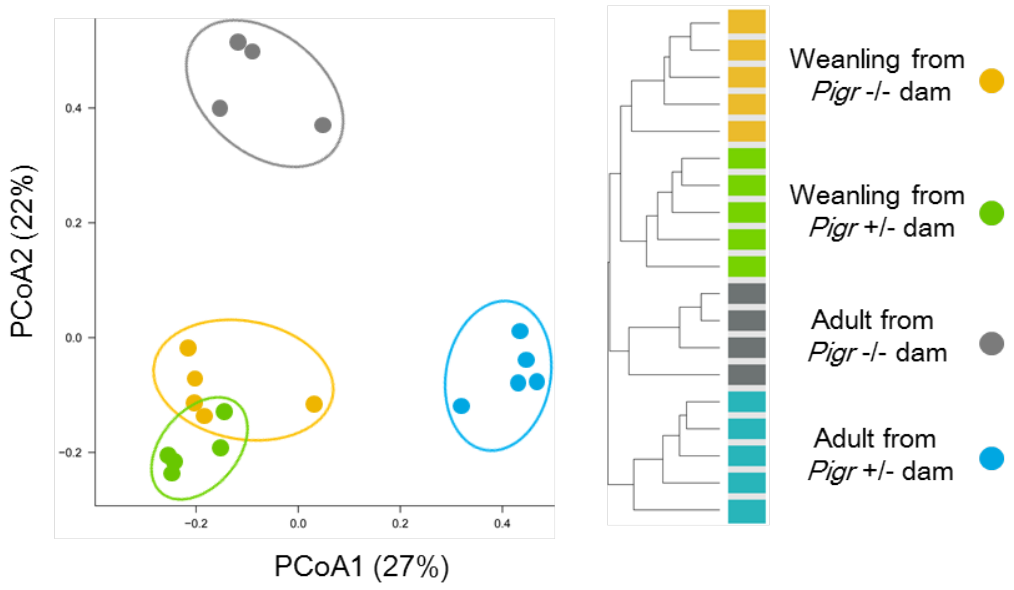
given timepoint. Scale bar = 50μm. B) Quantification of culturable anaerobic and aerobic bacteria in MLNs of 21d neonates. Data represented on a $\log_{10}$ scale as mean ± SEM (n=6-15 mice from 3-4 litters). C) BLAST sequence alignment of purified DNA from primary aerobic bacterium isolated from 21d offspring of *Pigr* $^{-/-}$ dams aligned to *Ochrobactrum anthropi* genome segment 228910 through 229104. Sequences show 99% identity. D) Day 21 neonate fecal prevalence of *Ochrobactrum* genus as measured by 16S rRNA signal. Data represented as mean ± SEM, n=5.


A
Pigr +/- offspring from Pigr +/- mother
Pigr -/- offspring from Pigr +/- mother
Pigr +/- offspring from Pigr -/- mother
Pigr -/- offspring from Pigr -/- mother
Bacteria/stool
1.9E+11
1.6E+11
1.3E+11
9.5E+10
6.5E+10
3.5E+10
5.0E+09
20
30
40
50
60
70
Age of mice (days)
B
Offspring from Pigr -/- mother
Offspring from Pigr +/- mother
52_33_A
52_33_W
16_3_A
16_3_W
44_44_A
44_44_W
44_5_A
44_5_W
44_4_A
44_4_W
42_10_A
42_10_W
65_4_A
65_4_W
23_3_A
23_3_W
23_2_A
23_2_W
23_1_A
23_1_W
0.0
0.2
0.4
0.6
0.8
1.0
Proportion total fecal bacteria
W=21d old weanling
A=70d old adult
Phyla Detected
Firmicutes
Proteobacteria
Bacteroidetes
Actinobacteria
Tenericutes
Acidobacteria
Cyanobacteria
Chloroflexi
Spirochaetes
Other

C


Pigr +/- offspring from Pigr +/- mother
Pigr +/- offspring from Pigr -/- mother
Pigr -/- offspring from Pigr +/- mother
Pigr -/- offspring from Pigr -/- mother
Percent of total fecal bacteria
Age of mice (days)
Phyla Assayed
Firmicutes
Proteobacteria
Bacteroidetes
Actinobacteria
Other

D


PCoA2 (22%)
PCoA1 (27%)
Weanling from Pigr -/- dam
Weanling from Pigr +/- dam
Adult from Pigr -/- dam
Adult from Pigr +/- dam

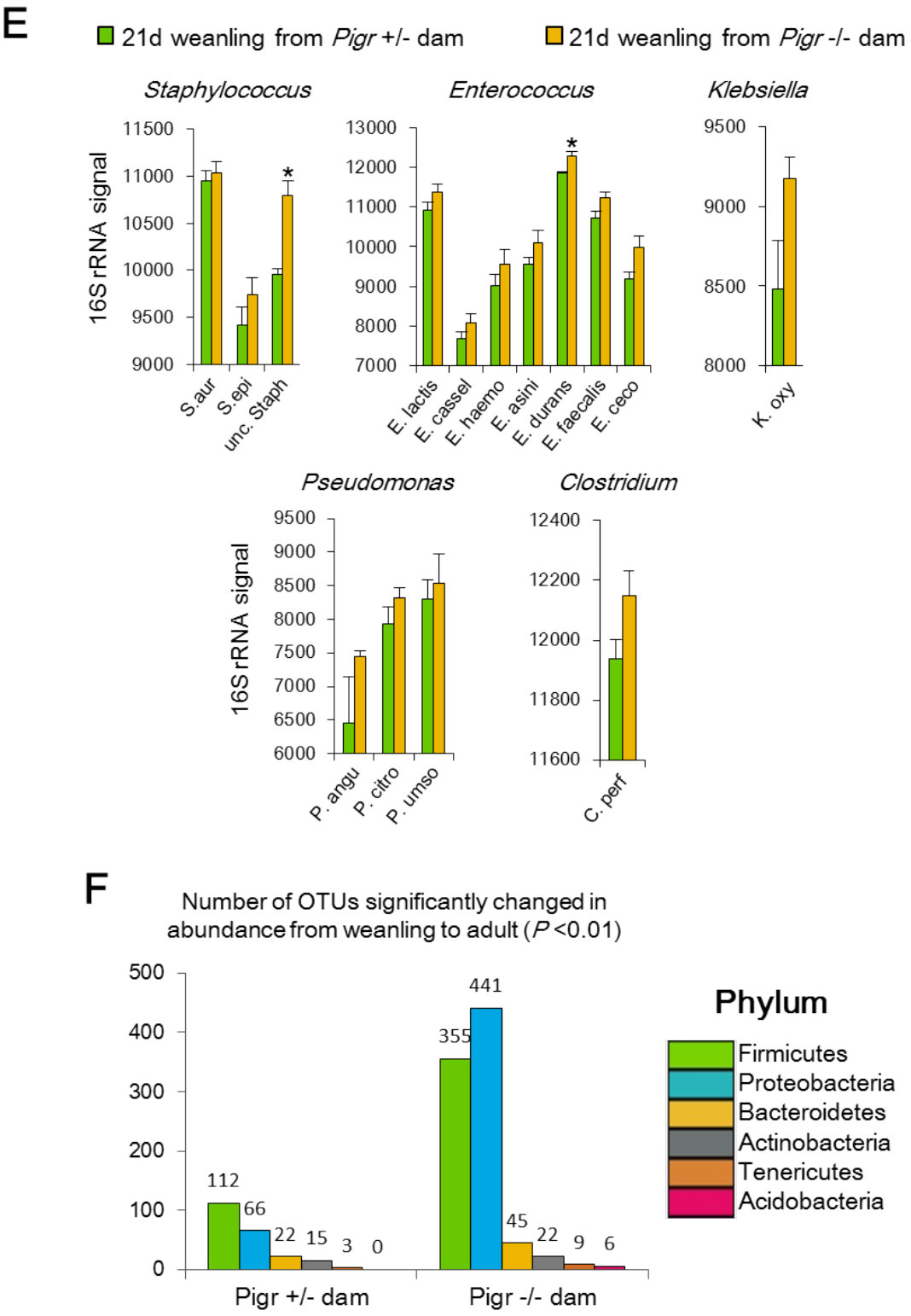

E
21d weanling from Pigr +/- dam
21d weanling from Pigr -/- dam
Staphylococcus
Enterococcus
Klebsiella
16S rRNA signal
11500
11000
10500
10000
9500
9000
13000
12000
11000
10000
9000
8000
7000
9500
9000
8500
8000
*
*
S.aur
S.epi
unc. Staph
E. lactis
E. cassel
E. haemo
E. asini
E. durans
E. faecalis
E. ceco
K. oxy
Pseudomonas
Clostridium
16S rRNA signal
9500
9000
8500
8000
7500
7000
6500
6000
12400
12200
12000
11800
11600
P. angu
P. citro
P. umso
C. perf
F
Number of OTUs significantly changed in abundance from weanling to adult (P <0.01)
500
400
300
200
100
0
112
66
22
15
3
0
355
441
45
22
9
6
Pigr +/- dam
Pigr -/- dam
Phylum
Firmicutes
Proteobacteria
Bacteroidetes
Actinobacteria
Tenericutes
Acidobacteria

Figure 5.3. Composition and stability of intestinal microbiota is highly dependent upon receiving passive SIgA during suckling. A) Total bacterial density per stool pellet as measured by real-time qPCR. Mice from SIgA-sufficient dams shown by solid bars and from SIgA-deficient dams in hashed bars. Stool densities more similar between mice with same maternal genotype compared to mice with same *Pigr* genotype. Statistically-higher bacterial numbers for mice from SIgA-sufficient dams at days 21 and 31. Data represented as mean from n=4-9 fecal samples from 2-3 litters per timepoint. *****, $p < 0.05$ comparing maternal genotypes. B) Phylochip microarray analysis of phyla richness for mouse stool samples from both maternal and both offspring genotypes (5 for each group). 'W' denotes 21d weanling, 'A' denotes 70d adult mouse. C) Real-time qPCR analysis of fluctuations from weaning at 21d of age to 70d in relative percentages of fecal bacteria within four dominant phyla of intestinal bacteria. (*Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria*). Data represented as mean from n=4-9 fecal samples from 2-3 litters per timepoint. D) Phylochip-derived principal coordinate (PCoA) analysis of mouse fecal samples by unweighted Unifrac presence/absence data showing fitted clustering of the four experimental groups and separation between weanling and adult samples. Dendogram to right shows hierarchical clustering of samples displayed on PCoA plot. PCoA1 explains 27% of varience and PCoA2 explains 22%. E) Signal strength from phylochip microarray for 21d weanlings for species associated within genera implicated in necrotizing enterocolitis (NEC): *Staphylococcus* (*S. aureus, S. epidermidis,* unclassified *S.*), *Enterococcus* (*E. lactis, E. casseliflavus, E.haemoperoxidus, E. asini, E. durans, E. faecalis, E. cecorum*), *Klebsiella* (*K. oxytoca*), *Pseudomonas* (*P. anguilliseptica, P. citronellolis, P. umsongensis*), *Clostridium* (*Clostridium perfringens*). F) Change in OTU abundance by phylum of fecal microbiota from paired samples from mice at 21 and 70d. Bar numbers indicate quantity of OTUs within given phylum with highly significant change ($P < 0.01$) in abundance from age 21d to 70d.

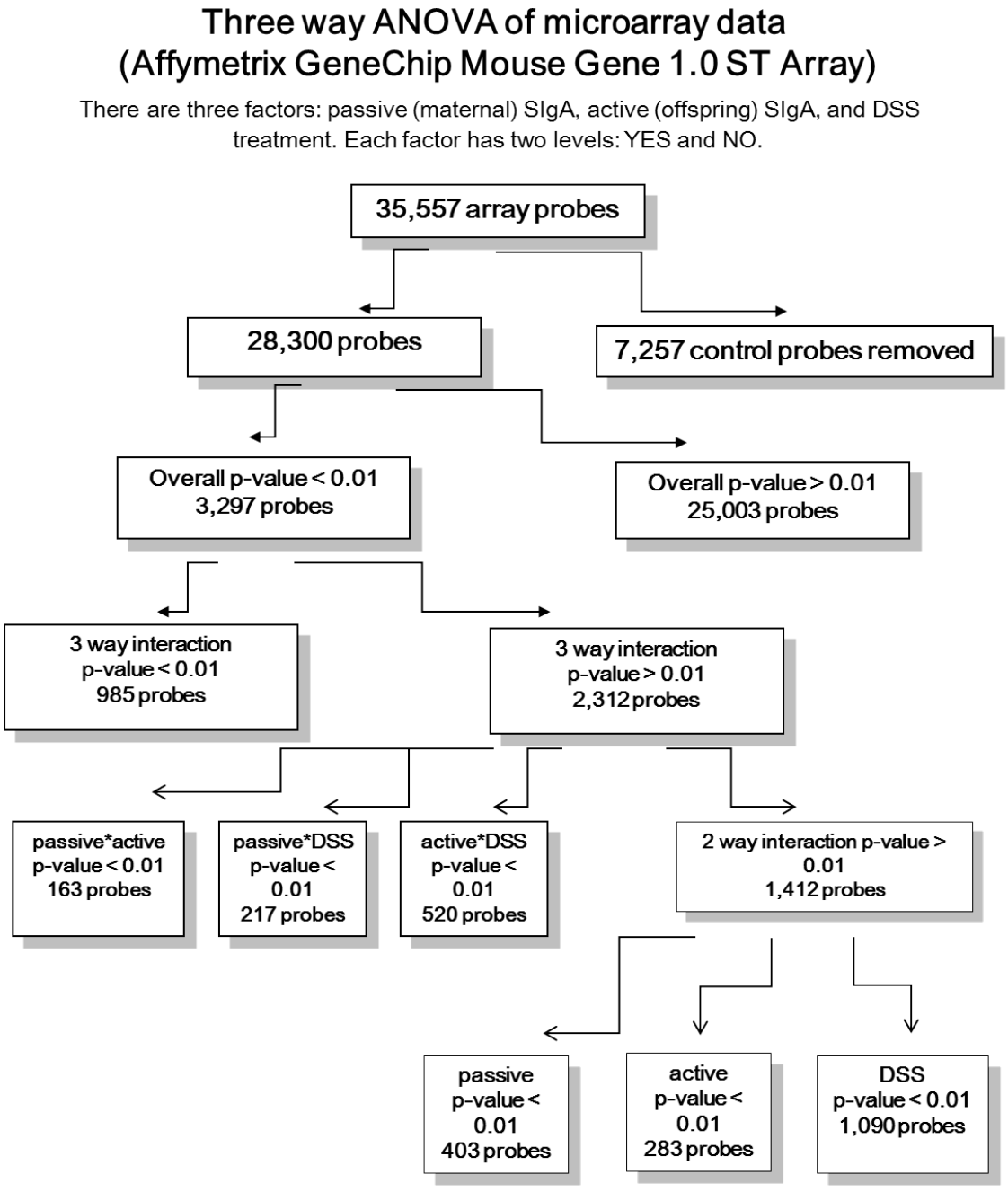


Figure 5.4. Flow chart for 35,557 probes in mouse gene expression microarray for isolated colonic epithelial cells from 8-10 week adult offspring from breeding scheme (Fig. 5.1A) with or without DSS treatment. Probes assort into bins depending upon depending on high statistical significance ('Yes' or 'No') of indicated factor or multifactor interaction. Analyses for this study focused on genes with 1 way interaction p-value < 0.01, for three independent factors in this study (lower right of flow chart).

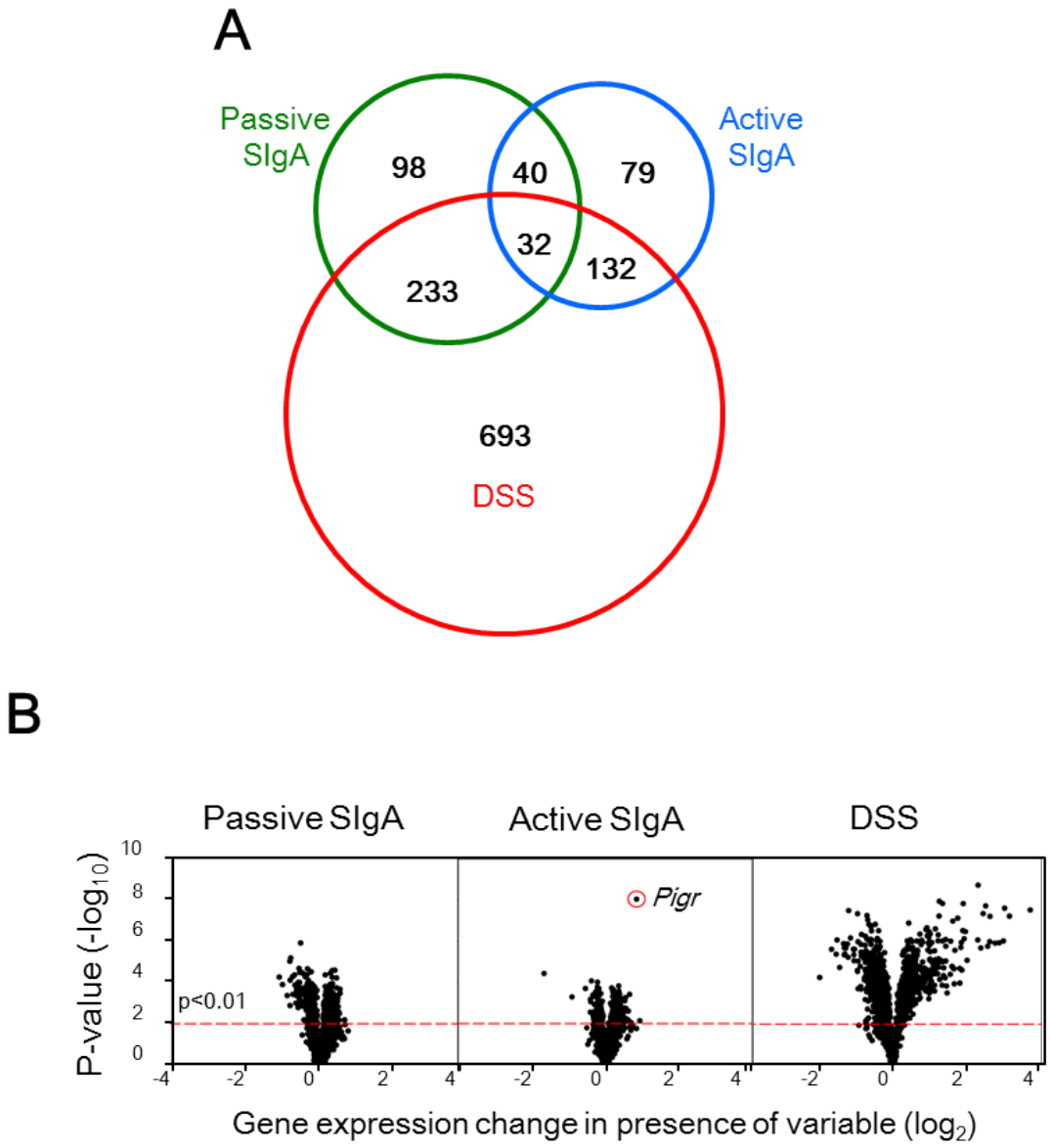


Figure 5.5. Receiving passive secretory IgA during suckling has more profound effect on colonic epithelial cell gene expression in adult mice than production of active secretory IgA. A) Proportional Venn diagram showing microarray analysis for each factor with quantities of highly significant ($p < 0.01$) changes in gene expression by 1 way interaction (see flow chart, Fig. 5.4). B) Volcano plots for each variable showing increases or decreases in gene expression with highly significant 2 way interaction ($p < 0.01$). y-axis plotted on $\log_{10}$ scale and x-axis on $\log_2$ scale.

Table 5.1: Indicated factor(s) upregulation (red) or downregulation (green) of genes with highly significant ($p < 0.01$) representation in biological pathway

Pathways affected by the presence of Passive SIgA		
Un-adjusted probability of seeing N or more genes in this Event by chance	Genes in query which map to this Event	Name of this Event
3.8e-04	Pold2, Pole2	Repair synthesis of patch ~27-30 bases long by DNA polymerase
3.8e-04	Pold2, Pole2	Repair synthesis for gap-filling by DNA polymerase in TC-NER
4.3e-04	Pold2, Pole2	Gap-filling DNA repair synthesis and ligation in GG-NER
4.3e-04	Pold2, Pole2	Gap-filling DNA repair synthesis and ligation in TC-NER
8.3e-04	Pold2, Pole2	Telomere C-strand (Lagging Strand) Synthesis
1.1e-03	Pold2, Pole2	Extension of Telomeres
1.9e-03	Pold2, Pole2	Global Genomic NER (GG-NER)
3.3e-03	Pold2, Pole2	Transcription-coupled NER (TC-NER)
3.6e-03	Arf1, Ap2a2	Nef Mediated CD4 Down-regulation
4.1e-03	Pold2, Pole2	Nucleotide Excision Repair
5.3e-03	Pold2, Pole2	Telomere Maintenance
5.7e-03	Arf1, Ap2a2	Nef-mediates down modulation of cell surface receptors by recruiting them to clathrin adapters
7.1e-03	Arf1, Ap2a2	The role of Nef in HIV-1 replication and disease pathogenesis
2.9e-03	Rps12, Rpl27a, Smg1	UPF1 Binds an mRNP with a Termination Codon Preceding an Exon Junction Complex
2.9e-03	Rps12, Rpl27a, Smg1	SMG1 Phosphorylates UPF1 (Enhanced by Exon Junction Complex)
3.4e-03	Rps12, Rpl27a, Smg1	Nonsense-Mediated Decay
3.4e-03	Rps12, Rpl27a, Smg1	Phosphorylated UPF1 Recruits SMG5, SMG7, SMG6, and PP2A
3.4e-03	Rps12, Rpl27a, Smg1	Nonsense Mediated Decay Enhanced by the Exon Junction Complex

Pathways affected by the presence of Active SIgA		
Un-adjusted probability of seeing N or more genes in this Event by chance	Genes in query which map to this Event	Name of this Event
9.5e-09	Olfr19, Olfr1385, Olfr582, Olfr706, Olfr171, Olfr1141, Olfr688, Olfr380, Olfr1090, Olfr1052, Olfr835	Olfactory Receptor - G Protein olfactory trimer complex formation
9.5e-09	Olfr19, Olfr1385, Olfr582, Olfr706, Olfr171, Olfr1141, Olfr688, Olfr380, Olfr1090, Olfr1052, Olfr835	Olfactory Signaling Pathway
3.0e-06	Olfr19, Olfr1385, Olfr582, Olfr706, Olfr171, Olfr1141, Olfr688, Olfr380, Olfr1090, Olfr1052, Olfr835	GPCR downstream signaling
5.1e-06	Olfr19, Olfr1385, Olfr582, Olfr706, Olfr171, Olfr1141, Olfr688, Olfr380, Olfr1090, Olfr1052, Olfr835	Signaling by GPCR
4.3e-04	Olfr19, Olfr1385, Olfr582, Olfr706, Olfr171, Olfr1141, Olfr688, Olfr380, Olfr1090, Olfr1052, Olfr835	Signal Transduction

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Pathways affected by the presence of DSS		
Un-adjusted probability of seeing N or more genes in this Event by chance	Genes in query which map to this Event	Name of this Event
6.9e-07	Psmd3, Nlrp3, Csf2rb, Cyba, Gbp2, Tyrobp, Stat1, Lcp2, Cd14, Il5ra, Sec61a2, Ube2s, Il6, Fcgr3, Lyn, Ifnar2, Ifitm1, Il1a, Icam1, Gbp4, Ifitm3, Tlr2, Ripk3, Lbp, Psmb3, Ripk1, Psmd2, Il1b, Calr, Eif4a3, Psmb9, Sar1b, Fcgr2b, Socs3, Eif4e3, Stat3, Ptafr, Gbp5, Irf8, Jak3, Cybb, Lgmn, Lilrb4, Zbp1, Ripk2, C3, Ncf1, Il1r2, Sh3kbp1, C1s	Immune System
1.7e-06	Eif4a3, Csf2rb, Gbp2, Stat1, Il5ra, Socs3, Eif4e3, Stat3, Ptafr, Irf8, Gbp5, Jak3, Il6, Lyn, Ifitm1, Ifnar2, Ripk2, Il1a, Icam1, Gbp4, Ifitm3, Il1r2, Il1b	Cytokine Signaling in Immune system
6.8e-06	Lyn, Csf2rb, Ripk2, Il1a, Stat1, Il5ra, Socs3, Stat3, Il1r2, Jak3, Il1b, Il6	Signaling by Interleukins
1.2e-05	Il1a, Il1r2, Il1b	Interleukin-1 receptor type 2 binds Interleukin 1
1.2e-05	Zbp1, Ripk1, Ripk3	DAI recruits RIP1 and RIP3
7.7e-05	Socs3, Stat3, Stat1, Il6	Interleukin-6 signaling
1.0e-04	Psmd3, Dapk2, Psmb9, Ptk2, H13, Tnf, Dynll1, Vim, Psmb3, Ripk1, Psmd2, Apaf1	Apoptosis
1.1e-04	Ncf1, Cyba, Cybb	Alkalization of the phagosomal lumen by NOX2
5.2e-04	Cd14, Lbp	LPS transferred from LBP carrier to CD14
5.2e-04	Cd14, Lbp	GPI-bound CD14 binds LPS
5.2e-04	Cd14, Lbp	Secreted CD14 binds LPS
5.2e-04	Stat3, Stat1	Phosphorylated STAT1, STAT3 form dimers
5.2e-04	Stat3, Stat1	STAT1/3 dimers translocate to the nucleus
6.1e-04	Stat3, Stat1, Il6	STATs bind gp130 phosphotyrosines
6.1e-04	Stat3, Stat1, Il6	Tyrosine phosphorylation of STATs by IL6 receptor
6.1e-04	Stat3, Stat1, Il6	Phosphorylated STATs are released
6.1e-04	Stat3, Stat1, Il6	Serine phosphorylation of STATs
6.1e-04	Ncf1, Cyba, Cybb	Cross-presentation of particulate exogenous antigens (phagosomes)
1.5e-03	Psmd3, Calr, Cyba, Psmb9, Psmb3, Ncf1, Psmd2, Sec61a2, Cybb	Antigen processing-Cross presentation
1.5e-03	Il1a, Il1b	Interleukin-1 receptor type 1 binds Interleukin 1
1.5e-03	Il1a, Il1b	Interleukin-1 family are secreted
1.7e-03	Lyn, Socs3, Stat3, Stat1	Growth hormone receptor signaling
1.7e-03	Zbp1, Ripk1, Ripk3	RIP-mediated NFkB activation via DAI
1.9e-03	Ifnar2, Ifitm1, Eif4a3, Icam1, Gbp2, Ifitm3, Gbp4, Stat1, Socs3, Eif4e3, Ptafr, Irf8, Gbp5	Interferon Signaling
2.2e-03	Lyn, Stat3, Stat1	Recruitment of STATs
2.2e-03	Lyn, Stat3, Stat1	Phosphorylation of STATs
2.2e-03	Lyn, Stat3, Stat1	Dimerization of STATs
2.2e-03	Lyn, Stat3, Stat1	Disassociation and translocation of STATs to the nucleus
2.9e-03	Lyn, Itga2, Fcer1g	Platelet Adhesion to exposed collagen
2.9e-03	Zbp1, Ripk1, Ripk3	DAI mediated induction of type I IFNs
2.9e-03	Zbp1, Ripk1, Ripk3	Cytosolic sensors of pathogen-associated DNA
3.0e-03	Plat, Pdgfc	Extracellular processing of novel PDGFs
3.0e-03	Cd47, Ptk2	Phosphorylation of ITIM in SIRP alpha
3.0e-03	Tyrobp, Sema6d	Sema6D binds PlexinA1-Trem2-DAP12
3.0e-03	Lyn, Fcer1g	GPVI binds Fyn and Lyn
3.0e-03	Il1a, Il1b	Interleukin-1 receptor type 1: Interleukin 1 binds Interleukin-1 receptor accessory protein, membrane associated isoform
3.0e-03	Il1a, Il1b	Interleukin-1 family precursors are cleaved by caspase-1
3.0e-03	Il1a, Il1b	Interleukin-1 processing
3.0e-03	Il5ra, Csf2rb	Interleukin-5:Interleukin-5 receptor alpha binds IL3RB:JAK2
3.4e-03	Xpo1, Psmd3, Psmb3, Dcp2, Psmd2, Psmb9, Dis3	Regulation of mRNA Stability by Proteins that Bind AU-rich Elements
5.0e-03	Ripk1, Tnf	TRADD:TRAF2:RIP1 complex dissociates from the TNF-alpha:TNF-R1 complex.
5.0e-03	Ripk1, Tnf	TNF:TNF-R1 binds TRADD, TRAF2 and RIP Complex
5.0e-03	Ifitm1, C3	C3d-complexed antigen binds to complement receptor
5.0e-03	Il1a, Il1b	IL1R1:IL1:IL1RAP binds MYD88 homodimer

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Pathways affected by the presence of DSS, continued		
Un-adjusted probability of seeing N or more genes in this Event by chance	Genes in query which map to this Event	Name of this Event
5.3e-03	Cd47, Tyrobp, Ptk2	Signal regulatory protein (SIRP) family interactions
5.8e-03	Atic, Ada, Xdh, Gda	Purine metabolism
6.4e-03	Il5ra, Csf2rb, Jak3	Phosphorylated SHC1 recruits GRB2:GAB2
6.4e-03	Il5ra, Csf2rb, Jak3	Phosphorylated SHC recruits GRB2:SOS1
6.4e-03	Il5ra, Csf2rb, Jak3	SHC1 mediates cytokine-induced phosphorylation of GAB2
6.4e-03	Il5ra, Csf2rb, Jak3	Phosphorylated SHC1 recruits SHIP
6.4e-03	Il5ra, Csf2rb, Jak3	The SHC1:SHIP1 complex is stabilized by GRB2
7.4e-03	Lyn, Fcer1g	Binding of GPVI:Fc Epsilon R1 gamma receptor complex with collagen
7.4e-03	Lyn, Fcer1g	Fyn/Lyn-mediated phosphorylation of FcR1 gamma
7.4e-03	Il1a, Il1b	IL1R1:IL1:IL1RAP:MYD88 homodimer binds IRAK4
7.4e-03	Stat3, Stat1	JAK2 phosphorylates STAT1/STAT3
7.4e-03	Stat3, Stat1	JAK2 binds STAT1/3
7.4e-03	Socs3, Ptafr, Irf8, Icam1, Gbp5, Gbp2, Gbp4, Stat1	Interferon gamma signaling
7.5e-03	Il5ra, Csf2rb, Jak3	SOS1 activates H-Ras
9.2e-03	Ifnar2, Ifitm1, Socs3, Irf8, Gbp2, Ifitm3, Stat1	Interferon alpha/beta signaling
1.9e-07	Acss2, Rbp2, Prkar2b, Ddc, Acer1, Slc27a1, Pccb, Pfkm, Itpr2, Pdk2, Pdxk, Adh1, Alb, Fmo1, Cyp2s1, Gpt, Acsl1, Aldh6a1, Papss1, Hexb, Lrat, Maob, Gnb5, Glul, Clps, Sult2b1, Slc46a1, Cth, Hk1, Slc6a8, Pnpo, Nnt, Csad, Gba2, Gstm1, Hsd3b7, Itpr1, Cps1, Sult1a1, Cdo1, Plcb1, Ccbl1, Pfkfb4, Pck1	Metabolism
2.7e-05	Stim1, Itpr2, Itpr1	Oligomerization of STIM1
2.3e-04	Rbp2, Lrat, Clps	Vitamin A uptake in enterocytes
3.6e-04	Cth, Csad, Cdo1	Degradation of cysteine and homocysteine
3.6e-04	Pdxk, Pnpo	Vitamins B6 activation to pyridoxal phosphate
3.6e-04	Rbp2, Lrat	Esterification of retinol
5.3e-04	Stim1, Itpr2, Itpr1	Elevation of cytosolic Ca2+ levels
1.1e-03	Itpr2, Itpr1	Transport of Ca++ from platelet dense tubular system to cytoplasm
1.1e-03	Itpr2, Itpr1	Binding of IP3 to IP3 receptor
1.1e-03	Itpr2, Itpr1	IP3 binds to the IP3 receptor, opening the endoplasmic reticulum Ca2+ channel
1.1e-03	Itpr2, Itpr1	Release of calcium from intracellular stores by IP3 receptor activation
1.1e-03	Itpr2, Itpr1	Opening of ER calcium channels by activated PKA
1.2e-03	Itpr2, Itpr1, Prkcd, Dgkh	Effects of PIP2 hydrolysis
1.2e-03	Fmo1, Acss2, Gstm1, Cyp2s1, Sult1a1, Sult2b1, Papss1, Adh1, Maob	Biological oxidations
1.3e-03	Itpr2, Prkar2b, Plcb1, Itpr1, Prkcd	PLC beta mediated events
1.4e-03	Itpr2, Prkar2b, Plcb1, Itpr1, Prkcd	G-protein mediated events
1.7e-03	Sult2b1, Papss1, Sult1a1	Cytosolic sulfonation of small molecules
2.7e-03	Itpr2, Prkar2b, Itpr1, Prkcd	DAG and IP3 signaling
3.2e-03	Glul, Ccbl1, Gpt	Amino acid synthesis and interconversion (transamination)
3.4e-03	Itpr2, Prkar2b, Itpr1, Prkcd	PLC-gamma1 signalling
3.4e-03	Itpr2, Prkar2b, Itpr1, Prkcd	EGFR interacts with phospholipase C-gamma
3.4e-03	Ddc, Glul, Cps1, Gpt, Cdo1, Aldh6a1, Cth, Slc6a8, Ccbl1, Csad	Metabolism of amino acids and derivatives
3.5e-03	Smad7, Smad6	I-SMAD competes with SMAD2/3 for type I receptor (TGFBR1)
3.5e-03	Smad7, Smad6	I-Smad competes with Co-Smad for R-Smad1/5/8
3.5e-03	Itpr2, Prkar2b, Itpr1, Prkcd, Fgfr3	Phospholipase C-mediated cascade
3.8e-03	Itpr2, Prkar2b, Itpr1, Prkcd	PLCG1 events in ERBB2 signaling
4.1e-03	Itpr2, Prkar2b, Plcb1, Itpr1, Prkcd, Gnb5	Opioid Signalling
5.2e-03	Kit, Grb7	Interaction of other adapter proteins with p-KIT
5.3e-03	Rhou, Rhot2, Rhoc	Rho GTPase:GTP activates downstream effectors
5.3e-03	Stim1, Itpr2, Itpr1	Platelet calcium homeostasis
6.2e-03	Slc46a1, Steap3, Atp6v1e1, Mcoln1	Iron uptake and transport
8.5e-03	Nr4a1, Itpr2, Prkar2b, Itpr1, Prkcd, Grb7	Downstream signal transduction
8.8e-03	Itpr2, Prkar2b, Itpr1, Gnb5	Regulation of Insulin Secretion by Glucagon-like Peptide-1
9.4e-03	Fmo1, Acss2, Cyp2s1, Adh1, Maob	Phase 1 - Functionalization of compounds
9.4e-03	Smad7, Smad6	I-Smad competes with R-Smad1/5/8 for type I receptor

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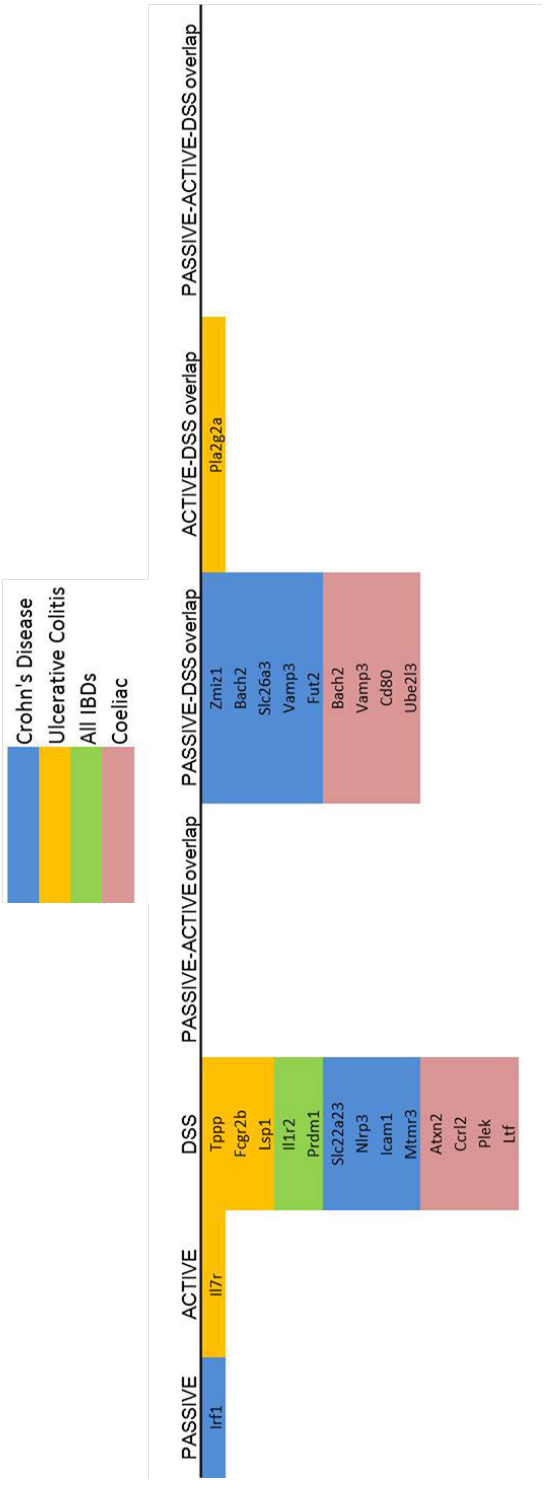
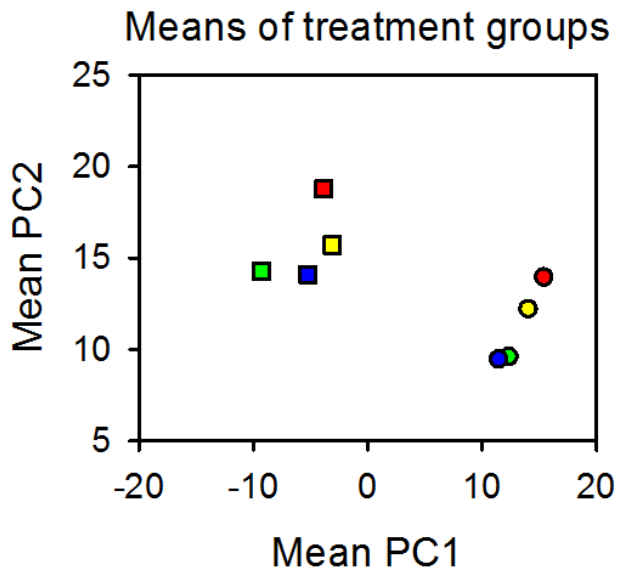
Pathways affected by the presence of Passive SIgA and DSS		
Un-adjusted probability of seeing N or more genes in this Event by chance	Genes in query which map to this Event	Name of this Event
1.3e-05	Rpn1, Ddost, Rpn2	Transfer of N-glycan to the protein
1.6e-05	Rpn1, B3gnt3, Alg9, Cct6a, Ddost, Alg8, Ssr3, Pigu, Spcs3, Sec61a1, Tuba1b, Hspd1, Rpn2, Alg3	Metabolism of proteins
9.0e-05	Rpn1, Alg9, Ddost, Alg8, Rpn2, Alg3	Asparagine N-linked glycosylation
1.7e-04	Rpn1, B3gnt3, Alg9, Ddost, Alg8, Pigu, Rpn2, Alg3	Post-translational protein modification
2.8e-04	Spcs3, Sec61a1, Rpn1, Ddost, Ssr3, Rpn2	Signal peptide cleavage from ribosome-associated nascent protein
4.5e-04	Spcs3, Sec61a1, Rpn1, Ddost, Ssr3, Rpn2	SRP-dependent cotranslational protein targeting to membrane
8.5e-04	Psme3, Ccnd1, Psmb2, Psmd14	Proteasome mediated degradation of Cyclin D1
9.2e-04	Psme3, Ccnd1, Psmb2, Psmd14	Ubiquitin-dependent degradation of Cyclin D1
9.2e-04	Psme3, Ccnd1, Psmb2, Psmd14	Ubiquitin-dependent degradation of Cyclin D
1.0e-03	Psme3, Ccnd1, Psmb2, Rrm2, Psmd14, Lin54	Mitotic G1-G1/S phases
1.1e-03	Psme3, Psmb2, Chek1, Psmd14	Ubiquitin Mediated Degradation of Phosphorylated Cdc25A
1.1e-03	Psme3, Psmb2, Chek1, Psmd14	p53-Independent DNA Damage Response
1.1e-03	Psme3, Psmb2, Chek1, Psmd14	p53-Independent G1/S DNA damage checkpoint
1.8e-03	Psme3, Psmb2, Chek1, Psmd14	G1/S DNA Damage Checkpoints
2.0e-03	Spcs3, Sec61a1, Rpn1, Ddost, Ssr3, Rpn2	Translation
2.2e-03	Alg9, Alg8, Alg3	Biosynthesis of the N-glycan precursor (dolichol lipid-linked oligosaccharide, LLO) and transfer to a nascent protein
2.6e-03	Sec61a1, Rpn1, Ddost, Ssr3, Rpn2	Translocation of signal-containing nascent peptide to Endoplasmic Reticulum
4.8e-03	Aaas, Eno1, Calm3, Prps1, Pklr	Metabolism of carbohydrates
6.6e-03	Psme3, Psmb2, Psmd14	Proteasomal clevage of exogenous antigen
6.7e-03	Psme3, Psmb2, Elavl1, Psmd14	Regulation of mRNA Stability by Proteins that Bind AU-rich Elements
7.1e-03	Psme3, Psmb2, Psmd14	Destruction of AUF1 and mRNA
7.4e-03	Srm, Mtap	Metabolism of polyamines
8.5e-03	Psme3, Psmb2, Psmd14	26S proteosome degrades ODC holoenzyme complex
8.5e-03	Psme3, Psmb2, Psmd14	Proteasomal cleavage of substrate
8.5e-03	Psme3, Psmb2, Psmd14	Proteasomal cleavage of substrate
9.0e-03	Psme3, Psmb2, Psmd14	Ubiquitinated geminin is degraded by the proteasome
9.0e-03	Psme3, Psmb2, Psmd14	Ubiquitinated Orc1 is degraded by the proteasome
9.0e-03	Psme3, Psmb2, Psmd14	Ubiquitinated Cdc6 is degraded by the proteasome
9.0e-03	Psme3, Psmb2, Psmd14	Proteolytic degradation of ubiquitinated-Cdc25A
9.0e-03	Psme3, Psmb2, Psmd14	Proteasome mediated degradation of PAK-2p34
9.0e-03	Psme3, Psmb2, Psmd14	Regulation of activated PAK-2p34 by proteasome mediated degradation
9.0e-03	Psme3, Psmb2, Psmd14	Proteasome mediated degradation of PAK-2p34
9.0e-03	Psme3, Psmb2, Psmd14	Proteasome mediated degradation of COP1
9.0e-03	Psme3, Psmb2, Psmd14	Cross-presentation of soluble exogenous antigens (endosomes)
9.5e-03	Psme3, Psmb2, Psmd14	CDK-mediated phosphorylation and removal of Cdc6
9.5e-03	Psme3, Psmb2, Psmd14	Regulation of ornithine decarboxylase (ODC)
9.5e-03	Psme3, Psmb2, Psmd14	Degradation of Ubiquitinated IkB (B cell)
3.8e-04	Foxo3, Akt3	AKT can phosphorylate forkhead box transcription factors
8.6e-04	Irs2, Foxo3, Akt3	PI3K/AKT activation
9.1e-04	Foxo3, Akt3	AKT phosphorylates targets in the nucleus
1.4e-03	Foxo3, Akt3, Erbb3	PI3K events in ERBB2 signaling
1.9e-03	Akt3, Erbb3	Downregulation of ERRB2:ERBB3 signaling
2.2e-03	Nr1d1, Nr3c2, Vdr	Formation of NR-MED1 Coactivator Complex
2.2e-03	Nr1d1, Nr3c2, Vdr	Nuclear Receptor transcription pathway
2.3e-03	Nr1d1, Ccrn4l	BMAL1:CLOCK/NPAS2 Activates Circadian Expression
5.1e-03	Irs2, Foxo3, Akt3, Plekhg5, Kalrn	Signalling by NGF
9.0e-03	Foxo3, Akt3	PIP3 activates AKT signaling

Pathways affected by the presence of Active SIgA and DSS		
Un-adjusted probability of seeing N or more genes in this Event by chance	Genes in query which map to this Event	Name of this Event
7.5e-03	Agpat3, Gpam	Triglyceride Biosynthesis
9.1e-03	Agpat3, Gpam, Ugt1a9	Fatty acid, triacylglycerol, and ketone body metabolism

Table 5.2: Known Human Gene Polymorphisms Associated with IBDs and Coeliac Disease that were Significantly Changed ($p < 0.01$) by Indicated Factor(s) (See Fig. 5.5A)



A

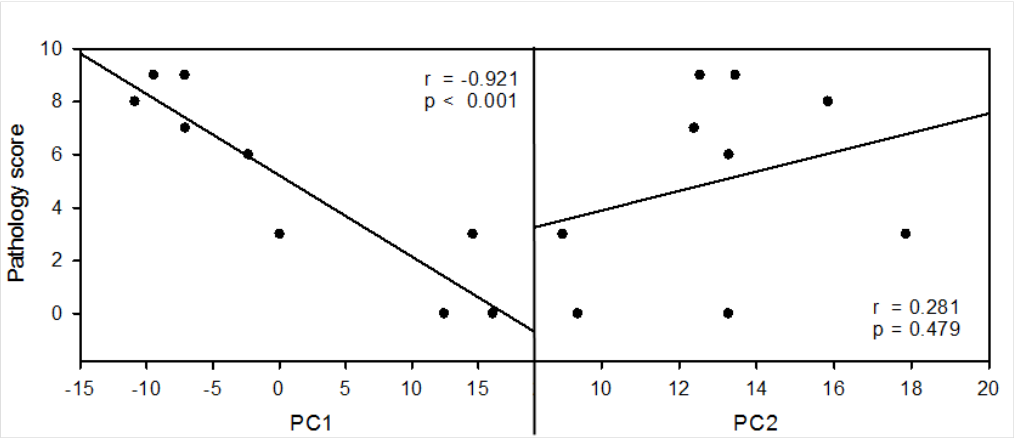


Dam *Pigr*	Pup *Pigr*	DSS
+/-	+/-	-
+/-	-/-	-
-/-	+/-	-
-/-	-/-	-
+/-	+/-	+
+/-	-/-	+
-/-	+/-	+
-/-	-/-	+

Analysis of Variance

Factor	P-Value PC1	P-Value PC2
Dam *Pigr* genotype	0.023	< 0.001
Pup *Pigr* genotype	0.652	0.263
DSS treatment	< 0.001	< 0.001

B



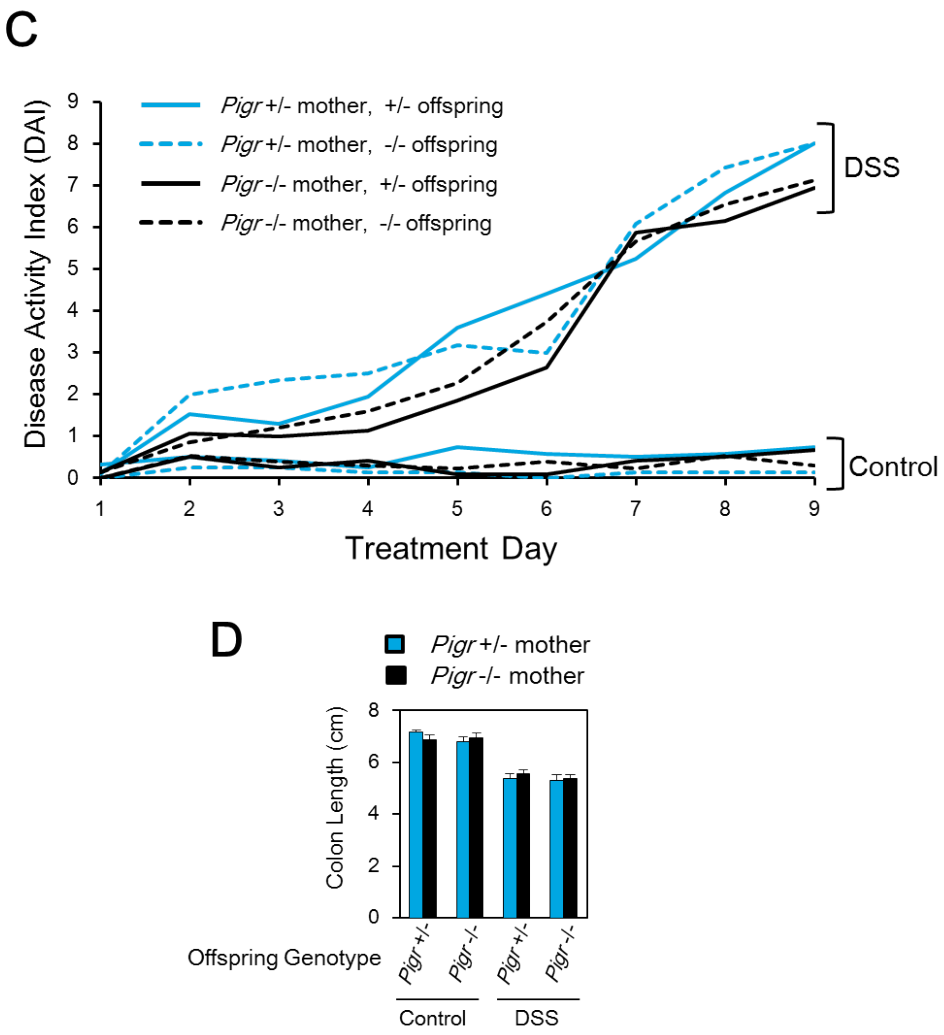


Figure 5.6. Colonic epithelial cell gene expression during experimental colitis more dependent upon receiving secretory IgA during suckling than ability of mouse to produce active secretory IgA. A) PCoA clustering of colonic epithelial cell gene expression, as determined from Nanostring nCounter survey, from 8-10week adult mice in breeding scheme with or without DSS treatment (n=6 per group). Analysis of variance table shows significant changes in both principal coordinates with respect to maternal genotype and DSS treatment, but not mouse genotype. B) Strong negative correlation of PCoA1 with microscopic pathological scoring of mouse colons C) Disease activity index (also, DAI) of mice during DSS regiment based on summation of scores for weight loss, stool consistency, and presence of occult or visible blood (1). No significant differences between maternal or offspring genotype for each timepoint. Data represent mean of n=8-15 mice from 5-8 litters per timepoint. D) Colon shortening as a result of DSS treatment (mean ± SEM, n=8-15 mice from 5-8 litters per timepoint).

Table 5.3: Selected Genes from 3-Way Factor Analysis Validated by Nanostring

PASSIVE	GO Biological Process Term
Herc2	protein modification process // spermatogenesis // protein ubiquitination // modification-dependent protein catabolic process // regulation of mitotic metaphase/anaphase transition
Arf1	transport // small GTPase mediated signal transduction // protein transport // vesicle-mediated transport
Ap2a2	transport // intracellular protein transport // endocytosis // protein transport // vesicle-mediated transport
Irf1	transcription // regulation of transcription, DNA-dependent // regulation of gene expression // CD8-positive, alpha-beta T cell differentiation
ACTIVE	**GO Biological Process Term**
Akt1	blood vessel development // placenta development // carbohydrate metabolic process // glycogen metabolic process // glycogen biosynthetic process // glucose metabolic process
DSS	**GO Biological Process Term**
Reg3g	acute-phase response // inflammatory response
Tppp	tubulin polymerization promoting protein // microtubule assembly
Tnf	negative regulation of transcription from RNA polymerase II promoter // regulation of protein amino acid phosphorylation // glucose metabolic process // induction of apoptosis
Smad7	transcription // regulation of transcription, DNA-dependent // transforming growth factor beta receptor signaling pathway
Socs3	regulation of protein amino acid phosphorylation // intracellular signaling cascade // negative regulation of signal transduction // modification-dependent protein catabolic process
Stat1	transcription // regulation of transcription, DNA-dependent // signal transduction // cytokine-mediated signaling pathway // lipopolysaccharide-mediated signaling pathway
Noxo1	superoxide metabolic process // cell communication
Sycn	exocytosis
Arf2	transport // small GTPase mediated signal transduction // protein transport // vesicle-mediated transport
Ccl3	chemotaxis // inflammatory response // immune response
Cxcl2	chemotaxis // inflammatory response // immune response
Il1a	fever // inflammatory response // immune response // cell proliferation // positive regulation vascular endothelial growth factor production // positive regulation of angiogenesis
Il1b	fever // inflammatory response // immune response // cell proliferation // positive regulation vascular endothelial growth factor production // negative regulation of glucose transport
Il1r2	receptor for IL1
Prdm1	in utero embryonic development // embryonic placenta development // maternal placenta development // transcription // regulation of transcription, DNA-dependent
Reg3b	acute-phase response // inflammatory response
Stat3	temperature homeostasis // eye photoreceptor cell differentiation // transcription // regulation of transcription, DNA-dependent
Tlr2	response to molecule of fungal origin // cell surface pattern recognition receptor signaling pathway // MyD88-dependent toll-like receptor signaling pathway // inflammatory response
Ppargc1a	transcription // regulation of transcription, DNA-dependent // respiratory electron transport chain // positive regulation of transcription
Icam1	T cell antigen processing and presentation // cell adhesion // leukocyte adhesion // cell-cell adhesion // regulation of cell adhesion // cell adhesion mediated by integrin
Nlrp3	apoptosis // activation of caspase activity // interleukin-18 production // interleukin-1 secretion // defense response to virus
PASSIVE-ACTIVE	**GO Biological Process Term**
Ccl5	chemotaxis // inflammatory response // immune response // response to cytokine stimulus // response to tumor necrosis factor
Syt7	plasma membrane repair // transport
PASSIVE-DSS	**GO Biological Process Term**
Vamp3	vesicle-mediated transport // calcium ion-dependent exocytosis
Slc26a3	transport // sulfate transport
Klf4	transcription // regulation of transcription, DNA-dependent // negative regulation of cell proliferation // epidermal cell differentiation // stem cell maintenance // cell differentiation
Foxo3	ovulation from ovarian follicle // initiation of primordial ovarian follicle growth // antral ovarian follicle growth // oocyte maturation // regulation of transcription, DNA-dependent
Atg7	liver development // protein amino acid lipidation // amino acid metabolic process // transport // autophagy // organelle organization // adult walking behavior
Il17rc	biological_process
Bach2	transcription // regulation of transcription, DNA-dependent
Zmiz1	vasculogenesis // in utero embryonic development // heart morphogenesis // transcription // regulation of transcription, DNA-dependent // vitellogenesis // cell aging
Fut2	carbohydrate metabolic process // protein amino acid glycosylation
Ube2l3	modification-dependent protein catabolic process // post-translational protein modification // regulation of protein metabolic process
Atg16l2	transport // autophagy // protein transport
ACTIVE-DSS	**GO Biological Process Term**
Pla2g2a	phospholipid metabolic process // negative regulation of cell proliferation // lipid catabolic process // somatic stem cell maintenance // regulation of growth // regulation of cell proliferation

Chapter 6

DISCUSSION AND FUTURE DIRECTIONS

The human body employs numerous redundant mechanisms in which to sense the bacterial inhabitants within the GI tract. These microbial colonizers are with us from the first seconds of life and persist in our bodies until we perish. This colonization is a potentially advantageous event for both host and immigrant. The immigrant benefits through the habitation of a niche environment which is rich in nutrients, warm, and at low oxidative stress. The profits gained by the human host are much more relevant in the field of the medical sciences and have multiple known avenues: higher caloric intake, stimulation of innate immune effectors, assisted maturation of the adaptive immune system, and colonization resistance of pathogens (162). With recent elucidation of the benefits exchanged between the two parties, their relationship nomenclature is quickly shifting from 'commensal', meaning two that eat from the same table (292), to 'mutualist', indicating exchanged services between the different organisms (96, 293, 294). Poor or contentious communication between these two parties is detrimental to both host and mutualist, and can quickly lead to the death of both organisms.

Along the entire length of the GI tract, microbes or their metabolic products first come into contact with the host at the GI epithelium. For this reason, the epithelial cell has the important role of being the "first responder" to microbial presence and deciding what actions to take not only for itself and other contiguous epithelial cells, but in the production of molecules that will affect both host and microbiota. The work presented in this dissertation focuses on mechanisms used by this cell type to appropriately respond to the continual company of bacteria at the oral and intestinal epithelium. We expound and add information to well-known mechanisms of recognition such as modulation of epithelial gene expression and cytokine secretion by bacteria and their products. Novel biological phenomena pertaining to mucosal immunology are also presented which include: inhibition of NF-κB signaling in oral epithelial cells by the hormone adiponectin, lowered expression of AdipoR2 in inflamed IBD patients' intestine, changes in crypt localization of epithelial pIgR expression patterns along the length of the colon, meticulous localization of the immune proteins SIgA and SC in the colonic outer mucus layer, necessity of SC binding to IgA in milk for transit through the entire GI tract, SIgA in hindrance of *Ochrobactrum anthropi* translocation to neonatal MLNs, a unique microbiota consortium as neonates and adults depending upon receiving passive SIgA, and maternally-

supplied passive SIgA and a stronger moderator of adult CEC gene expression versus endogenously-produced active SIgA.

Bacterial-induced regulation of intracellular signaling pathways, gene expression, and production of immune molecules in the epithelial cell. The current paradigm of the epithelial innate immune response to bacteria hinges around the concept of PRR-induced recognition of the microbe and the resulting activation of intracellular signaling pathways (46, 49, 295). Humans (296-298) and animals (299-301) deficient for these very basic bacterial recognition systems consistently present with increased susceptibility to excessive inflammation, especially at high bacterial-density mucosal sites along the GI tract. The direct microbial etiology for these inflammatory states is further supported by the antibiotic standard of care, which aids in amelioration of inflammation. Moreover, transgenic animals that would be susceptible to hyperinflammation under conventionally-raised conditions reliably show mitigated inflammation when reared under germ-free conditions (96, 150). But unabated inflammation is simply one way in which the host can inappropriately respond to the microbe. When reacting to microbial presence, the opposite of overreacting with injurious inflammation is not reacting at all. Severely immunocompromised humans and laboratory animals become systemic culture tubes for opportunistic bacteria and display strong predilections for bacteremia and associated infections (302). We propose a novel mechanism potentially utilized by human CECs *in vivo* in order to neither simply promote nor discourage a response to MAMPs from commensal bacteria. Because resident bacteria need to be responded to, but not overemphasized, we are labeling this phenomena as the "appropriate inflammatory" response. The molecule responsible for the initiation of the appropriate inflammatory response is the adipokine adiponectin, which is found at relatively high levels in human systemic circulation in the oligomerized high-molecular weight (HMWAd) form (169). By itself, HMWAd had little effect on human oral and intestinal epithelial cell lines, but in combination with multiple types of bacterial products which were capable of activation of multiple TLRs, the epithelial cells showed significant increases over sole TLR stimulation in gene expression and secretion for many pro-inflammatory cytokines and chemokines. In addition, the host-derived cytokine TNF was also able to synergize with HMWAd-induced signaling to further increase inflammatory gene expression and cytokine output over either of the stimulants alone. Similar findings of HMWAd as showing a pro-inflammatory nature has been seen in other human cell types (122, 123). At the epithelial interface, TNF is readily generated through PRR signaling, so HMWAd induction of the inflammatory response can be beneficial for the host, in the quick and sensitive detection and response to pathogenic or mutualistic microbes and their products. Concurrent with the increase in pro-inflammatory

mediators, the combination of TLR ligands and TNF with HMWAd and simply HMWAd by itself was able to increase production of molecules that function to shut off inflammatory signaling within the cell. Most strikingly in oral epithelial cells, gene expression of A20, which stymies the NF-κB heterodimer from entering the nucleus and inducing gene transcription, was increased by orders of magnitude with stimulation with HMWAd alone, or in combination with LPS or TNF. The NF-κB reverse shuttling protein, PPARγ was also found to be at significantly higher levels in intestinal epithelial cells following combined HMWAd and LPS stimulation. Data from other groups correlating low human serum levels of HMWAd with increased systemic presence of pro-inflammatory molecules in humans (210, 212, 303) shows the potential causative nature of one event leading to the other. In our experiments, we specifically saw the increase of NF-κB inhibitors in oral epithelial cells lead to reduced capacity of long-term continuation of the NF-κB signaling cascade. As copious research has shown the NF-κB pathway in the regulation of both pro- and anti-inflammatory signaling in epithelial cells (53), and since adiponectin receptors, TLRs, and the TNF receptor all utilize the NF-κB signaling cascade, perhaps continual intracellular interplay and pathway formatting exists when two or more of these receptor types are activated at the same time. Though tremendous increases in expression of A20 were seen in oral epithelial cells, we cannot exclusively attribute the decrease in NF-κB activity to this single regulator. Negative feedback in the CEC by the combined stimulation of adiponectin and Toll-like or TNF receptors could take numerous other forms. Future experiments should investigate expression levels for TLRs and TNFR1 following HMWAd pretreatment of the cells, since lower quantities of these receptors could lead the epithelial cells to more of an 'immune ignorant' state. We did not observe changes in expression of the two putative adiponectin receptors to our stimulants *in vitro*, but this does not rule out *in vivo* occurrence of this event. Additionally, the multitude of immune signaling regulators for TLRs (MyD88s, IRAK-M, TOLLIP, SIGGR, SOCS proteins) (56), TNFR1 (ARTS-1, TRAF1) (304, 305), and general NF-κB regulators (PI3K signaling, NIK, IKK-γ) (306) should also be assayed for abundance and intracellular activity. The concentrations of the stimulants used in this study were based upon normal physiological concentrations of HMWAd and typical amounts of TLR and TNFR ligands used for cell culture experiments. While we made concerted efforts to not present data on stimulatory (or inhibitory) capacity based upon variable concentrations, this is not representative of biological situations where HMWAd, TNF, and bacterial products are constantly in flux with proximity to epithelial cells. Variable concentrations of all three of these cell stimulants as well as time points for

stimulant exposure would give valuable information on the dynamics of physiological outcomes for different situations the host epithelium may be placed in.

Adiponectin was initially defined as a metabolic hormone in the late twentieth century with the striking finding that patients presenting as obese with or without metabolic syndrome comorbidity showed paucity of the protein in serum (307). This sole paradigm for adiponectin function persisted, even after the creation of a knockout (KO) mouse less than a decade ago (308). Only in the past few years has the adiponectin KO mouse been utilized for immunological studies, almost exclusively in the field of intestinal immunity (139, 141-143). Future experiments should selectively analyze epithelial cells along multiple bacteria-inundated sites of the GI epithelium in the adiponectin KO mouse to see if discriminatory activation or inactivation of inflammatory pathways occurs in the presence/absence of bacteria (even inoculation with specific mutualists or pathogens) when the animal lacks the adiponectin protein. In addition, our experiments clearly showed the abundant presence of both adiponectin receptors along the human GI epithelium, but selective downregulation of AdipoR2 in the inflamed colonic epithelium of the IBD patient. Our data showed statistical significance for AdipoR2 downregulation in both Crohn's Disease and ulcerative colitis patients, but we failed to assess protein levels for this receptor or other intracellular signaling molecules linked to adiponectin signaling. In addition, we also worked with a geographically homogenous patient population, and had a relatively low number of patients recruited for this study. Epidemiological studies should seek to assess if correlations exist between uncontrolled inflammatory events and the absence of HMWAd or its receptors in humans, especially if the state of inflammation is known to have at least a partial bacterial origin.

Depending on the contemporary working definition of bacterial species (also, OTU and phylotype), between 500 and 20,000 unique bacteria inhabit our intestinal tracts (309). In Chapter 4, we investigated the responsiveness of intestinal epithelial cells to an assortment of these microbes from the four dominant phyla which inhabit the GI tract. The HT-29 human intestinal epithelial cell line was selected for our studies due to its prolific use as a model of intestinal epithelial cells and its well-known regulation of pIgR production (57, 264, 265, 269). Using eight different species found regularly in human intestines and one pathogen, we found very few bacteria were able to stimulate gene expression and chemokine release from the HT-29 intestinal cells. In fact, of all bacteria used, only species within the family *Enterobacteriaceae* were able to provide any profound stimulation of gene expression in the HT-29 cell line, and constituted the majority of increased gene expression in another human intestinal epithelial cell line, CaCo-2 cells. While not a complete representation of intestinal epithelial cells in a higher

vertebrate, the common trend of unique bacteria being emphatically recognized by multiple intestinal cell lines points to a recognition priority of certain bacteria over others. Both the mutualist *E. coli* and pathogenic *S. typhimurium* share very common ligands for Toll-like receptors, including a hexyl-acetylated Lipid A moiety on their LPS (strong ligand for TLR4) and flagellin with TLR5 stimulatory capacity (47, 310). Multiple human enteric pathogens reside within the *Enterobacteriaceae* family (311), so it may be an evolutionary result of the default host survival mechanism for the intestinal epithelium to be able to quickly respond to interaction with bacteria from this group, whether friend or foe. The phenomena of endotoxin tolerance through PRR signaling relates to the concept of common mutualist/pathogen recognition by the host and may explain why mutualistic *Enterobacteriaceae* can be readily found in the oral and intestinal microbiota. Upon prolonged stimulation of PRRs, fresh receptor ligands are unable to elicit the magnitude of response at the transcriptional level as naïve cells seeing the stimulants for the first time (312, 313). Many of these "tolerizable" genes are pro-inflammatory, while "non-tolerizable" genes that are continually produced regardless of duration of PRR stimulation include many anti-inflammatory genes as well as AMPs (314, 315). It would then be advantageous to the host to foster a regulated population of *Enterobacteriaceae* lacking virulence factors, but still retaining MAMPs which would "tolerize" pro-inflammatory gene expression so excessive inflammation does not occur upon introduction of *Enterobacteriaceae* pathogens. This gives credence to the manufacturing of probiotic *Enterobacteriaceae*, such as *E. coli* strain Nissle (316), but due to inadequate education, the public's mind must change before their intestines can.

While the *Enterobacteriaceae* were strongly recognized by intestinal epithelial cells, the majority of other bacteria used in the study were seemingly ignored by the eukaryotic cells in both pro- and anti-inflammatory gene expression. In addition, when we performed co-stimulation experiments with *Ec*N in combination with another mutualist, we found no significant changes in pIgR or Il-8 gene expression over *Ec*N stimulation alone. Of course, this does not accurately represent the intestinal environment where hundreds if not thousands of bacteria and their MAMPs would be in close contact with the epithelial cell. Immune cells would also be in close proximity with the epithelial cell along the entire length of the GI tract. When stimulating the human THP-1 monocyte cell line with the same bacteria used for the HT-29 cells, we saw the ability of a more professional immune cell to respond to all bacteria used, regardless of phyla. With our epithelial cells, we also did not perform any repeated stimulations in this experimental model, so perhaps initial treatment with commensal(s) followed by stimulation with *Enterobacteriaceae* would show differing, and possibly more subdued gene expression patterns. For our study, a very limited number of genes were assayed for changes in expression, and more

thorough efforts have been made by other groups to characterize the stimulatory capacity of other mutualistic bacteria. However, the genes selected for our research are known to be some of the more important effectors produced by the intestinal epithelial cell. Specifically, the pIgR gene is almost exclusively expressed along the body's epithelia, which apparently correlates with higher vertebrates' necessity for IgA and IgM transport across the low-permeability epithelial barrier (99). However, pIgR orthologs have been found to be more evolutionarily ancient than IgA, so it is probable that pIgR not only plays a role in other Ig isotype transport, but also in other innate immune functions (112, 317). Consistent with previous findings from our group and others, elevated pIgR expression in intestinal epithelial cells required continuous, prolonged stimulation (255). We found that *Enterobacteriaceae* were unable to highly-upregulate pIgR expression if only in contact with epithelial cells for 3h, regardless if the epithelial cells were harvested immediately or 21h after contact with bacteria. This mirrors the situation within the host, where bacteria and their MAMPs are continuously present, thus continually promoting robust pIgR expression in the epithelium. If these products from *Enterobacteriaceae* had a strong affect upon pIgR production *in vivo*, it would be predicted that differential pIgR expression patterns on the underlying epithelium would be seen in connection with the overlying resident *Enterobacteriaceae* population. Our visualization of crypt pIgR expression in different locations of the colon showed a direct connection between pIgR expression on the apical crypt cells nearest the microbiota and the amount of resident *Enterobacteriaceae* (9, 38). In areas with fewer amounts of these bacteria, CECs producing pIgR were generally found in the middle or at the bottom of the crypt, indicating a mechanism of constitutive expression which may be independent from MAMP-induced signaling. Crypt apical epithelial cells will have the greatest access to *Enterobacteriaceae* MAMPs by their simple diffusion through the mucus layer. However, the CECs lining the deeper parts of the crypts will experience much less MAMP exposure due to the constricted nature of the crypt and the unidirectional flow of mucus continually pushing crypt contents into the colonic lumen. Being that this is a novel finding, this same imaging should be performed in germ-free mice to see the crypt pIgR localization patterns. Unfortunately, germ-free mice have altered crypt architecture (318), so interpretation of results might be difficult. Additionally, gnotobiotic mice which have been monocolonized with a single bacterial phyla/species/etc. might give additional information to the *in vivo* importance of the particular taxa on CEC pIgR patterning.

Localization of pIgR-derived products in the colonic mucus. With the recent advent of new tissue fixation methods and improved techniques, researchers are now able to see molecules, proteins, and cells *in situ* as never before. For our purposes, Carnoy's fixative

allowed preservation of the colonic mucus layer which would have normally been dehydrated by aldehyde-based fixatives (35). Conventional wisdom has assumed the distribution of SC, IgA, and SIgA throughout the mucus layer as providing a buffer zone to impede further bacterial migration through the mucus and contact with the epithelium (110, 149). We found SC, IgA, and SIgA all in high densities in only the outer mucus layer of mice. Similar imaging in human biopsies showed the same phenomenon of IgA migration to the outer mucus layer after departure from the epithelial cell. Of course, these immune molecules are forced to transit from the epithelial surface to the outer mucus layer by passing through the inner layer. In mouse colons, this was not visibly seen, likely due to the low production of IgA as a result of living in a sterile environment. However, when imaging human colon sections, a clear differentiation in color is seen between goblet cells secreting fresh mucus, and the mucus which has been in the lumen for a longer period of time. The lighter shade of the more aged mucus is likely due to diffuse IgA throughout the inner mucus layer which has not concentrated yet. Manual separation of the two mucus layers is possible, and proteomic analysis of the two layers has been published and shows IgA and SC in both layers (36, 244). However, direct quantification of these proteins in the two regions is not known and could further illuminate this discipline.

Our finding of the outer mucus-localization of these immune proteins immediately points to the other entity which inhabits this layer: the intestinal bacteria. Many other groups have shown the bacterial segregation to the outer mucus layer which we were also able to repeat in this study. Though SC, IgA, SIgA, and bacteria were all present in very high concentrations in the outer mucus layer, our findings using the *Pigr* $^{-/-}$ mouse showed that bacteria do not gain increased access to the inner mucus layer or the colonic epithelium in the absence of all three immune molecules. Again, these studies were performed in mice reared under mostly sterile conditions, so a heavier bacterial presence with the occasional pathogen may show more of a necessity for pIgR-derived products in bacterial exclusion. Studies in humans deficient for IgA are lacking, but inability to produce IgA is one of the most common immunodeficiencies and is many times asymptomatic (319). Individuals with this condition are more susceptible to recurrent bacterial infections along mucosal surfaces, especially in the upper respiratory tract (320). Infection studies have shown increased access of enteric pathogens to the epithelium and host in *Pigr* $^{-/-}$ mice (321), and amelioration of pathogen burden with the passive supplementation of IgA (322). Further infection studies should be performed to determine if this SIgA/SC protection strategy is pathogen-specific.

At birth, neonates are in a state of immunodeficiency due to their underdeveloped adaptive immune system. In nature, this is remedied by maternal supplementation of numerous

immune factors through the milk (80, 282). Both SC and IgA are at voluminous concentrations in colostrum and substantial amounts in mature milk, suggesting considerable biological importance for these proteins in the infant. The IgA^+ PCs in the mammary glands are immigrants from the intestinal mucosa, and secrete IgA that is polyclonal in nature for the intestinal microbiota (323). In our experiments, we observed that mouse neonates were unable to produce detectable amounts of IgA in stool samples until around 30 days after birth, likely because of the IgA^+ PC dearth. These low IgA levels are also seen in human infants (324), with a 3x-increase in fecal IgA of breastfed versus formula-fed neonates (325). During nursing, we found that neonates receiving passive SIgA showed complete transit of this molecule to the distal GI tract. However, IgA by itself was unable to make the complete GI voyage, assumedly due to degradation by digestive enzymes in the proximal intestine. Connecting the evidence that the maternal IgA^+ PCs in the mammary are secreting bacteria-directed antibodies, and that infants are unable to produce robust IgA responses, it is quite reasonable to assume maternally-supplied SIgA is going to have an effect on the intestinal microbiota. We found this to be the case in our experiments, with multiple analyses of the young mouse showing the palpable effect of presence (or absence) of passive SIgA. These two cohorts of mice each had unique microbiotas which were tightly grouped according to passive SIgA status. Around the time of weaning, the mice receiving passive SIgA had higher numbers of fecal bacteria and lower numbers of many bacteria within genera known to be a contributing factor to necrotizing enterocolitis (289). One of the most profound differences between these two groups of mice was the consistent presence of *Ochrobactrum anthropi* in the MLNs of the neonates not receiving passive SIgA. Again, this points to the maternal supplementation of SIgA as having inhibitory effects of opportunistic bacteria residing in the immature neonatal intestine. Human breastmilk is the best known remedy for prevention of NEC, though the direct inhibitory action of SIgA (or other milk antimicrobial molecules) has not been solidified as the prophylactic factor(s).

Consequences of an absent intestinal mucus layer. One of the hallmarks of intestinal health is the ability to keep bacteria segregated from the epithelium (149, 326). Many enteric pathogens are aware of this, and promote mucus demolition by mucin-degrading proteins and eliciting host inflammation (33, 182). Genetic alterations leading to the lack of an intestinal mucus layer promote spontaneous colitis in mice (88), though this has yet to be studied in the germ-free animal. In our studies, we noticed up to the time of weaning, neonates secrete mucus, but it fails to organize into structured layers, and was often seen detached from the intestinal epithelium. For the neonates, this absence of a secured mucus layer led to direct bacterial-epithelial cell interaction. No neonates in our experiments developed colitis, or any other signs of

overt internal inflammation that might have been caused by this intimate interaction. However, neonates not receiving passive SIgA almost always showed large amounts of *O. anthropi* in their MLNs, and it was never found in neonates gaining SIgA from their mothers. Since no differences in *O. anthropi* fecal abundance were seen in neonate treatment groups, passive SIgA supplementation likely was working by some type of mechanism to impede translocation across the epithelium. In humans, *O. anthropi* is considered an emerging opportunistic pathogen, and preterm infants have been found to be especially susceptible to infection of the peritoneal cavity (285). It would be worthwhile to assay mouse and human milk to see if antibodies exist against this specific bacterium, as it is known maternally-supplemented SIgA has been found to have specificity against bacterial species and strains of viruses (324). Our work should give even more credence to the idea of maternal milk as a prophylactic against bacterial invasion, especially in the immunocompromised infant.

When adult mice were genetically altered to not produce intestinal mucus, we found that the entire arrangement of the intestine was altered. Upon visual inspection, colonic crypts were greatly narrowed and elongated compared to the wild type mouse. Without a mucus layer to provide structure, SIgA and SC were found to be in diffuse globular arrangements. The most striking finding was the deep invasion of crypts by the intestinal microbiota, regardless of the presence of SIgA or pIgR/SC. Goblet cell atrophy is a common finding during severe inflammation in humans (171). Our findings would suggest that destruction of the intestinal mucus layer likely obliterates the assortments of molecules that home to this gel layer to provide immune functions. Once the scaffolding is broken, all homeostatic proteins secreted by the epithelium likely lose much of their effectiveness, and the microbiota is allowed to be in close contact with the epithelial cell. Furthermore, during goblet cell atrophy, the architecture of the crypt is also disrupted, which likely allows increased permeability of the epithelium. The SCFA butyrate is known to increase *MUC*2 expression in CECs (85), and butyrate enemas have been used for over 20 years to ameliorate disease in ulcerative colitis patients (327). Though butyrate is known to affect CECs in many ways, perhaps one mechanism to aid the colonic mucosa is to increase the amount of mucus filling goblet cells which would improve crypt integrity.

Assigning causality to bacterial-induced effects of epithelial gene expression which modulates intestinal bacterial communities which differentially stimulates epithelial cells. Within the organism, it is sometimes very difficult to assign succession to microbial-induced changes in gene expression. For instance, a factor common to all human inflammatory bowel diseases is the tremendous increase in quantities of chemokines, pro-inflammatory cytokines, growth factors, and heat-shock proteins produced by the epithelial cell (171). Another common

feature of IBDs is a dysbiosis of the intestinal microbiota, which is changed most severely during a flare of inflammation (9). These dysbiotic events are typically defined by an increase in the relative percentages of *Enterobacteriaceae* and a decrease in the once dominant phyla *Firmicutes* and *Bacteroidetes*. So, the immediate logical progression arises: 1) *Enterobacteriaceae* induce strong pro-inflammatory events in epithelial and professional immune cells that would be in the intestine; 2) IBD patients experience uncontrolled inflammation; 3) IBD patients see a dramatic increase in the relative percentage of *Enterobacteriaceae*; ergo 4) *Enterobacteriaceae* exacerbates inflammation in IBDs. However, an extensive list of IBD covariates and comorbidities now exist (Figure **1.1j**), and it would be short-sighted to purely label IBD-characteristic epithelial gene expression as being microbial. Equally, we would be naïve in simply stating that the intestinal dysbiosis is simply a consequence of the disease. We identified two unique populations of intestinal microbiota in adult mice which were directly associated with whether those mice did or did not receive SIgA in milk during nursing. In parallel, these two cohorts of mice also displayed differing patterns of gene expression in the epithelial cells of their colon. It is enticing to directly relate the two findings, with the change in passive SIgA-modulated intestinal microbiota at the causative factor for unique populations of CEC gene expression. Likewise, we could connect the findings by stating that reception of SIgA during nursing directly influenced CEC gene expression which led to the unique microbiota. No data in the field of immunology has been presented showing direct stimulation of epithelial cell signaling pathways by SIgA or IgA (in any multimeric form), whether maternally-provided or endogenously-produced. However, a clear connection has been made between the intracellular neutralization of MAMPs and dimeric IgA, which works by inhibiting the induction of PRR signaling cascades (105, 328). In addition, SIgA binding to bacterial motifs has been shown to also inhibit the extracellular capacity of bacteria to signal to epithelial cells (29). To connect the microbiota to presence (absence) of passive SIgA, we propose that passive SIgA subdues the early bacterial colonizers' ability to be recognized by intestinal epithelial cells, thereby inhibiting certain gene expression by never allowing it to happen. In addition to the direct effect passive SIgA would have on the intestinal microbiota (biofilm formation, etc.) (77), this early SIgA induced 'not-signaling' by the CECs would have results on maturation of the intestinal innate and adaptive immune systems. This gene expression from the reduced-signaling of epithelial cells, as well as dendritic cells and other immune cell types, would condition the intestinal environment, and further perpetuate the dialogue between the host and microbe. Other groups have presented convincing evidence that SIgA-bound bacteria are more readily taken up in M cells and antigen presenting cells, such as intestinal DCs (29, 112). This uptake will allow antigen presentation to mucosal T and B cells to aid in their

microbiota-specific maturation. In essence, we propose the change in CEC gene expression and microbiotas between our mice receiving and not receiving passive SIgA is simply because a different algorithm of languages was established in these mice, and continued into adulthood by perpetual cycling. In humans, the microbial consortium begins to be much more stable after the first year of life (1, 70), indicating that both the microbiota and immune system have become accustomed to each other. Early immune system patterning by the intestinal microbiota has been seen for mice artificially inoculated with different microbes soon after birth (71). The desire of the host intestinal mucosa to cultivate a beneficial microbiota is also supported by the evidence of factors secreted into the intestinal lumen to promote biofilm formation and other types of persistence mechanisms (147). Extension of this work should longitudinally follow humans exclusively breastfed and exclusively formula fed for the first 6 months of life to see if separations arise in adulthood. Additionally, other variables to be considered include the duration of breastfeeding, and if the children were ever supplemented with formula. **Figure 6.1** illustrates the perpetual microbe-intestinal cycle some of the languages used by each party.

Host and microbe both exist on the island that is the human body. Eons of time have established a mutualism that humanity is only now beginning to understand, but also disturb in alarming ways. As we continue to improve humanity's quality of life, we cannot forget our friends.

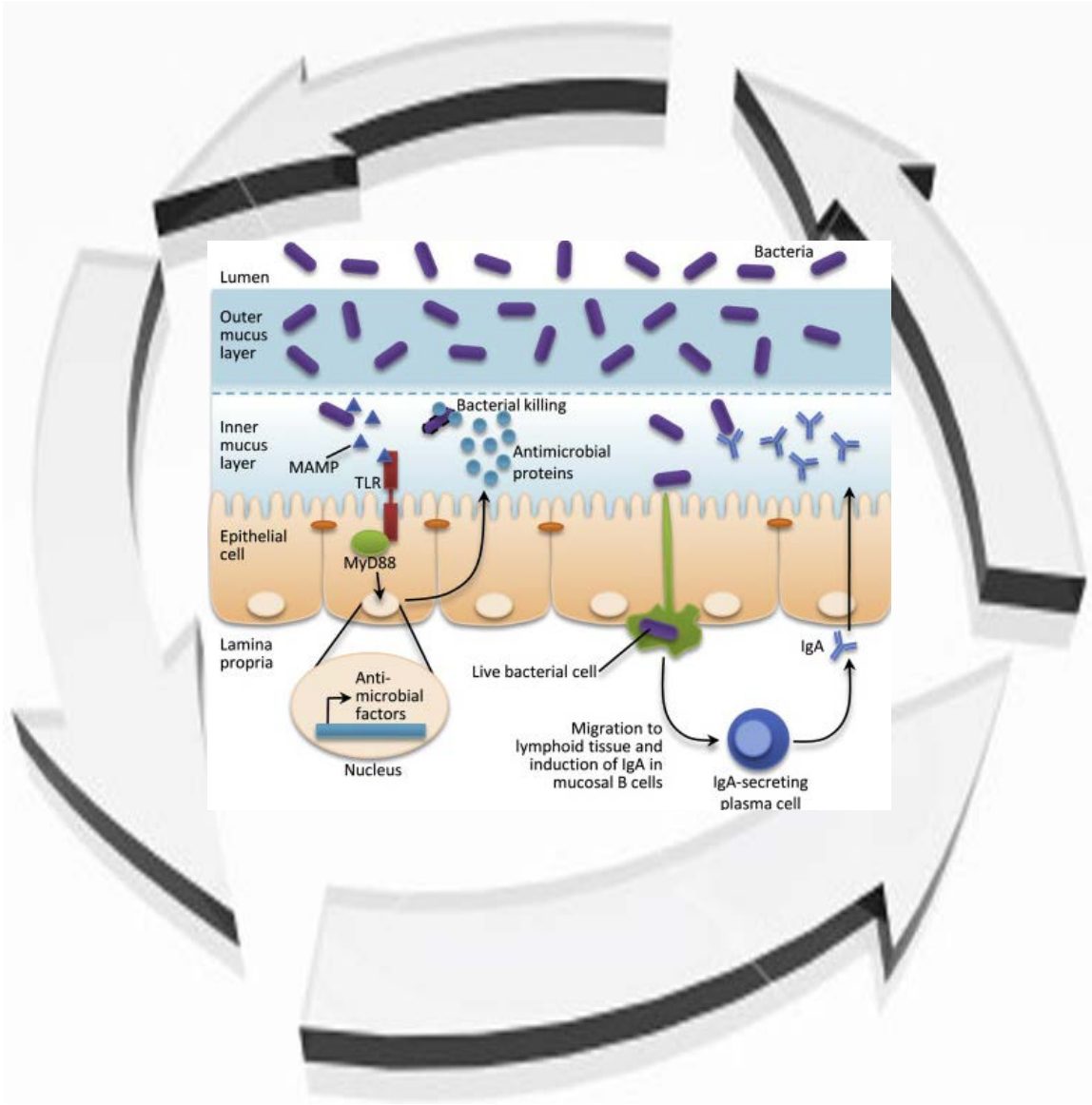


Figure 6.1 Perpetual cycle of host-bacterial dialogue. Intestinal recognition of bacteria through PRRs, dendritic cells, and other mechanisms leads to changes in gene expression and immune system maturation, which affects the microbiota……..*ad mortem.*

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EDUCATION

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PUBLICATIONS

Frantz AL, **Rogier EW**, Weber CR, Shen L, Cohen DA, Fenton LA, Bruno ME, Kaetzel CS. Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides. Manuscript accepted to *Mucosal Immunol.* PMID:22491177.

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