MICROBIOTA-INDUCED GUT FUNCTION AND IMMUNITY

THE ROLE OF INTESTINAL MICROBIOTA ON THE REGULATION OF GUT FUNCTION AND IMMUNITY

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– ABSTRACT –

Intestinal microbiota are key determinants of gut homeostasis and affect various gut physiological and immune processes. Co-evolution has enabled the host and intestinal microbes to exist in a mutualistic relationship. However, interactions between the host and its intestinal microbiota exist in a delicate balance between mutualism and pathogenicity. Maintenance or disruption of this balance depends on a complex interplay between the microbiota and the host, as well as other gut luminal factors, including diet, that are poorly understood. The main goal of this thesis has been to study the host-gut luminal interactions that regulate gut physiology and immunity. In particular, **Chapter 2** centers on investigating the effect of perturbing the intestinal barrier using a non-steroidal inflammatory drug on host-microbial and dietary interactions in a mouse model of gluten sensitivity. I demonstrated that indomethacin-induced increase in intestinal permeability is associated with altered intestinal microbiota composition, systemic antibody development against intestinal bacteria and a shift in immune responses to the dietary antigen, gluten. **Chapter 3** focuses on investigating whether modulation of the intestinal microbiota can affect the host's susceptibility to intestinal injury. I used mice with defective intracellular bacterial receptor signaling because discrimination between commensals and pathogens is, in part, achieved by a family of receptors that recognize conserved bacterial components. I demonstrated that the microbiota with which these mice are colonized influences the expression of RegIII-γ, a type of antimicrobial peptide, and susceptibility to intestinal injury. To gain further insight on the effect of microbiota on antimicrobial peptides, in **Chapter 4** we conducted a combination of gnotobiotic and

in-vitro experiments where we identified that specific components of the microbiota differentially regulate RegIII expression. Further examination showed that *MyD88 a*nd *Ticam1* genes, which are signaling adaptor proteins of pattern recognition receptors, are essential regulators of microbial–induced RegIII expression by intestinal epithelial cells. Collectively, the work presented in this thesis provides novel insight on the bi-directional interaction between the host and the gut luminal content as well as of potential beneficial effects of microbiota-modulating strategies in maintaining homeostasis and preventing disease.

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Helen Keller said: " *Life is either a great adventure or nothing*". Since I was young, I have always thrived for adventure, whether climbing the Himalayas, crossing the Sahara Desert or diving the Blue Holes. I used to think that adventures only came from conquering ultimate physical thrills. Unexpectedly, my graduate work turned out to be the biggest and most extraordinary adventure of my life - filled with lots of inspiration, perspiration, improvisation, aspiration, contemplation, exploration, frustration, desperation and last but not the least pure elation.

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– TABLE OF CONTENTS –

– LIST OF FIGURES –

– LIST OF TABLES –

– LIST OF ABBREVIATIONS AND SYMBOLS –

^{3}H	Thymidine
^{51}Cr	Chromium
Ab	Antibody
AGU	Axenic/ Gnotobiotic Unit
AmpR	Ampicillin Resistant
ANOVA	Analysis of variance
APC	Antigen presenting cells
ASF	altered Schaedler flora
Bb	*Bifidobacterium breve*
C	Celsius
CBA	Cytometric bead array
cDNA	Complementary DNA
CFA	Complete freund's adjuvant
CFU	Colony forming unit
Ci	Curie
cm	centimeters
ConA	Concanavalin A
Cox2	cyclooxygenase-2
CPM	Counts per minute
DAB	Diaminobenzidine
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
EC	epithelial cell
EM	Electron microscope
F4/80	Macrophage marker
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
FS	Frameshift
G	Conductance
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GF	Germ free
GI	Gastrointestinal tract
gm	gram
GS	Gluten sensitivity
H&E	Hematoxylin and eosin stain
HCD4	Human CD4
HCl	Hydrochloride
HLA	Human leukocyte antigen
hr	hour
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IF	Immunofluorescent
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IL	Interleukin
IL-7RA+	Lin–ckit+interleukin-7 receptor α-chain cells
ILF	Isolated lymphoid follicles
Indo	Indomethacin
ip	Intraperitoneally
kDa	kilodalton
KO	Knockout
L	Litre
LPL	Lamina propria lymphocytes
M cells	Microfold cells
MAMP	Microbe-associated molecular patterns
MCP-1	Monocyte chemotactic protein-1
mg	milligram
ml	millilitre
MLN	Mesenteric lymph nodes
mM	milliMolar
MPO	Myeloperoxidase
mS	Millisiemens
n	nano
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NLR	Nod-like receptors
NOD	Nucleotide-binding oligomerization domain
NSAID	Non-steroidal anti-inflammatory drug
OCT	Optimal cutting temperature

PAMP	Pattern associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Potential difference
pmol	picomolar
PP	Peyer's patches
PRR	Pattern recognition receptors
PT-G	Peptic-tryptic digests of gliadin
qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
REG	Regenerating islet-derived
RP	Repoopulate (balanced healthy microbiota)
RPMI	Rosell Park Memorial Institute
SD	Standard deviation
SEM	Standard error of mean
SFB	Segmented filamentous bacteria
SI	Stimulation index
SPF	Specific pathogen free
T-FRLP	Terminal restriction fragment length polymorphism
TCR	T-cell receptor
TG2	Transglutaminase 2
TGGE	Temperature gradient gel electrophoresis
TH	T helper
Ticam	Toll Interleukin receptor-containing adaptor molecule
TJ	Tight junction
TLR	Toll-like Receptors
TNF-α	Tumor necrosis factor alpha
T_{REG}	T regulatory cells
UC	Ulcerative colitis
WT	Wildtype
ZO-1	Zonula Occluden 1
α	Alpha
β	Beta
γ	Gamma
δ	Delta
Δ	Delta
κ	Kappa
μ	Micro

– CHAPTER 1 –

INTRODUCTION

1.1. Intestinal Microbiota

Immediately after birth, mucosal surfaces are colonized by a dense and complex microbial ecosystem, which is referred to as the *microbiota*. The GI tract is the largest mucosal surface in the body; as such, it represents a major site for microbial colonization and constitutes one of the largest microbial biospheres in the planet (Whitman et al., 1998). The bulk of microorganisms, which make up the intestinal microbiota are bacteria, however, representative eukarya, viruses, and even archaea are also present (Backhed et al., 2005; Eckburg et al., 2005; Lozupone et al., 2012). Approximately 100 trillion microbes, 1000 different microbial species and 7000 bacterial strains inhabit the human intestine (Eckburg et al., 2005; Gill et al., 2006; Whitman et al., 1998). Most of the bacterial species are permanent colonizers or autochthonous but some only exist transiently or allochthonous. The genes of intestinal microbiota, called the *microbiome*, exceed the total number of human eukaryotic genes (10^{12}) by a factor of at least 100 (Savage, 1977). Thus, humans have been described as a *supraorganism* (Ley et al., 2006; Turnbaugh et al., 2007), in which the intestinal microbiota is a critical factor that modulates physiological responses and disease susceptibility of the host.

1.1.1. Acquisition and Assembly of Intestinal Microbiota

Approximately 300 years ago, Van Leeuwenhoek first observed under a microscope the presence of microorganisms including bacteria and protozoans in fecal samples (Schierbeek, 1953). Since then, progress in understanding the intestinal microbiota has been hindered due to the fact that the majority of microbes are

unculturable (Eckburg et al., 2005). With the advent of high-throughput sequencing technologies in combination with advanced analysis methods (Table 1), we are beginning to understand more in-depth the composition, abundance and functionality of intestinal microbiota. Moreover, we are gaining insight on how the individual host acquires and develops a distinctive microbial profile.

Table 1.1.Techniques use to characterize GI microbiota composition and function.

Techniques	Sample	Description
Culture	Bacteria	Isolation of bacteria on selective media. Semi-quantitative. Used for phylotyping, functional assays, and genome sequencing.
qPCR	DNA	Amplification and quantification of 16S rRNA. Quantitative. Used to target specific bacterial groups/species.
DGGE/TGGE	DNA	Gel separation of 16s rRNA amplicon using denaturant/temperature. Semi-quantitative. Used for microbial community analysis.
T-RFLP	DNA	Restriction enzyme are use to digest the 16SrRNA amplicon and digested fragments are separated by gel electrophoresis. Semi-quantitative. Used for microbial community analysis.
FISH	DNA	Fluorescently labeled oligonucleotides probes hybridize with complementary target 16S rRNA amplicon and then analyzed by either microscope or flow cytometer. Semi-quantitative. Used to target specific bacterial groups/species.
Microarrays	DNA/RNA	Fluorescently labeled oligonucleotides probes hybridize with complementary target 16S rRNA amplicon and then analyzed by laser. Semi-quantitative. Used for gene expression profiling.
Cloned 16S rRNA gene sequencing	DNA/RNA	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis. Quantitative. Used for phylogenetic arrays, microbial community analysis, genome and metagenome sequencing.
Direct 16S rRNA amplicon sequencing	DNA/RNA	Massive parallel sequencing of partial 16s rRNA amplicons (e.g. 454 Pyrosequencing). Quantitative. Used for phylogenetic arrays, microbial community analysis, genome and metagenome sequencing.
Meta-genomics (Shotgun sequencing)	DNA/RNA	Massive parallel sequencing of the whole genome (e.g. 454 Pyrosequecing, Illumina). Quantitative. Used for phylogenetic arrays, microbial community analysis, genome and metagenome sequencing.
Meta-transcriptomics	RNA	Use for gene expression profiling, RNA sequencing, and microarrays, Phylogenetic arrays. Used to detect changes in community-wide gene expression profiles.

Techniques	Sample	Description
Meta-proteomics	Protein	Techniques used include Mass spectrometry. Used for proteomic profiling and identification of microbiome function.
Meta-bolomics	Metabolites	Used for metabolic profiling.

Abbreviations: rDNA, ribosomal DNA; qPCR, quantitative PCR; DGGE, denaturing gradient gel electrophoresis; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism, FISH, fluorescence *in situ* hybridization.

1.1.2. Microbiota along the Gastrointestinal Tract

The GI tract is divided into discrete sections, and each division has a unique digestive activity and function. In parallel with these distinct microenvironments, there is also a large variation in microbial structure across the length of the gastrointestinal tract (Figure 1.1). In the stomach, low pH maintains the microbial biomass relatively low and around 10^1 cells per gram of bacteria. Despite this, several bacteria including the pathogen *Helicobacter pylori,* which can colonize the stomach since childhood, have evolved to withstand the acidic environment of the stomach (Monstein et al., 2000). Bacterial density increases to 10^3 in the duodenum and then progress to $10^4 – 10^7$ bacteria per gram of contents in the jejunum and ileum (O'Hara and Shanahan, 2006; Walter and Ley, 2011). Small intestinal motility (the migrating motor complex), higher concentration of bile salts coupled with the presence of specialized epithelial cells such as Paneth cells (which release antimicrobial proteins) and lymphoid structures such as Peyer's patches, contribute to limit bacterial overgrowth in the small intestine. In contrast, many of the aforementioned properties are lacking in the colon. As a result, the density of bacteria in the colon exceeds 10^{12} cells per gram of content (Dethlefsen et al., 2007; Whitman et al., 1998). Most of the absorption and digestive activities occur in the small intestine while

water absorption takes place in the colon. Hence, density compartmentalization along the GI tract may indicate a mutualistic adaptation to ensure that the host salvages as much nutrients and energy from foods without serious competition from the intestinal microbiota.

In addition to density, the dominant types of microbes vary along the gastrointestinal tract. Most of the bacteria in the oral cavity are aerobic consisting of Streptococcus and Lactobacilli (Dewhirst et al., 2010; Stearns et al., 2011). On the other hand, the majority of small and large intestinal microbiota are strictly anaerobic bacteria (Walter and Ley, 2011). The small intestine is enriched for the *Bacilli* class of the *Firmicutes* and *Actinobacteria* while *Bacteroidetes* and the *Lachnospiraceae* family of the *Firmicutes* are more prevalent in colonic samples (Frank et al., 2007). Finally, there is also a great deal of latitudinal variation in microbiota composition; specifically, analysis of fecal microbiota revealed a different profile compared to mucosa-associated intestinal microbiota (Sekirov et al., 2010; Swidsinski et al., 2005a). Overall, intestinal microbiota has adapted to survive and thrive in specific functional and anatomical regions of the mammalian gut.

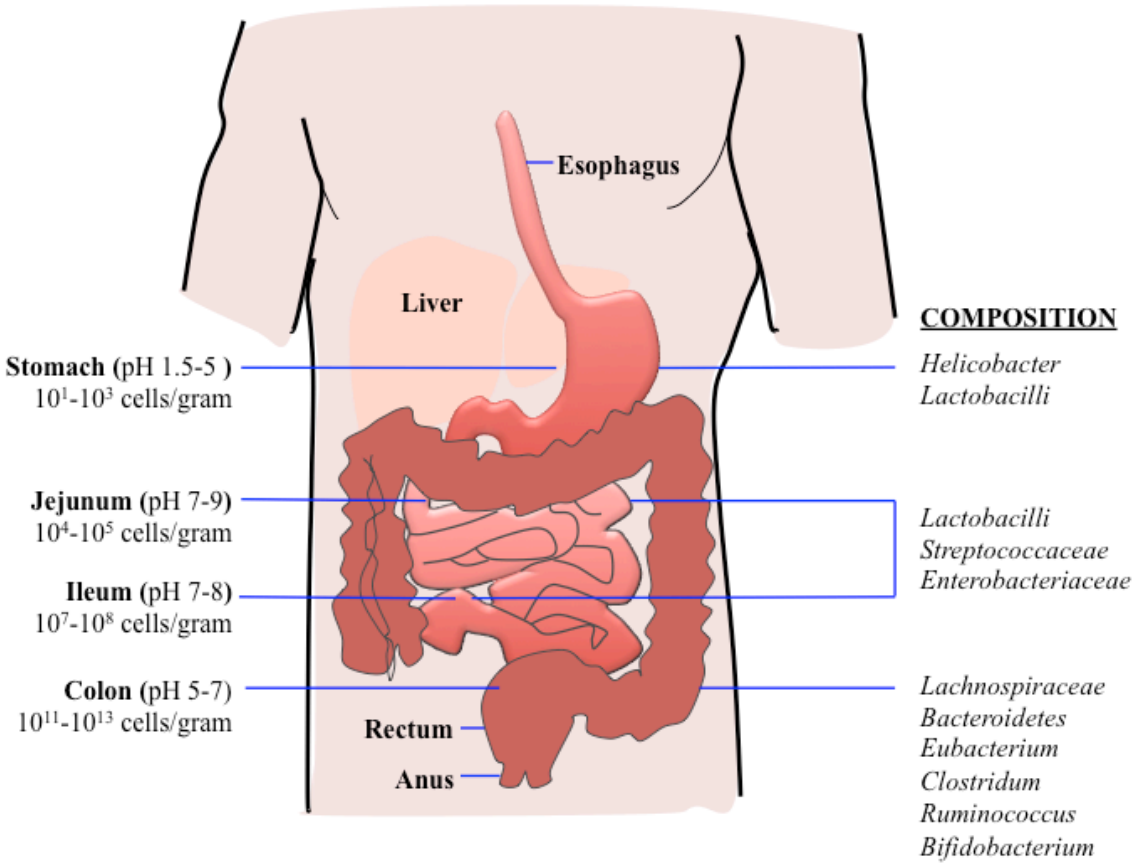

Figure 1.1. The GI tract and their inhabitants.
Variations in microbial numbers, dominant types of microbes and pH gradient across the length of the GI tract.

1.1.3. Intestinal Microbiota: More than a Gut Inhabitant

Years of co-evolution have allowed the host and the gut microbiota to peacefully coexist in a mutualistic relationship under steady state conditions. The host provides a safe niche, rich in nutrients to its resident bacteria. In return, intestinal microbes provide important functions to the host (O'Hara and Shanahan, 2006). These include salvaging nutrients for the host and conferring protection against infections by competing with

opportunistic pathogens both in resources and space (Benson et al., 2009; Hooper et al., 2002; Stecher and Hardt, 2008). Moreover, the gut microbiota provides pivotal stimuli and cues necessary to educate several aspects of host physiology and post-natal development (Round and Mazmanian, 2009; Smith et al., 2007).

1.1.3.1. Tools for Examining Host-Microbiota Interaction

Much of the supportive evidence describing the impact of microbiota to the host has been acquired from studies using germ-free animals. Germ-free animals are reared and maintained in sterile flexible film isolators under positive pressure with high efficiency particulate air filtered to control exposure to microorganisms, even viruses (Macpherson and Harris, 2004). These animals can be studied in its sterile state or they can be deliberately colonized with either single or complex groups of bacteria (Bry et al., 1996; Geuking et al., 2011; Hooper et al., 2001). The technology to colonize germ-free mice has been referred to as *gnotobiotic*, which is a Greek term meaning "known life". In a pioneering study, *Hapfelmeier et al* developed a technique to induce a reversible germ free colonization system in mice by mono-colonizing germ free mice with a mutant form of commensal *Escherichia coli* JM83 that lack the ability to synthesize prokaryotic-specific amino acids that can be grown in culture but do not persist *in vivo*. By using this system, one can decipher whether the impact of intestinal microbiota requires permanent colonization or not (Hapfelmeier et al., 2010). Additionally, humanized models have been described in which germ free mice are colonized with human-derived microbiota (Turnbaugh et al., 2009). Histological and molecular experiments performed on these

gnotobiotic animals illustrated that the presence of the microbiota causes extensive changes in host biology that include altered epithelial cell gene expression, changes in the composition and activity of the mucosal immune system, metabolism, angiogenesis, bile acid cycle, and intestinal motility (Table 1.2, Appendix 1). Interestingly, the impact of intestinal microbiota on host physiology is not limited to the GI tract, but rather extends systemically and influences the central nervous system and animal behavior (Collins and Bercik, 2009; Macpherson and Harris, 2004). Thus, intestinal microbiota has a critical role in shaping host physiology, making it a key player in the regulation of health and disease.

In recent years, insight into how intestinal microbiota influences metabolic signaling in mammalian hosts has been made possible by high throughput transcriptomic, proteomic and metabolic tools that utilize DNA/RNA microarrays, mass spectrometry and nuclear magnetic resonance spectrometry. When used in combination with gnotobiotic techniques, these tools can provide powerful information and detailed understanding on how microbiota shapes many aspects of host physiology and development (Holmes et al., 2011; Hooper et al., 2003; Peterson et al., 2007; Stappenbeck et al., 2002). Overall, studies utilizing these powerful microbiota-related techniques have established that intestinal microbiota co-evolved with mammals and that it can influence and complement mammalian biology in ways that are mutually beneficial.

Table 1.2. Select germ-free related phenotypes.

Description	Phenotype	Reference
Intestinal motility	Altered myenteric plexus	(Dupont et al., 1965)
	Altered transit time of contents	(Husebye, 1994)
Intestinal morphology	Decreased total intestinal mass and small intestinal surface area	(Meslin et al., 1973)
	Altered villus/crypt length and less lamina propria cellularity	(Glaister, 1973)
	Altered cecal characteristics	(Abrams et al., 1963)
Intestinal epithelial cell properties	Slower epithelial cell renewal/turnover	(Abrams et al., 1963)
Mucosal immunity	Decreased antimicrobial peptides expression	(Vaishnava et al., 2008)
	Decreased IgA	(Fahey and Sell, 1965)
	Very few plasma cells in small intestine	(Glaister, 1973)
	Fewer $CD8^+$T cells in intestinal epithelial lymphocytes with reduced cytotoxicity and $CD4^+$T cells in the lamina propria	(Round and Mazmanian, 2009)
Secondary lymphoid tissue	Decreased number and size of Peyer's patches	(Gordon, 1959)
	Smaller and decreased cellularity of MLN	(Glaister, 1973)
Systemic Immunity	Fewer germinal centers and plasma cells	(Gordon and Bruckner-Kardoss, 1961)

Adapted from (Smith et al., 2007).

1.2. Intestinal Homeostasis

Health, according to the World Health Organization, is *a state of physical, mental and social well-being and not merely the absence of disease or infirmity*. Healthy mammals coexist with trillions of bacteria that do not elicit an exaggerated immune response. As such, the relationship between the host and intestinal microbiota is often described as "homeostatic" under healthy conditions. Relative to other organs of the body, the GI tract is also exposed to a myriad of environmental antigens including those that are derived from food. Thus, the concept of intestinal homeostasis should also involve non-microbial antigens from dietary origin.

Although intestinal bacteria and host mostly live in a mutualistic relationship, the richness of microbes and their close proximity with the host presents a potential threat. Specifically, intestinal microbiota can cause opportunistic invasion of host's tissue that may result in the breakdown of host-intestinal microbiota relationship and contribute to pathologies including chronic inflammation and sepsis. To effectively minimize the adverse effects of intestinal microbiota to the host and maintain the beneficial nature of the intestinal host–microbiota relationship, the host has evolved with a series of multi-level and redundant adaptations.

1.3. Intestinal Immune System

The intestinal immune system plays a pivotal role in the maintenance of intestinal homeostasis. The intestinal tissue is armed with several immunological barriers, with distinct and tightly regulated immune mechanisms. These strategies have a crucial role in mounting an appropriate response against invading pathogens while maintaining tolerance against food antigens and a mutualistic relationship with the intestinal microbiota. Furthermore, specific anatomical adaptations limit microbial exposure to the systemic immune system and allow the immune effects caused by either accidental or non-accidental microbial penetration to be confined within the mucosal compartment. This is important since, unlike the mucosal immune system, the systemic immune system is neither "tolerant" nor "ignorant" to the intestinal microbiota. This is evident in studies showing that intravenous injection of one species of commensal bacteria induces a systemic immune response in the host (Konrad et al., 2006; Macpherson and Uhr, 2004b).

Thus, intestinal microbiota induces beneficial mucosal immune responses without activating pro-inflammatory systemic immune responses if efficiently contained within the gut. In summary, the intestinal immune system both physically and actively controls the exposure of intestinal microorganisms to host tissues and compartmentalizes the immune response within the mucosal site in healthy conditions. However, it is also important to note that systemic responses to commensals can be mounted in the event of a sufficiently important break in the intestinal barrier. It is unclear how breakdown of this homeostatic mechanism can contribute to cross sensitization to other luminal antigens and its relevance in disease.

1.3.1. Anatomy of the intestinal immune system

The gut-associated lymphoid tissue (GALT) can be functionally divided into effector and inductive sites (Figure 1.2). Effector sites include the epithelium and lamina propria. Different immune cells including lymphocytes, antigen-presenting cells (APCs), mononuclear phagocytes, granulocytes and innate lymphoid cells (ILC) are scattered throughout the lamina propria. The most common lamina propria lymphocytes are the plasma-producing B cells, the antigen-reactive T cells that mostly express T cell receptor (TCR)-αβ together with either one of the CD4 or CD8αβ co-receptors, and intraepithelial lymphocytes (IELs). T cells in the small intestine mostly express CD8αβ while most colonic T cells express CD4 co-receptors (Cheroutre and Madakamutil, 2004). Naïve $CD4^+$ T cells differentiate into T helper (Th) cells (Th1, Th2 and Th17) upon antigen presentation and activation by APCs. IELs are located between epithelial cells and mostly

comprise of T cells belonging to both TCR-γδ and TCR-αβ lineages. IELs are unique because they are antigen-experienced T cells but they do not require priming, and immediately release cytokines and cytotoxic granules to kill infected cells (Cheroutre et al., 2011). As such, IELs are categorized mostly as innate immune cells. Despite this, IELs still express typical T-cell activation markers, such as CD44, CD69 and CD103, and express effector cytokines, such as IFN-γ, IL-2, IL-4 or IL-17 (Cheroutre et al., 2011).

Inductive sites are organized tissues responsible for the generation of antigen specific immune responses, and involve IgA class switching and clonal expansion of B cell that occur in response to antigen specific T cell activation. Immune cells in inductive sites closely resemble cells from periphery whereas cells in the effector sites possess characteristics that seem unique to the mucosal site (Macpherson and Uhr, 2004a).

The inductive sites consist of the mesenteric lymph nodes (MLN), Peyer's patches (PP), isolated lymphoid follicles (ILF), colonic patches and cryptopatches. MLN are the largest lymph node in the body and formed before birth. It is believed that MLN are the firewall between mucosal and systemic immune system (Macpherson et al., 2009). This is exemplified by the observation that a healthy host has almost no systemic immune response against its own intestinal bacteria. However, upon MLN adenectomy, systemic ignorance is abolished (Macpherson and Uhr, 2004b). The PPs are macroscopic lymphoid aggregates that are found in the submucosa along the length of the small intestine. Aside from PPs, small lymphoid aggregates called ILF and cryptopatches, found throughout lamina propria, are also present in both small and large intestine of mice (Eberl, 2005). Similarly, isolated lymphoid structures are found in humans (Eberl, 2005; Lorenz and

Newberry, 2004; Moghaddami et al., 1998; Yeung et al., 2000). In contrast with MLN, PPs and lymphoid aggregates are formed after birth when the intestine begins to be colonized by the microbiota. Both PP and ILF are populated with different immune cells such as B cells, T cells and dendritic cells. On the other hand, cryptopatches contain dendritic cells and the recently identified Lin–cKIT+interleukin-7 receptor α-chain (IL-7Rα)+ cells (Eberl and Littman, 2004). IL-7Rα+ cells are also found in both ILF and PP (Eberl and Littman, 2004). Both PP and ILF are overlaid with specialized epithelial cells called Microfold (M) cells. Unlike any other epithelial cells in the intestine, these cells are unique because they are not lined with mucus and are able to take up luminal antigens from the gut lumen by endocytosis. Antigens are then transported across and taken up by antigen presenting cells such as dendritic cells. This mechanism is one way that allows the immune system to constantly sample luminal content and mount an appropriate response. Alternatively, antigen can be sampled directly in the lumen by specialized dendritic cells through extension of their dendrites (Niess et al., 2005). Collectively, the structure of gut-associated lymphoid tissues is organized in a specific manner to cater to its function.

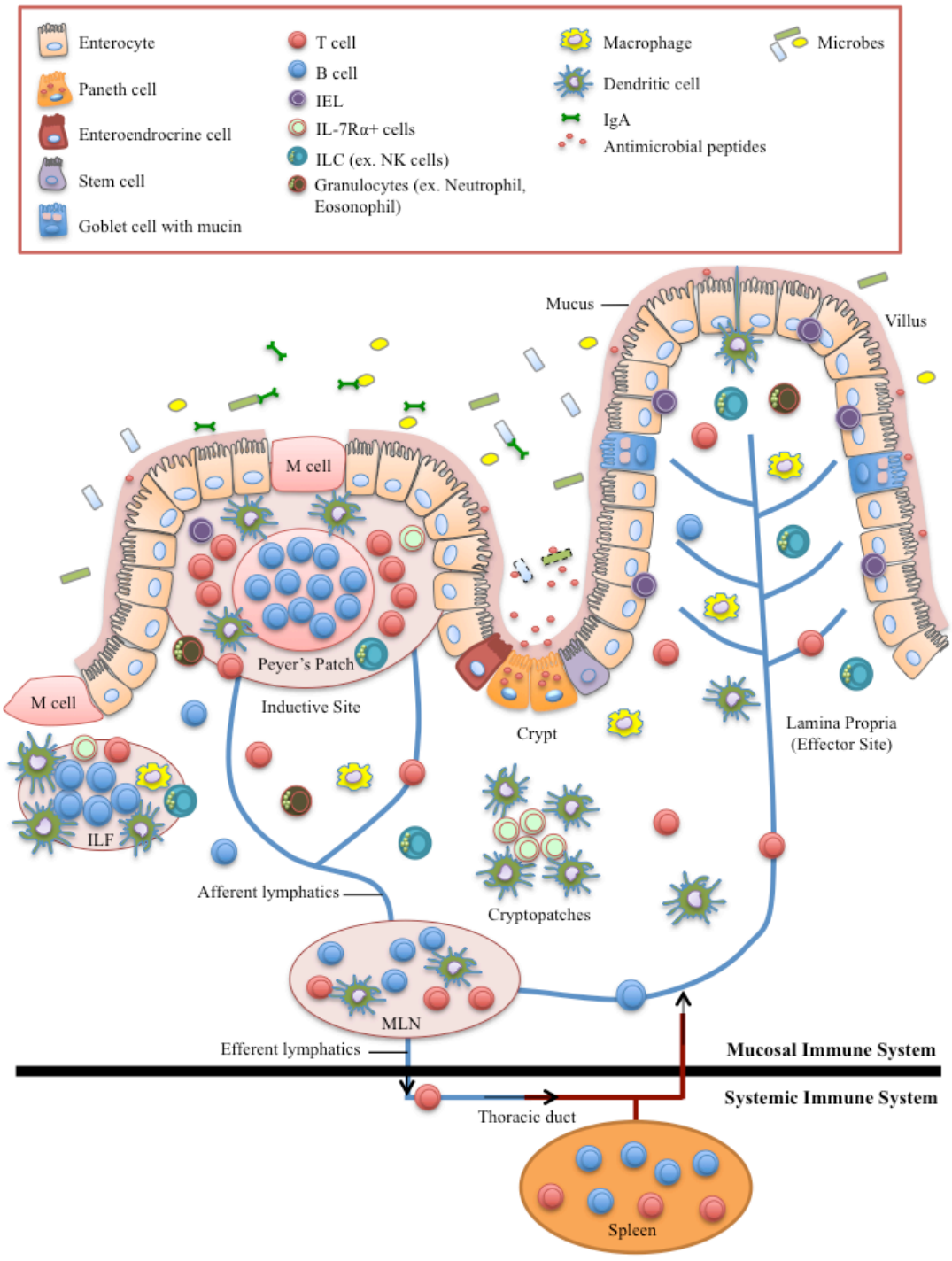

Figure 1.2. Schematic diagram of intestinal immune system.
The intestinal immune system is divided into inductive sites and into effector sites. Both Peyer's patches and the lamina propria are drained by afferent lymphatic that traffic to the MLN, which then connects with the systemic immune system via the thoracic duct.

1.3.2. Intestinal Epithelial Barrier

A key element of the mammalian intestinal strategy for maintaining homeostasis with the microbiota is to minimize contact between luminal microorganisms and the intestinal epithelial cell surface. The single layer of epithelial cells that lines the intestine exhibits numerous physical adaptations to separate the inside of the body from the dense population of microbes and potentially dangerous antigens in the lumen. In the intestine, there are five distinct epithelial lineages: enterocytes, M cells, goblet cells, Paneth cells and enteroendocrine cells (Figure 1.3). Each intestinal epithelial cell (IEC) is linked together by intercellular tight and adherens junctions that regulate paracellular traffic and keep cells polarized (Appendix I).

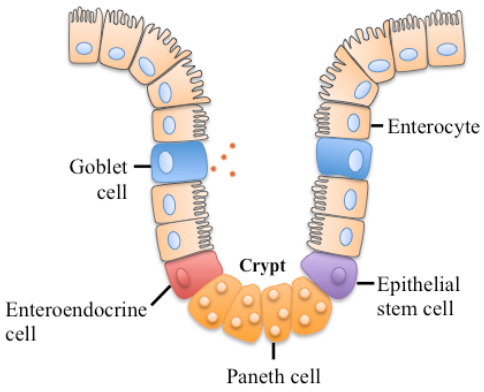

Figure 1.3. Different cell types in the intestine.
Paneth cells, which produce antimicrobial peptides; stem cells; mucin-producing goblet cells; hormone producing enteroendocrine cells and the absorptive enterocytes.

The intestinal epithelium mediates selective permeability through two major pathways: transcellular/transepithelial and paracellular transport (Figure 1.4). The transcellular pathway is an active, energy requiring process that entails movement across plasma membrane. On the other hand, paracellular permeability is associated with

transport in the space between two adjacent epithelial cells and is regulated by junctional complexes composed of tight junctions (zonula occludens), intermediate or adherens junctions (cadherins bound to α, β, γ-catenins), and desmosomes (macula adherens and gap junctions). Apical junctional complexes are also important in the regulation of cellular proliferation, polarization and differentiation.

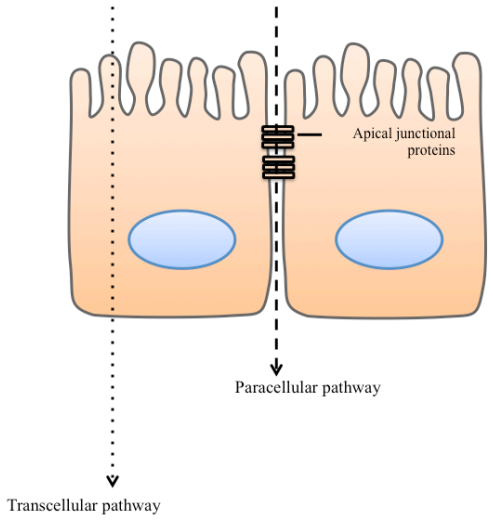

Figure 1.4. Transcellular and paracellular transport.
The intestinal epithelial cells restrict movement of luminal contents between cells (paracellular) and across cells (transcellular).

Intestinal permeability can be assessed *in vivo* and/or *in vitro*. Non-invasive *in vivo* techniques are based on the principles of differential urinary excretion of orally administered probes such as ^{51}Chromium (Cr)-EDTA. Several influencing factors such as gastric emptying, intestinal transit time and dilution of intestinal secretion may however affect the result of *in vivo* permeability (Arrieta et al., 2006). Intestinal segments can be isolated *in vitro* and allow for measurement of intestinal permeability with the advantage of excluding several factors associated with *in-vivo* permeability techniques. The Ussing

chamber apparatus is a physiological system that is commonly used to study the electrophysiology and permeability of isolated intestinal segments (Figure 1.5) (Clarke, 2009).

In addition to its function as a physical barrier, the intestinal barrier also possesses extrinsic adaptations to effectively protect the host's connective tissue from the external environment. The extrinsic barrier includes secreted gastric acid and digestive enzymes, which have bactericidal properties and the ability to degrade food. These secretions, with the help of peristalsis, which is the propulsion of luminal contents through the digestive, act to wash away bacteria and toxins. Specialized epithelial cells called goblet cells secrete a thick mucus layer, which assemble into approximately 150 μm thick on top of the epithelial surface. Two distinct strata can be found in the colon (Johansson et al., 2008). The outer layer contains large numbers of bacteria whereas the inner layer is relatively sterile. In contrast, the small intestine has a discontinuous and less well defined mucus layer. The mucus is secreted at the top of the crypts and then moves upward between the villi (Johansson et al., 2008). Antimicrobial proteins further hinder microbes that are able to penetrate the mucus layer. These natural antibiotics are members of several distinct protein families, including defensins, cathelicidins, proteases, and C-type lectins. The exact mechanism by which antimicrobial peptides exert their bactericidal effect has yet to be identified, but they seem to act through a variety of mechanisms including the killing of microbes through mechanisms that compromise bacterial cell wall integrity (Jager et al., 2010, 2013). Antimicrobial proteins are produced by various intestinal epithelial lineages, including enterocytes and Paneth cells, as well as innate

immune cells such as γδ-IEL (Mukherjee et al., 2008). There is great deal of variation in terms of antimicrobial expression. Some antimicrobial proteins are expressed constitutively while others are dependent on intestinal microbial colonization (Appendix 1).

Epithelial cells also transport IgA produced by B cells into the gut lumen. IgA deficiency is associated with increased penetration of bacteria into host tissues (Artis, 2008). Hence, IgA is an important factor in maintaining luminal compartmentalization of intestinal microbiota. The exact mechanisms by which IgA confines symbiotic bacteria to the intestinal lumen remain unclear, but may involve trapping of bacteria in the mucus layer, recruitment of complement with subsequent bacterial killing or promoting phagocytic clearance of organisms that have breached the epithelial barrier (Artis, 2008). Epithelial cells are also armed with antigen detection and immune signaling mechanisms, and in some cases can even act as antigen presenting cells for neighboring IEL. Overall, the epithelial barrier, along with its components and secretory IgA, operate together to confine symbiotic bacteria and other luminal antigens to the gut lumen and maintain a protected zone at the epithelial surface, thereby limiting bacterial and antigen penetration across the epithelial barrier.

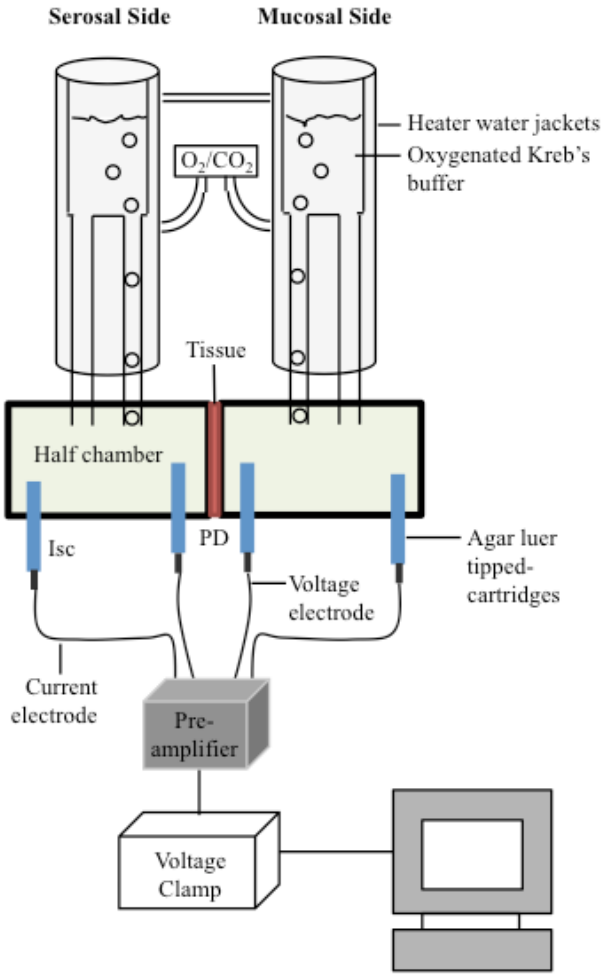

Figure 1.5. The Ussing chamber apparatus.
The Ussing chamber is composed of two separate chambers isolating the luminal and serosal aspects of a segment of the intestine. Oxygenated, warmed and isotonic buffers circulate separately in the two chambers allowing for administration of pharmacological agents and probes to an isolated aspect of the tissue. Electrodes in close proximity to the tissue measure potential difference (PD). To maintain PD at zero, a current equal and opposite to the current generated by net ion transport is injected into the tissue and is reported as the short-circuit current (Isc). Tissue permeability to ions is measured as tissue conductance (G), which is the inverse of resistance. The conductance reflects the permeability of tight junctions and cellular resistance of the tissue to the movement of ions. Permeability of the paracellular pathway can be determined using a small inert probe such as the radioactive probe ^{51}Cr-EDTA whereas transcellular movement across the epithelium can be measured using classic endocytosis tracers such as horseradish peroxidase (HRP), which moves via fluid-phase endocytosis when used at high concentrations.

1.3.3. Innate Pattern Recognition Receptors

The innate immune system provides a primary host response to microbial invasion, which induces an inflammatory process to localize the infection and prevent systemic dissemination of pathogens (Abreu, 2010). One key process in innate recognition of microbial antigens is mediated by pattern recognition receptors (PRR). PRRs include Toll-like receptors (TLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), RNA helicases, C-type lectin receptors, and cytosolic DNA sensors. These receptors are able to recognize evolutionary conserved pathogen-associated molecular patterns (PAMPs) expressed by various microorganisms (Table 1.3, Figure 1.6). The name PAMPs, however, is misleading as symbiotic microorganisms similarly express these conserved microbial patterns. Therefore, it has been proposed to change the name to microbe-associated molecular patterns (MAMPs) (Ausubel, 2005). Both epithelial and immune cells express PRRs. (Figure 1.6). Once activated, PRRs trigger sequential activation of intracellular signaling pathways and lead to induction of a range of cytokines and chemokines that promote immune and physiological responses. Due to the close proximity of IEC with the intestinal microbiota, expression of PRRs by IEC is spatially and regionally localized to minimize inflammatory response under normal physiological condition (Abreu, 2010). For instance, both Nod1 and Nod2 proteins are expressed inside the cytoplasm of IEC while some TLRs are only expressed at the basolateral side of IEC. PRR signaling facilitates the differentiation of T cells and B cells to establish antigen-specific adaptive immunity. Furthermore, emerging evidence supports the concept that signaling through PRRs is one way intestinal microbiota directly

communicates with the host to inform the immune system that they are present in the lumen and thereby maintain gut homeostasis (Abreu, 2010). For example, studies have demonstrated that a lack of constitutive TLR signaling driven by the commensal microbiota results in disruption of epithelial barrier integrity, which leads in turn, to increased susceptibility of the host to acute injury (Cario et al., 2007). In conclusion, innate PRRs are crucial components of host immunity that not only protect the host from invading pathogens but also help the host maintain homeostasis.

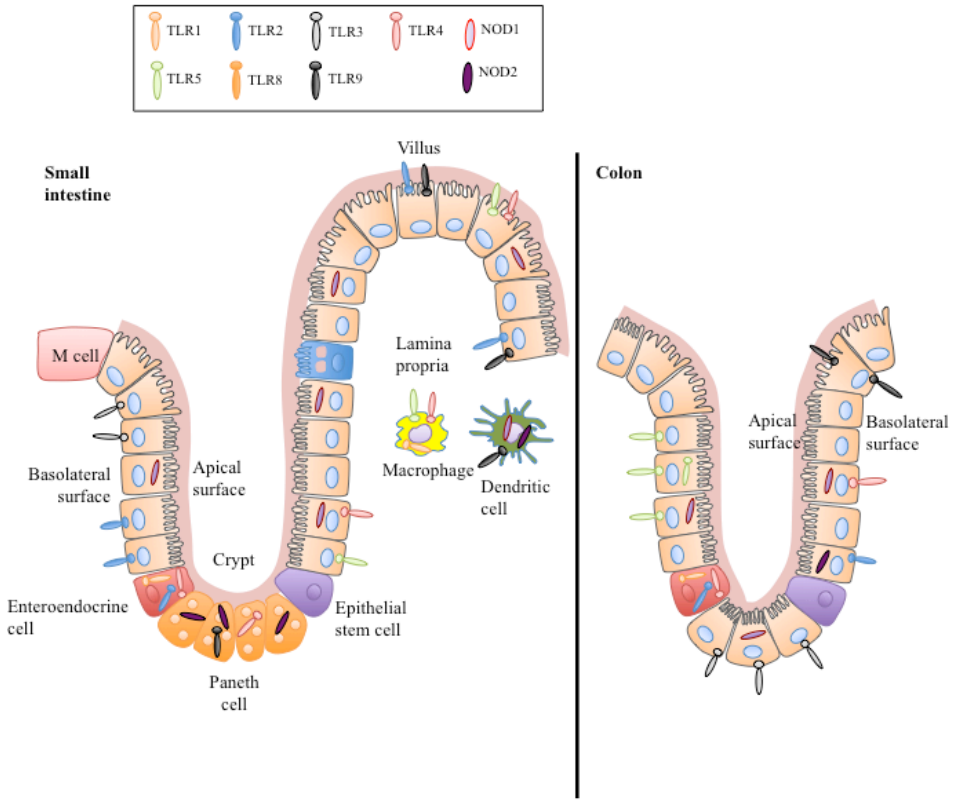

Figure 1.6. Distribution of TLR and NLR in the small and large intestinal epithelial cells. (Adapted from (Abreu, 2010)).
Microbe-associated molecular patterns are expressed by both resident intestinal microbes and pathogens. To minimize inflammatory response, PRRs are spatially and regionally expressed in IECs.

Table 1.3. Pattern Recognition Receptors in the intestine

PRRs	Ligands	Microorganisms Detected	Cell Types Expressed
TLR		Bacteria,	IEC, Macrophages,
TLR1	Triacyl lipoproteins	Viruses, Fungi	Dendritic cells,
TLR2	Peptidoglycan, lipoteichoic acid		Myofibroblast, Endothelial cells,
TLR3	Double stranded RNA, Poly(IC)		Polymorphonuclear cells
TLR4	LPS		
TLR5	Flagellin		
TLR6	Di-acyl lipo-peptides, Zymosan		
TLR7	Single-stranded RNA		
TLR8	Single-stranded RNA		
TLR9	CpG-ODN		
NLR		Bacteria, Viruses	IEC, Macrophages,
NOD1	Meso-DAP		Dendritic cells,
NOD2	Muramylpeptide		Myofibroblast,
NLRC4	Flagellin		Endothelial cells,
NLRP3, NLR6	Muramylpeptide, Bacterial RNA, crystals, mitochondria DNA		Polymorphonuclear cells
RNA Helicases		Viruses	IEC
RIG-I	Cytoplasmic dsRNA		
MIDA5	Cytoplasmic dsRNA		
C-type Lectins		Bacteria, Fungi,	IEC
CL-P1	D-galactose, L-and D-fructose, GalNac, T and Tn antigens	Viruses	
Monocoyte mannose receptor	Galactose/N-acetylglucosamine Mannose and/or N-acetylglucosamine		
Mannose-binding lectin	High-mannose-containing glycoproteins		
Ficolins	B-glucans		
DC-SIGN			
Dectins			
Cytosolic DNA sensors		Virus	IEC
DAI	DNA		
AIM-2	DNA		
RNA Polymerase III	DNA		
IFI16	DNA		

1.3.4. Innate and adaptive intestinal immunity operate to maintain intestinal homeostasis

Occasionally, intestinal resident microbes are able to breach the epithelial layer particularly when the intestinal epithelial barrier is compromised (Arakawa et al., 2012). Furthermore, some components of intestinal microbiota, referred to as pathobionts, have the potential to become opportunistic (Round and Mazmanian, 2009). Innate and adaptive immune cells work hand in hand to make sure host-microbiota homeostasis is maintained (Cong et al., 2009; Rimoldi et al., 2005; Round and Mazmanian, 2009). In healthy host, innate immune cells such as macrophages scattered throughout the lamina propria rapidly respond to occasional microbial penetration and invasive pathobionts. They then send signals to the adaptive immune cells, which then provide a slow but specific response (Hooper and Macpherson, 2010). In the lamina propria, B and T cells mainly provide the adaptive response (Hooper and Macpherson, 2010). Recently, pathobionts like segmented filamentous bacteria (SFB) have been shown to potently induce Th17 cells (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Induction of Th17 may be the host response to combat invasive pathobionts. On the other hand, symbionts such as the polysaccharide A-expressing *Bacteroides fragilis* or altered Schaedler flora (ASF), which contains eight bacterial species, induce T-regulatory (T_{REG}) cells, which are important component of immune system that regulate Th cells and maintain intestinal homeostasis (Atarashi et al., 2011; Geuking et al., 2011; Round et al., 2011). Overall, innate and adaptive immunity are critical in keeping intestinal homeostasis. It is possible, however, that immune

responses may become inefficient in controlling pathobionts; alternatively, immune responses may become overwhelmed leading to uncontrolled immune responses and development of intestinal inflammation particularly in immune defects or deficiency setting.

1.4. Dysregulation of Host-Intestinal Microbiota Interactions

Host-intestinal microbiota interaction has a crucial role in the maintenance of normal physiology and gut homeostasis. Despite the multiple levels of protection, the symbiotic relationship of the host and intestinal microbiota can shift into a dysfunctional interaction. This can have detrimental effects on the host and can lead to the development of disease. This breakdown in homeostasis can be brought upon by changes in the host, such as genetic predisposition or concomitant morbidities; in the environment, such as exposure to infections, dietary triggers, psychological stress and drugs that affect the intestinal microbiota or both.

Early environmental and epigenetic factors, such as the method by which babies are delivered and whether babies are breastfed or formula-fed, have been implicated in shaping the intestinal microbiota profile of an individual. Thus, it is possible that changes in gut microbial composition in early life can influence the risk for developing disease later in life (Gareau et al., 2007; Varghese et al., 2006).

In adulthood, the composition of the intestinal microbiota is relatively stable, however it still undergoes dynamic and transitory changes throughout life triggered by diet, hygiene habits and antibiotic exposure (Nicholson et al., 2012). Accumulating

evidence associates compositional shifts in members of the microbiota with certain chronic inflammatory disorders in the gastrointestinal tract, including Inflammatory Bowel Disease (IBD) and celiac disease. The alteration in microbiota composition has recently been termed "dysbiosis" and the functional implications of these shifts are still poorly understood. Although an individual with mutations in genes involved in immune regulatory mechanisms or pro-inflammatory pathways could exhibit unrestrained inflammation in the intestine, not all individuals that are genetically predisposed develop a disease, suggesting that chronic inflammation is multifactorial. One unsolved question is whether dysbiosis plays a causative role in disease pathogenesis, or is a mere consequence of disease. In this thesis, I investigated two models of chronic gut inflammation: Gluten Sensitivity (GS), which affects the proximal small intestine, and IBD, which affects the distal small intestine and colon. GS is clearly related to a dietary trigger in which the role of cross sensitization to microbiota is unknown. On the other hand, the trigger for the development of inflammation in IBD is unknown but increasing evidence suggests that it involves an exaggerated immune response to intestinal microbiota.

1.4.1. Celiac disease and gluten sensitivity

Celiac disease is an autoimmune disorder triggered by the ingestion of gluten in genetically predisposed individuals. It is a genetic-linked disorder, occurring in people who carry the human leukocyte antigen (HLA)-DQ2 or HLA-DQ8 genotypes. Upon gluten consumption, undigested gliadin fragments, which are the water insoluble storage

proteins in gluten, translocate across the intestinal epithelium into the lamina propria where it triggers mucosal immune response and subsequent inflammation. The enzyme transglutaminase 2 (TG2) is released upon the first signs of inflammation and amplifies the immune response to gliadin; it forms covalent bonds with gliadin and deaminates it, producing a negatively charged gliadin peptide, which enhances antigen presentation.

Clinically, the disease is characterized by the blunting of villi in the small intestine, antibody production against gliadin and TG2, and the activation of a large number of innate and adaptive immune cells. Celiac disease, however, has a spectrum of disease presentation, as with any other chronic diseases. Furthermore, there is a spectrum of disorders related to the ingestion of gluten. In 1991, Richard Logan first published the concept of celiac iceberg, which refers to the concept that the tip of an iceberg represents the number of symptomatic cases whereas the area below the waterline represents gluten sensitive or potential celiac disease cases, in a given population at a particular point in time (Figure 1.7). The milder forms of gluten sensitivity (GS), termed by some non-celiac GS, have been described as a gluten-related condition in which patients have gastrointestinal symptoms, in the absence of specific celiac disease markers. Some of these patients exhibit mild intraepithelial lymphocytosis in the absence of TG2 antibodies, and may also exhibit anti-gliadin antibodies and HLA-celiac associated genes, making it difficult to differentiate them from potential celiacs (Verdu, 2011; Verdu et al., 2009). There is currently a number of vague and often confusing terms currently being used in literature to define gluten sensitivity (Ludvigsson et al., 2013). In attempt to solve this problem, a recent consensus meeting (Oslo Definition) took place in attempt to re-define

terms related to celiac disease and gluten sensitive disorders (Ludvigsson et al., 2013). Despite this effort, the topic continues to be controversial and many question remain. In particular, it is unclear whether and how gluten sensitive and potential celiac disease patients transition to symptomatic celiac disease.

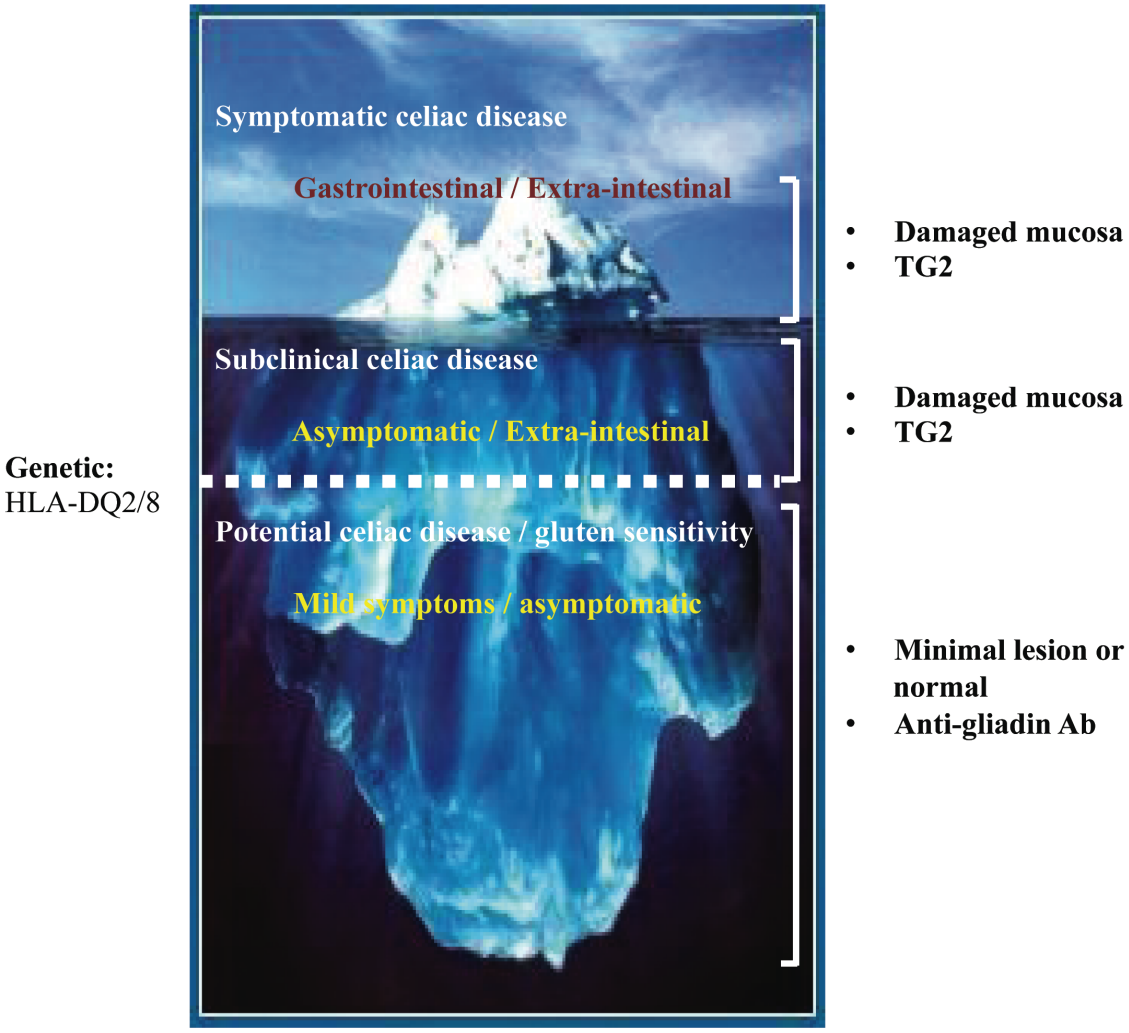

Figure 1.7. Spectrum of celiac disease and gluten related disorders.
Ab = antibodies.

Recently, compositional changes in intestinal microbiota have been described in celiac disease. Specifically, studies have revealed an increase in the quantity of pro-inflammatory bacteria (*Bacteriodes*) and a decrease in anti-inflammatory bacteria (*Bifidobacteria*). Interestingly, in patients that are treated for celiac disease (gluten-free diet), *Lactobacilli* species become more prevalent than in untreated individuals (De Palma et al., 2009). *Staphylococci* species have also been demonstrated to be more prevalent in individuals with celiac disease (De Palma et al., 2012; De Palma et al., 2010; Pozo-Rubio et al., 2012). Whether these changes in intestinal microbiota composition are cause or effect of disease is unknown. Moreover, it remains to be determined whether intestinal microbiota play a role in the onset of celiac disease or promotes the progression from a state of potential celiac disease to overt celiac disease.

Since the 1980's, animal models have been used to understand the pathogenesis of celiac disease (Table 1.4). However, significant advancement towards generating robust and useful animal models has only been made in the last few decades. These animal models include transgenic mice that carry the human gene that predisposes celiac disease (HLA-DQ8 and HLA-DQ2). Our laboratory has adapted the HLA-DQ8 model as a mouse model to study immune and physiological changes induced by gluten before the onset of overt intestinal inflammation (Verdu et al., 2008). The model can be used to investigate environmental factors, such as the intestinal microbiota, which may be involved in the development of disease. The next section (**Chapter 3**) will provide experimental details regarding the HLA-DQ8 model.

Table 1.4. Select animal models of celiac disease

Animal Model	Description	Reference
Dog (Irish setter)	Pups administered with wheat containing diet spontaneously develop MHC II independent partial villus atrophy and IEL infiltration.	(Batt et al., 1987)
Monkeys (rhesus macques)	Rhesus macques spontaneously develop villus atrophy and anti gliadin gliadin IgA and IgG when maintained on gluten containing diet.	(Bethune et al., 2008)
Rats (Germ-free Wistar AVN)	Administration of gliadin to germ-free after birth until 63 years old led to the shortening of villi, crypt hyperplasia, and increased number of o/$CD4^+$ TCR αβ T cells.	(Štepánková et al, 1996)
Non-obese diabetic (NOD) mice	NOD mice spontaneously develop CD3+ IEL and villi shortening when maintained on standard wheat containing food.	(Maurano et al., 2005)
Rag1$^{-/-}$ mice	*Rag1*$^{-/-}$ mice that received $CD45RB^{lo}$-$CD25^-$cells spontaneously develop lymphocytic infiltration of the villi and lamina propria, crypt hyperplasia and villus atrophy when maintained under gluten containing food.	(Freitag et al., 2009)

1.4.2. Inflammatory Bowel Disease

Inflammatory Bowel Disease can be subdivided into two different types: Crohn's disease and ulcerative colitis (UC). These chronic diseases represent lifelong, relapsing inflammatory conditions that can manifest as disease restricted to the colonic mucosa in ulcertative patients, or disease affecting both the colonic and ileal mucosal layer and epithelium in Crohn's patients. The etiology of IBD is not completely understood but believed to involve genetic, immunological, environmental and microbial factors.

Genome-wide association studies have revealed more than 99 unique IBD susceptibility associations (Anderson et al., 2011; Franke et al., 2010). Among the number of genes that have been identified as susceptibility loci for Crohn's disease, *NOD2* was the first identified susceptibility gene for Crohn's disease within the IBD susceptibility locus on chromosome 16, which is the locus most strongly linked to the

disorder (Hugot et al., 2001; Ogura et al., 2001). Unlike the results obtained for *NOD2*, studies that were conducted to investigate the association of *NOD1* polymorphism with IBD reported conflicting results (Hampe et al., 2007; McGovern et al., 2005a)

It is generally accepted that IBD results from aberrant immune responses to the intestinal microbiota in genetically predisposed hosts. The mechanism by which the intestinal micorbiota promotes inflammation in IBD has been the subject of much research in recent years. The importance of the microbiota in IBD has been highlighted in several studies showing severely altered intestinal microbial community structure in patients, compared to healthy controls. One of the most consistent findings related to dysbiosis in IBD is an overall reduction in the diversity of bacterial species. Studies investigating mucosa-associated microbiota showed larger numbers of bacteria in biopsies from Crohn's disease patients compared to controls but their biodiversity were reduced (Mylonaki et al., 2005). Similarly, studies in patients with ulcerative colitis showed a lower diversity of fecal microbiota (Sokol et al., 2008). Bacterial species abundance assessed by Illumina-based metagenomic sequencing can differentiate between healthy subjects and patients with IBD. Moreover the average bacterial gene number for IBD patients was lower compared to that of healthy subjects, indicating a reduction in bacterial diversity in IBD (Qin et al., 2010). This reduction in diversity may, however, be related to the inflammation seen in IBD as there are differences in microbial communities populating inflamed and non-inflamed tissues from patients with Crohn's disease and ulcerative colitis, with decreased biodiversity in inflamed tissue (Sepehri et al., 2007). Thus, dysbiosis in IBD be simply a consequence of inflammation and not

necessarily involve in the development of IBD. Nevertheless, it remains possible that inflammation-induced dysbiosis may perpetuate an existing inflammation.

Recent observations show that a number of genes associated with IBD could influence the structure of the intestinal microbial community and subsequently affect the outcome of inflammation. For instance, *Nod2* was described as an important regulator of commensal intestinal microbiota in mice; *Nod2*-deficient mice display impaired ability to kill mice, increased load of commensal bacteria and are more susceptible to be colonized by pathogenic bacteria (Petnicki-Ocwieja et al., 2009a; Rehman et al., 2011). Furthermore, compared to wild-type mice, *Nod2*-deficient mice display increased numbers of *Firmicutes* and *Bacteroidetes* (Petnicki-Ocwieja et al., 2009a; Rehman et al., 2011). Interestingly, an increased abundance of bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* was similarly observed in Crohn's disease patients homozygous for the NOD2 frameshift mutation (Rehman et al., 2011). These observations suggest that while inflammation may cause dysbiosis, genetics may influence microbiota composition and thus dysbiosis may also precede inflammation.

Aside from lower bacterial diversity, the microbiota of IBD patients show less *Firmicutes,* particularly *Faecalibacterium prausnitzii*, *Lactobacilli,* and *Bifidobacteria* compared to healthy individuals (Sokol and Seksik, 2010; Sokol et al., 2009; Swidsinski et al., 2005b). These bacteria may have anti-inflammatory properties or affect secretion of antimicrobial peptides that limit colonization of pathogenic bacteria; thus their underrepresentation may contribute or drive inflammatory responses. In contrast some pro-inflammatory bacteria are increased in IBD. Patients with active Crohn's disease had

larger fecal populations of *Escherichia coli* than patients with quiescent disease or normal controls, with a specific increase in entero-adherent invasive *E. coli* (Schultsz et al., 1997). Patients with ulcerative colitis were also reported to have higher concentrations of bacteria, particularly anaerobes such as *Bacteroides* and *Clostridiaceae* (Mylonaki et al., 2005; Schultsz et al., 1997). Overall, reduced diversity, increased temporal instability as well as increased proportion of pro-inflammatory (*Bacteroides fragilis*, Adherant-invasive *E. coli*) versus anti-inflammatory bacteria (*Faecalibacterium prausnitzii, Bifidobacteria*) are recurrent findings in IBD patients (Martinez et al., 2008).

Over the years, several animal models of colitis have been generated to study the pathogenesis of IBD (Mizoguchi and Mizoguchi, 2010; Sollid and Johansen, 2008); these models can be categorized into different types according to the type of reagents/infection used to induce the intestinal inflammation or defect in immunity/physiology (Table 1.5). Although these animal models cannot completely represent the full complexity of intestinal pathology observed in either Crohn's disease or ulcerative colitis, they can exhibit some of IBD disease aspects. One of the most commonly used models is the chemical model of dextran sulfate sodium (DSS) where the chemical damage of the epithelial layer leads to acute infiltration of inflammatory cells and intestinal microbial invasion which drives inflammation (Wirtz et al., 2007). The next section (**Chapter 5 and 6**) will provide experimental details regarding the DSS colitis model.

Table 1.5. Select animal models of IBD

Type of Inflammation	Animal Models
Chemical	Dextran sulfate sodium colitis, Trinitrobenzenesulfonic acid (TNBS)/Ditrinitrobenzenesulfonic acid (DNBS) colitis, Oxazolone colitis
Infection	*Salmonella, Citrobacter rodentium*
Epithelial integrity/permeability defect	DN N-cadherin transgenic mice, Multiple drug resistant gene deficient mice, IKK-γ (NEMO)/IKKαβ deficiency in IEC, SAMP1/YitFc mice
Innate immune defect	STAT3 deficiency in myeloid cells, A20 deficient mice
Adaptive immune defect	IL-10 deficient mice, $TNF^{\Delta ARE}$ mice, CD45RBHi transfer model, STAT4 transgenic mice, TCRα chain−/− mice

– CHAPTER 2 –

THESIS SCOPE AND OBJECTIVES

2.1. Thesis Scope

From birth, the intestinal microbiota intimately interacts with the host and plays an essential role in shaping the host physiology. Several factors including genetics, diet and medication may however influence host-microbiota interactions. Disruptions in the symbiotic relationship between the host and its intestinal microbiota may lead to disease. Understanding the complex interplay between host-gut luminal factors is relevant to decipher the mechanistic basis of the increasing prevalence of gastrointestinal diseases, particularly those that have been associated with alterations of the intestinal microbiota. I believe this knowledge will help develop new preventative strategies and therapeutic opportunities for these diseases.

Therefore, the main goal of my thesis was to investigate the role of host-intestinal microbiota interactions in the maintenance of homeostasis and disease pathogenesis in genetically susceptible hosts. Specifically, I examined the role of host-gut luminal interaction in the development of diseases that affect the upper and lower GI tract respectively: *gluten sensitivity* and *IBD* (**Chapter 3** and **4**). For this, I employed two different models of disease with a direct human genetic link: gluten-sensitization in HLA-DQ8 mice for gluten sensitivity and induction of DSS colitis in *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice for IBD. Furthermore, I examined the role of host-gut luminal interaction in the regulation of intestinal antimicrobial peptide, which is an important component of innate immunity (**Chapter 5**).

During my thesis, I generated data that further explore the role of intestinal microbiota in health and disease. I developed a gnotobiotic humanized-mouse model of

IBD by colonizing germ free mice with microbiota, harboring highly dysbiotic communities, isolated from patient with active IBD. Preliminary results from this study are discussed under *unpublished data* in **Chapter 6** of this thesis. Finally, I have published a peer-reviewed review paper in *Pharmacological Research* (**Appendix I**), which explores the pathological and therapeutic implications of modulating intestinal microbiota composition.

2.2. Thesis Objectives

The major findings in this thesis are organized into three data chapters, each of which contributes to the thesis goal. The specific objectives that are addressed in each chapter are described below:

Objective 1 To examine the impact of intestinal barrier dysregulation induced by non-steroidal anti-inflammatory drugs on host-microbial interactions and sensitization to gluten in HLA-DQ8 mice. As an undergraduate student at McMaster University, I participated in the original characterization and establishment of this mouse model, which was published in *Am J Physiology* (Verdu et al., 2008). Results pertaining to this objective have been published in the following manuscript:

Chapter 3 Natividad JM, Huang X, Slack E, Jury J, Sanz Y, David C, Denou E, Yang P, Murray J, McCoy KD, Verdu EF. *Host Responses to Intestinal Microbial Antigens in Gluten Sensitive Mice*. PLoS One. 2009 Jul 31;4(7):e6472. doi: 10.1371/journal.pone.0006472.

Objective 2 To examine whether signaling through *Nod1* and *Nod2* receptors, which are intracellular pattern recognition receptors, play a role in susceptibility to intestinal injury in mice and explore whether modulation of intestinal microbiota composition can influence the outcome of intestinal injury in mice lacking *Nod1* and *Nod2* signaling. Results pertaining to this objective have been published in the following manuscript:

Chapter 4 Natividad JM, Petit V, Huang X, de Palam G, Jury J, Sanz Y, Philpott D, Garcia Rodenas CL, McCoy KD, Verdu EF. *Commensal and probiotic bacteria influence intestinal barrier function and susceptibility to colitis in Nod1-/-; Nod2-/- mice. Inflamm Bowel Dis*. 2012 Aug;18(8):1434-46. doi: 10.1002/ibd.22848. Epub 2011 Dec 11.

Objective 3 To elucidate the differential role of specific components of intestinal microbiota on the regulation of antimicrobial RegIII. Results pertaining to this objective have been summarized in the following manuscript:

Chapter 5 Natividad JM*, Hayes CL*, Motta JP, Jury J, Galipeau HJ, Philip V, Garcia-Rodenas CL, Bercik P, Verdu EF. *Differential induction of antimicrobial RegIII by intestinal microbiota and Bifidobacterium breve.* In preparation for submission.

The work conducted in each manuscript required a collaborative effort with several colleagues resulting in multiple authors. Specific details regarding authorship can be found on the *Preface* of each chapter.

– CHAPTER 3 –

HOST RESPONSES TO INTESTINAL MICROBIAL ANTIGENS IN GLUTEN-SENSITIVE MICE

Host Responses to Intestinal Microbial Antigens in Gluten-Sensitive Mice

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The material presented in this chapter was published in PlosOne 4(7): e6472 doi:10.1371/journal.pone.0006472, 2009.

Preface: *The research presented in this manuscript was conducted from September 2007 to May 2009. I am the primary author of the paper. My supervisor, Dr. E.F. Verdu and I designed the experiments, analyzed the data, wrote the manuscript and addressed the reviewers' concerns. My supervisor and I performed the majority of the experiments with technical assistance from the following people: X. Huang (technician) provided us with experimental assistance with animal work and splenocyte proliferation, J. Jury (technician) provided us with experimental assistance with intestinal permeability experiment, E. Slack (post-doctoral fellow) established the protocol for antibody analysis against cecal content, P. Yang (collaborator) provided us with assistance with electron microscopy, E. Denou (post-doctoral fellow) provided us with experimental assistance with the microbiota analysis. Y. Sanz performed and generated the quantitative data for the intestinal microbiota composition presented in Figure 3.12. C. David (collaborator) provided us the transgenic mice. J. Murray and K.D. McCoy (collaborators) contributed valuable scientific input and critically appraised the manuscript.*

Summary and central message: This article examined the influence of intestinal barrier dysfunction by a non-steroidal drug, indomethacin, in immune responses to the intestinal microbiota antigens and gluten in a model of gluten sensitivity using HLA-DQ8/HCD4 mice. Gluten sensitive HLA-DQ8/HCD4 mice exhibit neuromuscular and epithelial barrier dysfunction in the absence of mucosal atrophy (Verdu et al., 2008). The model allows investigation of pathogenic mechanisms triggered by gluten before the onset of a chronic lesion. Concomitant treatment with indomethacin and gluten led to more pronounced barrier dysfunction, compromised mucosal containment of the microbiota and elevated pro-inflammatory response of gluten specific T-cells in HLA-DQ8/HCD4 mice. These findings suggest that environmental factors that alter the intestinal barrier can influence the nature of the host immune response to gluten. Bystander luminal antigens from the intestinal microbiota may contribute to enhance inflammatory responses to dietary antigens such as gluten by gaining access to the submucosal and systemic compartments. This mechanism could contribute to sensitization to gluten and onset of severe form of gluten sensitivity in genetically predisposed hosts.

Title: Host responses to intestinal microbial antigens in gluten-sensitive mice.

Authors: Jane M Natividad[1], Xianxi Huang, Emma Slack, Jennifer Jury, Yolanda Sanz[2], Chella David[3], Emmanuel Denou, Pinchang Yang[4], Joseph Murray[5], Kathy D McCoy and Elena F Verdú.

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Short Title: NSAID and gluten sensitivity

Key words: intestinal permeability, NSAID, food sensitivity, intestinal microbiota

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3.1. Abstract

Background and Aims: Excessive uptake of commensal bacterial antigens through a permeable intestinal barrier may influence host responses to specific antigen in a genetically predisposed host. The aim of this study was to investigate whether intestinal barrier dysfunction induced by indomethacin treatment affects the host response to intestinal microbiota in gluten-sensitized HLA-DQ8/HCD4 mice.

Methodology: HLA-DQ8/HCD4 mice were sensitized with gluten, and gavaged with indomethacin plus gluten. Intestinal permeability was assessed by Ussing chamber; epithelial cell (EC) ultra-structure by electron microscopy; RNA expression of genes coding for junctional proteins by Quantitative real-time PCR; immune response by *in-vitro* antigen-specific T-cell proliferation and cytokine analysis by cytometric bead array; intestinal microbiota by fluorescence in situ hybridization and analysis of systemic antibodies against intestinal microbiota by surface staining of live bacteria with serum followed by FACS analysis.

Principal Findings: Indomethacin led to a more pronounced increase in intestinal permeability in gluten-sensitized mice. These changes were accompanied by severe EC damage, decreased E-cadherin RNA level, elevated IFN-γ in splenocyte culture supernatant, and production of significant IgM antibody against intestinal microbiota.

Conclusion: Indomethacin potentiates barrier dysfunction and EC injury induced by

gluten, affects systemic IFN-γ production and the host response to intestinal microbiota antigens in HLA-DQ8/HCD4 mice. The results suggest that environmental factors that alter the intestinal barrier may predispose individuals to an increased susceptibility to gluten through a bystander immune activation to intestinal microbiota.

3.2. Introduction

Celiac disease is an immune-mediated enteropathy triggered by the ingestion of gluten containing cereals, and in particular gliadin, the storage protein in wheat. It has recently been recognized that both the pathology and the clinical spectrum of celiac disease varies considerably from severe to subtle, and that the clinical expression is not restricted to the presence of mucosal atrophy (Cronin and Shanahan, 2003). The concept of gluten sensitivity (GS) incorporates a variety of pathologic, immunological, and clinical scenarios that may, or may not, form part of the "celiac" spectrum such as gluten-sensitive diarrhea, immunological mucosal response to gluten in family members of celiac disease, persistent positive specific serology for celiac disease in the absence of defined enteropathy, and subtle immunopathological changes in the intestine exposed to gluten. Typically, these disorders occur in individuals who carry the same HLA genotypes associated with celiac disease-DQ2 and DQ8 (Kaukinen et al., 2001; Marsh, 1992; Mino and Lauwers, 2003; Troncone et al., 2003; Verdu et al., 2009). This has led to the development of animal models of gluten-sensitivity that mimic certain aspects of gluten-induced pathogenesis (Black et al., 2002). HLA-DQ8/HCD4 or single HLA-DQ8 transgenic mice that are sensitized with gluten develop an immune response to gliadin that involves both the adaptive and innate immune system (Black et al., 2002; Marietta et al., 2004; Senger et al., 2005; Verdu et al., 2008). Although these gluten-sensitive mice do not spontaneously develop intestinal atrophy, they exhibit gluten-dependent changes in gut neuromuscular and epithelial secretory function (Verdu et al., 2008). This model has

proven useful for the preclinical testing of novel experimental therapies designed to block gluten-induced mucosal pathology (Pinier et al., 2009).

The presence of HLA-DQ2/DQ8 genes are necessary but not sufficient for the development of celiac disease (Green and Jabri, 2006), as up to 25-40% of general populations in United States carry these genes and eat gluten, but do not develop a celiac lesion (Green and Jabri, 2006; Rostom et al., 2006), thus raising the possibility of contributing environmental and genetic risk factors yet to be identified (Kagnoff, 2007). The net availability of gliadin to the lamina propria seems to be an important factor in the inflammatory response of celiac patients. The immobilization and haptenation of gluten components to extracellular matrix proteins by tissue transglutaminase aids and allows reservoirs of antigenically potentiated gluten components to reach increased concentrations *in vivo*, and may even induce a widespread mucosal response against auto-antigens (Dieterich et al., 2006). Indeed, celiac patients have been shown to increase systemic titers of IgA antibodies against collagen (Dieterich et al., 2006).

Under normal conditions, the intestinal epithelium acts as a protective barrier restricting transport of luminal antigens, and only allows small and selective quantities to permeate the mucosa (Arrieta et al., 2006; Fasano and Shea-Donohue, 2005; Macpherson and Uhr, 2004b). In contrast, increased intestinal permeability has been demonstrated in patients with active celiac disease (Bjarnason et al., 1985; Bjarnason and Peters, 1984) and their healthy relatives, suggesting that in a proportion of cases, intestinal barrier abnormalities may predate overt inflammation (Vanelburg et al., 1993). Altered barrier function could be a critical step in facilitating the host responses that contribute to the

clinical expression of gluten sensitivity. Thus, the present study was designed to investigate whether alteration of intestinal barrier function using the non-steroidal anti-inflammatory drug (NSAID), indomethacin, enhances gluten-induced epithelial injury and influences subsequent host responses to gut luminal antigens. Our results show that indomethacin enhances gluten-induced changes in the mucosa leading to increased IFN-γ release by gliadin-stimulated splenocytes and to systemic priming against intestinal microbiota antigens. In genetically predisposed hosts with long standing barrier abnormalities, this mechanism may lower the threshold of inflammatory responses to specific antigens.

3.3. Materials and Methods

Animals. All experiments were conducted with approval from the McMaster University Animal Care Committee. Male transgenic mice expressing HLA-DQ8 genes (HLA-DQA1*0301; HLA-DQb1*0302) in the absence of endogenous mouse class II genes or HLA-DQ8/HCD4 double transgenic mice were used (Black et al., 2002; Cheng et al., 2003). The mice were bred in a conventional specific pathogen free colony (SPF) at McMaster University and maintained for at least 2 generations prior to breeding on a gluten-free diet (Bio-Serv, New Jersey). Mice were used at the age of 8-14 weeks. Male C57BL/6 mice were purchased from Taconic (Hudson, NY, USA).

Sensitization protocol and indomethacin treatment. All mice were continuously fed with a gluten-free diet and water available *ad libitum*. Mice were sensitized by injecting

intraperitoneally (ip) 500 μg of gluten (Sigma-Aldrich, Ontario) dissolved in 0.02 mM acetic acid in 50 μl of Complete Freund's Adjuvant (CFA, Sigma-Aldrich, Ontario). One week after sensitization, gluten challenge was performed 3 times on a weekly basis by intragastric gavage, for 7 weeks, using 2 mg of gluten dissolved in 0.02 mM acetic acid. Indomethacin was administered by gavage (Ovation Pharmaceuticals, Ontario) (3.5 mg/kg) 24 hours before the gluten challenge. Control groups consisted of a) non-sensitized mice (CFA only) subsequently gavaged with rice cereal (2 mg/0.02 mM acetic acid), b) gluten-sensitized mice subsequently gavaged with gluten (2 mg/0.02 mM acetic acid) c) non-sensitized mice (CFA only) subsequently gavaged with indomethacin (3.5 mg/kg).

In vitro intestinal permeability. Two sections of jejunum from each mouse were used for Ussing chamber studies. Briefly, 5 cm of jejunum samples were collected and divided into 2 segments. Each segment was opened along the mesenteric border, flattened and mounted in an Ussing chamber with an opening of 0.6 cm^2. Tissues were bathed in oxygenated Krebs buffer containing 10 mM glucose (serosal side) or 10 mM mannitol (luminal side) at 37°C. After a 20-minute equilibration period, conductance (G: mS/cm2) were recorded. Mucosal to serosal transport of macromolecules was assessed by adding horseradish peroxidase (HRP; type II, Sigma-Aldrich, Ontario), a commonly used macromolecular marker, in the luminal side. Serosal samples (500 μl) were obtained at 30 minutes intervals for 2 hours. Intact HRP was assessed using a modified Worthington method with *o*-dianosidine dihydrochloride (Sigma-Aldrich, Ontario) as the substrate, and

mucosal to serosal fluxes were calculated according to standard formulae and expressed as pmol/cm^2/hr.

Examination of epithelial cell injury. Jejunal sections were obtained and immediately fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 2 hours, transferred to sodium cacodylate buffer and stored at 4°C overnight. Tissues were subsequently processed for electron microscopy, and photomicrographs were prepared. Ultra-structural epithelial damage was evaluated in enterocytes by the presence of alterations in brush border, mitochondrial edema and tight junction (TJ) morphology. Epithelial damage was determined by transmission electron microscope (JEOL, Tokyo) of enterocytes in sections from 4-6 animals in each of the 4 study groups. The number of mitochondria with disrupted cristae within the apical region of enterocytes were counted on coded 5000x magnification photomicrographs measuring a total of 300 μm^2 (125-250 mitochondria/ photomicrographs, 5 photomicrographs/mouse, 6 mice/group) using Adobe CS3 Extended (Adobe Systems Incorporated, California). The fraction of altered mitochondria, defined as number of mitochondria with disrupted cristae divided by total number of mitochondria in a view, was calculated. Mitochondria on the edge of micrographs were excluded for evaluation, since neither their boundaries nor area could be accurately determined. The fraction of disrupted TJ structure was calculated as total altered TJ divided by total number of TJ evaluated in 20 fields per mouse in a blinded manner (3-20 tight junctions/field, 60-400 tight junctions/mouse, 4 mice/group). A field is defined as one square in the EM grid, measuring 8100 μm^2.

Apical junctional analysis by quantitative real-time PCR. Total RNA from a 30-60 mg proximal small intestine section was isolated using RNeasy mini kit (Qiagen, Ontario). cDNA was synthesized from 2 μg of purified total RNA using M-MLV reverse transcriptase (Invitrogen, Ontario). Quantitative real-time PCR was performed with 1:20 dilutions of cDNA. The reaction consisted of iQ^{TM} SYBR Green Supermix (Bio-rad, Ontario) for quantitative PCR, primers at 0.5 μM, and 1 μl of cDNA. Amplification was performed using iQ5 Real-Time Detection System at 95°C for 3 min followed by 37 cycles at 94°C for 15 s, 55°C (ZO-1) or 58°C (E-Cadherin) or 59°C (GAPDH) for 20 s, and 72°C for 25 s. Q real-time PCR was performed using the following primers: *ZO-1* 5'-AGGACACCAAAGCATGTGTGAG-3' and 3'-GGCATTCCTGCTGGTTACA-5'; *E-Cadherin* 5'-GCACATATGTAGCTCTCATC-3' and 5'CCTTCACAGTCACACACATG-3'. *Gapdh* 5'-CCATGGAGAAGGCTGGGG-3' and 5'-CAAAGTTGTCATGGATGACC-3' was used as housekeeping gene. CT values reported by iQ5 software were used in the study. PCR efficiencies for each amplicon were determined by making 10-fold serial dilutions of cDNA; the cDNA was then amplified using primers to both the gene of interest and housekeeping gene. Relative expression levels were calculated using the Pfaffl method (Pfaffl, 2001; Pfaffl et al., 2002), with efficiency correction for each primer set, using REST software (Pfaffl et al., 2002). A melting curve analysis was performed by heating the reactions from 50º to 99ºC in 0.2ºC intervals while monitoring fluorescence.

Splenocyte proliferation, cell cultures and cytokine analysis. Peptic-tryptic digests of gliadin (PT-gliadin) was prepared as described previously (Lindfors et al., 2008). Spleen cells were harvested and cultured (4 x 10^5 cells/well) in 96-well tissue culture plates at 37ºC, 5% CO_2 for 72 h in the presence or absence of 500 μg/ml PT-gliadin and/or 5 μg/ml indomethacin. The cultures were pulsed with 1 μCi/well [^{3}H]-thymidine for 18 h. The cultured cells were harvested onto glass fibre filters using Filtermate harvester (Cambridge Technology, Massachusetts). The radioactivity incorporated was determined with a Beta Scintillation Counter (Beckman, California). Results are expressed as stimulation index (SI) and calculated as: SI= (mean cpm of triplicate cultures containing antigen)/ (mean cpm of cells cultured with medium alone).

Splenocyte supernatants were collected 48 h after incubation with or without PT-gliadin and/or indomethacin. The presence of pro-inflammatory cytokines in the supernatant was measured using pro-inflammatory CBA kit (BD Bioscience, California) and analyzed using BD FACSarray Bioanalyzer System (BD Bioscience, California).

Fluorescent in situ hybridization (FISH). Oligonucleotide probes are summarized in Table 3.1. The group-specific probes were labeled at the 5'-end with fluorescein isothiocyanate (FITC), showing green fluorescence. EUB 338 probe, targeting conserved sequences within the bacterial domain, was used as positive control (Amann et al., 1990). NON EUB 338 probe was used as negative control to eliminate the background fluorescence (Wallner et al., 1993). Both control probes were labelled at the 5'end with either the indocyanine dye Cy3, showing red fluorescence, or with FITC. Aliquots of 36

µl fixed samples were incubated with 4 µl of each fluorescent probe (50ng/µl) in hybridization solution (10 mM Tris–HCl, 0.9 M NaCl, pH 8.0, and 10% [w/v] sodium dodecyl sulphate) at appropriate temperature (45-50ºC) overnight. Afterwards, bacterial cells were incubated with 400 µl washing solution (10 mM Tris–HCl, 0.9 M NaCl, pH 8.0) at 50 ºC for 30 min to remove non-specific binding of the probes. Hybridized cells were finally pelleted by centrifugation (12 000 g for 5 min) and resuspended in 400 ml of PBS for flow cytometry detection. Bacterial groups were enumerated by combining each FITC-labelled group-specific probe with the EUB 338-Cy3 probe, and expressed as a ratio of cells hybridizing with the FITC-labelled specific probe to cells hybridizing with the EUB 338-Cy3 probe. This proportion was corrected by subtracting the background fluorescence obtained with the negative control probe NON EUB 338 (Sanz et al., 2007; Sokol et al., 2006). Flow cytometry detections were performed using anEPICS® XL-MCL flow cytometer (Beckman Coulter, Florida, USA) as previously described (Sanz et al., 2007). This instrument is equipped with two light scatter detectors that measure forward (FSC) and side scatter (SSC) and fluorescence detectors that detect appropriately filtered light at green (FL1, 525 nm) and red-orange (FL3, 620 nm) wavelengths. The event rate was kept at the lowest setting (200-300 events per second) to avoid cell coincidence. A total of 15, 000 events were recorded in a list mode file and analyzed with the System II V.3 software (Beckman Coulter).

Table 3.1. Oligonucleotide probes and hybridization conditions used in FCM-FISH analysis of intestinal bacteria.

Probe	Target bacterial group	Sequence (5'-3')	References
EUB338	Domain bacteria	GCTGCCTCCCGTAGGAGT	(Nadal et al., 2007)
NON338	Negative control	ACATCCTAC GGGAGG C	(Sanz et al., 2007)
Bif164	*Bifidobacterium*	CATCCGGCATTACCACCC	(Langendijk et al., 1995)
Lab158	*Lactobacillus-other lactic acid bacteria*	GGTATTAGCA(C/T)C TGT TTC CA	(Harmsen et al., 2000)
Bac303	*Bacteroides-Prevotella*	CCAATGTGGGGGACCTT	(Manz et al., 1996)
Eco11513	*Escherichia coli*	CACCGTAGTGCCTCGTCATCA	(Poulsen et al., 1994)
Elgc01	*Clostridium leptum*	GGGACGTTGTTTCTGAGT	(Franks et al., 1998)
Erec0482	*Eubacterium rectale/Clostridium coccoides*	GCTTCTTAGTCAGGTACCG	(Hold et al., 2003)
CHis150	*Clostridium histolyticum*	TTATGCGGTATTAATCT(C/T)CCT TT	(Bullock et al., 2004)
CLis135	*Clostridium lituseburense*	GTTATCCGTGTGTACAGGG	(Bullock et al., 2004)
SBR	*Sulphate-reducing bacteria*	TACGGATTTCACTCCT	(Bullock et al., 2004)

Immunohistochemistry for macrophages. Immunostaining for macrophages was performed on paraffin sections using a monoclonal antibody recognizing the F4/80 antigen (Bercik et al., 2002). The primary antibody rat anti-mouse (1:2000; Serotec, London) was followed by biotinylated polyclonal goat anti-rat antibody (1:200; Cederlane Laboratories, Ontario) and then streptavidin/HRP (1:300; Dako cytomation, Ontario). Antibodies were visualized by DAB and counterstaining with Mayer's hematoxylin. Negative controls were performed in the absence of primary antibody.

Statistical Analysis. Statistical analysis was performed using ANOVA with post-hoc test for simple and multiple comparisons, respectively. Nonparametric statistical significance of relative RNA expression was calculated with REST software (Pfaffl et al., 2002) by a

pairwise fixed reallocation randomization test with 50,000 repeats. Data were presented as means ± standard error (SEM).

3.4. Results

Gluten sensitization and indomethacin treatment led to retardation of weight gain. Gluten sensitized mice and non-sensitized mice treated with indomethacin exhibited a mild retardation of weight gain after 7 weeks compared to non-sensitized controls. Gluten-sensitized mice treated with indomethacin exhibited a more severe retardation of weight gain after 7 weeks, compared to all groups (Figure 3.1). These results suggest delayed thriving in mice treated with both gluten and indomethacin.

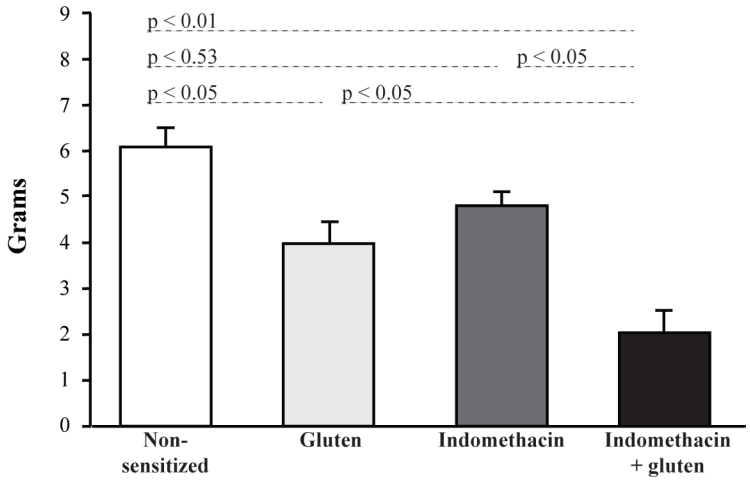

Figure 3.1. Weight gain over 7-week period.
Both gluten-sensitized and indomethacin treated mice exhibited a decreased rate of weight gain compared to non-sensitized controls. Gluten-sensitized mice treated with indomethacin exhibited more pronounced weight gain retardation compared to controls and to gluten-sensitized and indomethacin treated mice. n=10/group. Data are mean ±SEM

Indomethacin increased tissue conductance and permeation of macromolecules across epithelium in gluten-sensitized mice. In order to determine the effects of gluten sensitization and indomethacin treatment on intestinal permeability, tissue conductance and HRP flux were measured in segments of small intestine. Gluten-sensitized mice treated with indomethacin exhibited a significant increase in small intestinal tissue conductance compared to non-sensitized controls and indomethacin alone-treated mice (Figure 3.2A). HRP flux, a measurement of transcellular macromolecular transport, was elevated in all groups compared to non-sensitized controls. Gluten sensitization and indomethacin treatment, however, led to the highest increase in HRP flux with approximately 2.5 fold increase compared non-sensitized controls (Figure 3.2B). The potentiation of intestinal permeability changes by indomethacin was not observed in C57BL/6 mice sensitized with gluten, stressing the importance of the DQ8 transgene in the model (Figure 3.3).

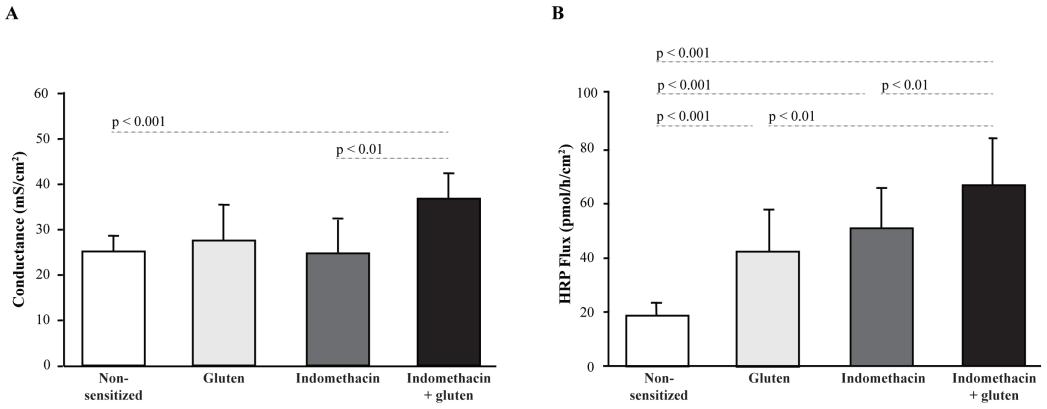

Figure 3.2. Intestinal barrier measurements.
(A) Gluten-sensitized mice treated with indomethacin showed a significant increase in tissue conductance. (B) HRP flux (transcellular permeability) increased significantly in all treatment groups compared to non-sensitized controls, however the highest values were observed in gluten plus indomethacin treated mice. n=10 mice/group. Data represent the means ± SEM of 10 mice/group.

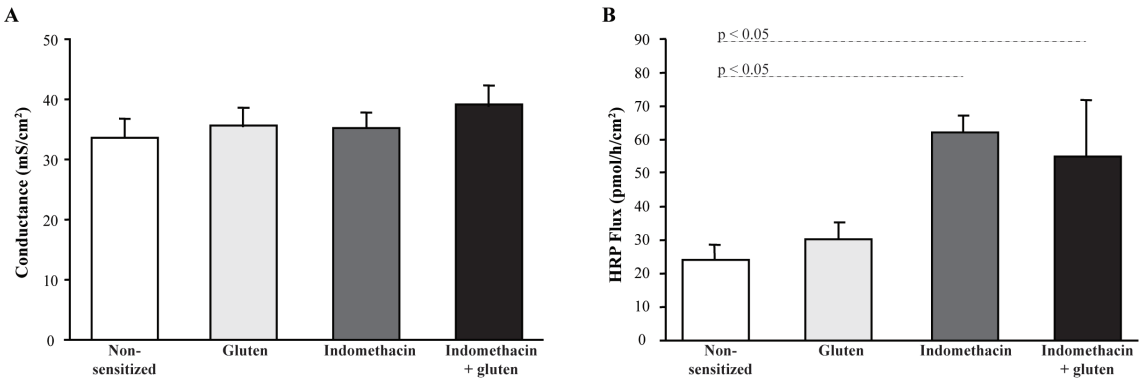

Figure 3.3. Conductance and HRP flux in C57BL/6 mice.
(A) Gluten and/or indomethacin treatment did not lead to changes in tissue conductance. (B) HRP flux was increased in indomethacin treated mice, but not in gluten sensitized mice without indomethacin. n=10 mice/group. Data are mean ± SEM.

Indomethacin led to epithelial ultra-structural damage in gluten-sensitized mice. Intestinal morphology was analyzed using electron microscopy. No mitochondrial abnormalities were detected in non-sensitized controls or in gluten-sensitized mice without indomethacin (Figure 3.4A-B). Mitochondrial abnormalities were observed in mice treated with indomethacin alone (Figure 3.4C) and in gluten-sensitized mice treated with indomethacin (Figure 3.4D). We quantified the proportion of mitochondria with disrupted cristae in a defined area with approximately the same number of mitochondria (Figure 3.4E). Gluten-sensitized mice treated with indomethacin, had a higher proportion of damaged mitochondria than gluten-sensitized mice without indomethacin. Epithelial cell edema and disrupted microvilli were observed in tissues obtained from gluten-sensitized plus indomethacin treated mice but not from the rest of the groups (Figure 3.5). These marked ultra-structural changes were not observed in gluten-sensitized (Figure 3.5C) and indomethacin alone-treated mice (Figure 3.5D). Altered junctional ultra-

structure was more pronounced in tissues from gluten-sensitized plus indomethacin-treated mice (Figure 3.5E-G) compared to non-sensitized (Figure 3.5A-B), gluten-sensitized (Figure 3.5C) and indomethacin alone-treated mice (Figure 3.5D).

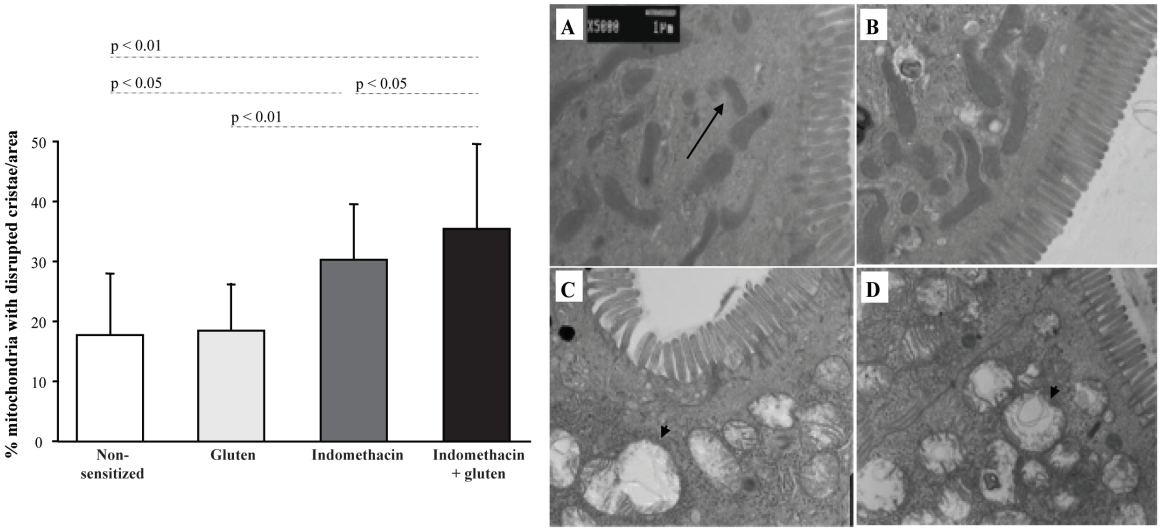

Figure 3.4. Evaluation of mitochondrial disruption.
Indomethacin increased the fraction of disrupted mitochondria. Gluten sensitization plus indomethacin treatment further increased the proportion of altered mitochondria. n=6 mice/group. Data represent the means ± SEM of 6 mice/group.
(A) Control mouse, arrow: normal mitochondria; (B) Gluten-sensitized mouse; (C) Indomethacin-treated mouse, arrowhead: mitochondria with disrupted cristae; (D) Indomethacin-treated plus gluten-sensitized mouse, arrowhead: mitochondria with disrupted cristae.

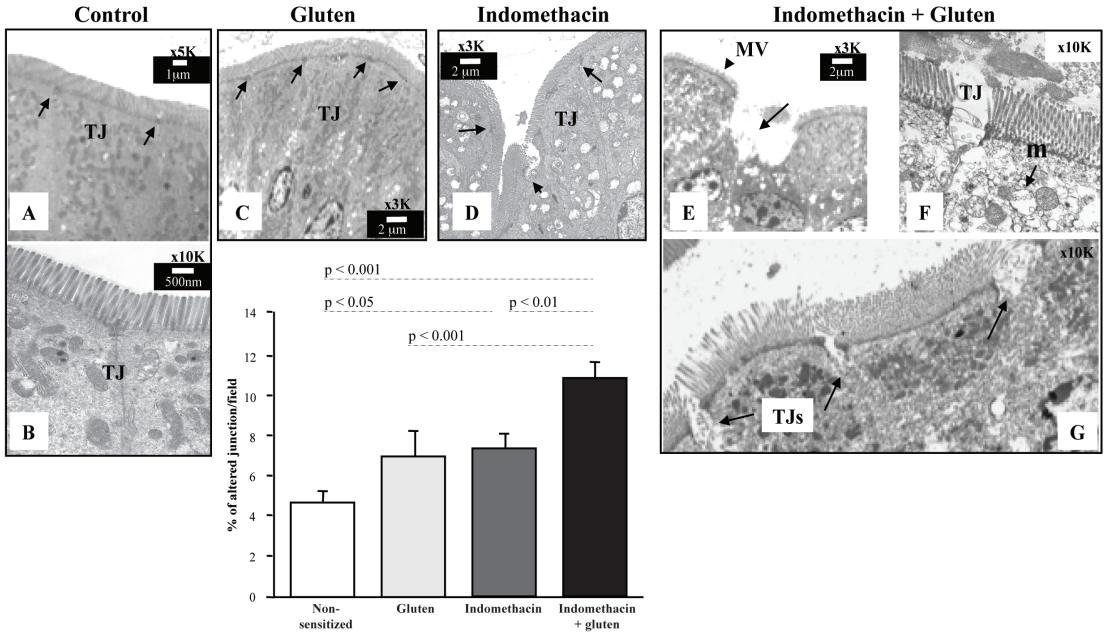

Figure 3.5. Apical epithelial cell structural abnormalities.
A significant proportion of altered TJ was observed in gluten-sensitized plus indomethacin-treated mice. Indomethacin alone also increased the proportion of altered TJ but to a lesser extent than indomethacin plus gluten. Gluten sensitization alone tended to increase the proportion of altered TJ but this did not achieve statistical significance (p=0.09 vs non-sensitized controls). Data represent the means ± SEM of 4 mice/group.
(A-B) Representative pictures of a control mouse, arrow: tight junction (TJ) with preserved structure; (C) Gluten-sensitized mouse, arrow: TJ with preserved structure; (D) Indomethacin-treated mouse showing one altered TJ (arrowhead) and 2 junctions with normal structure (arrows); (E-G) Gluten plus indomethacin treated mouse; (E) arrowhead: microvilli (mv) height reduction, arrow: apical epithelial cell destruction; (F) Altered TJ, arrow: mitochondria (m) with disrupted cristae; (G) Several altered TJs.

Concomitant treatment with indomethacin and gluten led to reduction of E-Cadherin mRNA expression. The changes in the ultra-structure of the tight junctions prompted us to investigate whether there were alterations in RNA expression of epithelial adherens and tight junctional proteins. Gluten-sensitized and indomethacin alone-treated mice showed reduced E-Cadherin RNA expression by a mean factor of 0.762 and 0.533

respectively, but this did not achieve statistical difference relative to non-sensitized controls (Figure 3.6). In contrast, expression of E-Cadherin RNA was markedly down regulated by 2.75 fold compared to non-sensitized mice, in gluten-sensitized plus indomethacin-treated mice. Gluten sensitization and indomethacin did not affect significantly tight junction ZO-1 relative RNA expression (Figure 3.7).

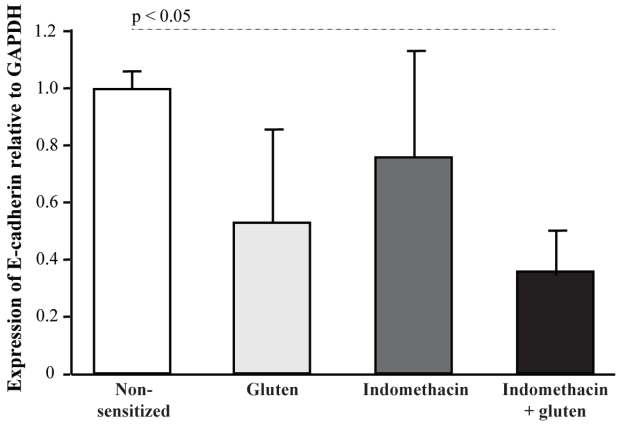

Figure 3.6. RNA level of E-cadherin relative to control (non-sensitized).
Gluten-sensitized and indomethacin alone-treated mice showed a trend for decreased expression of E-Cadherin relative to non-sensitized controls. Gluten-sensitized plus indomethacin-treated mice showed marked down-regulation of E-cadherin RNA level relative to non-sensitized controls. Data represent the means ± SEM of 6 mice/group.

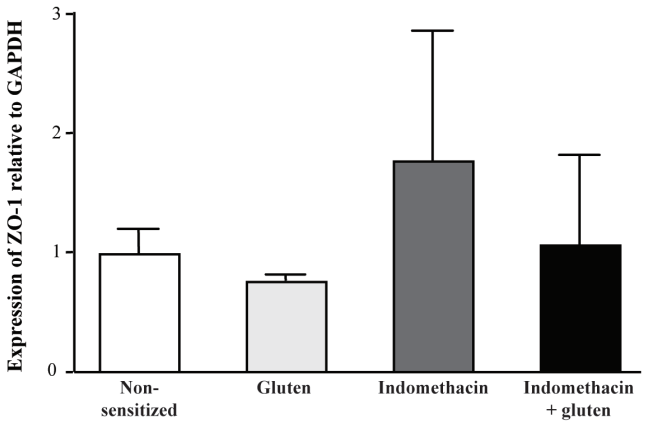

Figure 3.7. ZO-1 RNA expression.
No significant differences were seen when RNA expression for each treatment group was analyzed relative to non-sensitized controls.

Indomethacin treatment affected the release of IFN-γ by splenocytes from gluten-sensitized mice after in vitro challenge with PT-gliadin. In order to assess whether the increase in permeability and the damage to the intestinal structure in gluten-sensitized mice after treatment with indomethacin led to an increase in the systemic immune response to gliadin we analyzed antigen-specific proliferation and cytokine production of splenocytes. Increased T cell proliferation after incubation with PT-gliadin was observed in gluten-sensitized mice, but not in non-sensitized controls (Figure 3.8). Differences in proliferation did not reflect cell death or an inability to proliferate as polyclonal stimulation with ConA led to equal responses in all groups (data not shown). Surprisingly, indomethacin-treatment of gluten-sensitized mice did not exhibit higher levels of antigen-specific proliferation compared to gluten-sensitized mice that were not given indomethacin (Figure 3.8). *In-vitro* incubation of splenocytes from gluten-sensitized mice with indomethacin did not increase cell proliferation (Figure 3.9).

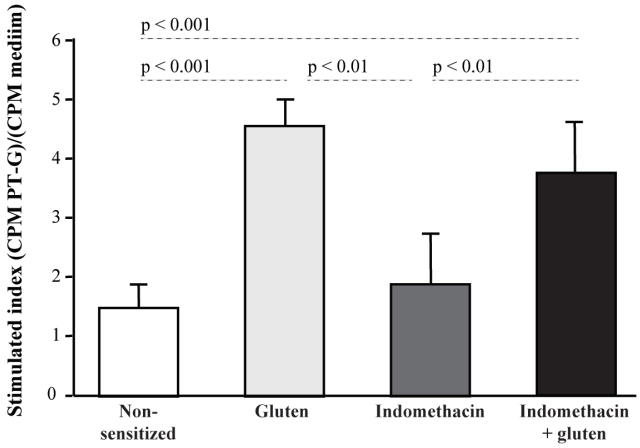

Figure 3.8. Splenocyte proliferation after incubation with PT gliadin.
Splenocytes from gluten-sensitized mice treated with or without indomethacin exhibited increased proliferation compared to non-sensitized controls. Data represent the means ± SEM of 6 mice/group.

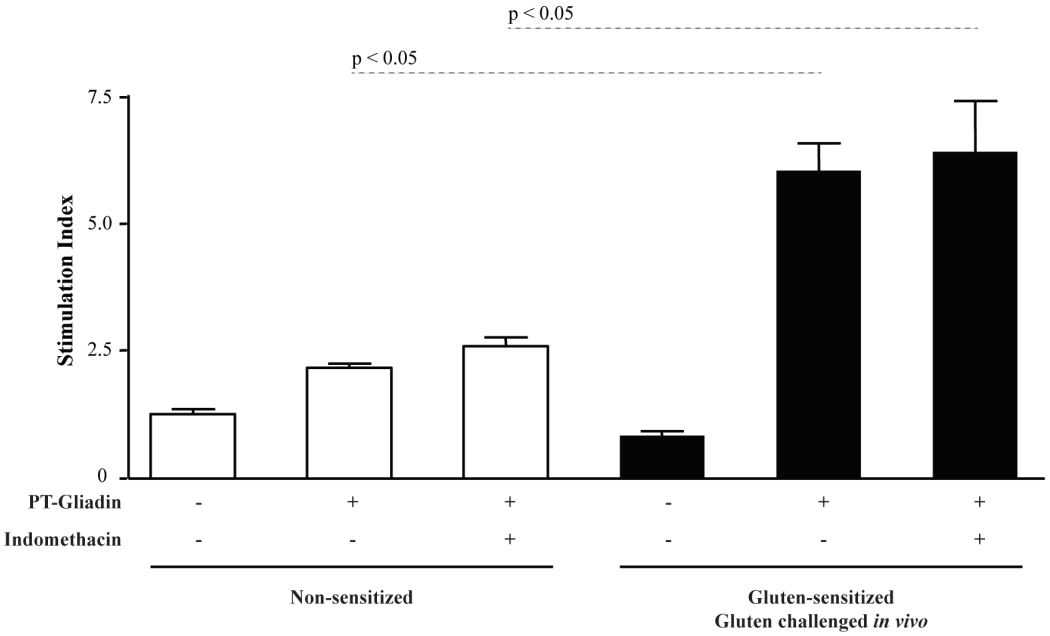

Figure 3.9. Splenocyte proliferation after incubation with PT-gliadin and/or indomethacin.
Stimulation with indomethacin alone did not increase splenocyte proliferation in gluten-sensitized mice. *In-vitro* stimulation with both PT-gliadin and indomethacin, did not further enhance cell proliferation compared to PT-gliadin alone. n=5/group. Data are mean ±SEM.

To further assess the systemic immune response IL-12, IFN-γ and IL-10 levels in the supernatant of the PT-gliadin stimulated splenocytes cultures were determined (Figure 3.10). Whilst IL-12 was not induced above media alone IL-10 levels were slightly increased in the culture supernatant of splenocytes from gluten-sensitized and indomethacin-treated gluten-sensitized splenocytes after *in vitro* stimulation with PT-gliadin, although the increases were not statistically significant.

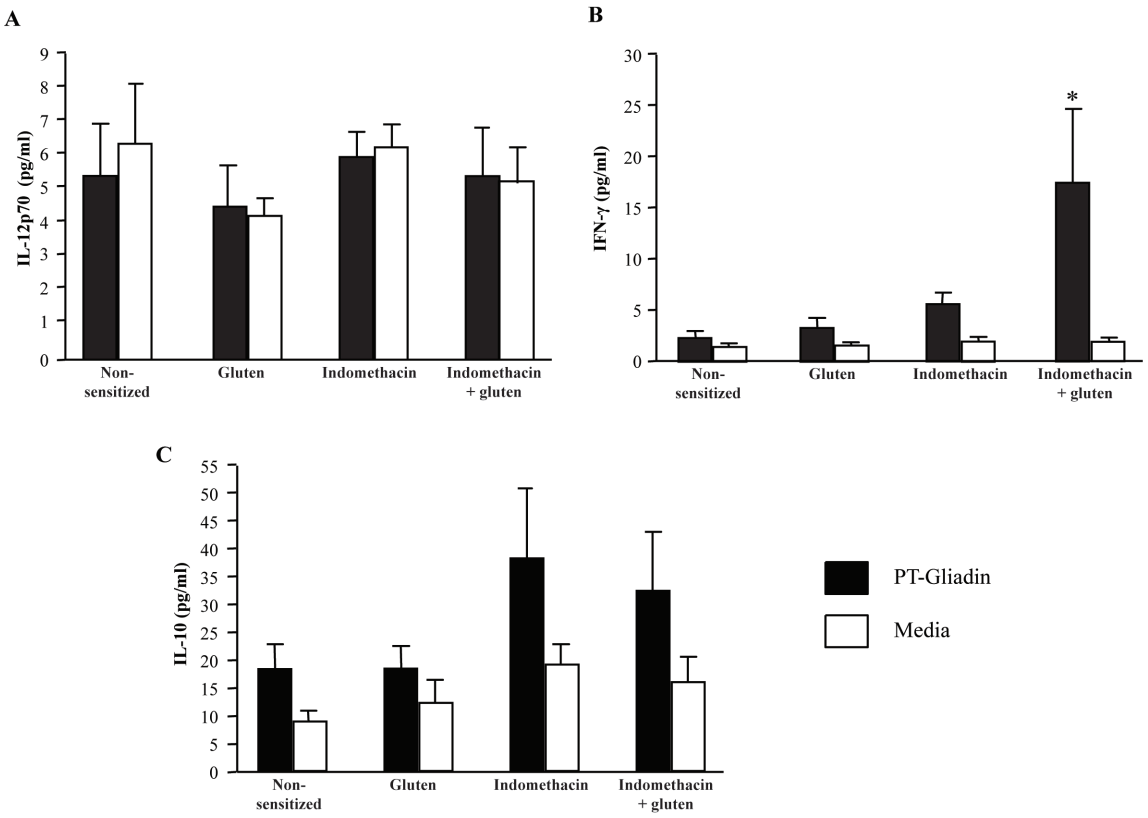

Figure 3.10. Cytokines in supernatant of splenocyte cultures after incubation with PT-gliadin (black) or medium (white).
Culture supernatants from gluten-sensitized plus indomethacin (Indo) treated mice showed increased IFN-γ (*p<0.01 vs all groups). Cultured splenocytes from gluten-sensitized mice, with or without indomethacin showed a trend for increased IL-10 release after PT-gliadin stimulation. Data represent the means ± SEM of 6 mice/group.

In contrast, indomethacin treatment of gluten-sensitized mice led to a significant increase in IFN-γ production in response to PT-gliadin stimulation. *In-vitro* incubation of splenocytes from gluten-sensitized mice with indomethacin did not increase IFN-γ production (Figure 3.11).

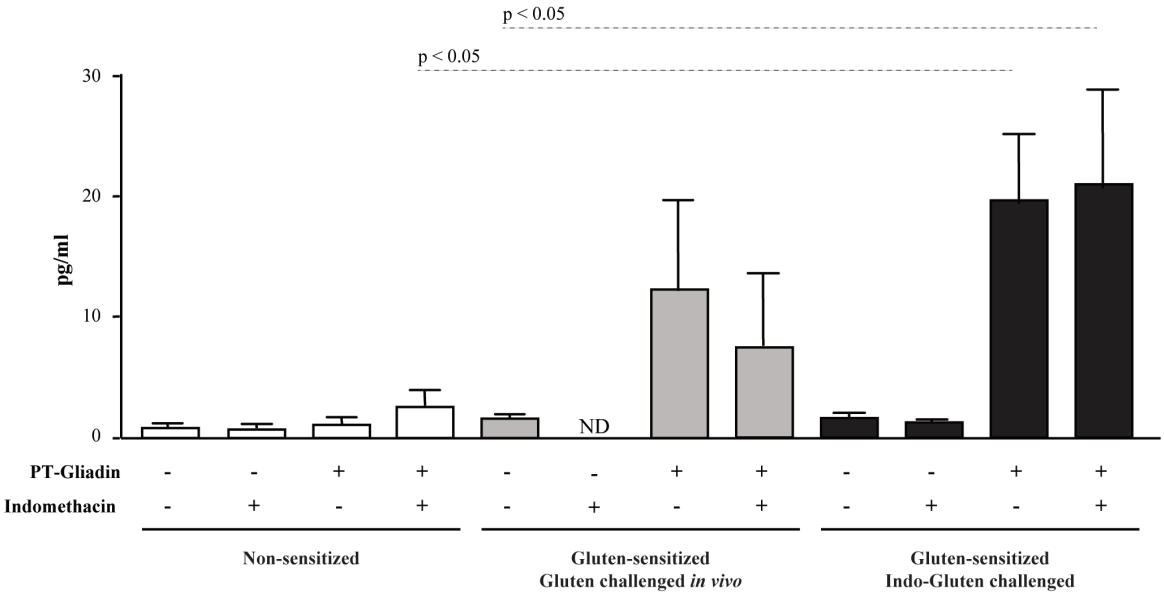

Figure 3.11. IFN-γ levels in supernatant of cultured splenocytes after incubation with PT-gliadin and/or indomethacin.
Stimulation with indomethacin alone did not increase IFN-γ production in gluten-sensitized mice. *In-vitro* stimulation with PT-gliadin and indomethacin did not increase IFN-γ levels compared to PT gliadin alone. n=5/group. Data are mean ±SEM. ND = not detectable.

Gluten and indomethacin led to changes in intestinal microbiota composition. We next analysed whether gluten sensitization or indomethacin treatment could lead to changes in the composition of the intestinal microflora. Gluten-sensitized mice showed a significant decrease of gut bacterial proportions of *E. coli* and *E. rectale-Clostridium* groups, as

compared to control mice. Indomethacin-treated mice also showed reductions in *E. coli* proportions, but increases in those of *Bacteroides-Prevotella* group. Gluten-sensitized mice treated with indomethacin showed the most remarkable alterations in the intestinal microbiota, characterized by reductions in the relative abundance of all bacterial groups analysed as compared with control mice (Figure 3.12).

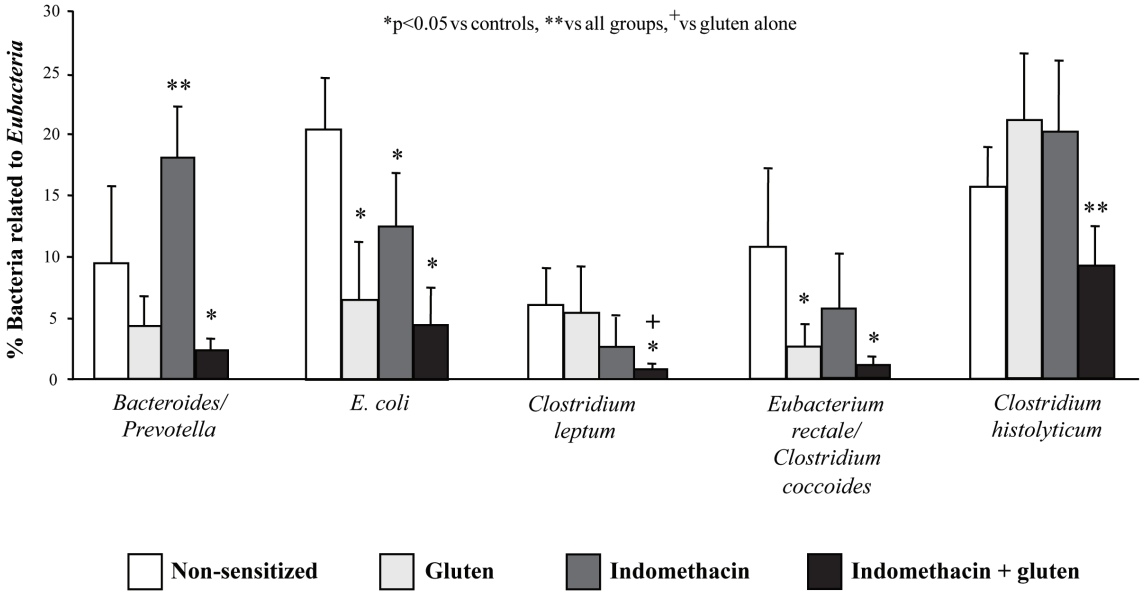

Figure 3.12. Microbiota composition.
Using 9 different oligonucleotide probes and fluorescent *in situ* hybridization (FISH), microbial profile was investigated in the distal jejunum of sensitized mice with and without indomethacin. The results indicate a significant perturbation in the proportions of microbiota investigated in all 3 treatment-groups when compared to non-sensitized controls, and remarkably in the gluten-sensitized plus indomethacin group. These differences achieve statistical significance in *Bifidobacteria* (*p=0.04 vs controls, +p=0.03 vs gluten) and *Clostridium leptum* cluster (both *p=0.02 vs controls and gluten sensitized, **p=0.04 vs indomethacin) compared to gluten-sensitized alone. Data represent the means ± SEM of 6 mice/group.

Indomethacin led to systemic priming against intestinal microbiota in gluten-sensitized mice. Previous data suggests that bacterial translocation beyond the mucosal immune system is necessary for systemic priming to intestinal commensals (Matysiak-Budnik et al., 2008). To determine if the increased conductance and HRP flux induced by gluten-sensitization and indomethacin was accompanied by loss of the host's normal systemic ignorance to the intestinal microbiota, we measured specific IgM antibody responses to culturable aerobic or anaerobic commensals. Non-sensitized mice showed no evidence of IgM specific antibodies against aerobic and anaerobic commensal flora as assessed by flow cytometric analysis of anti-bacterial IgM responses (Figure 3.13-3.14). Treatment with either gluten or indomethacin alone led to the production of very low titers of IgM antibodies directed against a subset of culturable bacteria. Gluten sensitization plus indomethacin treatment, however, resulted in induction of strong specific IgM responses directed against 40-80% of culturable bacteria. The anti-bacterial IgM induced was specific to the commensal microflora of the host and did not bind to *Salmonella*, which these mice have never been exposed to (Figure 3.15). These data indicate that the combination of gluten-sensitization and increased intestinal permeability as induced by indomethacin treatment increased systemic priming to the commensal microflora.

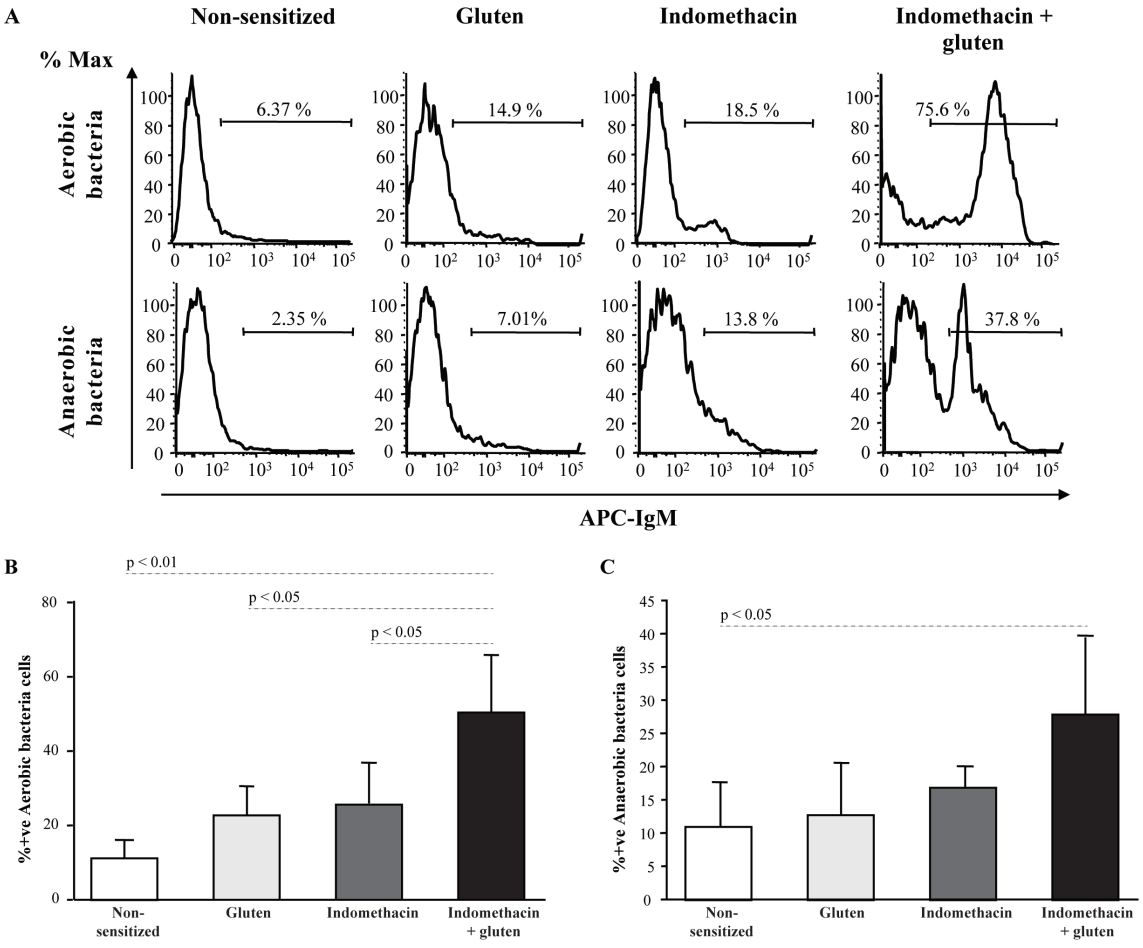

Figure 3.13. Systemic antibodies against commensals.
Serum from indomethacin and gluten treated mice showed significant positive serum antibodies against their aerobic and anaerobic intestinal microbiota. (A) Representative FACS histogram from each treatment gated on IgM^+ cells; (B) Proportion of IgM^+ aerobic bacterial cells for each treatment groups; (C) Proportion of IgM^+ anaerobic bacterial cells for each treatment groups. Data represent the means ± SEM of 6 mice/group.

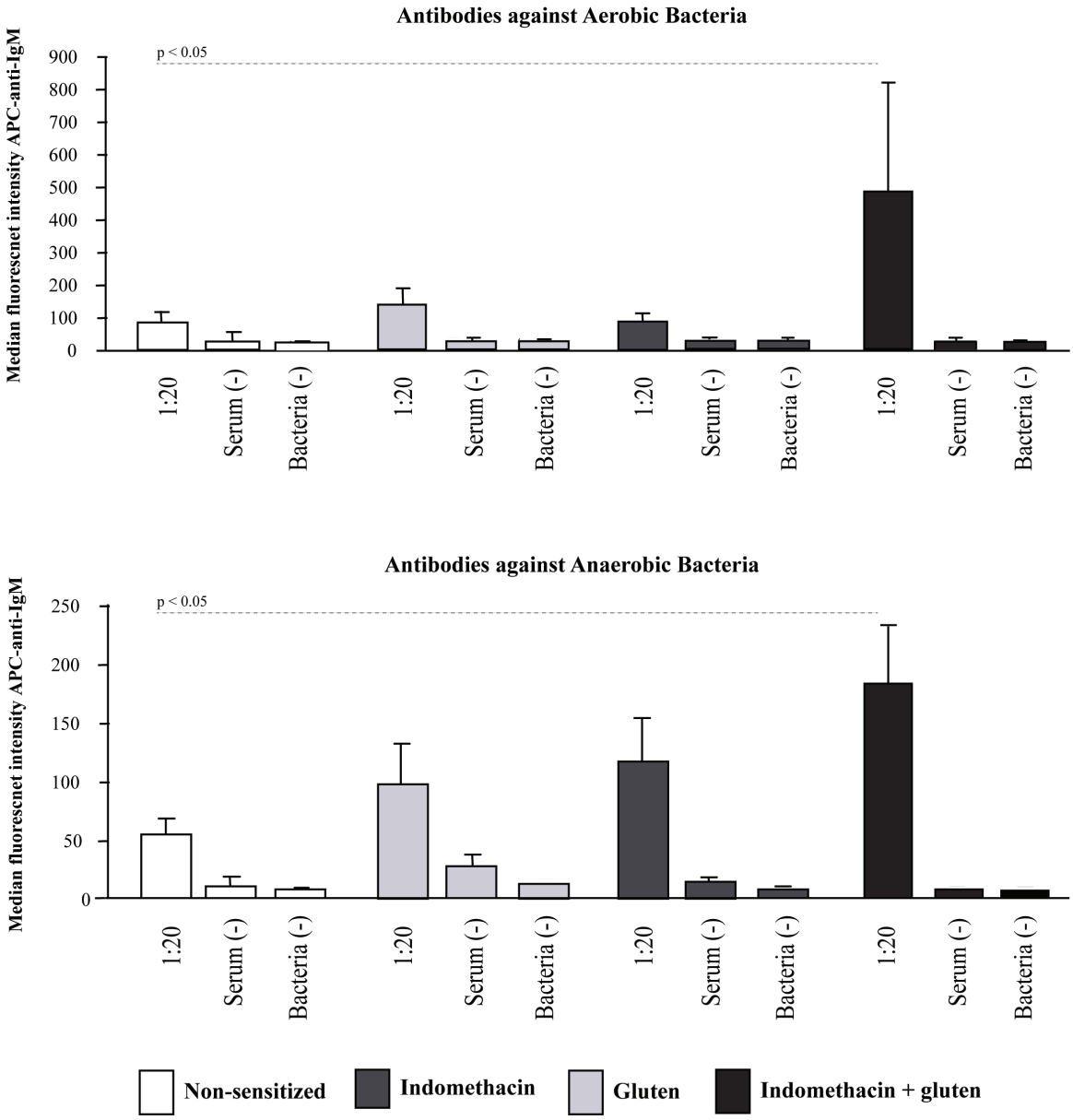

Figure 3.14. Systemic antibodies against commensals.
Gluten-sensitized plus indomethacin-treated mice exhibited increased serum antibodies against aerobic and anaerobic bacteria as assessed by median fluorescent intensity signal of APC-labelled anti-IgM (1:20 serum dilution). Negative controls include serum (-): no serum and bacteria (-): no bacteria. n=6 mice/group. Data are mean ± SEM.

A Negative antibodies against *Salmonella* M556 in Indomethacin + gluten treated mouse

B Positive antibodies against *Salmonella* M556 in *Salmonella* infected mouse

Figure 3.15. Positive and negative systemic antibodies against commensals.
(A) *Salmonella* M557, which is a pathogen not present in our HLA-DQ8/HCD4 mice colony, was stained with serum antibodies from indomethacin plus gluten treated mice. Results show the absence of positive antibodies against *Salmonella*, thus the specificity of the technique. (B) *Salmonella* M557 was stained with serum antibodies from *Salmonella* M557 infected mice. Results show the absence of positive antibodies against *Salmonella*.

3.5. Discussion

The aim of this study was to investigate whether modulation of the intestinal barrier by an environmental trigger can affect host responses to luminal antigens in a genetically predisposed host. Our results demonstrate that gluten sensitization and long-term gluten challenge in HLA-DQ8/HCD4 mice alters intestinal permeability as assessed by increased transcellular macromolecular transport and a tendency for higher conductance values (paracellular pathway). This is in agreement with previous reports showing that gluten peptides have the ability to rapidly disrupt the apical junctional structure (Clemente et al., 2003; Drago et al., 2006; Fasano et al., 2000; Sander et al., 2005) and can also be transported by an abnormal transcellular route (Matysiak-Budnik et al., 2008). Administration of indomethacin to gluten-sensitized mice led to more pronounced barrier dysfunction, which was accompanied by a mild pro-inflammatory shift with marked

changes in EC ultra-structure, reduced E-cadherin mRNA levels in the proximal small intestine and generation of systemic antibody responses to intestinal microbiota.

Indomethacin has been used at higher doses as a model of inflammatory bowel disease (IBD) (Porras et al., 2006; Sigthorsson et al., 2002), and has been shown to compromise epithelial barrier integrity and function, induce motility changes, and promote small intestinal bacterial overgrowth and translocation (Berg, 1995; Porras et al., 2006). In the present manuscript, low-dose indomethacin induced permeability changes without producing macroscopic or microscopic ulcers. However, ultra-structural observations revealed epithelial cell abnormalities characterized by mitochondria with disrupted cristae in mice receiving indomethacin. Mucosal toxicity induced by gluten in HCD4/DQ8 mice was potentiated by indomethacin, as shown by more marked elevation of HRP flux and a significant increase in tissue conductance. After administration of indomethacin, C57BL/6 mice exhibited increased HRP flux, but no change in tissue conductance. Gluten sensitization however, did not induce barrier dysfunction in C57Bl6 mice, emphasizing the relevance of the DQ8 transgene in the model (Figure 3.3). Electron microscopy examination in gluten-sensitized HCD4/DQ8 mice treated with indomethacin revealed more structural abnormalities in the apical region of the epithelium compared to gluten alone-treated mice. Furthermore, RT-PCR analysis demonstrated reduced E-cadherin RNA levels in gluten-sensitized plus indomethacin treated mice. E-cadherin is required for TJ formation and there is growing evidence for its role as modulator of TJ and intestinal barrier function (Guo et al., 2003; Sander et al., 2005). E-cadherin expression is reduced in children with celiac disease and gliadin has been shown to alter

its expression (Barshack et al., 2001). Our results support the hypothesis that both gluten and indomethacin play a role in the expression of E-cadherin, and that this effect is potentiated in a genetically susceptible host when both agents are administered together. Marked reduction of E-cadherin may constitute a mechanism for the enhanced barrier dysfunction observed in gluten-sensitized and indomethacin-treated HCD4/DQ8 mice.

The marked changes in barrier function in gluten-sensitized plus indomethacin-treated mice were accompanied by increased IFN-γ production in splenocyte cultures after incubation with PT-gliadin. These results suggest a shift towards a mild systemic pro-inflammatory state. Previous studies have shown that cyclooxygenase-2 (COX-2)-dependent arachidonic acid metabolites are important in the maintenance of intestinal immune homeostasis, particularly in the immunoregulation of dietary antigens (Newberry et al., 1999). Consequently, COX-2 inhibitors such as indomethacin may exacerbate the immune response to dietary antigens (Newberry et al., 1999). Our results using *in vitro* incubation of splenocytes with PT-gliadin and indomethacin, however, do not support a direct effect of indomethacin on splenocyte proliferation and IFN-γ release. Thus, we hypothesize that the shift in the immune response may be due to an enhanced uptake of luminal contents, including commensal bacteria, through a more structurally damaged and permeable epithelium.

The intestinal epithelium regulates permeation of luminal antigens and excessive immune activation within the mucosa (Fasano and Shea-Donohue, 2005; Yu and Perdue, 2000). The marked barrier defect in gluten-sensitized mice treated with indomethacin may not only allow an increased influx of gliadin peptides across the epithelium but also

of other luminal antigens such as intestinal microbiota with potential bystander, pro-inflammatory effects. Germ-free rats have been reported to have a higher threshold for intestinal damage after indomethacin administration compared to specific pathogen-free (SPF) rats (Robert and Asano, 1977). Since inhibition of prostaglandins in the absence of an intestinal microbiota is less severe, the results raise the hypothesis that intestinal bacteria potentiate the development of indomethacin-induced mucosal lesions. Thus, dysmotility induced by gluten sensitization (Verdu et al., 2008) or indomethacin (Porras et al., 2004), and/or the ability of indomethacin to induce small intestinal dysbiosis (Dalby et al., 2006; Porras et al., 2004) may facilitate bacterial translocation. Due to a severely impaired intestinal barrier in both gluten-sensitized and indomethacin-treated mice, increased permeation of luminal bacteria may disturb the natural commensal homeostasis in the gut promoting a pro-inflammatory response. SPF mice have been shown to be systemically ignorant to their intestinal microbiota due to the geographic and functional separation between the mucosal and systemic immune system by the mesenteric lymph nodes (MLN) (Macpherson and Uhr, 2004a; Macpherson and Uhr, 2004b). Our results show, however, that a low level of systemic priming against intestinal microbiota occurs in SPF mice treated with either gluten or indomethacin alone. Gluten-sensitized mice, in which barrier function is further perturbed by indomethacin treatment, show dramatic systemic priming to their intestinal microbiota. These data therefore imply that gluten-sensitization, in combination with indomethacin treatment, results in decreased mucosal containment of the commensal flora. NSAIDs have been shown to reduce the phagocytic properties of macrophages (Furst et al., 2005). Thus, we

acknowledge that it is possible that in addition to changes in intestinal barrier function, indomethacin may have a dual effect by inhibiting macrophage function, allowing the persistence of live bacteria, and facilitating a systemic immune response against intestinal microbiota. $F4/80^+$ cell counts in the lamina propria of gluten plus indomethacin-treated mice were significantly increased (Figure 3.16), however macrophage function was not assessed. The exact identities of the commensals to which gluten and indomethacin-treated mice are systemically primed in this model are not yet known but the absence of IgM binding to *Salmonella*, known to be absent from the commensal flora of our mice, strongly suggests the specificity of the IgM antibodies against commensal flora in our mice (Figure 3.15). The clinical relevance of the loss of systemic ignorance against the intestinal microbiota remains to be established, however, systemic priming to the commensal flora represents a significant shift in the normal relationship between host and commensal bacteria (Macpherson and Slack, 2007). Consequently, this may indicate a novel mechanism that could contribute to the progression of disease in a gluten-sensitive host. On the other hand, specific IgM against flora may be part of a protective mechanism mounted by the immune system to limit subsequent translocation and widespread inflammation. Additional host factors, such as an underlying immune dysbalance, may play a role in determining whether this mechanism will become maladaptive and contribute to widespread inflammation. A recent epidemiological study has determined that consumption of non-steroidal anti-inflammatory drugs (NSAIDs) is a risk for the development of irritable bowel syndrome (Ford et al., 2008). No epidemiological studies

to date have investigated whether a history of NSAID consumption is also a risk factor for the protean clinical expression in gluten sensitivity.

Although the role of the intestinal microbiota in other chronic diseases of the gut is clearly established [for review see (Tlaskalova-Hogenova et al., 2004)] little is known about the role of abnormal immune responses to commensals in gluten and other food intolerances. Recent findings, however, report presence of rod-shaped bacteria in the mucosa of active and non-active celiac patients but not in healthy controls (Forsberg et al., 2004). A study in patients with celiac disease revealed the presence of serological responses to microbial antigens, such as anti-*Saccharomyces cerevisiae*, anti-I2 (*Pseudomonas fluorescens*) and anti-ompW, compared to healthy controls. Interestingly, microbial seropositivity was also present in gluten-sensitive patient with no evidence of active CD. However, increasing age was associated with sero-reactivity for anaerobic bacteria, possibly reflecting exposure to different environmental antigens with longer duration of disease (Ashorn et al., 2008). The disappearance of anti-*Saccaromyces cervisiae*-antibodies (ASCA) after a gluten-free diet suggests that healing of mucosal lesions is related to microbial sero-markers (Mallant-Hent et al., 2006). However, a causal relationship between gut dysfunction, symptoms and microbial sero-responses in celiac disease remains to be determined. It is possible that accumulated bacterial products have a bystander effect and lower the threshold for immune cell activation (Sollid and Gray, 2004). To this respect, a study in DQ8 mice has shown that oral challenge with *Lactobacillus casei* at the time of mucosal sensitization with gliadin and cholera toxin exacerbates the Th1 response induced in the model (D'Arienzo et al., 2008). Thus, it is

possible that dysbiosis or shifts in the composition of the intestinal microbiota at the time of gluten sensitization, and not necessarily the presence of a pathogen, contribute to enhance gluten-induced immune responses. An altered microbiota composition has been reported in patients with celiac disease compared to healthy controls (Nadal et al., 2007; Sanz et al., 2007). In this study, we observed significant alterations in the composition of the small intestinal microbiota in gluten-sensitized mice treated with indomethacin. It is unclear, however, if these changes are primary or secondary to the functional gut abnormalities observed in the model (Verdu et al., 2008).

In conclusion, our findings suggest that an environmental alteration of the intestinal barrier plays a critical role in determining host immune responses to gluten and intestinal microbiota antigens. Bystander luminal antigens such as components of the intestinal microbiota may contribute to enhance inflammatory responses to dietary antigens such as gluten. This mechanism may become important in genetically predisposed hosts with longstanding barrier abnormalities. The results warrant further investigations on the interactions between host genotype, diet, and intestinal microbiota.

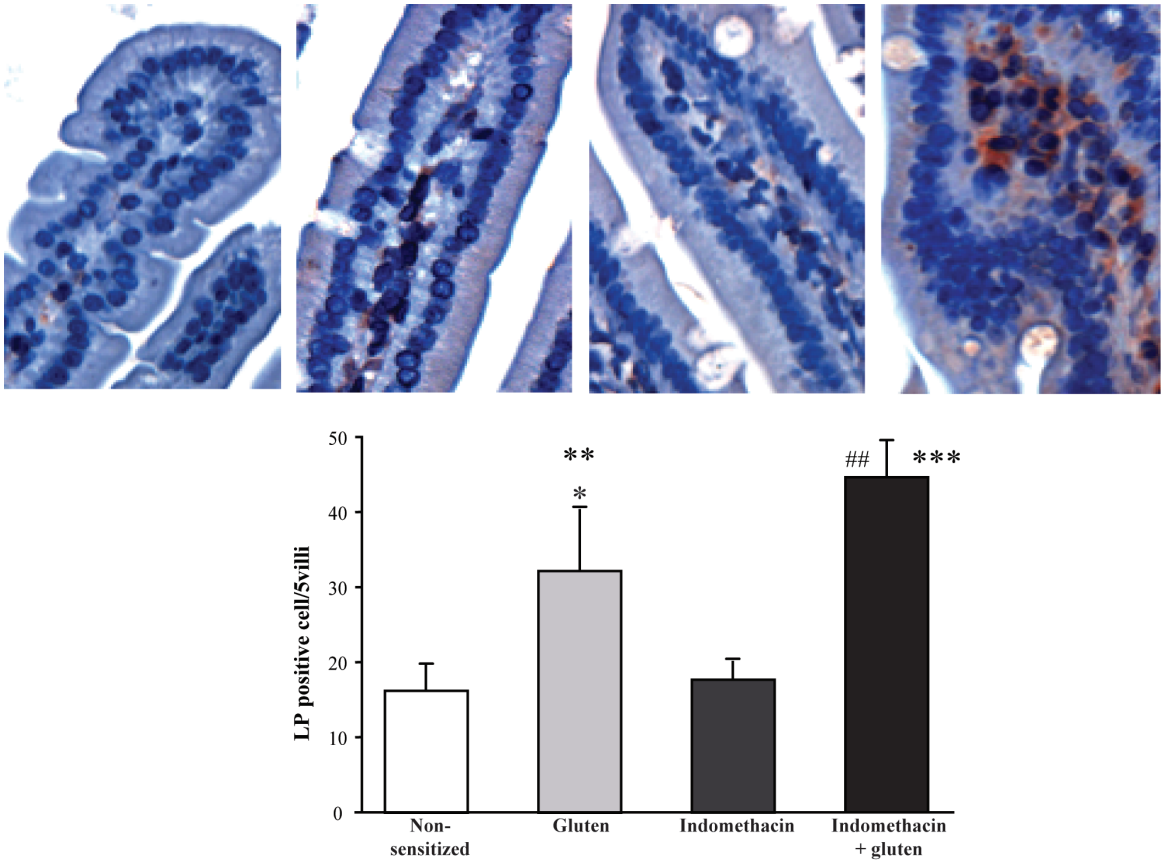

Figure 3.16. Immunohistochemistry for F4/80+ cells. Staining for F4/80+ was increased in gluten sensitized mice.
Infiltration of F4/80+ cells was most marked in gluten-sensitized mice treated with indomethacin. n=6 mice/group. Data are mean ±SEM. *p<0.05 vs non-sensitized, **p=0.03 vs indomethacin, ***p=<0.05 vs non-sensitized, ##p<0.05 vs indomethacin. Representative picture of macrophage infiltration in the lamina propria from (A) control mice (B) gluten-sensitized mice (C) indomethacin-treated mice (D) gluten-sensitized plus indomethacin treated mice.

3.6. Acknowledgements

We acknowledge Markus Geuking, Julia Cahenzli and Giada de Palma for their excellent assistance.

– CHAPTER 4 –

COMMENSAL AND PROBIOTIC BACTERIA INFLUENCE INTESTINAL BARRIER FUNCTION AND SUSCEPTIBILITY TO COLITIS IN *NOD1*$^{-/-}$;*NOD2*$^{-/-}$ MICE.

Commensal and probiotic bacteria influence intestinal barrier function and susceptibility to colitis in *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice.

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Preface: *This study was conducted from September 2009 to December 2011. I am the primary author of the paper. My supervisor and I designed the experiments, analyzed the data and wrote the manuscript. I performed all the experiments with experimental assistance from X. Huang (technician) and J. Jury (technician). V. Petit (post-doctoral fellow) performed the RT-qPCR experiment for RegIII-γ and provided the quantitative data for Figure 4.2F, 4.9D and Figure 4.14B. G. de Palma (post-doctoral fellow) performed the DGGE and PCR microbiota analysis, and provided the quantitative data presented in Table 4.2 and Figure 4.1. D. Philpott (collaborator) provided the mice. C.L. Garcia Rodenas provided the probiotic biomass. Y. Sanz, and K.D. McCoy K (collaborators) contributed valuable scientific input and critically appraised the manuscript.*

Summary and central message: This article explores the role of the intestinal microbiota in the maintenance of health and development of inflammation in a genetically predisposed host. Polymorphisms in Nucleotide oligomerization domain (*Nod*) receptors have been associated with IBD. The exact mechanisms by which *Nod* mutations confer susceptibility to IBD are not clearly understood. In the context of colitis and *Nod* mutations, it is unknown whether genetic risk can be modulated by environmental factors. This article investigated whether modulation of the intestinal microbiota influences intestinal barrier function and colitis susceptibility in *Nod1* and *Nod2* deficient mice. Under specific pathogen free (SPF) conditions, and compared to heterozygote littermate controls, *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice displayed increased colonic paracellular permeability, paralleled by decreased expression of E-cadherin, decreased RegIII expression, and increased susceptibility to experimental colitis. Mice colonized with the altered Schaedler flora, which is composed of eight defined and benign bacteria, as well as supplementation of SPF mice with *Bifidobacterium breve*, normalized antimicrobial RegIII expression and reduced the increased susceptibility to experimental colitis of *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. These findings provide support for specific probiotics and colonization strategies with intestinal microbiota with "low colitic capacity", as prophylactic measures for chronic intestinal inflammation or IBD relapses in genetically susceptible individuals.

Title: Commensal and probiotic bacteria influence intestinal barrier function and susceptibility to colitis in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice

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Key Words: Intestinal Barrier Function, RegIII-γ, Intestinal Microbiota, altered Schaedler flora, *Bifidobacterium breve*

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4.1. Abstract

Background: The intestinal microbiota regulates key host functions. It is unknown whether modulation of the microbiota can affect a genetically determined host phenotype. Polymorphisms in Nucleotide oligomerization domain (*Nod*)-like receptor family confer genetic risk for IBD. We investigated whether the intestinal microbiota and the probiotic strain *Bifidobacterium breve* NCC2950 affect intestinal barrier function and responses to intestinal injury in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.

Methods: Specific-pathogen free (SPF) *Nod1*$^{-/-}$*;Nod2*$^{-/-}$mice and mice gnotobiotically derived with altered Schaedler flora (ASF) biota were used. SPF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ littermates (generated by crossing SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$and germ-free C57BL/6 mice) and ASF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice were used as controls. SPF mice were gavaged daily with 10^9-CFU *B. breve* for 14 days before colitis induction. Denaturing Gradient Gel Electrophoresis (DGGE) and real time PCR were used to assess microbiota composition. Intestinal permeability was assessed by *in vitro* and *in vivo* techniques. Expressions of epithelial apical junction proteins, mucin and antimicrobial proteins were assessed by qRT-PCR and immunofluorescence. Responses to intestinal injury were investigated using an acute experimental model of colitis.

Results: Under SPF conditions, *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice had increased paracellular permeability, decreased E-cadherin and lower colonic antimicrobial RegIII-γ expression compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ littermate controls. These changes were associated with

increased susceptibility to colitis. ASF colonization or *B.breve* supplementation normalized RegIII-γ expression and decreased susceptibility to DSS colitis in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.

Conclusions: The intestinal microbiota influences colitis severity in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. The results suggest that colonization strategies with defined commensals or exogenous specific probiotic therapy may prevent intestinal inflammation in a genetically predisposed host.

4.2. Introduction

Accumulating evidence suggests that inflammatory bowel disease (IBD) involves an aberrant host response to the intestinal microbiota, which most likely stems from a complex interplay between genetic and environmental factors (Halfvarson et al., 2007; Halme et al., 2006; Xavier and Podolsky, 2007). Defective bacterial sensing through Nod1 and Nod2 receptors has been implicated in chronic intestinal inflammation (Hugot et al., 2001; Lu et al., 2010; McGovern et al., 2005b; Ogura et al., 2001) and increased susceptibility to intestinal infection (Chamaillard et al., 2003; Geddes et al., 2010; Geddes et al., 2011; Viala et al., 2004). These receptors recognize specific bacterial peptidoglycan moieties, and promote NF-κB-dependent pro-inflammatory response upon activation (Chen et al., 2009). The precise role of Nod1 and Nod2 receptors in gut inflammation is not well understood but considering their role as bacterial sensors (Franchi et al., 2009; Fritz et al., 2006), they likely contribute to the development of dysregulated immune responses to intestinal bacteria. It has been proposed that in addition to their role as bacterial sensors, Nod1 and Nod2 receptors help maintain homeostasis. For instance, they contribute to adaptive lymphoid follicle genesis (Barreau et al., 2007; Bouskra et al., 2008), regulation of Th2 responses (Fritz et al., 2007; Magalhaes et al., 2008), expression of anti-microbial peptides by Paneth cells (Kobayashi et al., 2005b; Petnicki-Ocwieja et al., 2009b), crosstalk with Toll-like receptors (Barreau et al., 2010; Watanabe et al., 2008), and regulation of transcellular permeability in Peyer's patches and ileum (Barreau et al., 2010; Barreau et al., 2007). However, it is still unknown whether these functions can be modulated by manipulation of the intestinal microbiota.

In this study, we investigated whether commensal and one specific probiotic bacterium influence intestinal barrier function and susceptibility to experimental colitis in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. We used conventionally raised (specific pathogen free, SPF) *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice, devoid of the most common murine pathogens, and *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ derived gnotobiotically with a defined microbiota altered Schaedler flora (ASF) (Dewhirst et al., 1999). SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice were also supplemented with the probiotic bacterium *Bifidobacterium breve* NCC2950 before colitis induction.

4.3. Materials and Methods

Specific Pathogen Free Mice. Double *Nod1* and *Nod2* knockout (*Nod1*$^{-/-}$*;Nod2*$^{-/-}$) mice on a C57BL/6 background, backcrossed at least 10 generations were originally from Dr. D. Philpott (University of Toronto). *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice were originally generated by crossing fully backcrossed single Nod1 knockout mice (*Nod1*$^{-/-}$) and single Nod2 (*Nod2*$^{-/-}$) knockout mice and then mating the SPF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ heterozygote offspring together (Geddes et al., 2011), SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice were then bred and maintained at McMaster University Central Animal Facility. To ensure a high similarity in microbiota profiles between *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice and controls, F1 heterozygous controls (*Nod1*$^{+/-}$*;Nod2*$^{+/-}$) were generated by crossing SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ female with germ-free C57BL/6 male. Mice were used at the age of 6-9 weeks. All experiments were conducted with approval from McMaster University Animal Care Committee.

Gnotobiotic rederivation of Nod1$^{-/-}$;Nod2$^{-/-}$ mice. The ASF comprised of *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Bacteroides distasonis*, a spiral-shaped bacterium, and four fusiform EOS (for Extremely Oxygen Sensitive) bacteria (Dewhirst et al., 1999). ASF-colonized *Nod1$^{-/-}$;Nod2$^{-/-}$* mice were obtained by 2-cell embryo transfer as described previously (Slack et al., 2009). Briefly, 4-6 weeks old SPF *Nod1$^{-/-}$;Nod2$^{-/-}$* female mice were super-ovulated, injected with human chorionic gonadotropin (VWR, Canada), and paired with *Nod1$^{-/-}$;Nod2$^{-/-}$* stud males. Two days later, plugged females were euthanized and the embryos were flushed out of the oviducts. The fertilized 2-cell embryos were then transferred into pseudo-pregnant ASF Swiss Webster recipient females, from the standard colonies in the Axenic/ Gnotobiotic Unit (AGU) at McMaster University. The mice were reared and maintained under gnotobiotic conditions and thus, were free of opportunistic bacteria such as *Enterococus faecalis*, segmented filamentous bacteria and *Escherichia coli*.

Microbiota analysis. DNA extraction and DGGE analysis were done as previously described (Nadal et al., 2007; Sanz et al., 2007). Briefly, PCR fragments of 200 bp representing total fecal bacterial were amplified from total DNA (80 ng) with the universal primers HDA1-GC(CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAGACTCCTACGGGAGGCAGCAGT) and HDA2 (GTATTACCGCGGCTGCTGGCAC) at 59ºC as annealing temperature. Similarities between DGGE profiles obtained with universal primers were determined by using the

Dice coefficient and the unweighted-pair group method with arithmetic average (UPGMA) clustering algorithm. Differences in species composition in every DGGE profile of different mice groups were expressed in terms of prevalence and analyzed statistically by χ^2 tests using STATGRAPHICS software (Manugistics, Spain).

Intestinal paracellular permeability. *In vitro* permeability was assessed by Ussing Chamber technique as previously described (Slack et al., 2009a; Verdu et al., 2008). Briefly, baseline conductance (mS/cm^2) was recorded after 20-minute equilibrium period. Mucosal to serosal transport of macromolecules was assessed by adding 6 μCi/ml radioactive ^{51}Cr-EDTA (Perkin-Elmer, Canada), an inert paracellular probe, in the luminal buffer. ^{51}Cr-EDTA the flux was calculated and expressed as percent recovery of the hot sample per square centimeter per hour.

In-vivo permeability was assessed by gavaging the mice with 20 μCi of ^{51}Cr-EDTA in 0.2 ml of sterile water. Mice were then kept in metabolic cages and given *ad libitum* access to food and water. After 24 hours in the metabolic cages, urine was collected, and percent recovery of ^{51}Cr-EDTA in the urine was calculated.

Apical junctions, mucin and anti-microbial expression. RNA expression of apical junctional proteins, mucin, and anti-microbial proteins was assessed using quantitative RT-PCR. Briefly, total RNA from a 30 mg colon section was isolated using RNeasy mini kit (Qiagen, Canada). RNA integrity was assessed using Agilent 2100 Bioanalyzer (Applied Biosystems-Ambion, USA). cDNA was synthesized from 1 μg of isolated total

RNA using iScript reverse transcriptase (Bio-Rad, Canada). Real-time PCR was performed with 1:20 dilutions of cDNA in triplicate. The reaction consisted of iQ^{TM} Eva Green Supermix for qPCR (Bio-Rad, Canada), primers at 0.5 μM (Table 4.1), and 1 μl of cDNA. Amplification was performed using iQ5 Real-Time Detection System (Bio-Rad, Canada). GAPDH was used as housekeeping gene. Relative expression levels were analysed using the $2^{-\Delta\Delta CT}$ method.

Table 4.1. quantitative PCR primers.

Gene	Primer Sequences
E-cadherin	5'-GCACATATGTAGCTCTCATC-3' 5'-CCTTCACAGTCACACACATG-3'
RegIII-γ	5'-TTCCTGTCCTCCATGATCAAAA-3' 5'-CATCCACCTCTGTTGGGTTCA-3'
IL-22	5'-CATCCACCTCTGTTGGGTTCA-3' 5'-TCCGAGGAGTCAGTGCTAAA-3'
GAPDH	5'-CCATGGAGAAGGCTGGGG-3' 5'-CAAAGTTGTCATGGATGACC-3'

Protein expression of apical junctional proteins was evaluated using immunofluorescence. Colon samples were embedded in Tissue-Tek OCT (Sakura, USA). Frozen sections were then cut (5 μm), fixed with 3% PFA for 15 min at 20°C, and blocked with PBS/bovine serum albumin at 2% for 20 min. Samples were immune-stained overnight with E-cadherin antibody (1:4000 dilution, BD Pharmaceutical, USA) and one hour with secondary antibody (1:400 dilution, Molecular Probes, USA). Negative controls (isotype control antibody, BD Pharmaceutical, USA) were included in all experiments. Representative pictures from each animal were taken with the same

exposition time. Quantification of E-cadherin fluorescence staining intensity was performed using ImageJ software.

Experimental colitis. Dextran Sulfate Sodium (DSS, MPP Biomedicals, Canada) was administered at different doses (2%, 3.5%, 5%) in drinking water for 5 days. On the sixth day, DSS was removed and replaced with water for 2 more days. Mice were sacrificed on day 8. Disease severity was evaluated using a modified scoring system described by Cooper (Cooper et al., 1993) that includes rectal and gross colonic bleeding (Bercik et al., 2002). Disease severity was scored from 0-9 using the following criteria: rectal bleeding (0-3), rectal diarrhea (0-3), and colonic bleeding (0-3).

Colonic myeloperoxidase (MPO), an index of granulocyte infiltration, activity was measured according to the technique described by Bradley (Bradley et al., 1982). Histological (microscopic score) assessment was graded on a scale from 0 to 4 described by Cooper (Cooper et al., 1993) in H&E-stained sections.

Cytokine expression in the colon was measured using supernatants collected after 24-hour full-thickness organ culture as described previously (Wirtz et al., 2007). The presence of pro-inflammatory cytokines in the supernatant was measured using Mouse Inflammatory CBA kit (BD Biosciences, USA) and analyzed using FACSarray Bioanalyzer System (BD Biosciences, USA).

Bacterial translocation to the spleen was assessed by gavaging the mice with 10^{10} CFU of AmpR *Escherichia coli* K12 (generously provided by Dr. S. Hapfelmeier, Bern, Switzerland) (Hapfelmeier et al., 2004) 18 hours before sacrifice. Spleen was then

homogenized and plated in LB agar supplemented with ampicillin (100 μg/ml) for overnight culture at 37°C. Bacterial counts were normalized according to the weight of the tissues.

Probiotic administration. *Bifidobacterium breve* NCC2950 was obtained from the Nestle Culture Collection (Lausanne, Switzerland), and grown under anaerobic conditions in MRS supplemented with 0.05% (v/v) L-cysteine hydrochloride. After 18 hours at 37°C, bacterial cells were pelleted, and re-suspended at a concentration of 10^{10} CFU/ml in PBS with 10% (v/v) glycerol and kept in frozen aliquots until used.

Nod1$^{-/-}$*;Nod2*$^{-/-}$ mice were fed with *B. breve* or PBS with 10% glycerol daily for 14 days. The effects of *B. breve* on the intestinal barrier were evaluated 12-hours after the last gavage. To test the preventive effect of *B. breve* on DSS colitis, additional groups of *B. breve* or PBS/glycerol-fed mice were given 3.5% DSS after the 14 days of treatment.

Statistics. Data are presented as means with standard deviation (SD). Statistical testing was performed using Unpaired t-Test or analysis of variance (ANOVA) with Bonferonni post hoc test, as appropriate. p-value lower than 0.05 was considered as statistically significant.

4.4. Results

SPF Nod1*$^{-/-}$*;Nod2*$^{-/-}$ *mice have altered intestinal barrier function. The intestinal microbiota regulates several key host functions (Smith et al., 2007a; Vijay-Kumar et al.,

2010), hence a different microbiota may influence host phenotype. To delineate the role of Nod1 and Nod2 sensors in regulating intestinal barrier function without the difference in microbiota profile as a confounding factor, we generated a control group (F1 *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice) with a similar microbiota profile as our SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. To do this, we crossed germ-free C57BL/6 males with female SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. This procedure allowed germ-free male C57BL/6 and resulting F1 heterozygous litters (*Nod1*$^{+/-}$*;Nod2*$^{+/-}$) to be naturally colonized with SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ microbiota. Using DGGE analysis, we confirmed that SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ and F1 *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice had comparable microbiota profile with similarity index of 67-91%. Moreover, no differences were observed on the prevalence of various fecal bacterial species (*Bacillus* spp., *Bifidobacterium pseudomonas*, *Blautia hanseii, Clostridium* spp., *Escherichia coli, Enterococcus gallinarum, Lactococcus garvieae, Lactobacillus gasseri,* Lactococcus spp*., Parabacteroides distasonis, Proteus mirabilis* and *Staphylococcus* spp.) between the two groups (Table 4.2, Figure 4.1).

We investigated colonic permeability *in vitro* by Ussing Chamber technique and *in vivo* using metabolic cages. *In vitro* permeability measurements indicated a significant increase in colonic tissue conductance and ^{51}Cr-EDTA flux in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls (Figure 4.2A). Similarly, 24 hours after *in vivo* gavage with ^{51}Cr-EDTA, significantly more of the radioactive probe was recovered in the urine of SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls (Figure 4.2B).

Table 4.2. *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ and *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls showed similar prevalence of various fecal bacterial species.

Bacterial Species	-/- vs +/- (p-value)
Bacillus spp.	0.15
Bifidobacterium pseudomonas	0.28
Blautia hanseii	0.68
Clostridium spp.	1.00
Escherichia coli	0.06
Enterococcus gallinarum	0.44
Lactococcus garvieae	1.00
Lactobacillus gasseri	0.71
Lactococcus spp.	0.56
Parabacteroides distasonis	0.45
Proteus mirabilis	1.00
Staphylococcus spp.	0.44

Bacterial DNA was extracted from stool samples, amplified by PCR and then analyzed using DGGE. DGGE patterns were analysed and differences in species composition in every DGGE profile of different mice groups were expressed in terms of prevalence. ($p<0.05$; Fisher Test).

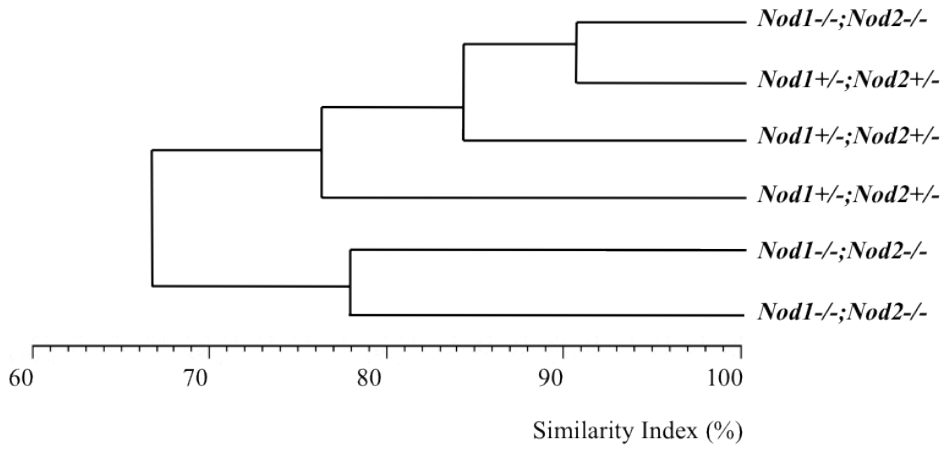

Figure 4.1. *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ and *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls have >67% similarity in fecal microbiota profiles.
Figures of the scale of the dendogram are percentages of similarity between DNA band profiles. Each end of the bracket represents one mouse.

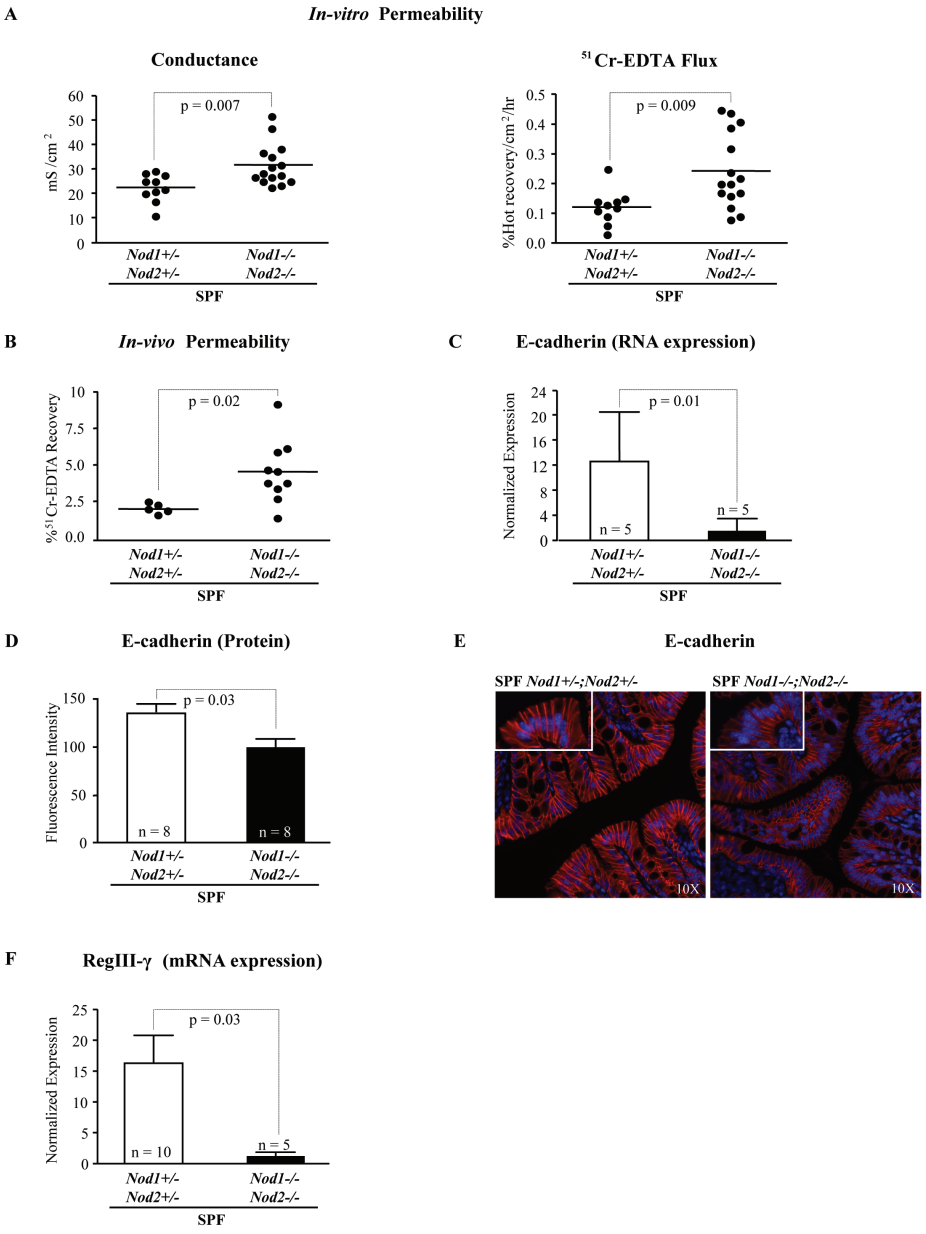

Figure 4.2. SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ exhibit altered intestinal barrier function.
(A) SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice exhibited increased tissue conductance and ^{51}Cr-EDTA flux. (B) After 24-hour *in vivo* challenge, SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice showed increased ^{51}Cr-EDTA percent recovery in the urine. (C) SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice exhibited decreased colonic mRNA E-cadherin expression compared to *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ controls. (D-E) SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ displayed decreased intensity of E-cadherin (TRITC: red) staining compared to *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ controls (Nuclei, DAPI: blue). (F) SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice showed decreased colonic mRNA RegIII-γ expression compared to *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ controls. Each bar represents mean ± SD. ($p<0.05$; Unpaired t-Test).

We then investigated whether the difference in intestinal permeability observed in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice was associated with altered epithelial apical junctions, which modulates paracellular permeability (Turner, 2009). Using quantitative RT-PCR, no differences were found in the relative expression of ZO-1, Claudin-2, Claudin-3, Claudin-4, Claudin-5, and Claudin 8 between the SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ and *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice (Figure 4.3). The bacterial sensing receptor TLR2 has been shown to modulate the tight junction protein ZO-1 (Cario et al., 2004). Thus, we also measured protein expression of ZO-1 but found no difference between SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice and *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls (data not shown). In contrast, mRNA expression of E-cadherin, an adherence junction protein, was down regulated in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice relative to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice Figure 4.2C. Decreased RNA expression of E-cadherin was paralleled by decreased protein expression of E-cadherin as evaluated by immunofluorescence Figure 4.2D. Specifically, disrupted membranous E-cadherin adhesion complex bordering apical enterocytes was observed in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls Figure 4.2E.

Epithelial cells also produce and secrete anti-microbial peptides and mucins, which together with tight junctions help fortify the intestinal barrier (Turner, 2009). mRNA expression of trefoil factor, Muc1, Muc2, Muc3, Muc4 and Muc13 was similar in the two experimental groups Figure 4.4. In contrast, we found that the anti-microbial lectin RegIII-γ was significantly down regulated in the colon of SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ compared to *Nod*$^{+/-}$*;Nod*$^{+/-}$ controls Figure 4.2F.

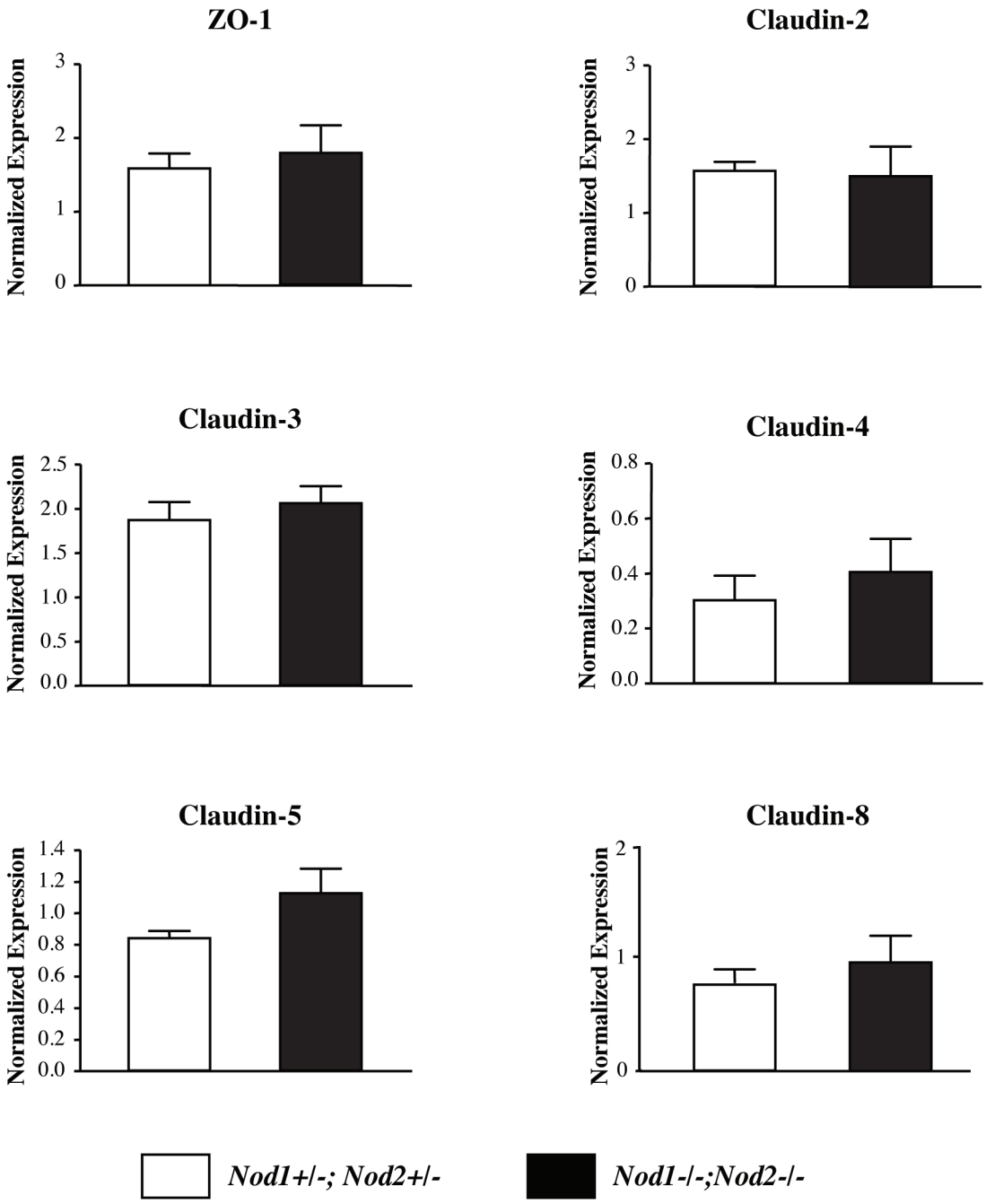

Figure 4.3. SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ and *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ littermate controls exhibit similar levels of ZO-1, claudin-2, 3, 4, 5 and 8 mRNA expression in the colon.
Mean ± SD; n=5-8/group. ($p<0.05$; Unpaired t Test).

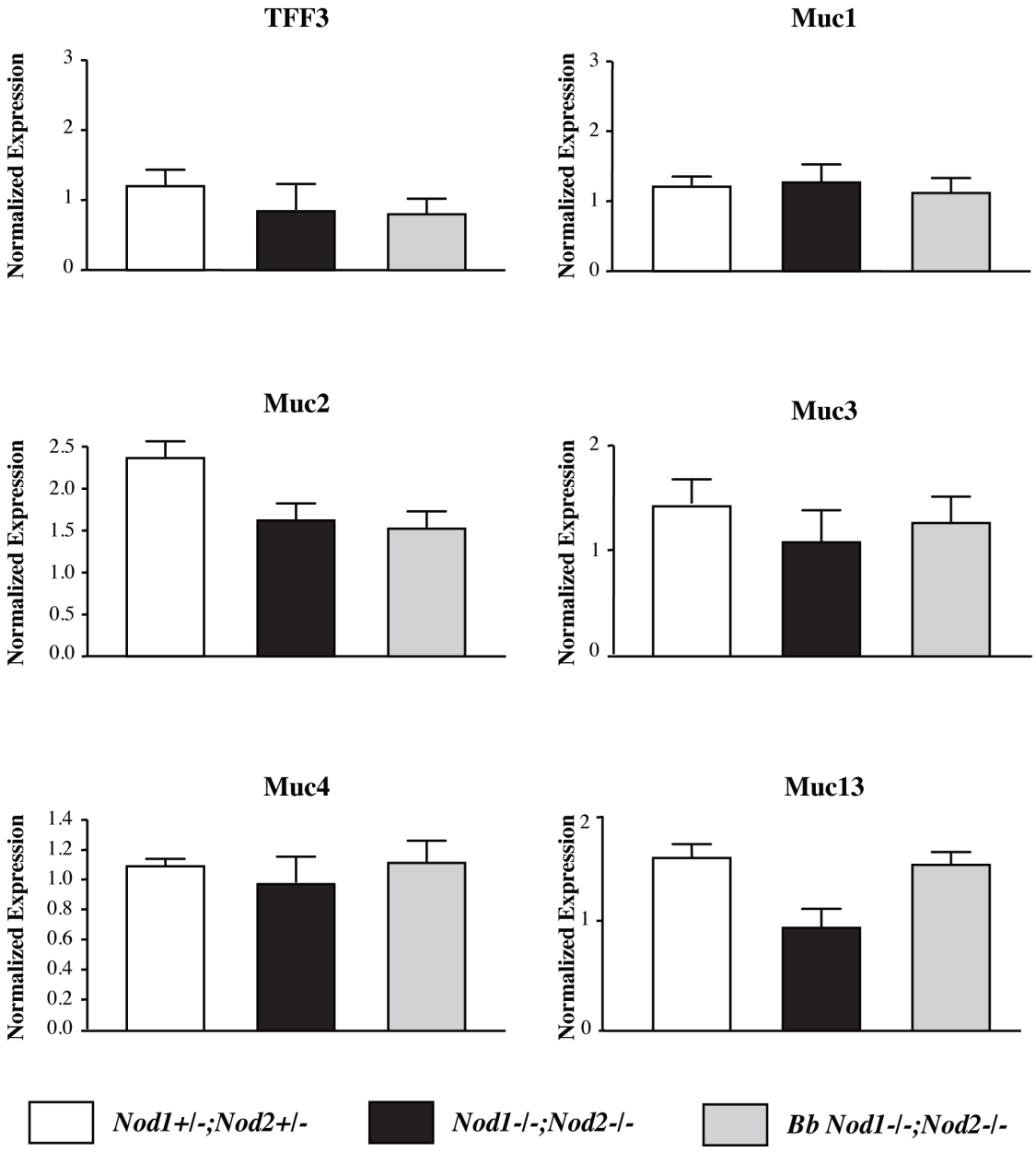

Figure 4.4. SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice exhibit no difference in mRNA mucus expression compared to *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ littermate controls.
Mean ± SEM; n=5-10/group. Expression was normalized to housekeeping gene GAPDH. ($p<0.05$; Unpaired t Test).

SPF Nod1*$^{-/-}$;*Nod2*$^{-/-}$ *mice are more susceptible to acute intestinal injury. Increased bacterial uptake through a dysfunctional intestinal barrier has been proposed as a

mechanism that perpetuates intestinal inflammation in IBD (Arrieta et al., 2006; Bouma and Strober, 2003; Macdonald and Monteleone, 2005). To investigate whether the intestinal barrier defects observed in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ were associated with spontaneous inflammation, inflammatory markers were investigated in naïve mice. Histological analysis of colonic samples and granulocyte infiltration revealed no signs of spontaneous inflammation in naïve SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice Figure 4.5. Similarly, both naive SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ and *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice showed low and comparable levels of IL-6, IL-10, MCP-1, IFN-γ, TNF-α, IL-12p70 in their colon (data not shown) and undetectable levels of translocating bacteria in their spleen (data not shown). This suggests that intestinal barrier dysfunction *per se* is insufficient to cause spontaneous intestinal inflammation in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.

We next investigated whether the phenotypic characteristics observed in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice were associated with increased susceptibility to acute intestinal injury using the DSS colitis model. At 5% DSS, we found that SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice had increased morbidity, as 46% of them reached clinical endpoint as early as day 4 of treatment (Figure 4.6A). We further analyzed bacterial translocation to the spleen by gavaging the mice with the commensal bacterium *E. coli* K12 and we found that 67% of SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice that completed the 5% DSS cycle showed detectable numbers of the bacterium in their spleen Figure 4.6B. No differences were found in the *E. coli* counts in cecal content and mesenteric lymph nodes between groups (data not shown). These data suggest that SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice have a compromised ability to properly maintain intestinal bacteria within the mucosal compartment during 5% DSS.

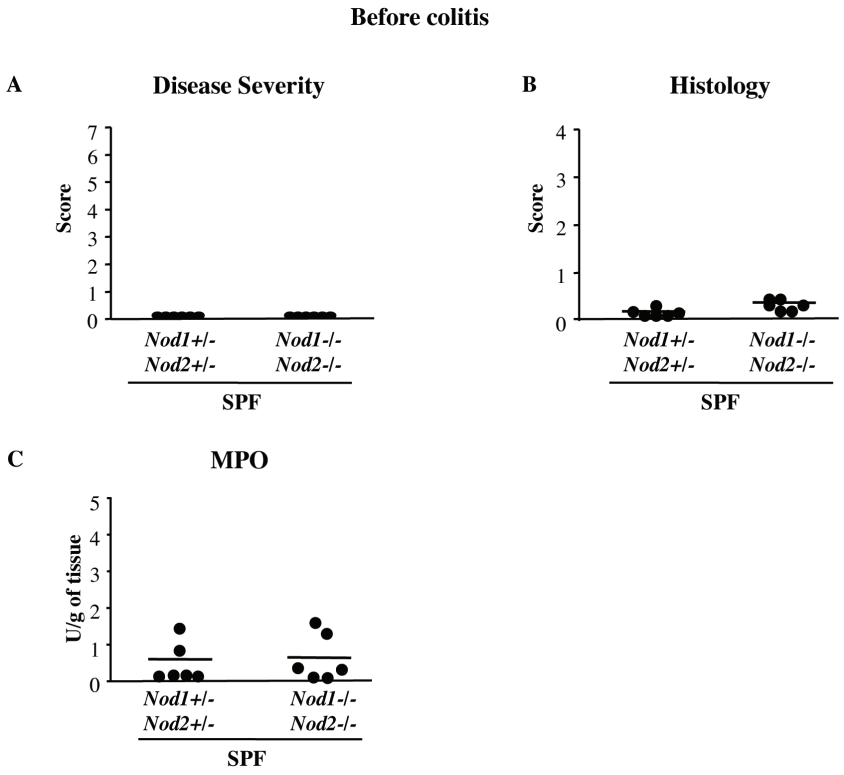

Figure 4.5. Naïve SPF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ and *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ controls exhibit no signs of inflammation.

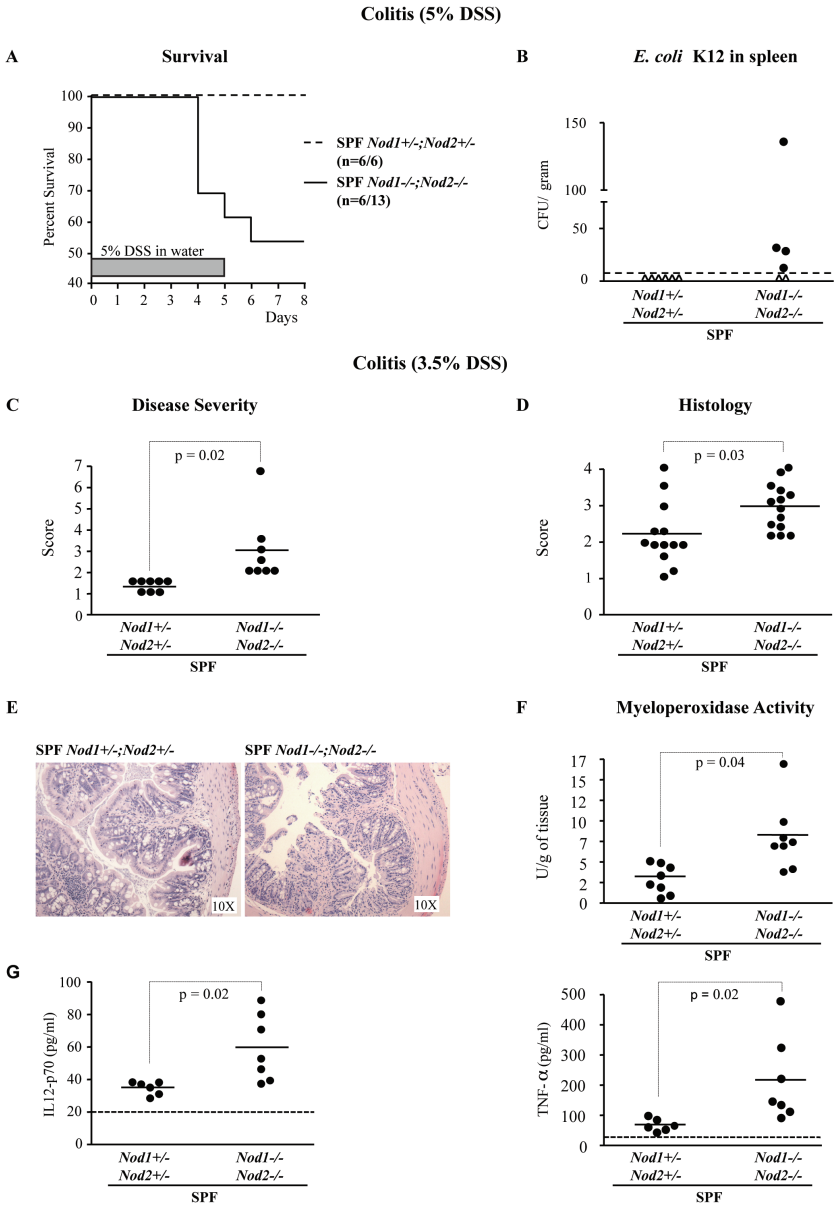

Figure 4.6. SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice have increased colitis severity.
At 5% DSS, SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice exhibited (A) increased morbidity and (B) detectable levels of AmpR *E. Coli* K12 in their spleen (open triangle: mice with undetectable levels of AmpR *E. Coli* K12 in the their spleen). At 3.5% DSS, SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice exhibited increased (C) disease severity scores and (D) microscopic colonic scores. (E) Representative pictures (hematoxylin and eosin) from SPF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ and *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice showed higher (F) MPO activity, (G) IL-12p70 and TNF-α levels in the colon compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls. ($p<0.05$; Unpaired t-Test). Statistical analysis in (B) was not performed due to all SPF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice having undetectable values.

To further evaluate the susceptibility of SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ to colitis, we performed a dose response experiment. At 2% DSS, no difference in inflammation was detected between SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice and controls (Figure 4.7). At 3.5% DSS, SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice showed increased severity of colitis compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls (Figure 4.6C). Loose stool and traces of blood were common features observed in the rectum of *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. Histological analysis in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice showed shortening of crypts with areas of total epithelial structure loss (Figure 4.6D-E). Increased MPO activity (Figure 4.6F) and IL-12p70 and TNF-α cytokine levels (Figure 4.6G) was observed in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$. However, at 3.5% DSS, *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice did not show increased detectable numbers of bacteria in their spleen compared to heterozygote controls (data not shown) nor significant overall weight loss (Figure 4.8). Taken together, the results suggest that there is a threshold above which homeostatic mechanisms in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice are overwhelmed resulting in enhance susceptibility to intestinal injury and bacterial translocation.

ASF colonization restores RegIII-γ expression and decreases susceptibility to colitis in Nod1*$^{-/-}$*;Nod2*$^{-/-}$ *mice. To investigate whether modulation of the microbiota influences intestinal barrier phenotype and susceptibility to DSS, we re-derived *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice into ASF conditions by using recipient pseudo-pregnant females with ASF biota from our standard colonies in the Axenic Facility. Quantitative PCR of DNA isolated from cecal contents confirmed successful ASF colonization of *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ litters (data not shown).

Under ASF conditions, we observed that tissue conductance and ^{51}Cr-EDTA Flux in naïve *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice remained increased compared to ASF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ (Figure 4.9A). Similarly, mRNA and protein expression of E-cadherin in the colon was decreased in ASF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls (Figure 4.9B-C). In contrast, there was normal expression of RegIII-γ in the colon of ASF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice (Figure 4.9D).

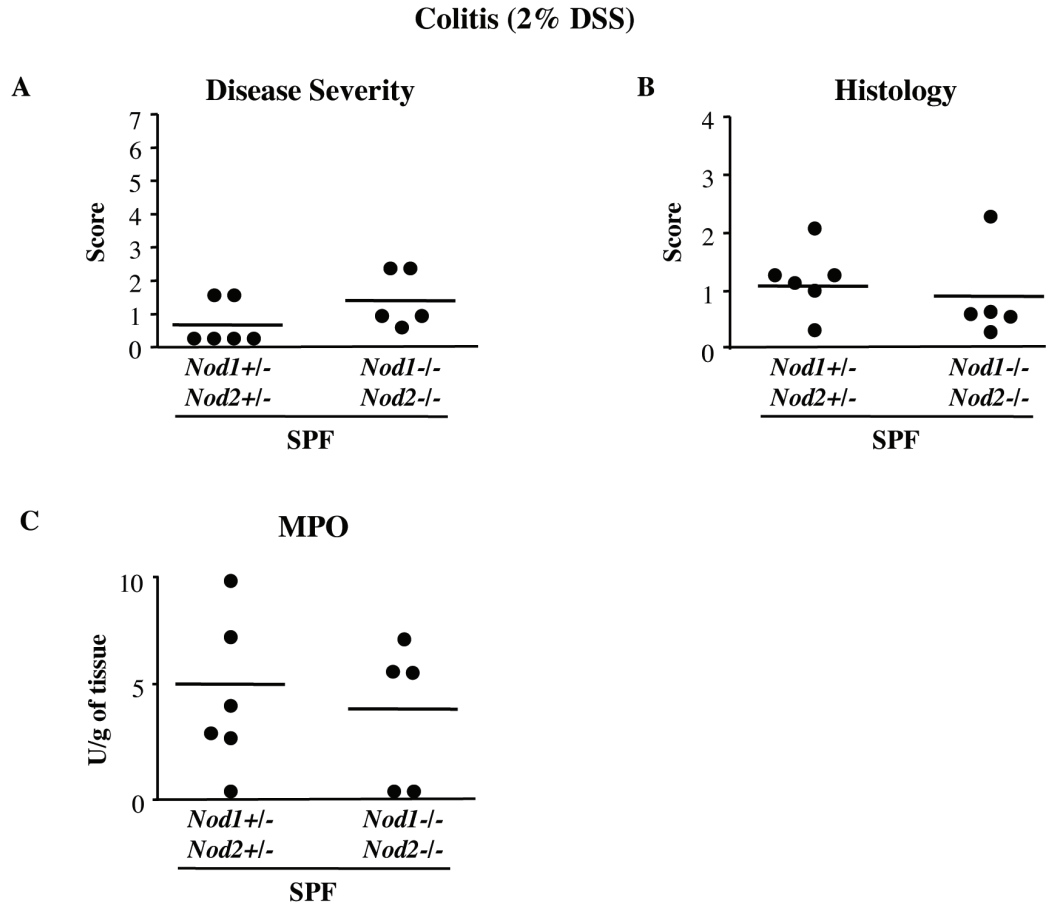

Figure 4.7. SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ littermate mice exhibit similar (A) disease severity scores, (B) colonic histological scores and (C) MPO activity in colonic samples compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ littermate controls at 2% DSS.
($p<0.05$; Unpaired t Test).

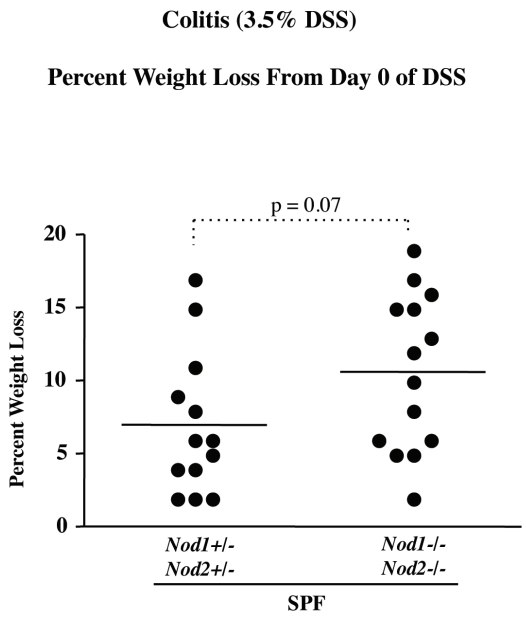

Figure 4.8. SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice did not exhibit significant weight loss compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ littermate controls at 3.5% DSS.
($p<0.05$; Unpaired t Test).

At 3.5% DSS, no difference in colitis severity was observed between ASF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ and *Nod1*$^{+/-}$*;Nod*$^{+/-}$ mice (Figure 4.10). IL-6, IL-10, MCP-1, IFN-γ, TNF-α, IL-12p70 levels were either low or below limit of detection in both groups (data not shown). Increasing the DSS dosage to 5%, increased the colitis severity in both ASF groups but no significant differences were observed between ASF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice and *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls (Figure 4.11). Likewise, the IL-6, IL-10, MCP-1, IFN-γ, TNF-α, IL-12p70 levels in the colon were comparable (Figure 4.12). Both groups showed 100% survival at 5% DSS. Thus, ASF colonization prevented the higher susceptibility to colitis observed in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.

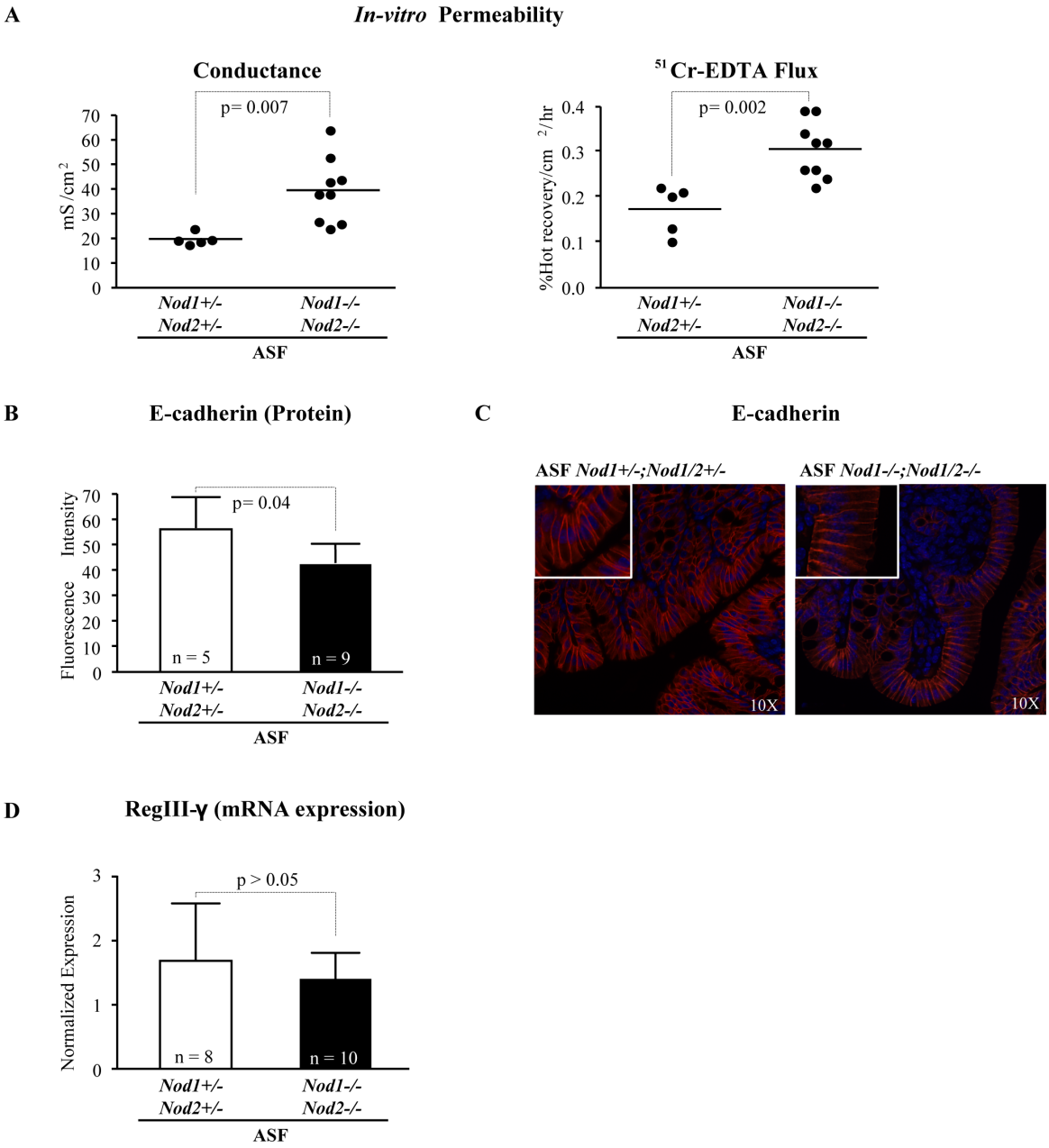

Figure 4.9. ASF colonization normalized RegIII-γ expression in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. (A) ASF-colonized *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice showed higher tissue conductance and ^{51}Cr-EDTA flux. (B-C) ASF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ exhibited decreased intensity of E-cadherin (TRITC: red) staining compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls. Nuclei (DAPI: blue). (D) ASF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice showed similar mRNA RegIII-γ expression compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls. Each bar represents mean ± SD. ($p<0.05$; Unpaired t-Test).

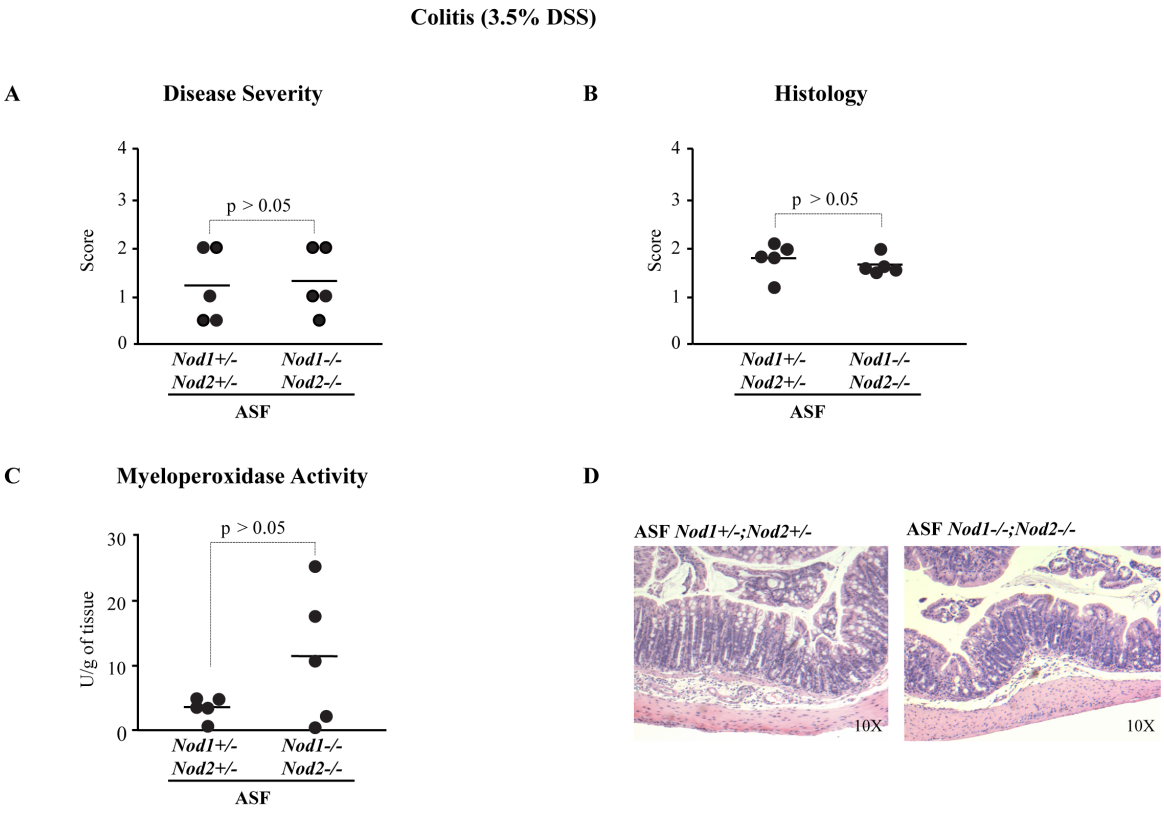

Figure 4.10. ASF colonization normalized colitis susceptibility in *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice.
At 3.5% DSS, ASF *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice exhibited similar (A) disease severity scores, (B) microscopic colonic scores, and (C) MPO activity in colonic samples compared to ASF *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ mice. (D) Representative pictures (hematoxilin and eosin) from ASF *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ and *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice.

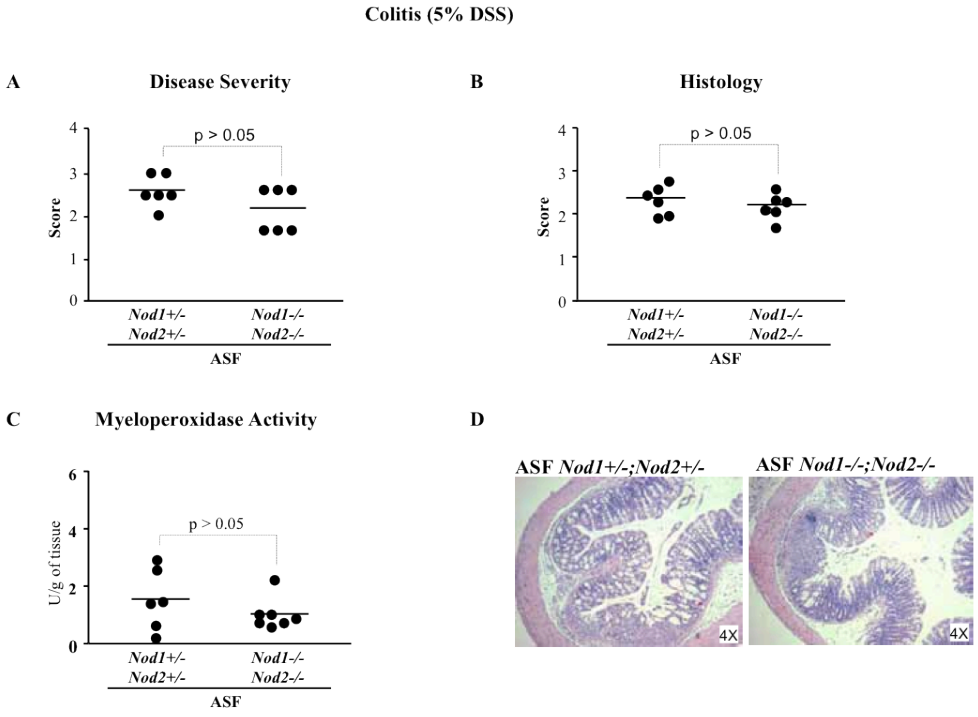

Figure 4.11. ASF colonized *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ and *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ littermate controls exhibit similar (A) disease severity scores, (B) microscopic scores, and C) MPO activity in the colon.
($p<0.05$; Unpaired t Test).

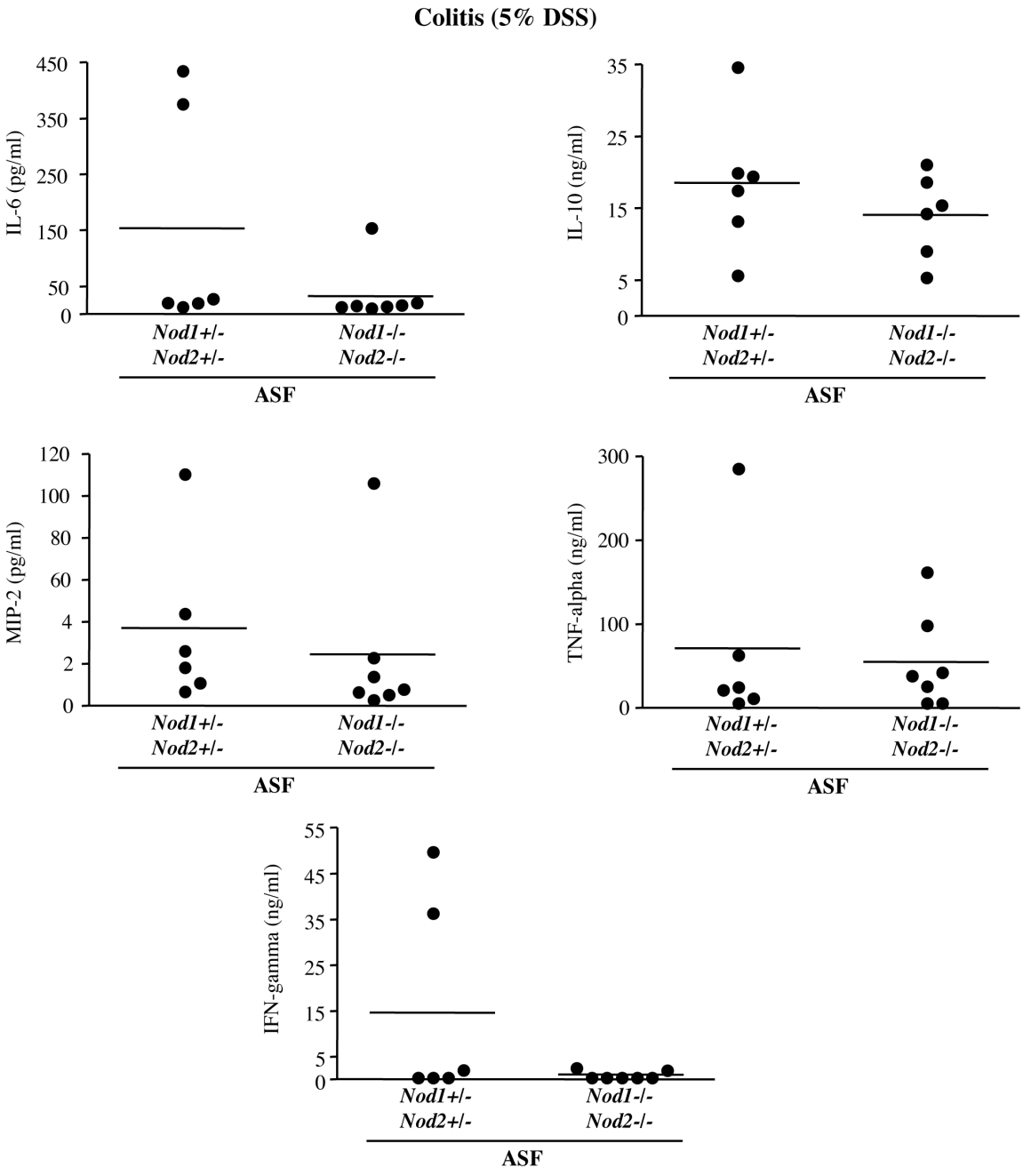

Figure 4.12. After 5% DSS, no significant difference in colonic pro-inflammatory cytokine levels were found between ASF colonized *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice and *Nod1*$^{+/-}$;*Nod2*$^{+/-}$ littermate controls.
IL-12p70 is below the limit of detection for both groups.

Bifidobacterium breve upregulates RegIII-γ expression and prevents increased susceptibility to colitis in SPF Nod1$^{-/-}$;Nod2$^{-/-}$ mice. To investigate whether the probiotic *Bifidobacterium breve* NCC2950 influences intestinal barrier function in SPF *Nod1$^{-/-}$;Nod2$^{-/-}$* mice, we gavaged the mice with *B. breve* NCC2950 for 14-days. *B. breve* treatment did not restore increased colonic ^{51}Cr-EDTA Flux and conductance in *Nod1$^{-/-}$;Nod2$^{-/-}$* mice (Figure 4.13A), however, it up-regulated the expression of RegIII-γ Figure 4.13B. Additionally, PBS/glycerol fed mice and *B. breve* treated *Nod1$^{-/-}$;Nod2$^{-/-}$* mice displayed comparable colonic TNF-α, IL-6, and IL-10 levels (data not shown) and colonic mRNA expression of IL-22 (Figure 4.14). IFN-γ, MIP-2 and IL-12p70 levels were either low or below the limit of detection in all groups (data not shown). Thus, Nod1 and Nod2 sensors are major determinant of paracellular permeability and apical junctional structure, whereas expression of antimicrobial lectins is also influenced by intestinal bacterial composition.

To investigate whether probiotics prevent the increased susceptibility to colitis in SPF *Nod1$^{-/-}$;Nod2$^{-/-}$* mice, we administered *B. breve* NCC2950 or PBS/glycerol, prior to colitis induction. At 5% DSS, prophylactic treatment with *B. breve* improved clinical disease activity in *Nod1$^{-/-}$;Nod2$^{-/-}$* (Figure 4.15A). *Nod1$^{-/-}$;Nod2$^{-/-}$* mice treated with *B. breve* further displayed a decrease in detectable numbers of AmpR *E. coli* in their spleen after 5% DSS compared to PBS/glycerol treated *Nod1$^{-/-}$;Nod2$^{-/-}$* mice (Figure 4.15B). Similarly, at 3.5% DSS, *Nod1$^{-/-}$;Nod2$^{-/-}$ B. breve* treated mice had attenuated colitis (Figure 4.15C-D), and showed lower levels of TNF-α and IL-6 in the colon compared to PBS/glycerol treated *Nod1$^{-/-}$;Nod2$^{-/-}$* mice (Figure 4.15E).

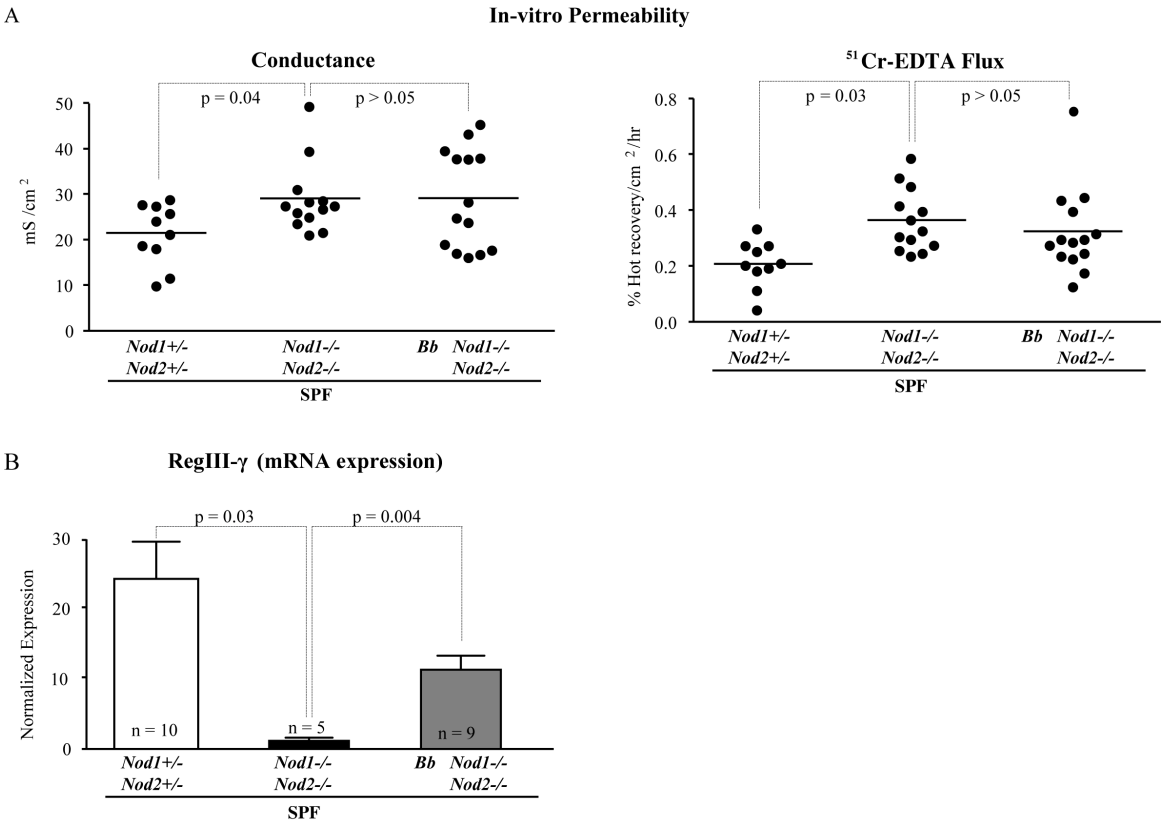

Figure 4.13. *B. breve* increased RegIII-γ expression in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.
(A) *B. breve* supplementation did not normalize tissue conductance or ^{51}Cr EDTA flux in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. (B) *B. breve* treated *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice had increased mRNA expression of RegIII-γ in the colon compared to PBS/glycerol treated *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. Each bar represents mean ± SD. ($p<0.05$; ANOVA followed by Bonferonni *post-hoc* test).

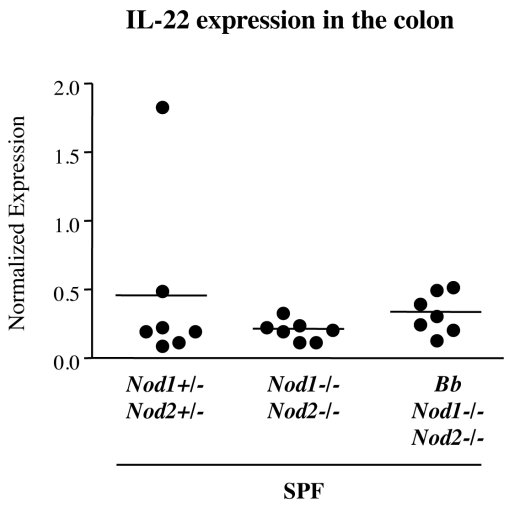

Figure 4.14. SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ and *B. breve* (Bb) treated *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice exhibit no difference in mRNA IL-22 expression compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ littermate controls.

Expression was normalized to housekeeping gene GAPDH. ($p<0.05$; Unpaired t Test).

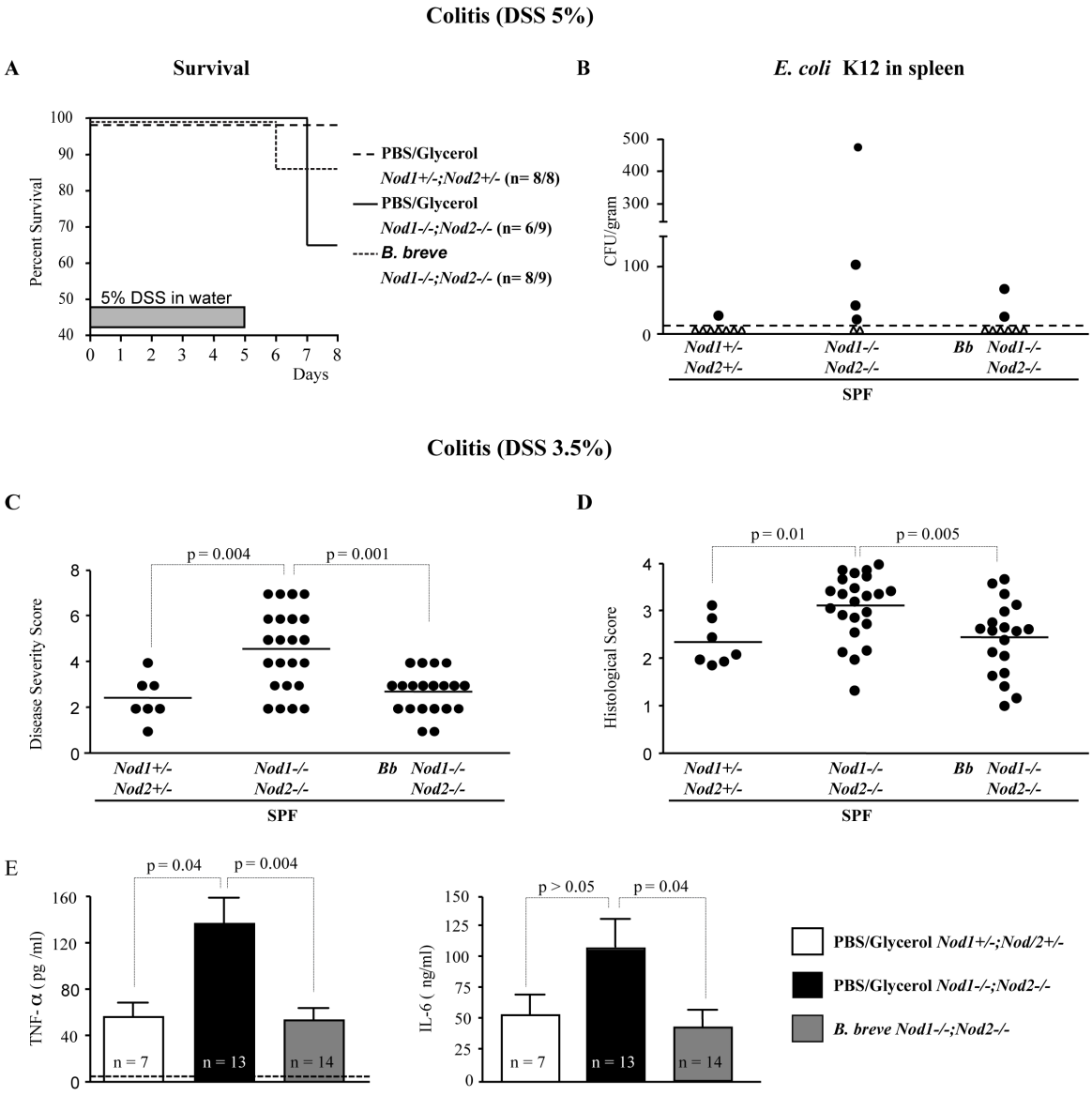

Figure 4.15. *B. breve* prevented colitis susceptibility in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.
At 5% DSS, prophylactic treatment with *B. breve* (A) decreased morbidity and (B) decreased numbers of detectable AmpR *E. Coli* K12 in the spleen of SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice (open triangle: mice with undetectable levels of AmpR *E. Coli* K12 in the their spleen). At 3.5% DSS, *B. breve* treated *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice exhibited decreased (C) disease severity, (D) microscopic score, and (E) colonic TNF-α and IL-6 expression compared to PBS/glycerol treated *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. Each bar represents mean ± SD. ($p<0.05$; ANOVA followed by Bonferonni *post-hoc* test). Statistical analysis in (B) was not performed due to the fact that only one out of eight SPF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice had detectable values.

4.5. Discussion

In addition to genetic predisposition (Halme et al., 2006) (Halfvarson et al., 2007), environmental factors are an important parameter in susceptibility to IBD. It is unknown whether genetic risk can be decreased through modulation of these factors. We sought to determine whether mice deficient for *Nod1* and *Nod2* sensors exhibit changes in intestinal barrier and/or responses to intestinal injury that are influenced by their microbial composition, and whether these can be modulated by colonization with a defined microbiota or by supplementation with a specific probiotic. We found that SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice had colonic barrier dysfunction and increased susceptibility to intestinal injury after DSS colitis. ASF colonization or supplementation of SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice with *B. breve* NCC2950, restored some parameters of intestinal barrier function and decreased colitis susceptibility.

Growing evidence suggests that chronic inflammation is in part driven by the microbiota, and both *Nod1* and *Nod2* sensors are associated with IBD susceptibility (Halme et al., 2006). Hence, we used mice that lack intracellular *Nod1* and *Nod2* to understand how these bacterial recognition systems interact with intestinal bacteria to regulate intestinal barrier homeostasis and intestinal injury. A role of intestinal barrier dysfunction as a mechanism for increased bacterial uptake and trigger for aberrant immune response in IBD has been suggested, but not yet established (Arrieta et al., 2006; Bouma and Strober, 2003; MacDonald and Pettersson, 2000). Increased intestinal permeability has been demonstrated in patients prior to IBD diagnosis (Irvine and Marshall, 2000), during active disease, and before relapses (Arnott et al., 2000; Wyatt et

al., 1997; Wyatt et al., 1993). Clinical studies have also shown that IBD patients carrying mutations in *Nod2* gene and their healthy relatives have increased intestinal permeability (Buhner et al., 2006; D'Inca et al., 2006), suggesting a link between systems controlling intracellular bacterial recognition and barrier function. Experimental data further revealed that single *Nod1* or *Nod2* knockout, maintained under SPF conditions, exhibit different intestinal barrier phenotype and altered responses to experimental colitis compared to wild type controls (Barreau et al., 2007; Chen et al., 2008; Petnicki-Ocwieja et al., 2009). A recent study has raised the possibility that defects in the Nod2 receptor predisposes the host to be colonized by a particular intestinal microbiota (Petnicki-Ocwieja et al., 2009), which can influence host phenotype (Smith et al., 2007). For these reasons, we generated a control group with similar SPF microbiota profile as our *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice by crossing germ-free C57BL/6 males with female SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.

Most of the preceding studies investigating the role of Nod2 receptors in intestinal barrier regulation have focused on the small intestine (Barreau et al., 2010; Barreau et al., 2007; Kobayashi et al., 2005a; Petnicki-Ocwieja et al., 2009), while little is known about the role of Nod1 receptors in colonic barrier homeostasis. Hence, in our study, we investigated colonic barrier function and found that SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice displayed increased colonic paracellular permeability compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ littermate controls. *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice also had decreased expression of adherens junction E-cadherin, which is known to regulate the apical tight junctions(Groschwitz and Hogan, 2009), and are important in the maintenance of gut homeostasis (Hermiston and Gordon, 1995; Riethmacher et al., 1995). We found that colonic RegIII-γ expression was

significantly down regulated in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice. RegIII-γ and its human homologue, HIP/PAP, are c-type lectins that bind to peptidoglycan moieties of bacteria and have direct bactericidal activity, particularly against Gram-positive bacteria (Cash et al., 2006). Interestingly, it has been shown that both single *Nod1* or *Nod2* knockout mice are more sensitive to *Listeria monocytogenes* infection compared to wild type counterparts (Brandl et al., 2007; Mosa et al., 2009), and RegIII-γ has been shown to be protective against this pathogen (Brandl et al., 2007). It is tempting to hypothesize that reduced RegIII-γ expression associated with Nod1 and Nod2 deficiencies likely contribute to increased susceptibility of single *Nod1* or *Nod2* knockout to *L. monocytogenes* infection. Intestinal bacteria can activate the expression of RegIII-γ in the Paneth cells and small intestine (Cash et al., 2006). Similarly, IL-22 has been shown to induce RegIII-γ gene expression by intestinal epithelial cells (Zheng et al., 2008). However, we found that under naïve conditions, SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice express the same level of IL-22 compared to heterozygote controls, suggesting that IL-22 is not a major determinant of the altered RegIII-γ expression in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice under naïve conditions. It has also been shown that RegIII-γ expression is MyD88, but not, Nod2 dependent (Brandl et al., 2007). Our results, however, suggest that Nod sensors, perhaps Nod1, also influence colonic RegIII-γ expression. Further experiments in single Nod1 deficient mice are needed to confirm this hypothesis. Altogether the data support a role of Nod receptors in regulating key aspects of the intestinal barrier.

Despite the innate immune defects (Fritz et al., 2006) and barrier abnormalities reported here, spontaneous inflammation was not observed in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.

This suggests that intestinal barrier dysfunction *per se* is insufficient to cause spontaneous intestinal inflammation in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. It is possible that alternative pathways involving TLR receptors and/or compensatory adaptive immune responses help to maintain homeostasis in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. This has been previously demonstrated in mice lacking complete TLR signaling (Slack et al., 2009). Similarly, we found that under naïve conditions, SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice had increased serum antibodies reactive against their cecal bacteria compared to *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ controls without a significant increased in bacterial translocation in the spleen (Figure 4.16). This is similar to the described adaptive serum compensatory mechanism previously observed in mice without TLR receptor signaling (Slack et al., 2009). Hence, due to the redundant homeostatic mechanisms, it is likely that combination of several factors is required to initiate intestinal inflammation in a genetically predisposed host with Nod mutations. It cannot, however, be ruled out that intestinal barrier abnormalities in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ play a permissive role in response to intestinal injury. Indeed, we reported here that there is a concentration threshold (3.5% DSS), above which higher susceptibility to colitis in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice was observed. At 5% DSS, we recovered more live bacteria from the spleens of *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice compared to heterozygote controls. Enhanced splenic bacterial translocation in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice during DSS colitis may be due to increased leakage through a more marked intestinal barrier defect in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. This may lead to inadequate containment of the translocating bacteria, when adaptive serum compensatory mechanisms are overwhelmed (Slack et al., 2009).

To investigate whether modulation of the microbiota can influence the phenotype and colitis susceptibility observed in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice, we used two different strategies. First, we re-derived *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice into ASF conditions. Both the original Schaedler's flora and its modified version, referred to as "altered Schaedler flora" was designed to standardize the microbiota of germ free mice (Dewhirst et al., 1999). Despite lowering the intestinal microbial diversity, *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice displayed increased permeability. In contrast, the level of expression of RegIII-γ in ASF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ was comparable to that in ASF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$. Moreover, no differences in colitis severity were observed between the two groups even when the concentration of DSS was increased to 5% DSS, suggesting that genetic defect alone was not sufficient to induce increased DSS colitis. Thus, the presence of "colitogenic" microbiota seems to be required to promote different antimicrobial peptide phenotype and responses to intestinal injury in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ *mice*. A recent study in rodents with a mutation in the Crohn's disease-related gene Atg16L1 showed altered host phenotype and response to DSS only in the presence of specific murine norovirus infection (Cadwell et al., 2010). Thus, environmental factors that include the microbiota and pathogens, can lead to more severe intestinal disease in genetically susceptible hosts.

Increased temporal instability and decreased number of bacterial species, with reduction of certain beneficial bacteria (dysbiosis), have been demonstrated in patients with IBD (Frank et al., 2007; Sartor, 2009; Sokol et al., 2008). Specific probiotic supplementation protects against experimentally induced colitis (Sokol et al., 2008). It is, however, unknown whether probiotic treatment is equally effective in mice deficient for

intracellular Nod signaling. Hence, our second strategy involved oral supplementation with the probiotic *B. breve* NCC2950 in adult SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. *B. breve* NCC2950 was selected for its anti-inflammatory properties determined *in vitro* using peripheral blood mononuclear cells (unpublished data). The probiotic did not normalize increased colonic permeability in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. Similarly, ASF colonization did not normalize changes in colonic permeability in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. These results suggest that the lack of Nod1 and Nod2 genes may have a direct impact on the regulation of paracellular permeability in the colon. Despite the lack of colonic permeability improvement, *B. breve* administration improved the outcome of DSS colitis in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice. No significant changes in cytokine profile were observed after the 14-day supplementation of *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ with *B. breve* or PBS/glycerol. Thus, it is unlikely that the preventative action of *B. breve* is mediated by a modulation of cytokine profile. We found that *B. breve* markedly restored colonic RegIII-γ expression, suggesting that antimicrobial molecules in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice can be modulated, not only by ASF colonization, but by supplementation with a specific probiotic as well. It is possible that the up-regulation of RegIII-γ in *B. breve* treated mice contributed to enhance mucosal containment of opportunistic bacteria, fortify epithelial barrier function, and improve colitis outcome. This is supported by decreased translocation of AmpR *E. coli* to spleen after DSS in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice treated with *B. breve*. The exact mechanism by which *B. breve* up-regulates the expression of RegIII-γ in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice remains to be determined. As MyD88-dependent sensing of intestinal microbiota by epithelial cells have been previously shown to directly instruct RegIII-γ expression, it is possible that *B.*

breve signals through this pathway (Brandl et al., 2007). Alternatively, it is possible that *B. breve* signals through MyD88-dependent receptors expressed by other innate immune cells such as dendritic cells (Sanos et al., 2011). This could lead to molecular cues that instruct the expression of IL-22 by innate lymphocytes which, in turn, induce RegIII gene expression (Sanos et al., 2011). We did not find significant up-regulation of IL-22 after *B. breve* treatment in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice, suggesting that IL-22 does not play a significant role in *B. breve* up-regulation of RegIII-γ expression in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice (Figure 4.14). Finally, it is possible that *B. breve* exerts it effect through overall modulation of the microbiota. However, we did not find significant changes in microbial communities before and after *B. breve* administration, making this hypothesis unlikely and supporting the notion of a specific effect mediated by *B. breve* (Figure 4.17).

In conclusion, in a controlled microbiota environment, we showed that Nod1 and Nod2 receptors in mice play a critical role in the regulation of colonic paracellular permeability and susceptibility to colitis. Normalization of colitis susceptibility in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice was achieved by colonization with a microbiota devoid of pathogens and opportunistic bacteria (ASF) as well as by specific probiotic supplementation. The underlying mechanism may involve normalization of RegIII-γ expression after ASF colonization or *B. breve* administration. Our results support the potential value of specific probiotics, or colonization strategies with a microbiota of "low colitic capacity", as prophylactic measures to reduce IBD risk or prevent relapses in genetically susceptible individuals.

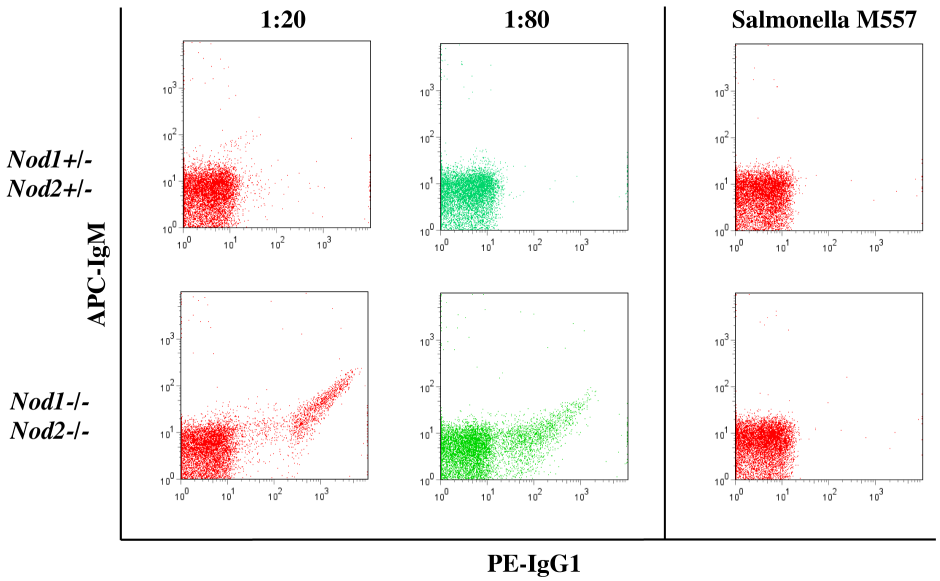

Figure 4.16. Presence of reactive serum IgG1 and IgM antibodies against cecal bacteria in SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice.
Cecal contents from each mouse were cultured overnight in LB broth at aerobic condition (37^{O}C). Cultured cecal bacteria were then stained with serum, counterstained with specific antibody isotype, and then analyzed by FACS. *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ littermate controls showed zero or low levels of serum IgG1 antibodies reactive against their cecal bacteria. In contrast, *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice showed IgG1 antibodies reactive against their cecal bacteria. Serial dilutions of the serum confirmed that the positive IgG1 antibodies against cecal bacteria are not the result of changes in the population of the bacteria. Using *Salmonella typhimurium* M557, a pathogen absent in our SPF colony, as a negative control, we confirmed that IgM and IgG1 antibodies are not natural antibodies that have the ability to cross-react to a wide variety of microbial bacteria.

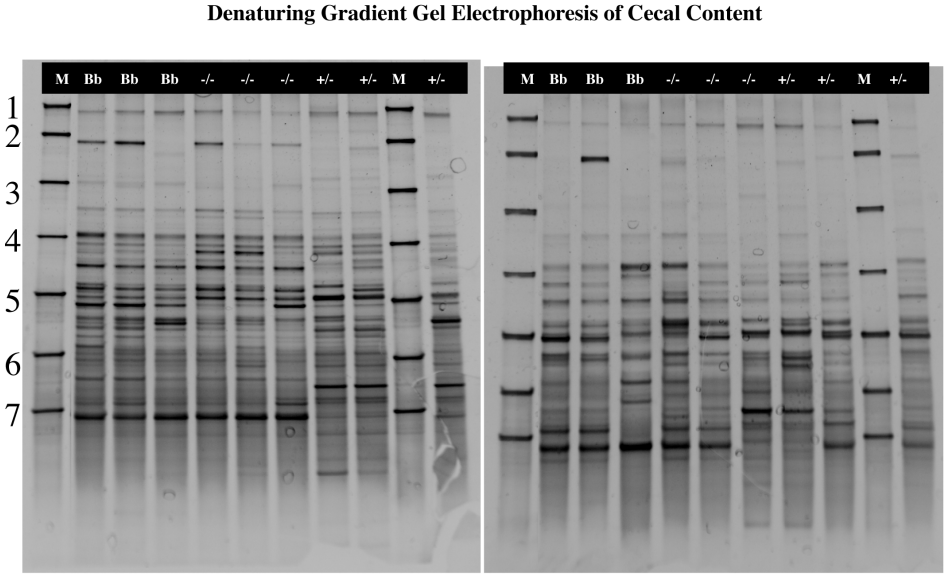

Figure 4.17. No significant changes in cecal microbiota profiles were detected between *Bifidobacterium breve* treated SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice (Bb) and PBS/glycerol treated SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice (-/-).
Similarly, no significant changes in cecal microbiota profiles were detected between PBS/glycerol treated SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice and PBS/glycerol treated SPF *Nod1*$^{+/-}$*;Nod2*$^{+/-}$ mice (+/-). Each lane in the DGGE represents one mouse. Markers (M): 1) *Bacteroides acidofaciens*, 2) *Lactobacillus johnsonii*, 3) *Lactobacillus helveticus*, 4) *Lactococcus lactis*, 5) *Bifidobacterium breve*, 6) *Bifidobacterium longum*, 7) *Bifidobacterium lactis*.

4.6. Acknowledgements

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– CHAPTER 5 –

DIFFERENTIAL INDUCTION OF ANTIMICROBIAL REGIII BY INTESTINAL MICROBIOTA AND BIFIDOBACTERIUM BREVE NCC2950.

Differential induction of antimicrobial RegIII by intestinal microbiota and *Bifidobacterium breve* NCC2950.

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Preface: *The research presented in this manuscript was conducted from May 2012 to June 2013. I share the first place authorship with C. Hayes. C.L. Hayes (graduate student), my supervisor and I designed the experiments, analyzed the data and wrote the manuscript. I performed majority of the experiments with technical assistance from the following people: J. Jury (technician) provided us with experimental assistance, J.P. Motta provided experimental assistance with immunoflourescence staining of RegIII proteins, H.J. Galipeau (graduate student) and V. Philip (graduate student with Drs. P. Bercik & S.M Collins) provided assistance in mono-colonizing the germ-free mice. C.L. Garcia-Rodenas provided the probiotic biomass. H. Kiyama provided the antibodies against RegIII. P. Bercik contributed valuable scientific input and critically appraised the manuscript.*

Summary and Central Message: This article examines the mechanism by which a specific component of the intestinal microbiota differentially regulates the expression of the antimicrobial RegIII protein. Our results suggest that members of the intestinal microbiota induce RegIII proteins differentially. Moreover, the probiotic bacterium *Bifidobacterium breve* NCC2950 stimulates intestinal innate defense through up-regulation of inducible RegIII proteins through MyD88-Ticam pathway and occurs at the level of the intestinal epithelial cell. Overall, our results suggest that specific induction of RegIII by *B. breve* NCC2950 may enhance the mucosal barrier and increase resistance to inflammatory injury.

Title: Differential induction of antimicrobial RegIII by intestinal microbiota and *Bifidobacterium breve* NCC2950.

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Short Title: Microbiota and *B. breve*-induced RegIII expression.

Key words: Intestinal microbiota, *B. breve,* RegIII proteins.

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5.1. Abstract

The intestinal microbiota is a key determinant of gut homeostasis, which is achieved, in part, through regulation of antimicrobial peptide secretion. The aim of this study was to determine the efficiency by which members of the intestinal microbiota induce the antimicrobial peptide REGIII and elucidate the underlying pathways. We showed that germ-free mice have low levels of REGIII-γ in their ileum and colon compared to mice with different intestinal microbiota backgrounds. Colonization with a microbiota of low diversity (altered Schaedler flora) did not induce the expression of REGIII-γ as effectively as a complex community (Specific Pathogen Free). Monocolonization with the probiotic *Bifidobacterium breve*, but not with the non-probiotic commensal *Escherichia coli* JM83, up-regulated REGIII-γ expression. Induction of REGIII-γ by *B. breve* was abrogated in mice lacking MyD88 and Ticam1 signalling. Both live and heat-inactivated *B. breve,* but not spent culture media from *B. breve,* induced the expression of REGIII-α, the human ortholog and homolog of REGIII-γ, in human colonic epithelial cells (Caco-2). Taken together, the results suggest that REGIII-γ expression in the intestine correlates with the richness of microbiota composition. Also, specific bacteria such as *Bifidobacterium breve* NCC2950, effectively induce REGIII production in the intestine via *MyD88-Ticam1* pathway. Treatment with this probiotic may enhance the mucosal barrier and protect the host from infection and inflammation.

5.2. Introduction

Antimicrobial peptides secreted by intestinal immune and epithelial cells are important effectors of innate immunity. These endogenous peptides are induced during exposure to enteric pathogens in an attempt to protect the host from infection (Muniz et al., 2012). It is increasingly apparent that antimicrobial peptides also play an essential role in the maintenance of intestinal homeostasis, by limiting microbial-epithelium interactions and preventing unnecessary microbe-driven inflammation (Hooper, 2009). This is particularly important in the distal gut where microbiota load and density are high.

The intestinal microbiota consists of a complex community of bacteria with varying physiological and immune modulating capacity (Round and Mazmanian, 2009). A balanced composition of symbionts and pathobionts is thought to stimulate homeostatic responses in the host (Round and Mazmanian, 2009), while shifts in this balance (dysbiosis) have been associated with inflammatory disorders of the gut, such as inflammatory bowel disease (IBD) (Sokol et al., 2008). Recently, it has been shown that the intestinal microbiota provides pivotal stimuli and cues necessary for the induction of antimicrobial peptides (Mukherjee et al., 2008).

Regenerating islet-derived (REG)III proteins, which belong to the family of C-type lectins, are one class of antimicrobials that are expressed in the intestine. In mice, three distinct classes of *RegIII, α, β and γ*, have been identified. In contrast, only *REGIII-α* and *γ* have been identified in humans. Human *REGIII-α*, also known as hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (HIP/PAP), shares 67% homology with murine *RegIII-γ*, while human *REGIII-γ* shares 68% homology with

murine *RegIII-β*. REGIII proteins bind to the peptidoglycan moieties of bacteria and induce damage to the bacterial cell wall (Cash et al., 2006; Lehotzky et al., 2010; Mukherjee et al., 2009). Different intestinal cell types express REGIII proteins (Cash et al., 2006; Ismail et al., 2011). We have previously shown that modulating intestinal microbiota composition, either by colonizing with microbiota devoid of pathobionts or supplementation with a probiotic bacteria, affects the expression of *RegIII-γ* in mice lacking intracellular microbial recognition receptors (Natividad et al., 2012). However, it is unclear whether specific component(s) of the intestinal microbiota differentially and directly regulate the expression of REGIII proteins by various intestinal cell types.

Here, we sought to determine whether different components of the intestinal microbiota and specific probiotics differ in their capacity to stimulate the expression of antimicrobial peptide REGIII by intestinal epithelial cells. For this, we investigated the effects of colonization with both diverse communities and specific components of the microbiota on REGIII-γ expression by ileal and colonic epithelial cells. REGIII-γ levels were quantified in whole ileal and colonic tissue of germ-free (GF) mice, and in mice colonized with specific pathogen free (SPF) microbiota, altered Schaedler flora (ASF), commensal *Escherichia coli* JM83, or the probiotic *Bifidobacterium breve* NCC2950. The importance of Toll-like receptor (TLR) signalling in this process was also investigated using GF *MyD88*$^{-/-}$*Ticam1*$^{-/-}$ mice. Futhermore, *REGIII-α* expression in human colonic epithelial cells was also quantified after stimulation with *E. coli* or *B. breve*.

5.3. Materials and Methods

Mice. *MyD88*$^{-/-}$*Ticam1*$^{-/-}$ mice on a C57BL/6 background were kindly provided by Prof. B.A. Beutler (La Jolla, CA, USA). SPF C57BL/6 mice were purchased from Taconic. Germ-free C57BL/6 and *MyD88*$^{-/-}$*Ticam1*$^{-/-}$ mice were rederived at McMaster University Axenic Gnotobiotic Unit by axenic two-cell embryo transfer technique previously described (Slack et al., 2009). Germ-free mouse colonies were maintained in flexible film isolators at the AGU and germ-free status was routinely confirmed by a combination of culture-and non-culture based techniques in fecal and cecal contents (Slack et al., 2009). Periodic serological testing was also performed for viruses, parasites and known pathogens (Charles Rivers laboratories). All mice had unlimited access to autoclaved food and water. Both male and female mice were used at the age of 8-12 weeks. All experiments were carried out in accordance with the McMaster University animal utilization protocols.

Bacterial Strains. *Bifidobacterium breve (B. breve)* NCC2950 was obtained from the Nestlé Culture Collection (Lausanne, Switzerland), and grown under anaerobic conditions in MRS supplemented with 0.05% (v/v) L-cysteine hydrochloride. *Escherichia coli* JM83 (American type culture collection, ATCC) was grown under aerobic conditions in Luria Broth (LB, Oxoid) media. After 18 hours at 37^{O}C, bacterial cells were pelleted, washed in PBS and re-suspended at a concentration of 10^{10} Colony-forming unit (CFU)/ml in PBS with 20% (v/v) glycerol, and kept in frozen aliquots until used. Growth media was spun down and sterile filtered to generate the spent culture medium (SCM). The heat-

inactivated (HI) formulation was prepared by incubating 50ml aliquots of *B. breve* cells at 90°C for 1 hour.

Colonization and Monocolonization Experiments. Monocolonization of GF mice was performed in dedicated flexible film gnotobiotic isolators as previously described (Slack et al., 2009). Briefly, *Bifidobacterium breve* or *Escherichia coli* JM83 cultures were prepared and imported into the isolator following strict aseptic procedures. 10^9 CFU of bacteria of each preparation was gavaged into the stomach of each mouse. Three weeks after monocolonization, mice were used for experiments. Monocolonization was confirmed at sacrifice by plating both the fecal and cecal contents in de Mann-Rogosa-Sharpe (MRS, Oxoid) agar supplemented with 0.05% (v/v) L-cysteine hydrochloride (Sigma-Aldrich) and mupirocin (Sigma-Aldrich) under anaerobic conditions for *B. breve* and LB agar under aerobic conditions for *E. coli*. ASF consisted of *Lactobacillus (L.) acidophilus, L. salivarius, Bacteroides distasonis*, a spiral-shaped bacterium, and four fusiform extremely oxygen sensitive bacteria (Dewhirst et al., 1999). ASF colonization was achieved by co-housing germ-free mice with an "ASF-colonizer" from McMaster's standard colony and was confirmed by a combination of culture and molecular techniques (Natividad et al., 2012).

Colonic Epithelial Cell Line Assays. The Caco-2 cell line was obtained from the ATCC and used from passage 19-25. Cell monolayers were maintained in cell medium consisting of Dulbecco's modified Eagle medium, 20% heat-inactivated fetal bovine

serum (FBS, PAA), and 1% MEM nonessential amino acids (Gibco) supplemented with 2mM glutamine and cultured in a humidified atmosphere with 5% CO_2. For stimulation experiments, cells were seeded in 24-well tissue culture plates and used at 70-80% confluence. Cells were stimulated with live or HI *B. breve* or live *E. coli* at cell-to-bacterium ratios of 1:10 and 1:100 or SCM (10% v/v). PBS/glycerol (10% v/v) was used as negative control while IL-22 (10ng/ml; R&D Systems) was a positive control. All stimulations were performed in FBS-free medium with 1% penicillin-streptomycin (pen-strep) for 4 to 24 hours. After stimulation, cells were washed twice with PBS containing pen-strep and 50μg/ml gentamicin, and stored at -80°C until used.

RNA Isolation and Quantitative Real-Time PCR. Total RNA from the ileum and colon of mice or cell lines were isolated using an RNeasy Mini Kit (Qiagen). Potential DNA contamination was removed by column DNAse treatment (Qiagen). RNA quantity and integrity was checked with NanoDrop (Thermo Scientific) and agarose gel electrophoresis. Only samples with intact RNA were used for subsequent cDNA synthesis with iScript reverse transcriptase (Bio-Rad). 500μg of input RNA was used for each sample. Quantitative real-time PCR was performed on iQ5 Real-Time Detection System (Bio-Rad) with SSofast Evagreen Supermix (Bio-Rad). Primers used were as follows: murine *RegIII-γ* fwd, 5'-CGTGCCTATGGCTCCTATTGCT-3'; murine *RegIII-γ* rev, 5'-TTCAGCGCCACTGAGCACAGAC-3'; human *REGIII-α* fwd, 5'-TATGGCTCCCACTGCTATGCCT-3'; human *REGIII-α* rev, 5'-TCTTCACCAGGGAGGACACGAA-3'; *GAPDH* fwd 5'-

CCATGGAGAAGGCTGGGG-3', *GAPDH* rev 5'-CAAAGTTGTCATGGATGACC -3'. The iQ5 manager software (Bio-Rad) was used to calculate the relative fold change in expression normalized to *GAPDH* expression by the $2^{-\Delta\Delta CT}$ method. All procedures were performed according to the manufacturer's instructions.

Immunofluorescence for RegIII-γ Proteins. REGIII-γ protein expression in the mouse intestine was evaluated using immunofluorescence in formalin fixed, paraffin-embedded tissue sections. Sections were cut (5µm), deparaffinised, then blocked with phosphate-buffered saline (PBS) / bovine serum albumin at 2% / Tween 20 at 0.05% for 1 hour. Samples were stained overnight (4°C) with rabbit anti-mouse REGIII-γ antibodies (1:100 dilution, kindly provided by Dr. Kiyama (Ampo et al., 2009) and 1 hour with secondary antibody at room temperature (goat anti-rabbit IgG H+L; 1:250 dilution, Molecular Probes), mounted in Prolong Gold with DAPI. For quantification, specific fluorescence intensity from three different microscopic fields per animal was acquired using Nikon Eclipse 90i. All individual fields have been normalized by using the same fluorescence acquisition settings and by the surface of autofluorescent tissue. Specific REGIII-γ fluorescence staining was quantified using ImageJ software (NIH) and was reported per unit surface of tissue. Data were represented as fold increase of signal intensity compared to control group (germ-free C57BL/6 mice), which was arbitrary reported as 1.

Statistics. Data are presented as either dot plots or bar graph (mean ± SD). Statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by

the Bonferronni test, or the two-tailed Student *t* test, when applicable. All statistical testing was performed using GraphPad Prism 6 (GraphPad Software Inc.). $p<0.05$ is considered significant.

5.4. Results

Germ-free mice had lower REGIII-γ expression than SPF-colonized mice. **Germ-free mice had lower REGIII-γ expression than SPF-colonized mice.** Different regions of the gastrointestinal tract have unique microenvironments characterized by specialized cell types, microbial diversity and load. Thus, we first sought to examine ileal and colonic expression of REGIII-γ in the absence of microbiota, and whether microbiota with different levels of diversity had an impact on its expression. We measured *RegIII-γ* in the ileum and colon of GF-, ASF-colonized, and SPF C57BL/6 mice by quantitative real-time PCR. GF mice showed significantly lower *RegIII-γ* expression than SPF mice (Figure 5.1). REGIII-γ protein expression was also examined by immunofluorescence (IF) staining. Consistent with RNA results, REGIII-γ protein expression was significantly lower in GF mice compared to SPF mice and the pattern of expression was different in the ileum from that in the colon (Figure 5.2). In the ileum of SPF mice, REGIII-γ was expressed by IECs located both at the crypt and the villi, with the highest fluorescence often localized within the crypt. On the other hand, colonic expression of REGIII-γ in SPF mice was specifically expressed by colonocytes. Overall, our results show that REGIII-γ expression varies between the ileum and colon and is influenced by the type of microbiota it is colonized with.

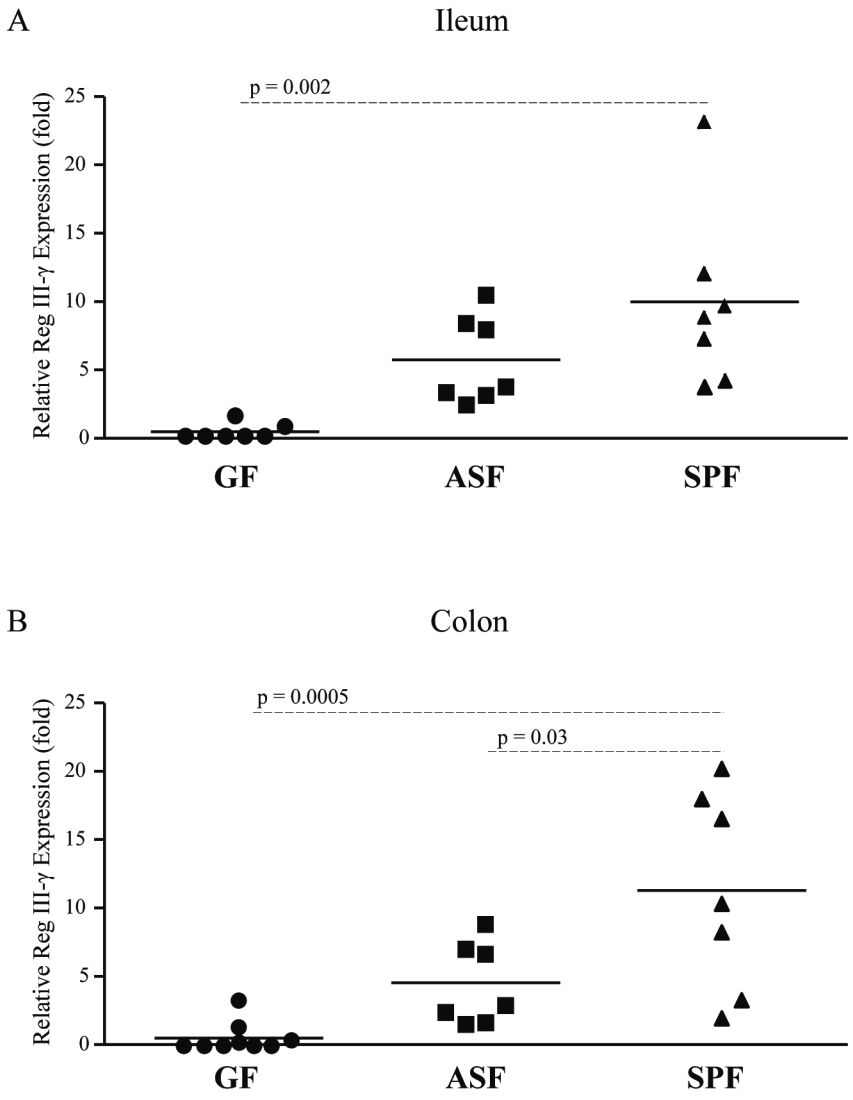

Figure 5.1. *RegIII-γ* RNA expression in (A) ileum and (B) colon of GF, ASF-colonized and SPF mice.
SPF mice had higher *RegIII-γ* RNA expression than ASF colonized mice. Total RNA from ileum and colon of 8-12 week old mice were extracted, and *RegIII-γ* expression was measured by RT-qPCR. RNA expression was normalized to *Gapdh* and shown relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data pooled from 2-3 independent experiments. *p*-values are calculated using ANOVA followed by Bonferroni post-hoc test.

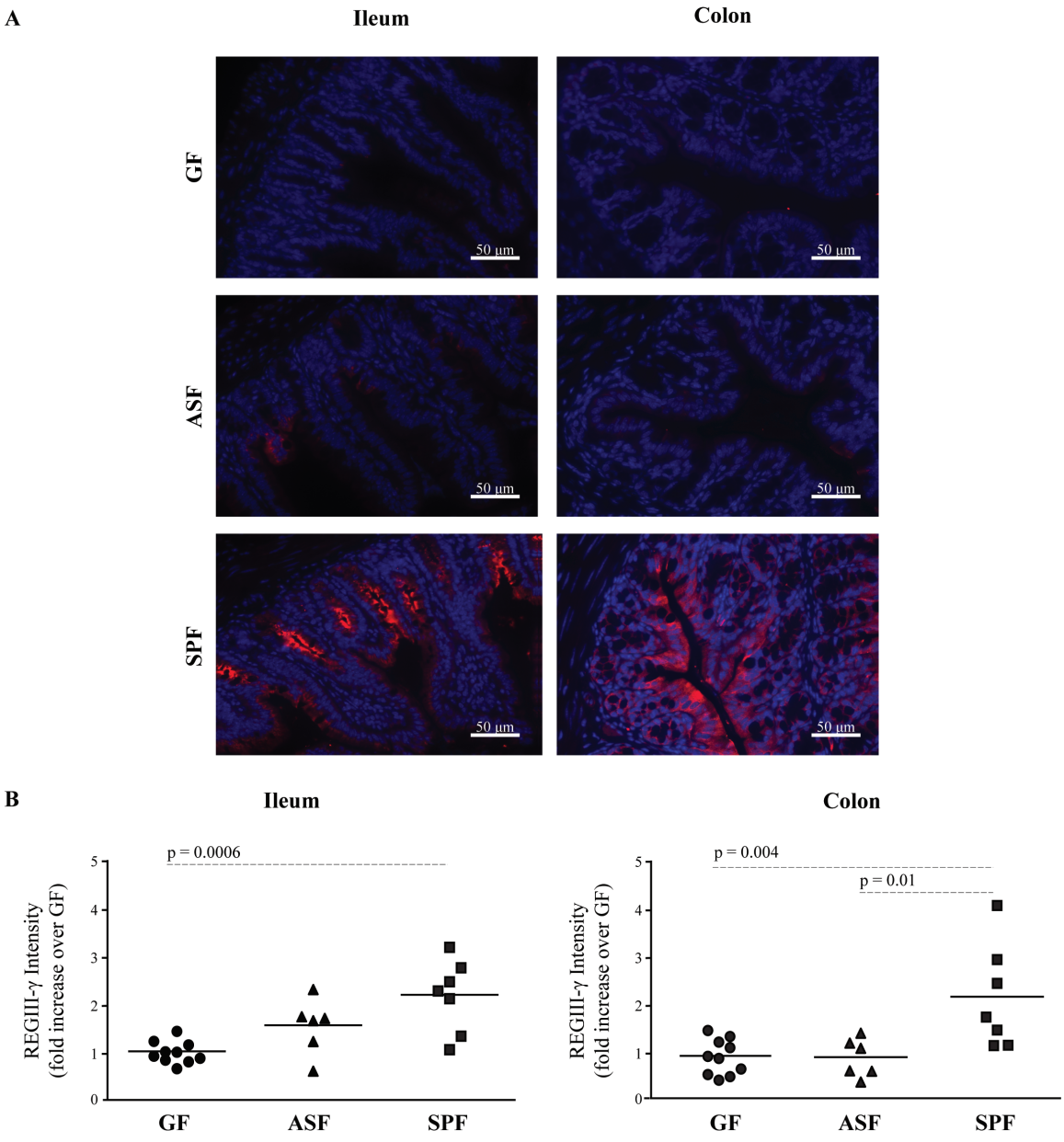

Figure 5.2. REGIII-γ protein expression in ileum and colon of GF, ASF-colonized and SPF mice.

SPF mice had higher REGIII-γ protein expression in ileum and colon than GF mice. (A) Representative immunofluorescence staining (anti-REGIII-γ; red) in each group. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). (B) Quantification of REGIII-γ staining intensity was expressed as fold increase relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data pooled from 2-3 independent experiments. *p*-values are calculated using ANOVA followed by Bonferroni post-hoc test.

Monocolonization with *B. breve* but not *E. coli* increased RegIII-γ expression. There are important inter-individual differences in gut microbial communities (Dewhirst et al., 2010; Stearns et al., 2011), and this may have functional implications. We investigated this using a model of gnotobiotic mono-colonization. The effects of *Bifidobacterium breve* NCC2950, previously found to restore REGIII in mice lacking Nod signalling (Natividad et al., 2012), and of *Escherichia coli* JM83 on REGIII expression in the intestine were explored. Three weeks after monocolonization, mice were aseptically exported from flexi-film isolators and tissue collected to measure REGIII-γ RNA and protein expression. Successful monocolonization was confirmed by selective plating of both fecal and cecal contents collected 21-days post-colonization and by FISH staining (Figure 5.3). *B. breve*- and *E. coli*-monocolonized mice, displayed similar bacterial loads in stool and cecal content (Figure 5.4). However, only *B. breve*-monocolonized mice exhibited higher expression of ileal and colonic REGIII-γ compared to GF controls, suggesting that induction of REGIII-γ was strain specific (Figure 5.5-5.6).

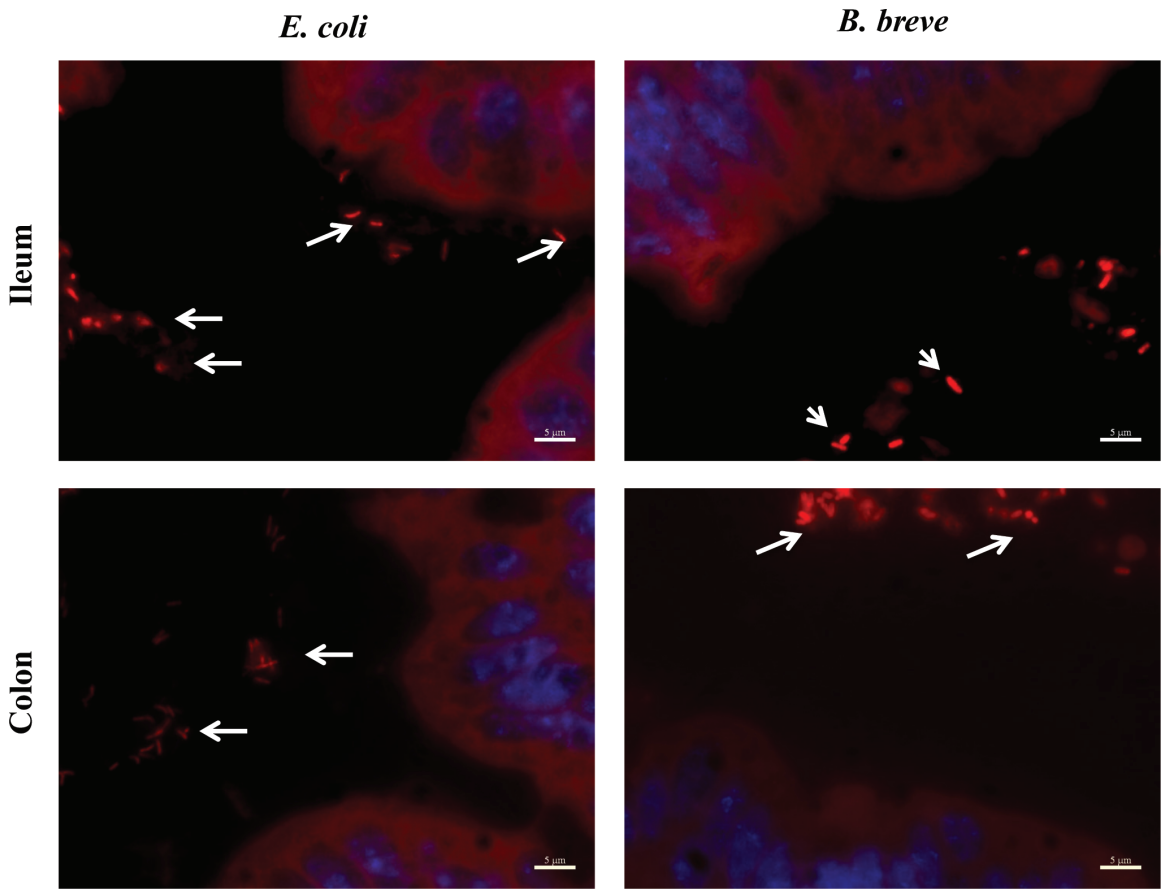

Figure 5.3. Visualization of bacterial localization relative to the epithelial surface by FISH.
Closer association of bacteria with the epithelial surface was observed in the ileum of *E. coli*-monocolonized mice compared to *B. breve*-monocolonized mice. Intestinal sections were fixed in Carnoy for 24 hours then paraffin embedded. Sections were hybridized to a probe (EUB 338, GCTGCCTCCCGTAGGAGT) that recognizes the 16S rRNA genes of all bacteria (red), and counterstained with DAPI to visualize nuclei (blue). Arrows indicate the bacteria.

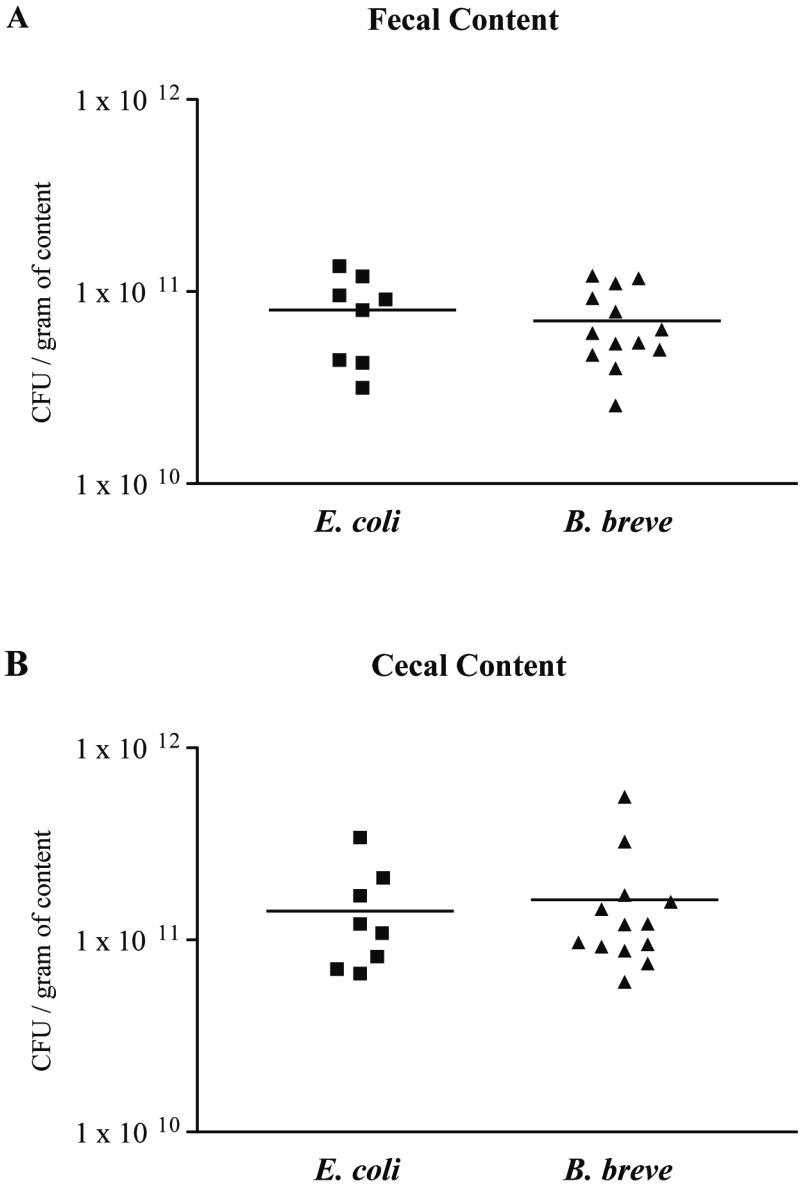

Figure 5.4. Bacterial load in the (A) feces and (B) cecum of *B. breve*- and *E. coli*-monocolonized mice.
GF mice were monocolonized with either *B. breve* or *E. coli.* 21 days post-monocolonization, the density of bacteria in the stool and cecal content was determined by selective plating. Data are pooled from 2-3 independent experiments. CFU, colony-forming units.

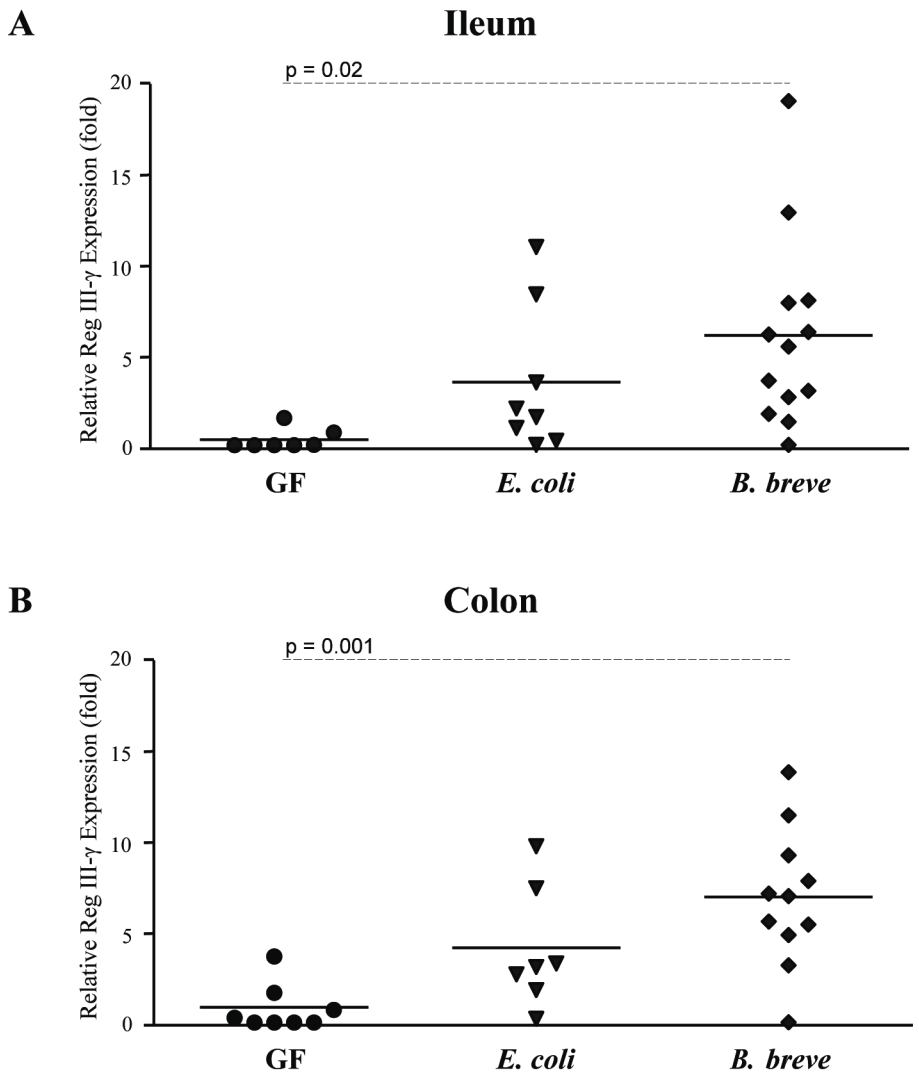

Figure 5.5. *RegIII-γ* RNA expression in (A) ileum and (B) colon of GF, *B. breve*- and *E. coli*-monocolonized mice.
Monocolonization with *B. breve* induced higher *RegIII-γ* RNA expression than monocolonization with *E. coli*. 21 days post-monocolonization, total RNA from ileum and colon were extracted, and *RegIII-γ* expression was measured by RT-qPCR. RNA expression was normalized to *Gapdh* and shown relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data pooled from 2-3 independent experiments. *p*-values are calculated using ANOVA followed by Bonferroni post-hoc test.

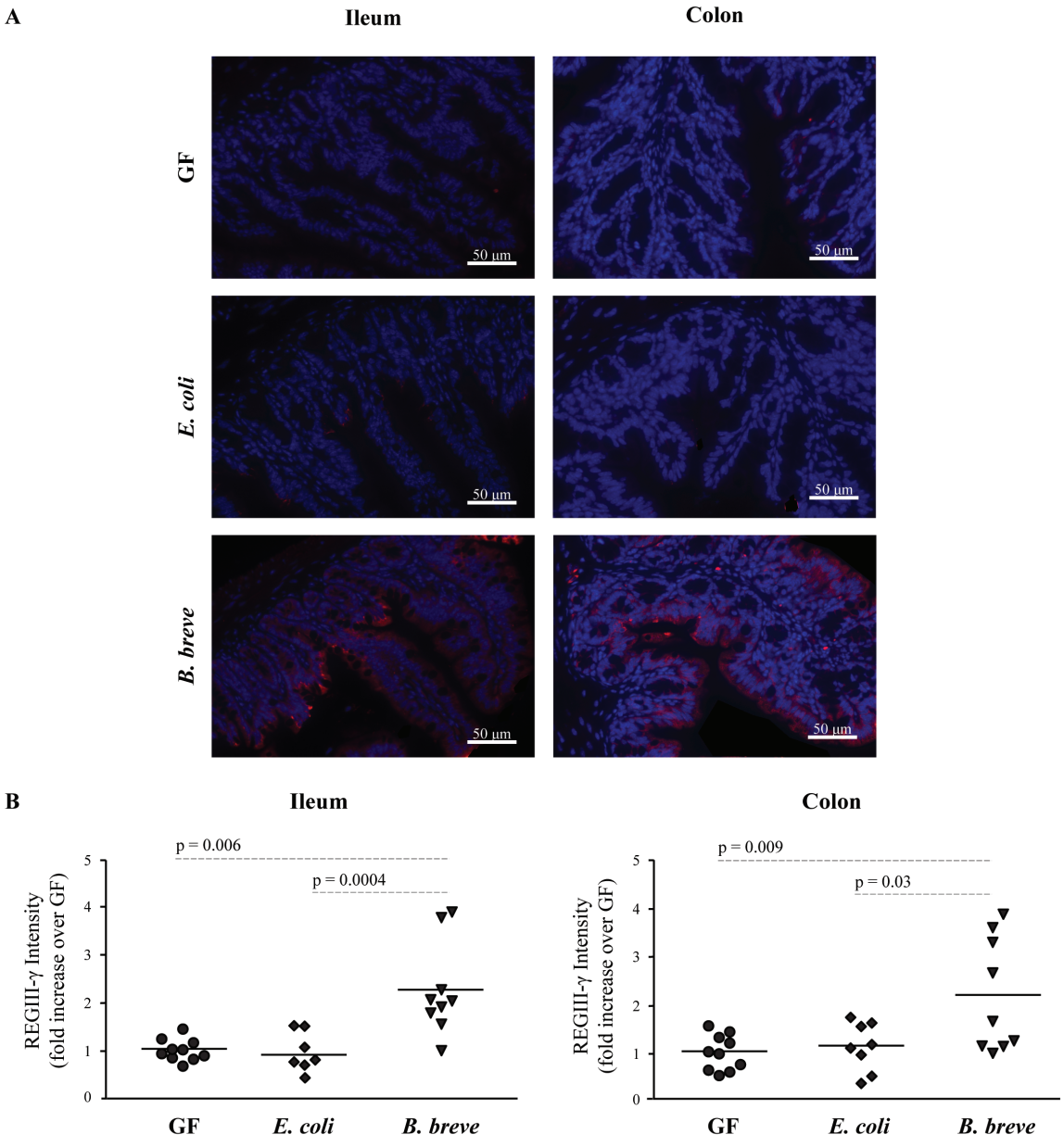

Figure 5.6. REGIII-γ protein expression in ileum and colon of GF, *B. breve*- and *E. coli*-monocolonized mice.

B. breve-monocolonized mice showed higher REGIII-γ protein expression compared to *E. coli*-monocolonized mice. (A) Representative immunofluorescence staining (anti-REGIII-γ; red) in each group. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). (B) Quantification of REGIII-γ staining intensity was expressed as fold increase relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data pooled from 2-3 independent experiments. *p*-values are indicated in the figure and calculated using ANOVA followed by Bonferroni post-hoc test.

Monocolonization with *B. breve* did not increase RegIII-γ expression in *MyD88*$^{-/-}$*;Ticam1*$^{-/-}$ mice. To determine the host factors that govern the *B. breve*-induced REGIII-γ expression, we used mice that lack myeloid differentiation primary response 88 (*Myd88*) and TIR-containing adaptor molecule (*Ticam1*) genes, which are relevant downstream signalling adaptor proteins for all TLRs. Similar to wild type GF C57BL/6, GF *MyD88*$^{-/-}$*;Ticam1*$^{-/-}$ mice displayed low levels of ileal and colonic REGIII-γ expression. Levels of REGIII-γ expression in *MyD88*$^{-/-}$*;Ticam1*$^{-/-}$ mice, however, remained unchanged after *B. breve* monocolonization (Figure 5.7-5.8). Together, these results suggest that microbial induction of REGIII-γ is mediated by MyD88-Ticam dependent pathways, such as TLR signalling.

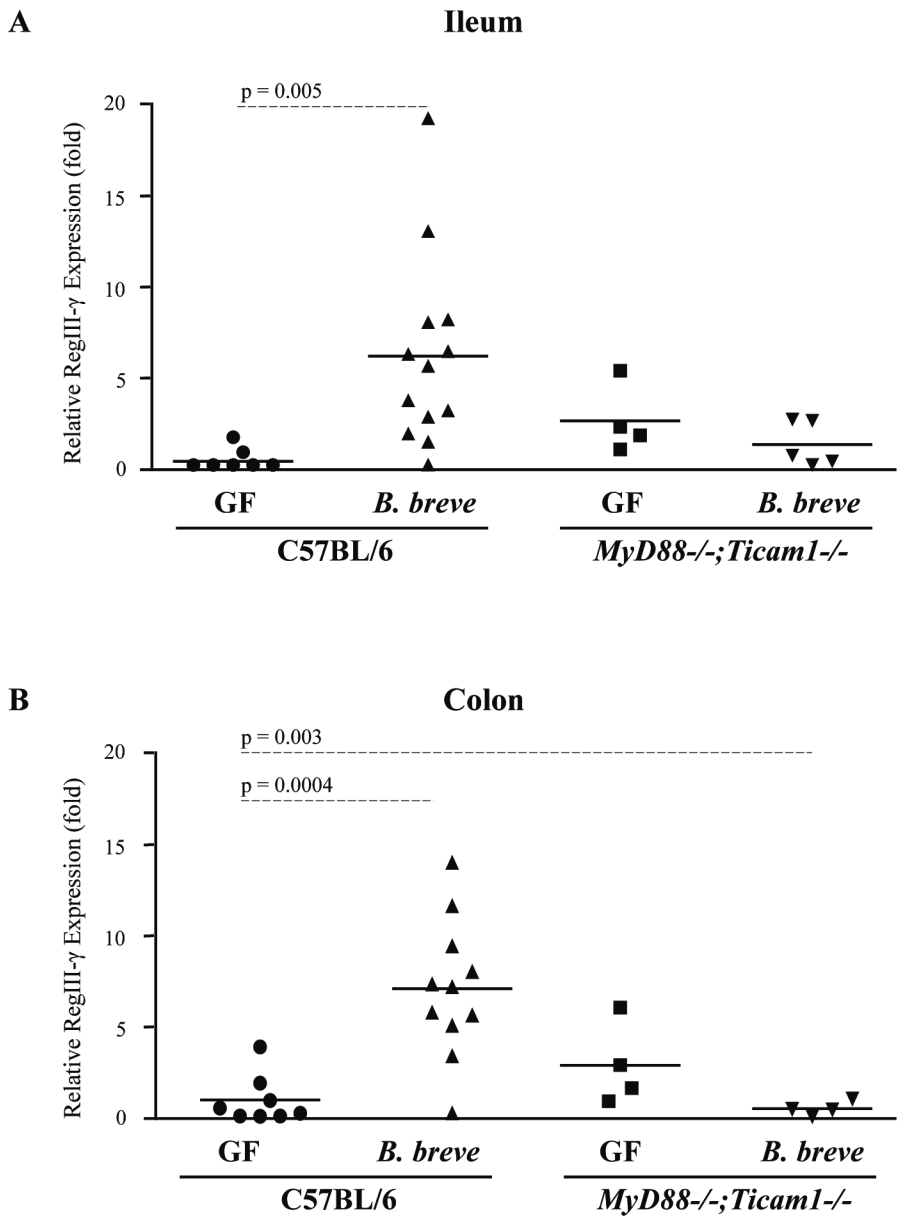

Figure 5.7. *RegIII-γ* RNA expression in (A) ileum and (B) colon of *Myd88$^{-/-}$;Ticam$^{-/-}$* mice monocolonized with *B. breve*.

B. breve-monocolonized *Myd88$^{-/-}$;Ticam$^{-/-}$* mice displayed similar *RegIII-γ* RNA expression compared to GF *Myd88$^{-/-}$;Ticam$^{-/-}$* mice. 21 days post-monocolonization, total RNA from ileum and colon were extracted, and *RegIII-γ* expression was measured by RT-qPCR. RNA expression was normalized to *Gapdh* and shown relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data pooled from 2-3 independent experiments. *p*-values are calculated using ANOVA followed by Bonferroni post-hoc test.

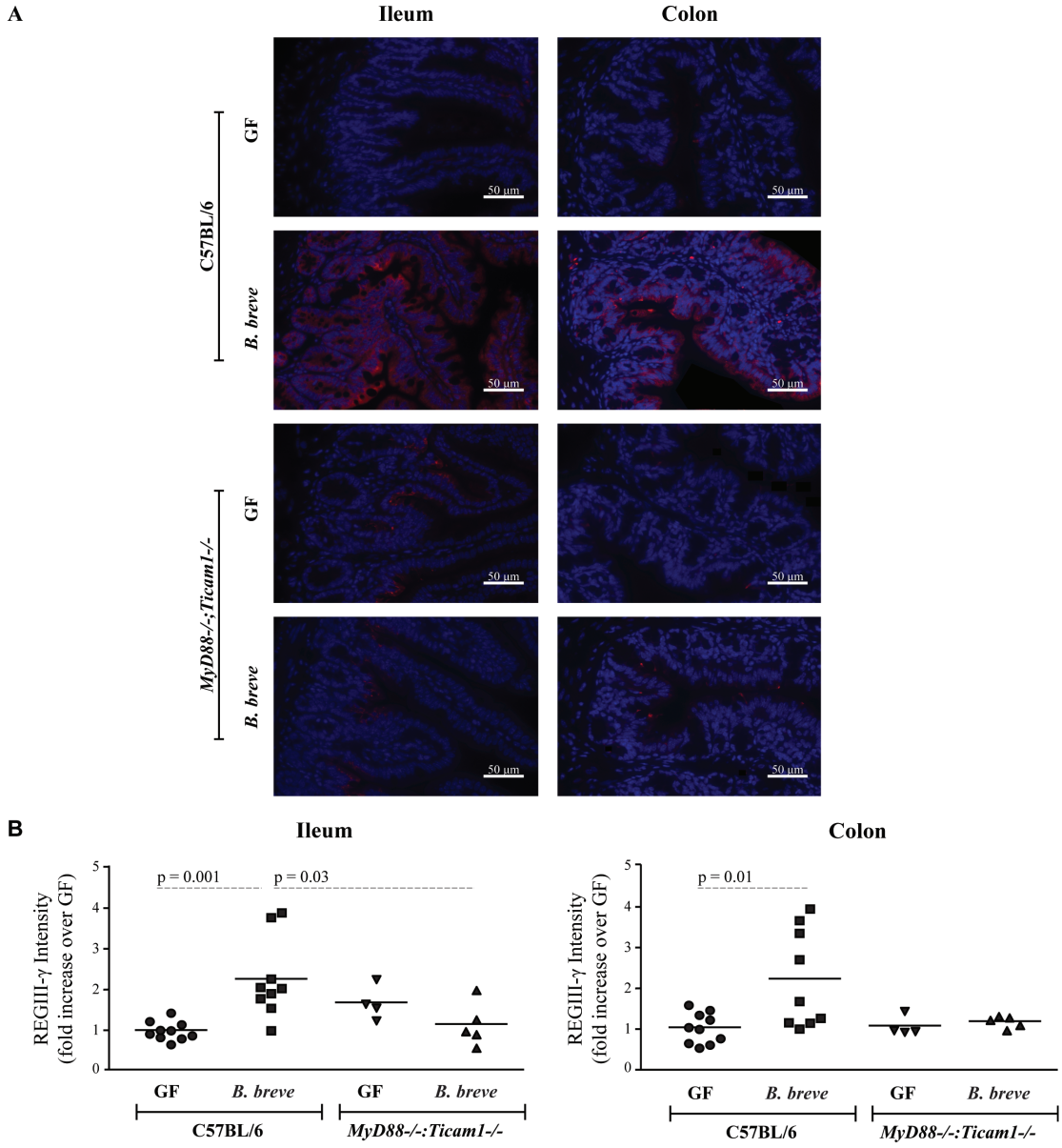

Figure 5.8. REGIII-γ protein expression in (A) ileum and (B) colon of *Myd88*$^{-/-}$*;Ticam*$^{-/-}$ mice moncolonized with *B. breve*.

B. breve-monocolonized *Myd88*$^{-/-}$*;Ticam*$^{-/-}$ mice displayed similar *RegIII-γ* protein expression compared to GF *Myd88*$^{-/-}$*;Ticam*$^{-/-}$ mice. (A) Representative immunofluorescence staining (anti-REGIII-γ; red) in each group. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). (B) Quantification of REGIII-γ staining intensity was expressed as fold increase relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data pooled from 2-3 independent experiments. *p*-values are calculated using ANOVA followed by Bonferonni post-hoc test.

Live *B. breve* induced the expression of RegIII-α in human colonic epithelial cells. To quantify *REGIII-α*, the human ortholog and homolog of murine *RegIII-γ,* we performed *in vitro* experiments using Caco-2 and HT-29 human colon epithelial cell lines. To optimize the expression of *REGIII-α* induced by bacteria, cells were incubated for 2, 4, 6, and 24 hours at cell-to-bacterium ratios of 1:10 and 1:100. Results show that 4 hours of incubation at a cell-to-bacterium ratio of 1:100 led to the highest *REGIII-α* expression (Figure 5.9). Using these parameters, the amount of *REGIII-α* expression was measured, and our results showed that colonic epithelial cells significantly upregulated expression in response to *B. breve* stimulation, but not to *E. coli* (Figure 5.10).

We then asked whether *B. breve* actively secreted metabolites able to upregulate *REGIII-α* expression from IECs. For this, Caco-2 cells were stimulated with live, HI and SCM formulations of *B. breve.* Our results showed that only live and HI preparations induced higher *REGIII-α* expression compared to the PBS/glycerol control, suggesting that the whole *B. breve cell*, is required for the induction, independently of its metabolic activity (Figure 5.11).

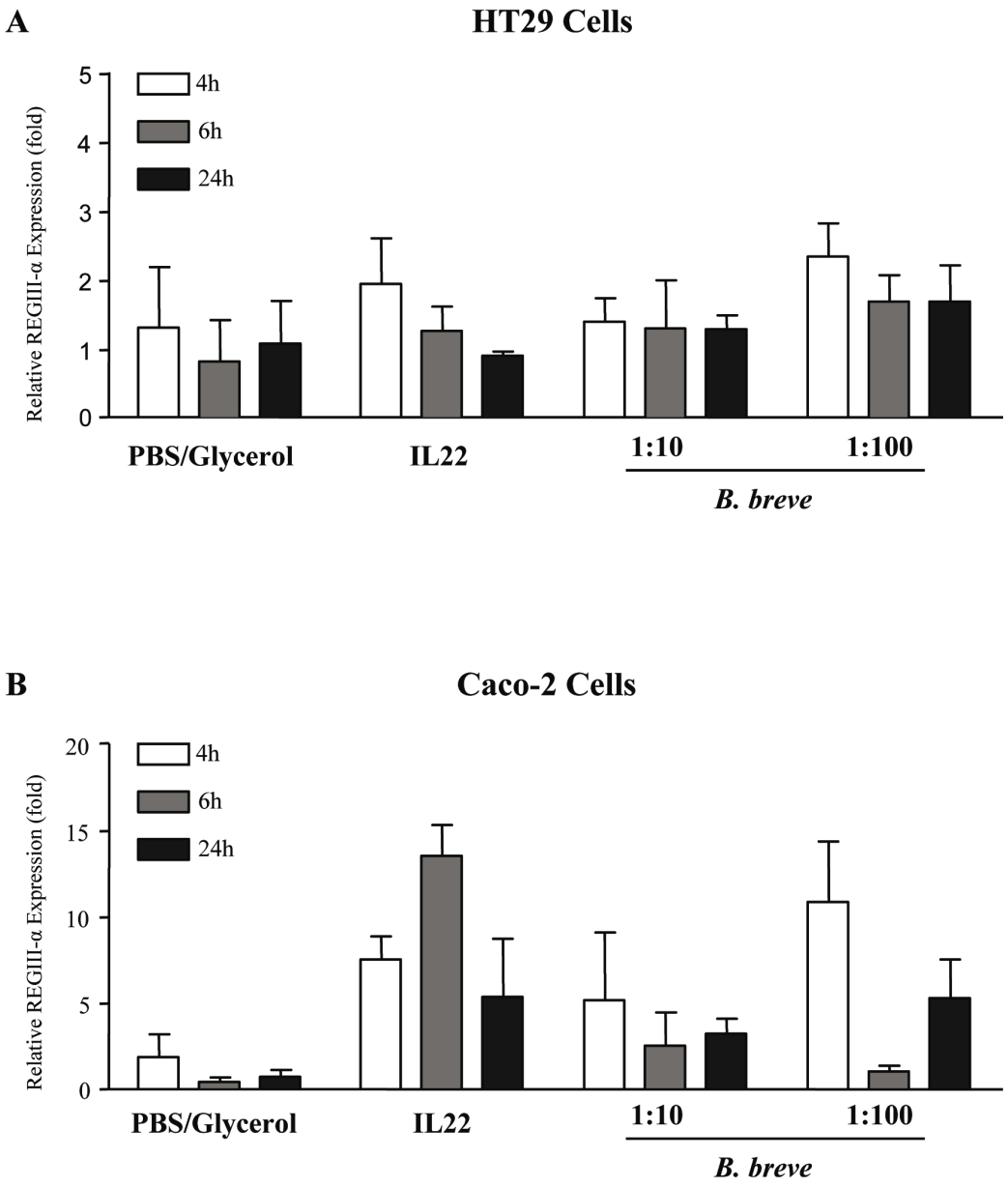

Figure 5.9. *B. breve* induced *REGIII-α* in (A) HT29 and (B) Caco-2 cells in a dose- and time-dependent manner.
Caco-2 and HT-29 cells were stimulated with IL-22 or *B. breve*.
REGIII-α expression was measured at different time points by RT-qPCR. Data shown represent one independent experiment. RNA expression was normalized to *GAPDH* and shown relative to the mean of PBS-glycerol stimulated cells (all time-points), which is set to 1.

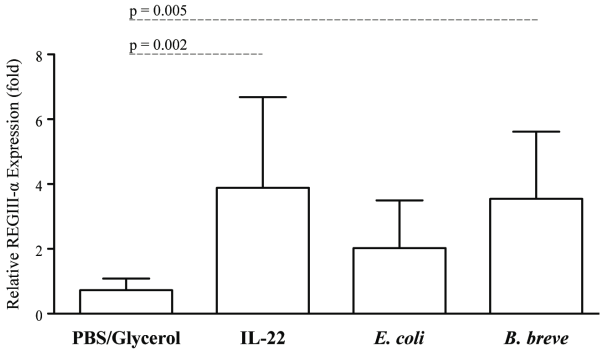

Figure 5.10. *REGIII-α* RNA expression in Caco-2 cells stimulated with IL-22 (positive control), *B. breve* or *E. coli*.
Both IL-22 and *B. breve* induced RegIII-α RNA expression in Caco-2 cells. Total RNA from stimulated cells was extracted, and *REGIII-α* expression was measured by RT-qPCR. RNA expression was normalized to *GAPDH* and shown relative to the mean of PBS-glycerol stimulated cells, which is set to 1. Data were pooled from 3-4 independent experiments, 2-4 wells per group, and shown in bar graph (mean ± SD). *p*-values are calculated using ANOVA followed by Bonferroni post-hoc test.

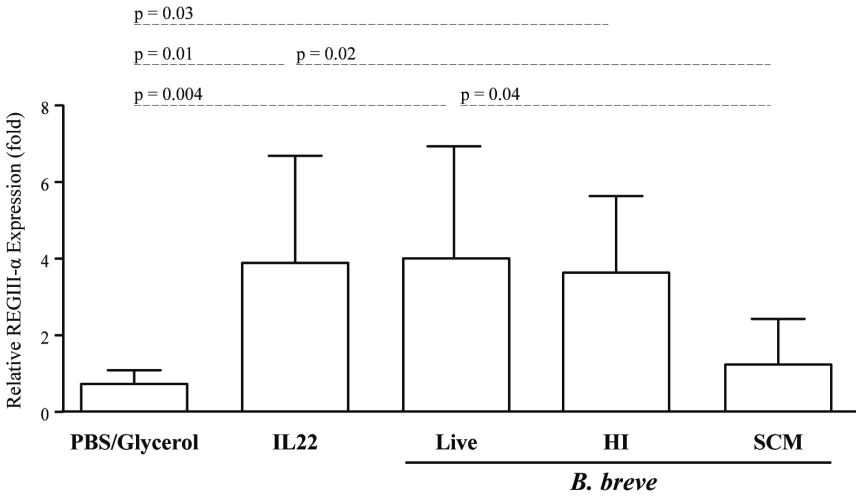

Figure 5.11. *REGIII-α* RNA expression in Caco-2 cells stimulated with live, heat-inactivated (HI) and spent culture medium (SCM) *B. breve*.
Both live and HI *B. breve* (live or HI) induced *REGIII-α* RNA expression in Caco-2 cells. Total RNA from stimulated cells was extracted, and *REGIII-α* expression was measured by RT-qPCR. RNA expression was normalized to *GAPDH* and shown relative to the mean of PBS-glycerol stimulated cells, which is set to 1. Data were pooled from 3-4 independent experiments, 2-4 wells per group, and shown in bar graph (mean ± SD). *p*-values are calculated using ANOVA followed by Bonferroni post-hoc test.

5.5. Discussion

Antimicrobial REGIII proteins play an important role in maintaining gut homeostasis through spatially segregating bacteria, preventing potentially harmful immune responses, and protecting the host from infection (Brandl et al., 2007; Vaishnava et al., 2011; Zheng et al., 2008). In parallel with previous findings (Bohn et al., 2006; Ogawa et al., 2003; Ogawa et al., 2000; Vaishnava et al., 2008), this study shows that the intestinal microbiota affects the level of REGIII expression in the intestine. However, the level of expression differs depending on the region of the gut examined and the nature of microbiota to which it is exposed. We demonstrated that the probiotic *Bifidobacterium breve* NCC2950, but not the commensal *Escherichia coli* JM83, significantly induced REGIII expression *in vivo* in mice and *in vitro* in human intestinal cell line, and this upregulation was independent from the metabolic activity of the strain and mediated through MyD88-Ticam1 signalling. Collectively, these results indicate that regulation of REGIII depends on the richness and specific components of the intestinal microbiota.

Colonization with the community of eight strains of bacteria that compose the ASF did not induce the same level of REGIII-γ expression as observed in SPF mice. Colonization with ASF has previously been shown to effectively reverse GF-related phenotypes (Smith et al., 2007); however, ASF-induced phenotypes are not always identical to those found in SPF mice (Geuking et al., 2011; Slack et al., 2009). This may be related to variability in SPF composition; the SPF mice used in this study came from Taconic which, unlike SPF mice from other suppliers, contain segmented filamentous bacteria (SFB), a potent inducer of T-helper (Th)17 cells (Ivanov et al., 2009). IL-22

produced by Th17 cells (Dong, 2008) induces the expression of REGIII-γ in both murine and human colonic epithelial cells (Geddes et al., 2011; Zheng et al., 2008). In fact, monocolonization with SFB led to increased REGIII-γ production comparable to that of ASF-colonized BALB/c mice (Keilbaugh et al., 2005). It has been shown that reducing microbiota diversity with broad-spectrum antibiotics decreases REGIII-γ expression (Brandl et al., 2007; Vaishnava et al., 2008). Thus, in conjunction with previous work, our results support the notion that microbiota composition, especially with regards to the presence of pathobionts, is an important factor in REGIII-γ regulation.

We have previously shown that supplementation of *B. breve* upregulated REGIII-γ in *Nod1*$^{-/-}$*; Nod2*$^{-/-}$ mice with lower baseline expression of this antimicrobial peptide (Natividad et al., 2012), but the mechanisms underlying this specific probiotic stimulation were unclear. In this study we monocolonized wildtype C57BL/6 mice with *B. breve* or *E. coli* and found that *B. breve*, but not *E. coli*, significantly increased REGIII-γ levels in the ileum and colon. Although ileal bacterial counts were not performed, fecal and cecal content showed similar bacterial loads of *B. breve* and *E. coli*, suggesting the effects on REGIII-γ expression were not due to differential capacity of these two strains to colonize the gut. It should be noted that although *B. breve* stimulated REGIII-γ, the level of induction was lower than in SPF mice. In accordance with our findings, monocolonization with *Bacteroides thetaiotamicron,* but not with non-invasive *Listeria innocua,* resulted in increased REGIII-γ expression in the small intestine that did not reach the level seen in SPF mice (Cash et al., 2006). Others have also determined the effect of specific mono- or dicolonizations on REGIII-γ production in the colon and

cecum with variable results (Keilbaugh et al., 2005; Sonnenburg et al., 2006). Overall the data support the conclusion that the net effect of intestinal bacteria on REGIII-γ expression will be modulated by the presence of specific strains in the microbiota that include both commensals and potential pathobionts. One important aspect in colonization studies relates to variability in experimental design and the time point of tissue sampling. Dynamic changes in the microbiota load and diversity, as well as in immune responses, occur immediately after colonization (El Aidy et al., 2012). We chose to evaluate REGIII-γ at a steady-state (21 days) post-colonization (Turnbaugh et al., 2009) since we were interested in defining REGIII responses under stable conditions. Indeed, REGIII levels have been shown to peak 4 days post-colonization in the small intestine and stabilize by day 16. Therefore, in addition to strain specificity, the time point chosen to determine colonization effects in antimicrobial peptides should be carefully defined.

Many cell types in the gastrointestinal tract are capable of producing REGIII proteins, including intestinal epithelial cells (IEC) and γδ intraepithelial lymphocytes (Cash et al., 2006; Ismail et al., 2009). Studies have proposed that IEC, particularly enterocytes, are producers of REGIII-γ in the colon (Keilbaugh et al., 2005; Matsumoto et al., 2012). In this study we used immunofluorescence to investigate the main source of REGIII-γ after microbial exposure *in vivo*. We found that IECs are the cell types that predominantly express REGIII-γ in both the ileum and colon. Furthermore, our *in vitro* studies confirmed that incubation of human colonic epithelial cells with *B. breve,* but not *E. coli,* induced expression of REGIII-α, the human ortholog and homolog of REGIII-γ.

There is evidence to suggest REGIII-γ expression in the intestine is regulated by MyD88 mediated TLR signalling (Frantz et al., 2012; Gong et al., 2010; Larsson et al., 2012; Vaishnava et al., 2008; Vaishnava et al., 2011). *B. breve* has previously been shown to stimulate TLR2/MyD88 responses in CD103+ dendritic cells (DC). Thus, it is probable that *B. breve* potentially induces responses in epithelial cells through TLR2/MyD88 signalling as well (Jeon et al., 2012). We found that REGIII-γ expression in *MyD88-Ticam1* double knockout mice monocolonized with *B. breve* was low and comparable to that of GF mice, indicating *B. breve*-induced REGIII-γ production requires TLR signalling. Direct signalling of *B. breve* through epithelial TLRs is consistent with the epithelial cell-autonomous model of REGIII-γ expression (Sanos et al., 2011). The results may also explain our previous results in *Nod1*$^{-/-}$*; Nod2*$^{-/-}$ mice in which *B. breve* led to normalization of REGIII-γ expression, likely through preserved TLR signalling in these mice. Recently, a new model of REGIII-γ production that involves IL-22 has been proposed in which luminal bacteria interact with TLR expressed by DC leading to release of cytokines which then primes innate lymphoid cells (ILC) to release the cytokine IL-22 (Sanos et al., 2011). In our previous study, we did not detect an increase in IL-22 after *B. breve*-induced REGIII-γ expression (Natividad et al., 2012). We propose that *B. breve*-induced REGIII-γ expression may occur in the absence of IL-22 supplementation through an epithelial cell-autonomous manner that involves the *MyD88-Ticam1* pathway, although in this case, induction may be more moderate than in the presence of IL-22.

A number of studies have demonstrated that anti-inflammatory effects of probiotics can be elicited without live bacteria (Kverka et al., 2011; Rachmilewitz et al.,

2004; Sokol et al., 2008). Likewise, induction of antimicrobial human β-defensin 2, can be mediated by either live *Escherichia coli* Nissle 1917 or its bacterial structure flagellum (Schlee et al., 2007). Here, we examined different probiotic preparations on *REGIII-α* expression by colonocytes. We found that live and heat-inactivated *B. breve* increased *REGIII-α* expression, whereas the spent culture medium did not induce any changes. These results suggest that a specific component of the *B. breve's* structure, and not its secreted metabolites, is responsible for *REGIII-α* epithelial expression.

In conclusion, we demonstrated that the effects of the microbiota on REGIII expression in the intestine correlate with microbial composition, and that the effect is strain and formulation specific. We determined that the probiotic *B. breve* NCC2950 upregulates REGIII-γ expression through MyD88-Ticam1 signalling. We have previously shown that preventive administration of *B. breve* NCC2950 to genetically susceptible mice not only increased REGIII-γ expression, but also ameliorated the severity of subsequent colitis (Natividad et al., 2012). Based on these findings, we hypothesize that treatment with *B. breve* may regulate REGIII-γ production in a controlled manner that enhances barrier integrity and protects from inflammation. Our results support the use of microbiota modulating strategies to target homeostatic regulation of antimicrobial peptides. This could be of benefit for IBD patients, their first-degree relatives, as well as patients undergoing chemotherapy or radiation therapy to prevent intestinal injury.

5.6. Acknowledgements

This work was supported in entirety by a Grant in Aid from the Crohn's and Colitis Foundation of Canada (E. F. Verdu). Dr. Verdu holds a Canada Research Chair in Intestinal Inflammation, Microbiota and Nutrition. JP Motta holds a Canadian Institute of Health Research fellowship, and awards from Group for Research and studies on Mediators of Inflammation and CECED (Digestive epithelial cells study group).

We thank Dr. KD McCoy for the initial GF rederivation of *MyD88*$^{-/-}$*;Ticam1*$^{-/}$. We thank Joseph Notarangelo, Sarah Armstrong, and Sheryll Competente from the Axenic Gnotobiotic Unit at McMaster University for their assistance in gnotobiotic experiments. We thank Valerie Petit for helping us set-up the cell-culture experiments. We thank Dr. Hiroyuki Konishi for sending the antibodies against REGIII-γ.

– CHAPTER 6 –

DISCUSSION

6.1. Summary

Mammalian physiology is governed by complex interactions between factors encoded in the genome and environmental exposures. The impact of these interactions is most evident in the gastrointestinal tract, where digestion and absorption of dietary nutrients occur in the presence of the intestinal microbiota.

The famous French physiologist Claude Bernard suggested that, "the constancy of the internal milieu is a requisite for the maintenance of life." Walter Cannon, after him, was instrumental in coining the term *homeostasis*, initially referring to the tendency of mammals to maintain a constant internal environment through modification of behavior. For instance, shivering in cold weather contributes to maintenance of body temperature (Gross, 1998). Today the concept of homeostasis has been extended to immune and functional mechanisms that contribute to the maintenance of equilibrium and health. In the GI tract, host-microbial-dietary interactions are key processes necessary for normal gut development and function, while dysfunctional interactions can lead to the breakdown of homeostasis and disease.

The central theme of the research I carried out during my PhD training focused on understanding how gut luminal antigens interact with host genome-encoded processes to influence host physiology and pathophysiology. The results presented as part of **Chapter 3** revealed that the breakdown of intestinal barrier function by non-steroidal anti-inflammatory drugs was associated with changes in microbiota composition and increased translocation of intestinal bacteria to the lamina propria. This, in turn, influenced systemic immune responses to commensals and to gluten, which is the dietary

trigger in celiac disease (Figure 6.1). The results generated in **Chapter 4** revealed that experimental modulation of intestinal microbiota influences intestinal barrier function and impacts the severity of colitis in a mouse model that lacks intracellular pattern recognition receptors (Figure 6.2). Finally, the work undertaken as part of **Chapter 3** identified that microbiota-induced changes in the host, such as antimicrobial peptide secretion, are dependent on the presence of specific strains or groups of bacteria as well as the richness of microbial ecosystems (Figure 6.3). Taken as a whole, the research presented in this thesis contributes to our understanding of the role of intestinal microbiota in the regulation of health and disease. Although, much remains to be learned, my work offers preliminary insight in ways to manipulate the intestinal microbiota in order to prevent or treat chronic inflammation.

The key findings of my work have been discussed individually in each manuscript (**Chapter 3-5**). Accordingly, this last chapter will discuss potential interplay between studies, elaborate on the overall significance and implications of my research, identify limitations and potential areas for improvement as well as propose future directions.

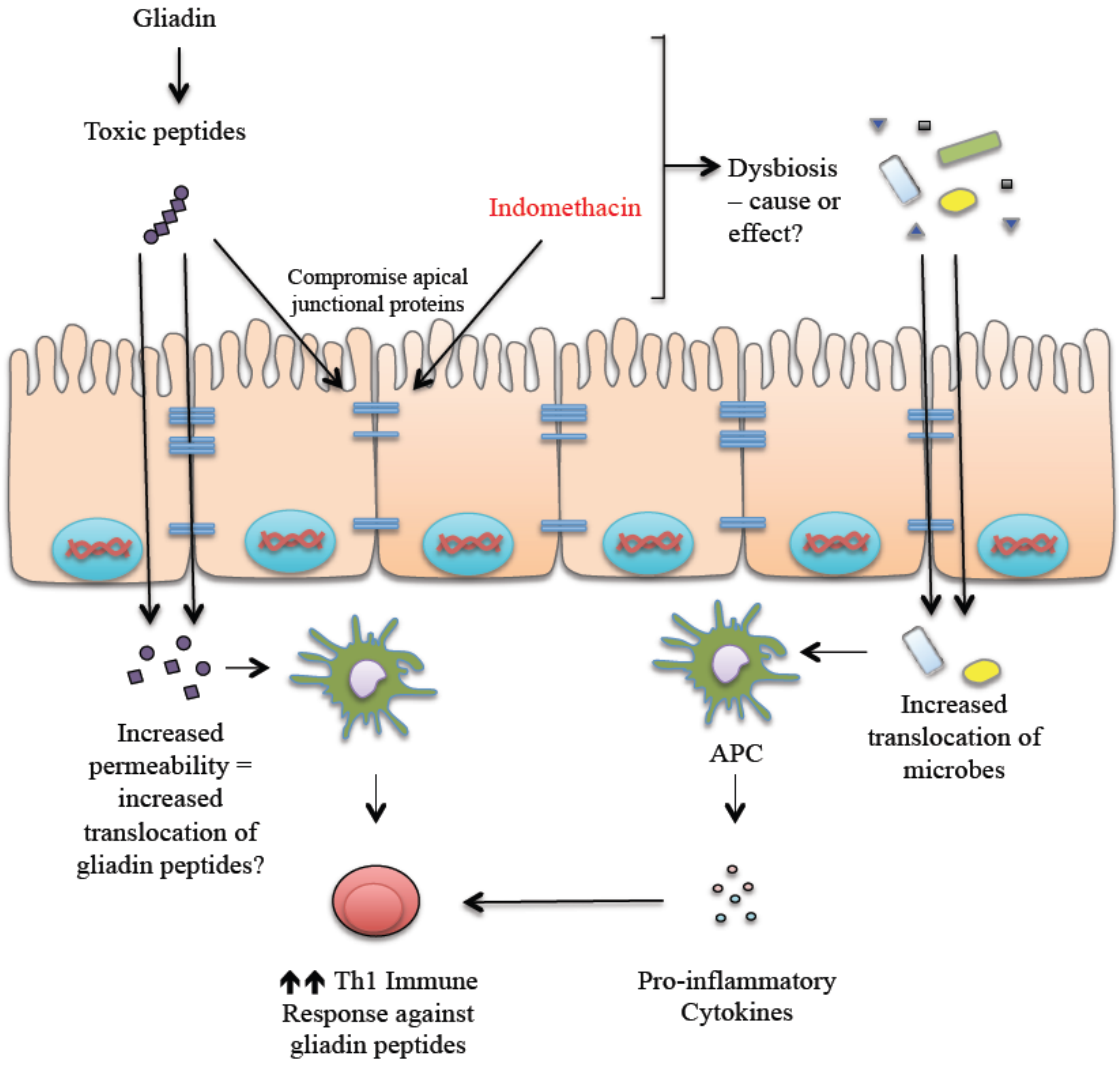

Figure 6.1. Host response to intestinal microbial antigens in gluten-sensitive mice.

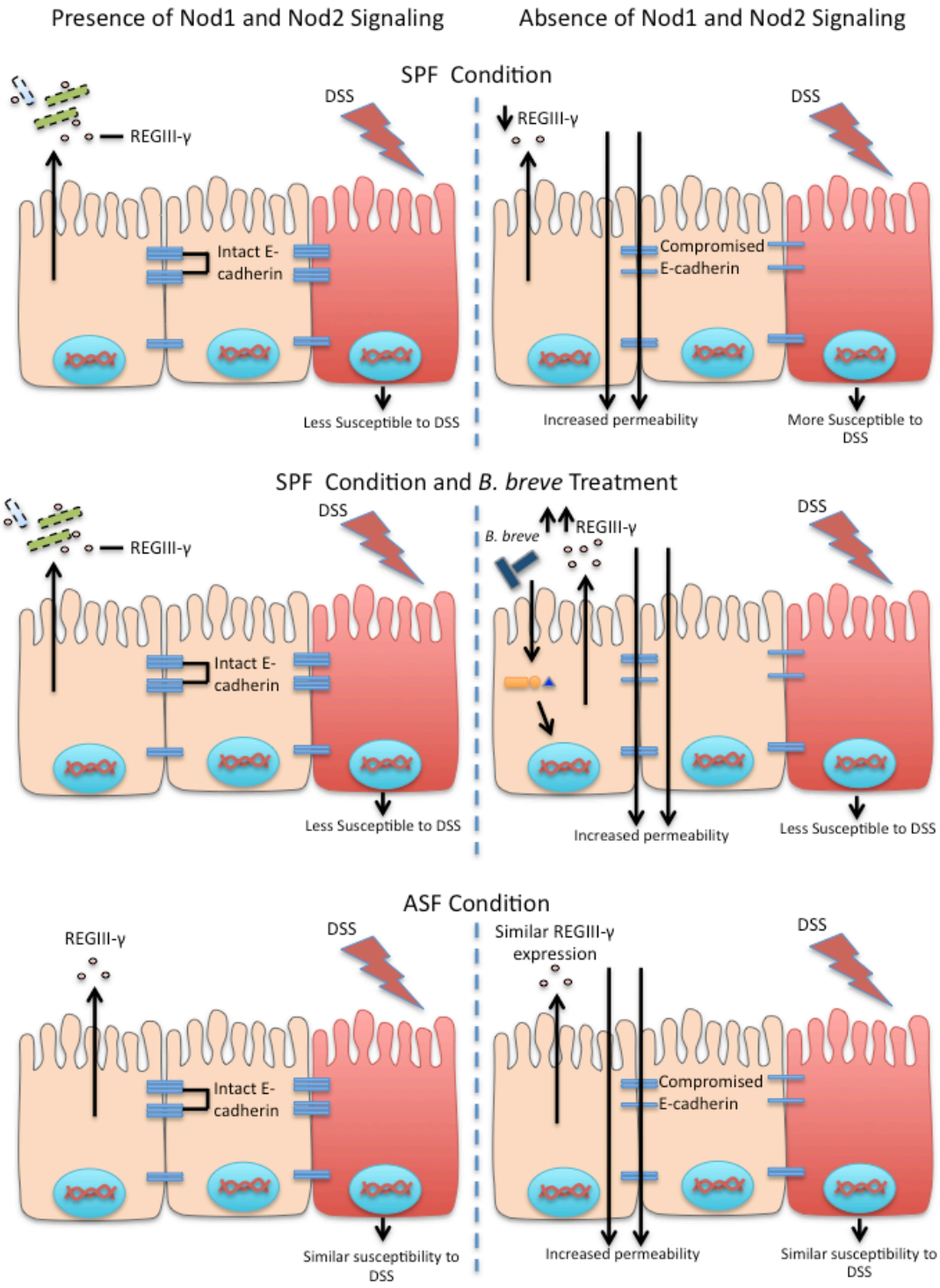

Figure 6.2. Commensal and probiotic bacteria influence intestinal barrier function and susceptibility to colitis in *Nod1*$^{-/-;}$ *Nod2*$^{-/-}$ mice.

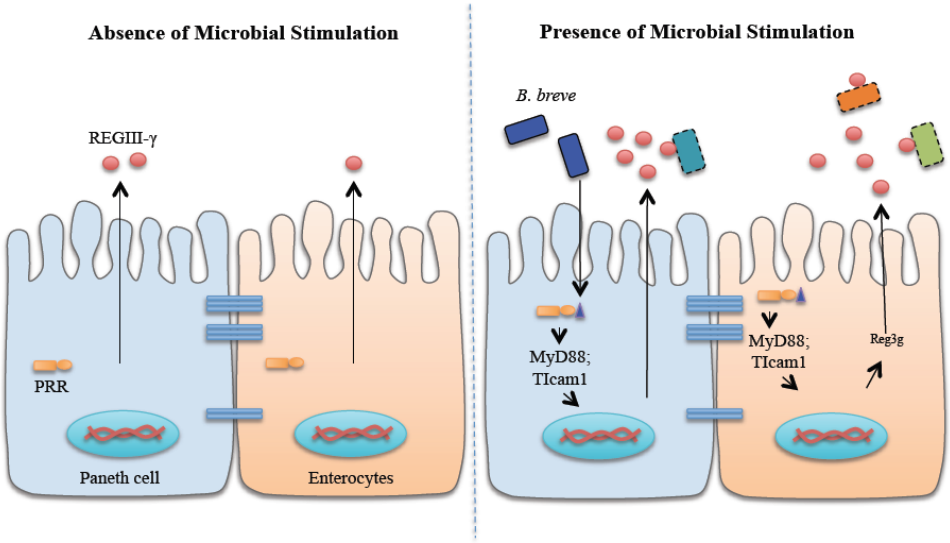

Figure 6.3. Differential induction of antimicrobial REGIII by intestinal microbiota and *Bifidobacterium breve* NCC2950.

6.2. Host-Microbial-Dietary Interactions: Key factor in the development of chronic gastrointestinal diseases.

In the last few decades, a substantial increase in the incidence and prevalence of chronic intestinal inflammation have been observed, particularly in industrialized and urban areas (Soon, 2012; Na, 2012; Rubio-Tapa, 2013). Although phenotypically different, most chronic intestinal inflammatory disorders share a common pathophysiological paradigm (Figure 6.4). For instance, the two models explored in this thesis, celiac disease and IBD, share three main pathophysiological factors: host genetics, environment (including, but not exclusively to diet) and alterations in the composition of the intestinal microbiota. Understanding the relative contributions of each of these factors, as well as how they interact with each other, can provide insight into specific

disease pathogenesis that may ultimately be exploited to develop "druggable" targets or preventative strategies.

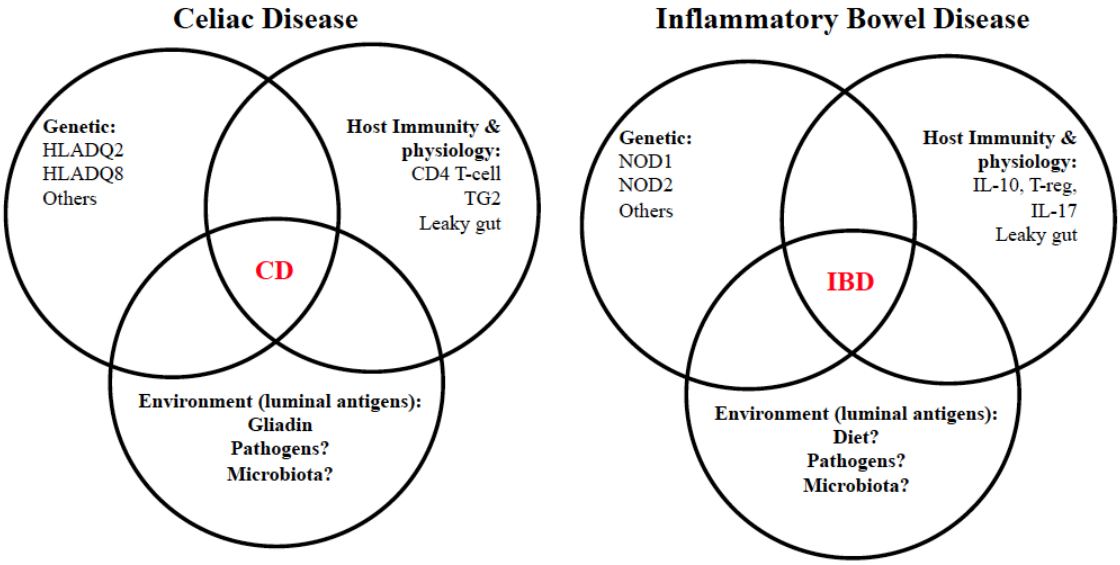

Figure 6.4. Pathophysiology of GI diseases.

6.2.1. Genetics: A necessary but insufficient factor in the development of chronic inflammation.

Genetic predisposition plays a significant role in the pathogenesis of both celiac disease and IBD. Celiac disease is associated with the expression of HLA-DQ2 and DQ8 molecules, encoded by the human leukocyte antigen, mainly in antigen presenting cells. Surprisingly, about 30% of the general population carries these genes, but only 1% develops the disease (Louka and Sollid, 2003; Okazaki et al., 2008). For IBD, genome wide association studies have revealed several associated genes; two of which belong to NOD family of receptors, which are intracellular pattern recognition receptors (Hugot et

al., 2001; Ogura et al., 2001). These IBD-related genes, however, only explain a small proportion of disease heritability (Adler et al., 2011; King and Ciclitira, 2000; Zuk et al., 2012). To understand how genetics play a role in the maintenance of homeostasis and development of chronic inflammation in the small intestine and colon, we used HLA-DQ8 transgenic mice (**Chapter 3)** and mice lacking Nod1 and Nod2 receptors, respectively (**Chapter 4)**. Induction of a gluten-sensitive phenotype in HLA-DQ8 transgenic mice required the use of an adjuvant to break the tolerance to gluten. Similarly, mice lacking Nod1 and Nod2 receptors did not spontaneously develop overt intestinal inflammation under basal conditions, and required the use of the chemical DSS to reveal their increased susceptibility to colitis. Such findings highlight the concept that genetic predisposition is insufficient for the spontaneous development of chronic intestinal inflammation. It is important to note that the lack of distinct effects of genetics under basal conditions does not implicate that these genetic factors do not contribute to susceptibility. It rather supports the multifactorial concept of these disorders, which has been termed by some the "perfect storm scenario" (Vaarala et al., 2008). Gene-to-gene interactions have been suggested in IBD while other non-HLA genes have been identified with celiac disease (Louka and Sollid, 2003; Okazaki et al., 2008). For instance, increasing evidence supports the interaction of Nod2 gene with another IBD susceptibility gene ATG161 (Cooney et al., 2010). Therefore, future experiments could investigate the relevance of these gene-to-gene interactions in defined animal models exposed to additional environmental factors. Interestingly, work at our laboratory has recently shown that the expression of HLA-DQ8 gene in non-obese diabetic (NOD) background

predisposes the mice to express a more severe form of gluten sensitivity, characterized by villous to crypt abnormalities not typically observed in the single HLA-DQ8 transgenic mice (Galipeau et al., 2011). Likewise, another study suggested that overexpression of IL-15 in HLA-DQ8 mice, may lead to an IL-15-driven breakdown of tolerance to gluten, similar to what has been described in a subset of patients with celiac disease (DePaolo et al., 2011). Still, the overall contribution of non-HLA genes, at least for celiac disease, is less than 1%, indicating that other factors may be involved in the disease development and/or progression (Louka and Sollid, 2003). Consequently, the influence of environmental factors as modifiers of genetic susceptibility continues to gain momentum, particularly those related to changes in the intestinal microbiome.

6.2.2. Intestinal microbiota: Key modulator of intestinal homeostasis

In adults, the composition of the intestinal microbiota is relatively individual and stable. However, it can undergo transient changes as a result of its interactions with pathogens, drugs, diet, hygiene habits and other environmental factors (Figure 6.5). The consequences of these interactions can vary markedly ranging from temporary changes in physiology to profound ecological disruptions in intestinal microbiota composition, leading to dysbiosis, which may be detrimental to the host. Not surprisingly, changes in intestinal microbiota composition, characterized by a shift in the relative abundance of pathobionts and symbionts, have been associated with GI diseases, including celiac disease, IBD, irritable bowel syndrome (IBS) as well as non-gastrointestinal diseases such as metabolic disease, type 1 diabetes, allergy, asthma, and neurological disorders. Despite

growing evidence-linking alterations in the microbiota to inflammatory diseases, it remains to be established whether these are primary or secondary events. Nonetheless, there is a bi-directional interaction between different components of the host-microbiome-environment interactions (**Chapter 3-4**). Variations on these interactions may explain the clinical observations that inflammatory diseases are heterogeneous, comprising of multiple different subtypes (Verdu et al., 2009).

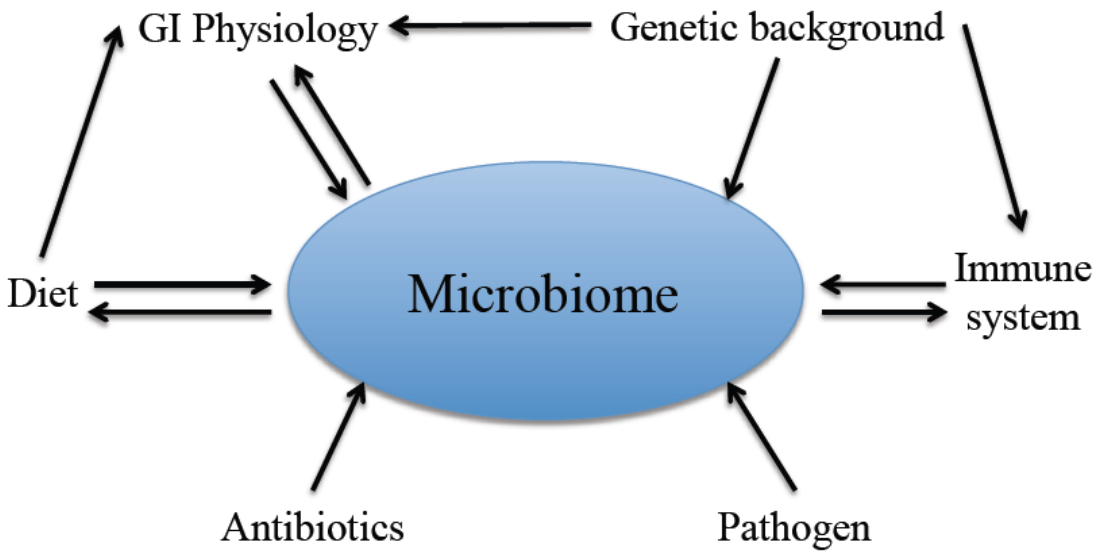

Figure 6.5. Factors that affect the microbiome.

In recent years, the concept that dietary factors affect the composition of microbial communities and functionality has emerged (Gordon et al., 2012; Maslowski and Mackay, 2011; Turnbaugh et al., 2009). For instance, a recent study by *Wu et al* showed that long-term diets based on either protein or carbohydrates were associated with the

enterotypes *Bacteroides* and *Prevotella,* respectively (Wu et al., 2011). Accordingly, the impact of diet on the intestinal microbiota may be an important environmental factor involved in the pathogenesis of disease states that show a rapidly increasing incidence in industrialized nations such as IBD. In **Chapter 2**, we showed for the first time that gluten sensitization and indomethacin treatment in HLA-DQ8 mice, led to changes in microbiota composition in the small intestine. Because the model used deliberate breakdown of tolerance to gluten using antigen and adjuvant, we hypothesized that the observed dysbiosis was secondary to gluten induced-hyper-contractility and dysmotility. Unlike IBD, detailed studies deciphering the possible involvement of intestinal microbiota in celiac disease have only emerged in the last five years. Nevertheless, results are promising and hint on the possible role of intestinal microbiota in celiac pathogenesis. Specifically, the presence of dysbiosis has been described in patients with active celiac disease. Moreover, studies in celiac patients have shown lower counts in anti-inflammatory commensal bacteria believed to play homeostatic roles, such as *Bifidobacteria* and *F. prausnitzii* (De Palma et al., 2009). Interestingly, similar findings have been observed in IBD patients, emphasizing the possible roles of these bacteria to dampen a wide range of inflammatory processes (Sokol et al., 2008). Increases in potentially pro-inflammatory bacteria of the *Bacteroides* and *E. coli* with virulence gene carriage have also been described in active celiacs (De Palma et al., 2009; Nadal et al., 2007). Rod-shaped bacteria, identified in the small intestine of children born during the Swedish celiac epidemic has been proposed as a risk factor contributing to the fourfold increase in disease incidence in children during that time (Forsberg et al., 2004).

Similarly, a recent study found an increased abundance of *Staphylococcus epidermidis* coding for methillicin resistance in celiac patients compared to healthy subjects (Sanchez et al., 2012). Interestingly, some of these changes did not revert after a gluten free diet and induction of clinical remission. Thus, it is possible that dysbiosis is a condition that develops early in the pathogenesis of gluten sensitivity and has a primary role in the development of full blown celiac disease.

There is evidence in the literature suggesting that genetics influence the composition of intestinal microbiota. For example, epidemiological studies have revealed that the microbiota of monozygotic twins closely resemble each other's compared to their marital partners (Zoetendal et al., 2012). Similarly, experimental evidence has shown that mice that lack specific pattern recognition receptors including Nod2 receptors predispose mice to have different microbiota profile than their wild type counterparts (Petnicki-Ocwieja et al., 2009). Changes in microbiota may then influence host physiology and susceptibility of the host to disease (**Chapter 4**). There is however a caveat in these animal studies; most of which did not account for the fact that wild type mice were reared in a different facility. Indeed, in **Chapter 4**, we were able to normalize microbiota composition of knockout and wild type mice using gnotobiotic techniques. Similarly, recent studies have shown that mice lacking either Nod1 or Nod2 receptors have the same microbiota composition *if co-housing and littermate controls are used* (Robertson et al., 2013). The results highlight the importance of defining microbial background composition when studying gene-related phenotypes. Indeed, we found that ASF-colonized mice induced a different antimicrobial expression phenotype compared to SPF

mice (**Chapter 5**). Likewise, in collaboration with Macpherson's group, our group has observed that mice lacking TLR signaling and colonized with low diversity microbiota (ASF) did not display increased intestinal permeability (Slack et al., 2009). This was in contrast with previous observations in SPF-mice lacking TLR signaling, which showed to have "a leakier gut" than their wildtype counterparts (Cario et al., 2004; Gibson et al., 2008). Thus, hygiene status and background microbiota may influence host phenotype independently of genotype. This does not exclude that genetics can influence microbiota composition, but it supports the notion that investigation of interactions between host genotype and microbiota must rely on carefully designed experiments that minimize microbiota differences due to "cage" or "facility" effects. Specifically, it emphasizes the importance of either using F2 littermate controls or gnotobiotic tools to fully decipher the effect of genotype-microbiota interaction in determining health and disease. Interestingly, a more recent study showed that faecal microbiota of children was no more similar to their mothers than to that of their biological fathers, and genetically unrelated but co-habiting mothers and fathers had significantly higher microbial similarity to one another than to members of different families (Yatsunenko et al., 2012). Accordingly, aside from genetics and kinship, environmental factors have a considerable effect on the composition of intestinal microbiota.

Future perspectives: While the animal models presented in **Chapters 3** and **4** provided proof of concept for the role of microbiota in the pathogenesis of both celiac disease and IBD, they did not establish a primary role for the intestinal microbiota in these disorders.

It is possible to colonize germ-free mice with either celiac patient- or IBD- derived microbiota. These humanized mouse models have been described to study obesity and malnutrition (Turnbaugh et al., 2009), although not in the context of either IBD or celiac disease, showing that the structure and diversity of the human microbiota was preserved following human microbiota transfer into germ-free mice (Turnbaugh et al., 2009). To determine a causal effect of a dysbiotic microbiota in gluten sensitization, it would be interesting to investigate whether HLA-DQ8 mice reared in germ-free conditions can be sensitized to gliadin and whether they express a more pro-inflammatory response compared to colonized mice. Also colonization with celiac derived-microbiota (dysbiotic) should be employed in gnotobiotic colonizations.

6.3. Intestinal barrier: Role in intestinal homeostasis.

Antimicrobial proteins such as RegIII proteins are innate immune effectors that perform key roles against invading pathogens (Brandl et al., 2007; Geddes et al., 2011). RegIII-γ proteins and other secreted antimicrobials are retained in the epithelial surface overlaying mucus (Meyer-Hoffert et al., 2008; Vaishnava et al., 2011). This distribution suggests that aside from acting as natural host antibiotics against pathogens, they also contribute to intestinal homeostasis by fortifying the mucus/epithelial barrier and preventing intestinal resident microbes from adhering or contacting with the epithelial cells, which can otherwise promote unnecessary immune responses. This is highly relevant as some components of intestinal microbiota have potential to become opportunistic. Intriguingly, IBD patients have been shown to harbor increased numbers of

mucosa-associated bacteria and adherent-invasive bacteria (Man et al., 2011); these bacteria colonize areas in close proximity to the intestinal epithelium, placing them in a prime position to promote or exacerbate inflammatory responses. It remains to be determined, however, whether IBD patients with higher mucosa-associated bacteria have less antimicrobial peptide. Nevertheless, impaired antimicrobial expression, either primary or secondary to disease, may lead to reduction of antibacterial activity, making susceptible individuals to become vulnerable to microbial-induced inflammation. Indeed, SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice have lower RegIII-γ expression in their colon; and this deficiency is associated with increased susceptibility to DSS colitis and increased bacterial translocation (**Chapter 4**). Oral administration of DSS is toxic to intestinal epithelial cells and induce epithelial damage; a break in the barrier can lead to exposure of lamina propria immune cells to pathobionts and subsequent induction of intestinal inflammation (Wirtz et al., 2007). Thus, it is possible that the inability to express sufficient RegIII-γ renders the SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice to be more susceptible to intestinal microbiota-induced inflammation during DSS. Consequently, these findings suggest that stimulation of RegIII-γ expression may be useful in designing therapeutic strategies to prevent the development and/or exacerbation of inflammation particularly in a genetically susceptible individual.

Antimicrobial peptides are differentially induced and secreted in response to a variety of stimuli (Mukherjee et al., 2008). Gnotobiotic studies have revealed that bacterial signals are needed for transcriptional regulation of RegIII-γ proteins in the small intestine (Cash et al., 2006). In accordance, with previous results (Cash et al., 2006), we

demonstrated intestinal bacteria have an important role in RegIII-γ regulation (**Chapter 3-4**). We further observed that RegIII-γ expression in intestinal epithelial cells correlated with the richness of microbiota composition and that effects of bacteria were strain specific. Specifically, we found that *Bifidobacterium breve* NCC2950, a probiotic bacterium, is more efficient in up-regulating RegIII protein than the commensal *Escherichia coli* JM83. Interestingly, administration of *B. breve* corrected the RegIII-γ deficiency; this was paralleled by decreased colitis susceptibility of SPF *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice to DSS colitis (**Chapter 3**). Hence, this indicates that *B. breve* induced RegIII-γ proteins may be helpful in maintaining intestinal health by protecting the vulnerable mucosal surface from microbial invasion and penetration.

It is important to note, however, that increased expression of RegIII and other antimicrobials have been described during active inflammatory bowel disease (IBD) in humans, and during colitis in murine models (Granlund et al., 2011; Ismail et al., 2009; Ogawa et al., 2003; Ramasundara et al., 2009; van Beelen Granlund et al., 2013). Similarly, increased antimicrobial expression has been observed in inflammation models induced by pathogens (Brandl et al., 2007; Geddes et al., 2010). Antimicrobial proteins such as RegIII-γ proteins have broad-spectrum antimicrobial activity (Cash et al., 2006). Thus, it is possible that increased expression during inflammatory situations is not always beneficial, as it could perturb intestinal microbiota composition by non-specifically killing commensals or reducing colonization resistance. A provoking hypothesis is that very marked stimulation of antimicrobial peptide secretion during an infectious episode could be deleterious and contribute to the dysbiosis observed in chronic inflammatory

disorders. In this scenario, dysbiosis could play a primary role in celiac disease, IBD and even IBS. This would also explain the epidemiological association of celiac disease, IBD and IBS onset after a gastrointestinal infection (Verdu and Riddle, 2012). In contrast, moderate production of antimicrobial peptides would fulfill a protective role by strengthening the barrier and segregating potentially invading pathobionts, without significantly perturbing the balance of the commensal microbiota.

6.4. Modulation of Intestinal Microbiota: Prevention and Treatment of Disease

Therapies that directly target the microbiota have been proposed for the treatment of IBD (Khan et al., 2011). Since deficiencies in the innate immune receptors Nod1 and Nod2 have been linked to IBD, we tested the hypothesis that modulating intestinal microbiota composition in mice lacking Nod-signaling would alter intestinal barrier function and increase susceptibility to chemically induced intestinal injury (**Chapter 4**). We used two strategies to alter community composition. First, we replaced the entire community with a well-defined microbiota that is free of pathobionts or opportunistic bacteria such as segmented filamentous bacteria. Second, we introduced probiotic bacteria, *Bifidobacterium breve,* into the intestinal microbial community of SPF mice. Our results revealed that changing intestinal microbiota background had an important effect on colitis susceptibility, although not in all phenotypic aspects of the *Nod1*$^{-/-}$; *Nod2*$^{-/-}$ mice (**Chapter 4**). Specifically, modulation of intestinal microbiota correlated with changes in the expression of the antimicrobial RegIII-γ proteins but not with colonic intestinal permeability. Changes in antimicrobial protein expression in mice lacking Nod-

signaling correlated with decreased susceptibility of mice to colitis. Overall, these results provide a rationale for modulating microbiota as a preventative treatment against colitis particularly in a genetically susceptible host.

Future perspectives: The neonatal stage represents the most critical period during which the host is influenced by the microbiota. Together with the postnatal development of the immune system, it is possible that deviations from the normal development of the microbiota during this early stage of life may affect host homeostasis and result in higher susceptibility to disease later in life. Indeed repeated use of antibiotics early in life have been associated with increased risk to develop IBD (Kronman et al., 2012). Moreover, decreased postnatal exposure to infection has been epidemiologically linked to a rise in allergic and inflammatory conditions. Strachan coined the term "hygiene hypothesis" in 1989 to explain this epidemiological observation. Following this line of thought, introduction of specific beneficial microbiota post or prenatally (the latter through the mother) may be advantageous in preventing inflammatory and allergic diseases. Hence, experiments that involve neonatal or perinatal colonization with beneficial probiotic bacteria such as *B. breve,* with known immunomodulatory effects, may be of interest. Recently, fecal transplantation has been suggested to treat *Clostridium difficile* infection and has been proposed as a strategy to displace the dysbiotic community found in IBD patients in hopes of treating inflammation (Allen-Vercoe and Petrof, 2013). One of the problems in the usage of a single probiotic is that it does not colonize permanently, at least if given as a supplement in adult life. Thus, the addition of known probiotic bacteria

in a more complex and stable consortium (as proposed in fecal transplants) may lead to prolonged colonization and stability of the transferred communities. This approach may help prevent IBD in populations at risk. The role of microbiota modulating strategies in celiac disease is in its infancy, however some studies in which our lab collaborated have proposed beneficial effects of probiotics in this disorder (Smecuol, 2013; unpublished results). Although provocative, supplementation with probiotics with specific immunomodulatory and barrier enhancing effects at an early developmental stage during introduction of gluten to the infant's diet, could one day be used as a strategy to prevent celiac disease in populations at risk.

6.5. Unpublished Results

The studies featured in **Chapters 4** and **5** of this thesis investigated the consequences of modulating the microbiota with a defined microbial ecosystem (ASF) or a specific probiotic, which targets the phenotype associated with the genetic risk. The underlying mechanism(s) by which colonization with a community of bacteria devoid of pathobionts and opportunistic bacteria reduces risk to colitis, remains unclear. More importantly, whether a defined, human-derived bacterial community can achieve similar effects is unknown. Microbiota-targeted therapies face major challenges; for instance, changes in the composition or function of the microbiota must be identified as a direct cause or contributor to disease. To address this, we have collected fecal sample from ulcerative colitis patient with active disease (patient information in Table 6.1). Using culture-based techniques, we observed that compared to fecal microbiota derived from a

healthy individual, UC derived microbiota displayed lower diversity and harbor highly dysbiotic communities (Figure 6.6). In particular, UC patient's microbiota displayed lower culturable bacterial strains that belong to the order *Clostridales*. To test the pathophysiological role of this dysbiotic community on host-microbiota interaction we colonized germ-free wildtype and *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice with these ecosystems. We used a balanced microbiota community as control, formulated by Dr. E. Vercoe from Guelph University, which is composed of 33 commensal species that were generally sensitive to a range of antimicrobials (Allen-Vercoe and Petrof, 2013; Petrof et al., 2013). This robust commensal community is able to withstand perturbations by antibiotics and has been shown to be effective in displacing and treating recurrent *Clostridium difficile* (Petrof et al., 2013). Interestingly, this community is able to colonize the human colon after transplantation, which is highly important as commercially available probiotics can only transiently colonize the intestine.

Table 6.1. Patient information of ulcerative colitis flare patient

Characteristics of Study Participants and Medical History	
Sex	Female
Race	First Nation
Marital Status	Married
Type of Inflammatory Bowel Disease	UC
Present state of health during stool collection (Active, Remission)	Active, 4 days prior to colectomy
Reason for visiting during stool collection	Collected during hospitalization
Age when first diagnosed	36 (now 48)
Have the patient undergone operation for the IBD? If yes, what kind?	Total colectomy 4 days later
Is the patient taking any medications during the time of stool collection? What kind?	Yes, IV steroids and 2 doses of Humira (1 and 2 weeks earlier)
Is the patient taking any steroids?	Yes, solumedrol
Phenotype location of disease?	Severe pancolitis
Diagnosis during time of visit?	UC
Other information	

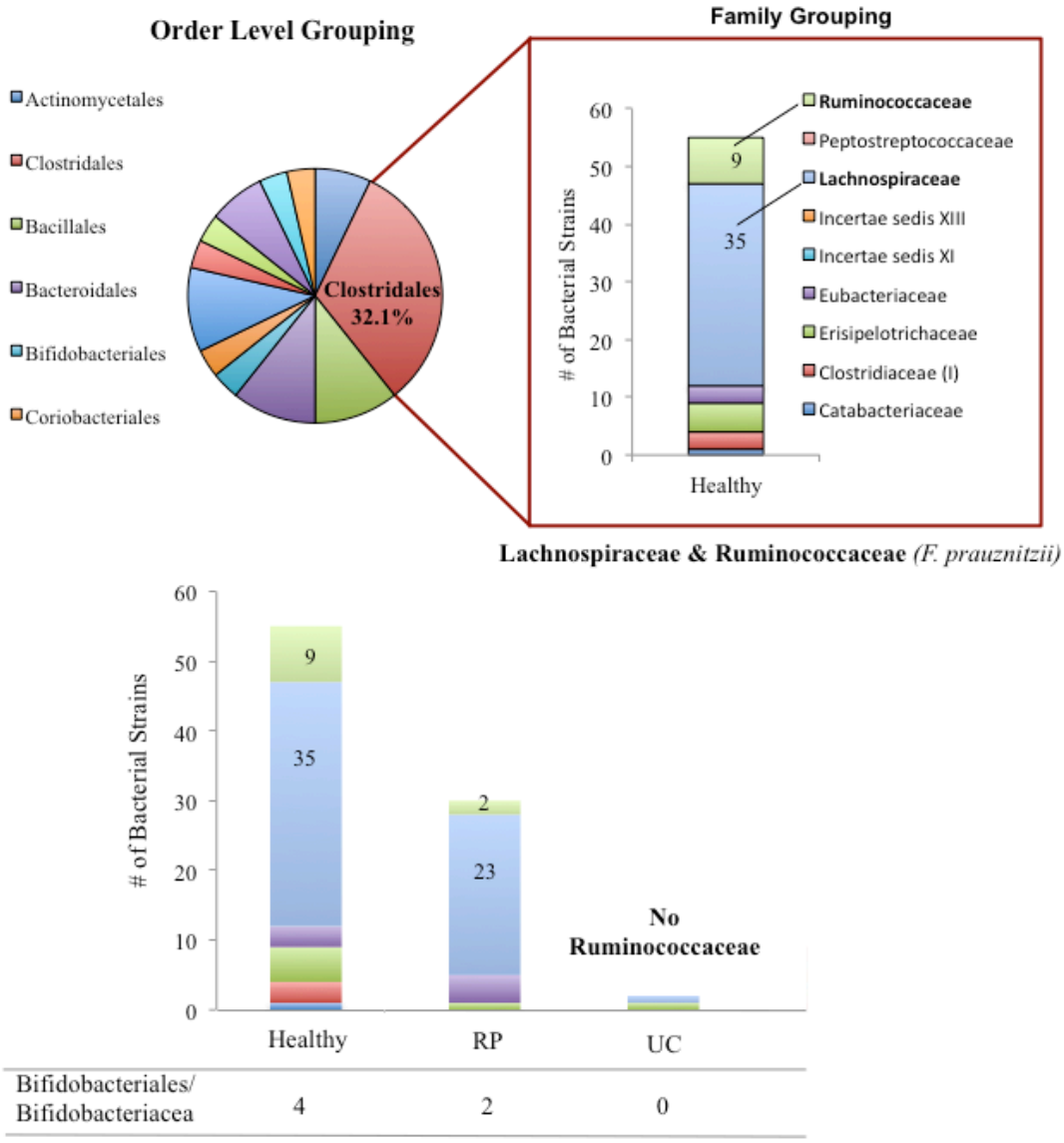

Figure 6.6. Bacterial ecosystems derived from the 2 patients with active ulcerative colitis (UC) is dysbiotic compared to ecosystems derived from a healthy subject and that of the stable experimental community termed RePOOPulate (RP).
In patients with active UC, there is lower diversity of bacterial families that include anti-inflammatory bacteria such as *Bifidobacteria* and *F. prauznitzii*.

Germ-free mice were colonized with freshly voided ulcerative flare stool samples (prepared anaerobically) and maintained in separate ventilated cages. Three-weeks post-colonization, immune responses, focusing on T helper responses, generated by these colonization strategies were evaluated. Regardless of the type of microbiota, intestinal colonization resulted in the activation and generation of colonic T_{REG}, both in wild type and mice lacking Nod-signaling (Figure 6.7). In addition to the induction of T_{REG}, there was an induction of T helper 17 (Th17) characterized by increased intracellular staining of IL17α (Figure 6.8). Compared to healthy-derived microbiota colonizations, mice that received ulcerative flare-microbiota showed increased Th17 immune response. This correlated with increased susceptibility of the mice colonized with ulcerative flare-microbiota to DSS colitis (Figure 6.9). This suggest that a dysbiotic community can trigger a pro-inflammatory immune response regardless of the genetic background of the mice, and this may influence the host response to experimentally induced colitis. Altogether, these preliminary data are exciting. Further studies will involve characterizing other immune changes that are different between mice colonized with healthy-derived microbiota and mice colonized with dysbiotic microbiota before and after colitis induction.

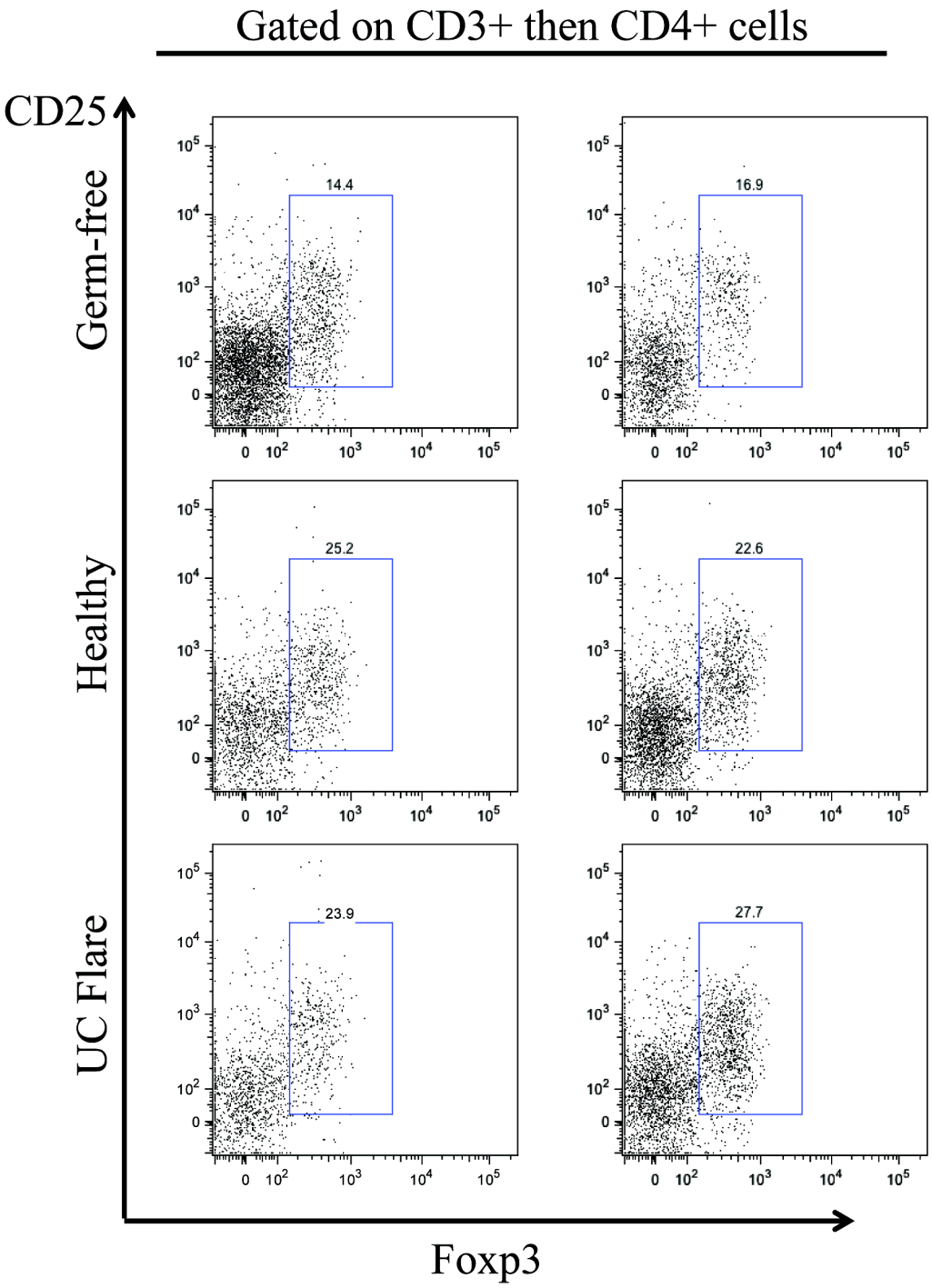

Figure 6.7. Colonization of germ-free mice with human derived microbiota induces a regulatory T-cell responses in the colon lamina propria.
Representative dot plots from pooled animals (n=3). Data shown are representative of 1-2 independent experiments

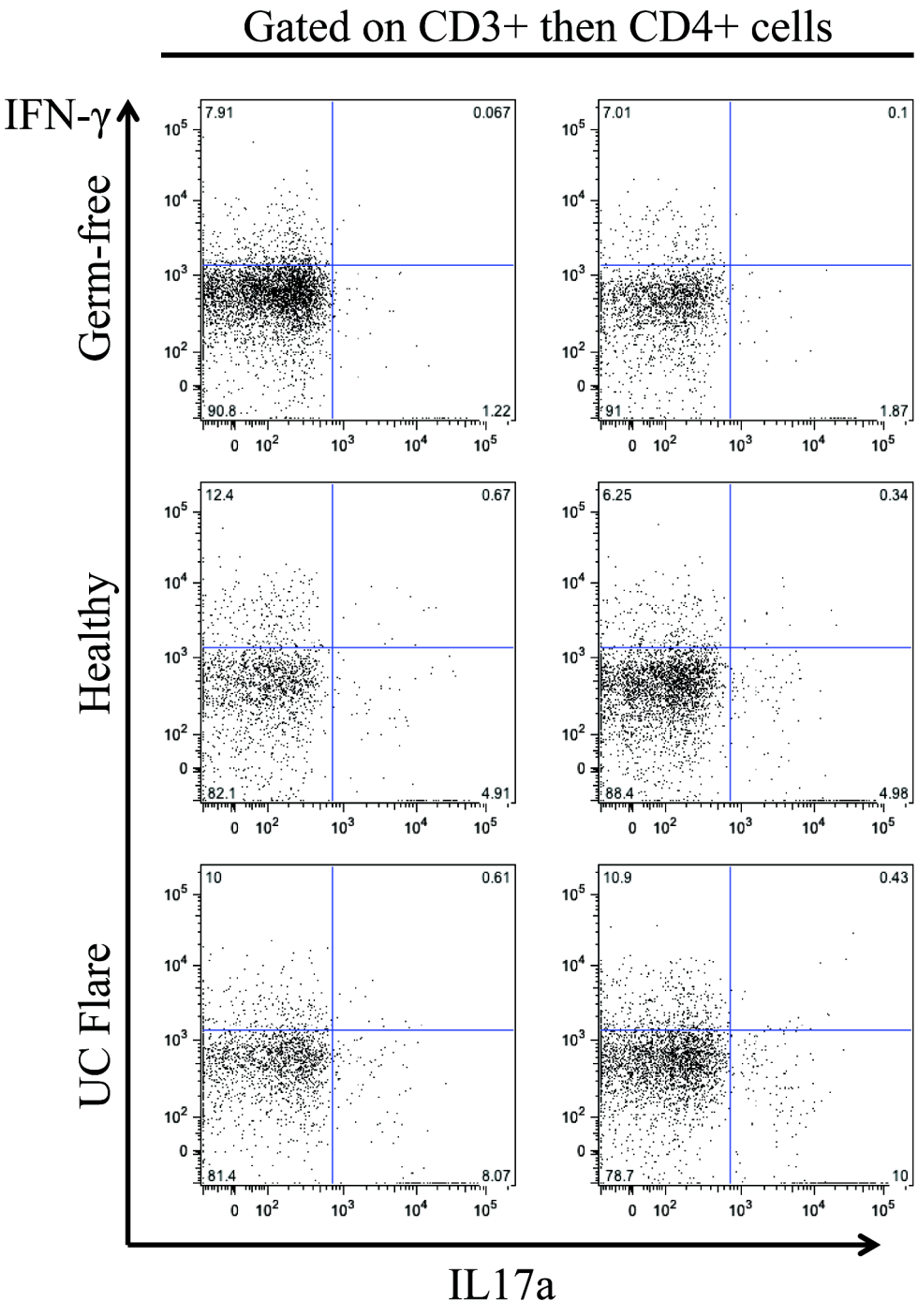

Figure 6.8. Colonization of germ-free mice with ulcerative colitis-patient microbiota but not healthy-derived microbiota induces a Th17 response in the colonic lamina propria.

Representative dot plots from pooled animals (n=3). Data shown are representative of 1-2 independent experiments

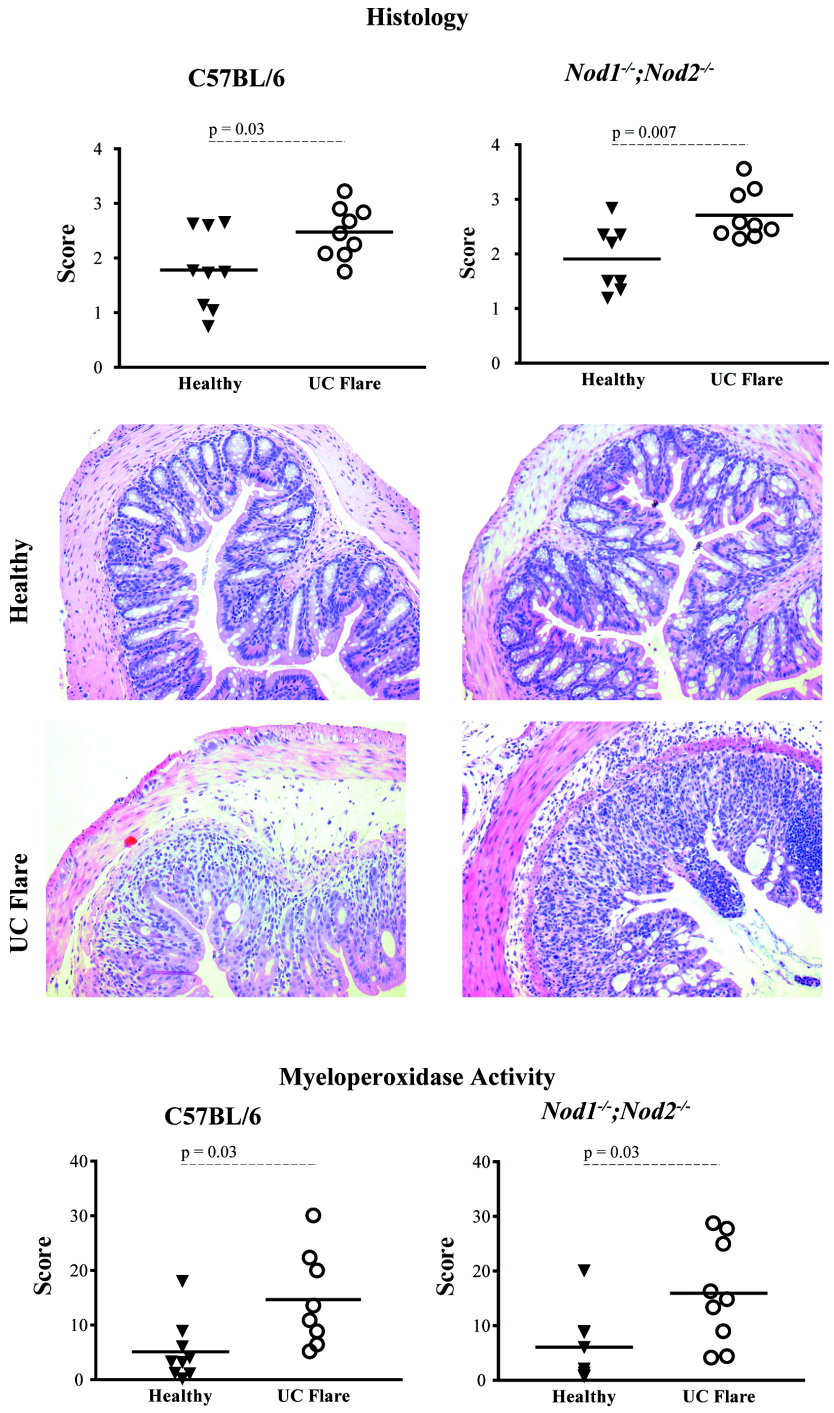

Figure 6.9. Germ-free mice colonized with ulcerative colitis-derived patient microbiota showed more susceptibility to DSS colitis compared to germ-free mice colonized with healthy-derived microbiota.

6.6. Conclusions

The work presented in this thesis reveals novel insights into diet-microbial-host interactions in the intestine. Further studies will aid in delineating their implication in the pathogenesis of inflammatory diseases such as celiac disease and IBD, with the aim to develop optimized or customized therapeutic or preventative approaches based on microbial modulation strategies.

– APPENDIX I –

MODULATION OF INTESTINAL BARRIER BY INTESTINAL MICROBIOTA: PATHOLOGICAL AND THERAPEUTIC IMPLICATIONS.

Modulation of Intestinal Barrier by Intestinal Microbiota: Pathological and therapeutic implications.

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Title: Modulation of Intestinal Barrier by Intestinal Microbiota: Pathological and therapeutic implications.

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Key Words: Intestinal barrier function, intestinal microbiota, cell renewal, apical junctional proteins, antimicrobials, mucin

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7.1. Abstract

Mammals and their intestinal microbiota peacefully coexist in a mutualistic relationship. Commensal bacteria play an active role in shaping and modulating physiological processes in the host, which include, but are not restricted to, the immune system and the intestinal barrier. Both play a crucial role in containing intestinal bacteria and other potentially noxious luminal antigens within the lumen and mucosal compartment. Although mutualism defines the relationship between the host and the intestinal microbiota, disruptions in this equilibrium may promote disease. Thus, alterations in gut microbiota (dysbiosis) have been linked to the recent increased expression of obesity, allergy, autoimmunity, functional and inflammatory disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). In this article, we review the evidence supporting a role of gut microbiota in regulating intestinal barrier function. We discuss the hypothesis that microbial factors can modulate the barrier in ways that can prevent or promote gastrointestinal disease. A better understanding of the role of the intestinal microbiota in maintaining a functional intestinal barrier may help develop targeted strategies to prevent and treat disease.

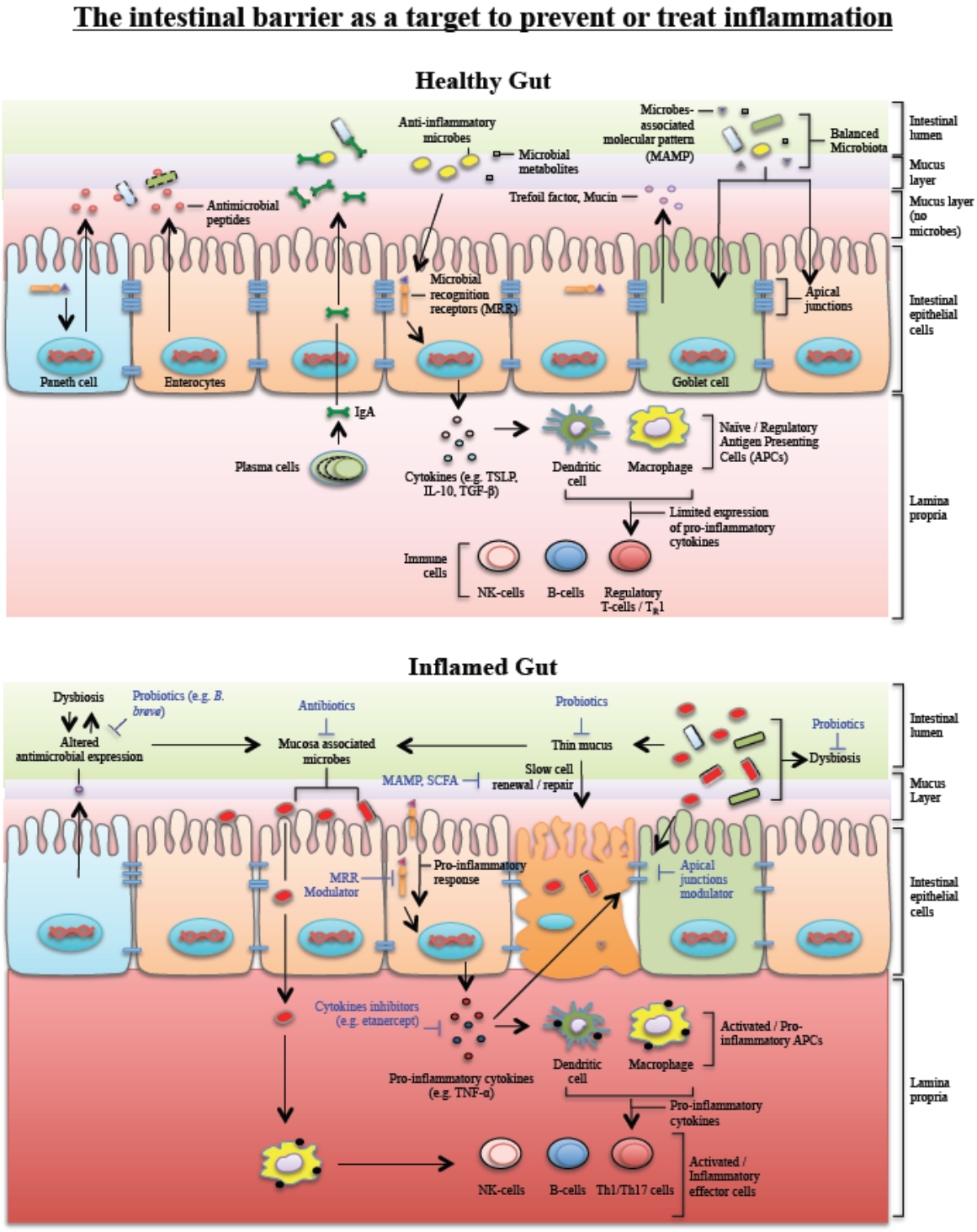

Figure 7.1. Graphical Abstract: The intestinal barrier as a target to prevent or treat inflammation.

7.2. Intestinal microbiota and intestinal barrier: Important determinants of host's homeostasis

There are more than 10^{14} resident bacteria in the gastrointestinal tract, that establish a life-long, bi-directional and symbiotic relationship with our cells (Hooper, 2009). This ecosystem, termed collectively the intestinal microbiota, plays a key role in the maturation of the immune system and in other physiological processes including neuro-motor and intestinal barrier function (Hooper et al., 2001; Smith et al., 2007). Conversely, alterations in gut colonization at the time of birth or disturbances of this complex ecosystem in adulthood may result in allergy and inflammation (Blumberg and Powrie, 2012).

The host has evolved with immune and physiological adaptations to maintain constant vigilance against potentially harmful luminal antigens while preventing the development of uncontrolled inflammation. As such, the intestinal barrier provides the first line of defense, by physically separating the internal milieu from the gut lumen and through active mechanisms such as microbial recognition, production of antimicrobial peptides, mucus, and secretion of antibodies (Figure 7.2).

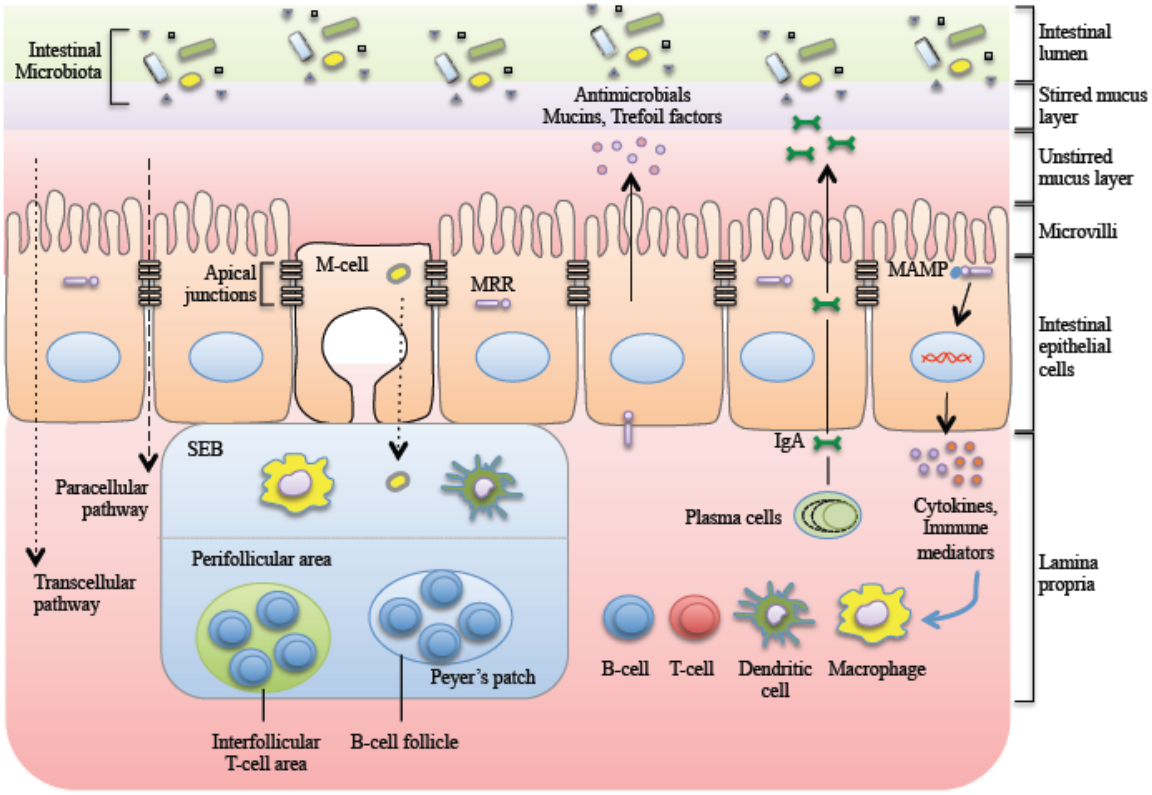

Figure 7.2. Intestinal barrier as modulator of intestinal homeostasis.
The intestinal barrier is equipped with several levels of defense mechanisms to limit luminal antigen translocation. This includes a single layer of semi-permeable epithelial cells, apical junctions (adherens and tight junctions proteins) that bind epithelial cells together and regulate paracellular antigen and molecule entry across the epithelium. Intestinal epithelial cells transport antigens and molecules from the lumen into the mucosa through the transcellular pathway. Specialized epithelial cells called M-cells mediate constant sampling of luminal antigens. Goblet cells, Paneth cells and enterocytes secrete mucins and antimicrobial peptides that assemble into a mucus layer. Mucins and antimicrobials are localized within the unstirred mucus layer. Intestinal epithelial cells secrete IgA, which are produced by plasma cells in the lamina propria. Intestinal epithelial cells are also equipped with a number of microbial recognition receptors (MRR) such as TLR and NOD-like receptors that can recognize specific microbial associated molecular patterns. (MAMP). Basal recognition of intestinal microorganisms by intestinal epithelial cells induces secretion of cytokines and other immune mediators, which may help in the induction a regulatory response against intestinal microbiota and maintenance of intestinal homeostasis.

Regulation of barrier integrity and function is mediated by endogenous and exogenous factors, such as cytokines, chemicals and drugs (Jacob et al., 2005; Madara and Stafford, 1989; Natividad et al., 2009; Taylor et al., 1998; Wirtz et al., 2007). Pathogens and their toxins also have the ability to directly or indirectly modulate the intestinal barrier (Berkes et al., 2003; Kalischuk et al., 2009; Sartor, 2006). Recently, commensal bacteria have been proposed as key modulators of intestinal barrier function (Hooper and Gordon, 2001; Smith et al., 2007).

A defect in intestinal barrier can lead to persistent immune activation. Indeed, intestinal barrier dysfunction has been suggested to play a pathogenic role in a number of intestinal diseases including celiac disease (CD), colorectal cancer, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Cerf-Bensussan and Gaboriau-Routhiau, 2010). Although the exact role of intestinal barrier dysfunction, particularly in the context of IBD pathogenesis, is still a matter of debate, animal models indicate that it could play a primary or adjuvant role. This does not necessarily imply that it is the sole factor causing disease, but that barrier dysfunction may exacerbate inflammation in the presence of other triggers and compromise mucosal healing after inflammation (Natividad et al., 2011).

7.2.1. Intestinal epithelial cell types, gut homeostasis and inflammation.

The intestinal barrier consists of a polarized and permeable monolayer of epithelial cells. There are four major intestinal epithelial cell types, which arise from the multi-potent stem cells within the crypt: (1) absorptive enterocytes which make up more than 80% of all epithelial cells; (2) mucous and trefoil factor producing goblet cells; (3)

hormone producing enteroendocrine cells; and (4) antimicrobial and growth factor producing Paneth cells.

With the exception of Paneth cells, all intestinal epithelial cell types are located in the small and large intestine and undergo continual renewal every 3-5 days (van der Flier and Clevers, 2009). Paneth cells, on the other hand, are primarily located at the base of small intestinal crypt and are renewed approximately every 18 to 23 days (Cheng and Leblond, 1974). The M (microfold) cells, characterized by the lack of microvilli, overlay the Peyer's patches found in small intestine as well as isolated lymphoid follicles (ILF) in the small and large intestine. Under homeostatic conditions, a wide range of microorganisms and macromolecules can gain entry through the M-cells ensuring constant sampling by immune cells (Kucharzik et al., 2000).

The development of knockout mice as well as the ability to *in-vivo* ablate the development of specific intestinal cell lineages have highlighted the role of intestinal cell types in maintenance of health. Murine atonal homologue 1 (Atoh1 or Math1) is an important transcription factor involved in differentiation of secretory intestinal epithelial cells. Math1 null mice lack goblet, enteroendocrine and Paneth cells in the intestine (Yang et al., 2001). Interestingly, homozygous Math1 knockout mice (*Math1*$^{-/-}$), but not heterozygous mice, die shortly after birth (Yang et al., 2001). Math1 is also essential for the development of certain neuronal cell types (Bermingham et al., 2001). Thus, decreased survival of *Math1*$^{-/-}$ mice cannot be solely attributed to the loss of secretory epithelial cells in the intestine. Instead, it has been suggested that respiratory failure due to loss of brainstem neurons underlie the premature death of *Math1*$^{-/-}$ mice (Bermingham

et al., 2001). The importance of secretory intestinal epithelial cells, however, has been underscored in mice that lack the Math1 gene in intestinal epithelial cells (Bermingham et al., 2001). These intestinal epithelial specific *Math1*$^{-/-}$ mice failed to survive past 14 days of age. It is unknown whether the intestinal microbiota influences the survival of mice lacking Math1. However, it is possible that the lack of secretory epithelial cells, such as Paneth cells, impairs antimicrobial defense leading to increased morbidity. It is unknown whether *Math1*$^{-/-}$ mice can thrive normally when maintained under germ-free conditions. An essential role of Paneth cells in limiting mucosal penetration of intestinal bacteria have been underlined by studies showing that upon oral challenge with pathogenic bacteria, Paneth cell-deficient transgenic mice (CR2-*tox176*) displayed increased numbers of live bacteria in mesenteric lymph nodes (MLN) compared to wild-type mice, suggesting (Garabedian et al., 1997; Vaishnava et al., 2008). The mechanism by which Paneth cells limit bacterial penetration may involve cell activation through pattern recognition receptors and secretion of antimicrobials (Vaishnava et al., 2008).

On the other hand, the importance of goblet cells in maintenance of health have been emphasized by experimental observation that mice that lack Kruppel-like factor 4 (Klf4) gene, a transcription factor necessary for terminal differentiation of goblet cells, die shortly after birth (Katz et al., 2005). *Klf4*$^{-/-}$ neonatal mice displayed goblet cell hypoplasia and abnormal expression of Muc2. These mice also displayed other abnormalities, which likely contributed to increased morbidity and mortality (Katz et al., 2005). In agreement with this, mice with specific intestine-epithelial deletion of Klf4 gene were viable but still displayed significant lower numbers of goblet cells and altered

intestinal epithelial homeostasis characterized by dysregulated cell proliferation, migration, differentiation and positioning of intestinal epithelial cells (Ghaleb et al., 2011). It remains to be determined whether the reduced numbers of goblet cells in *Klf4*$^{-/-}$ mice renders them susceptible to intestinal inflammation. In contrast, transgenic mice in which 60% of goblet cells were ablated by the expression of an attenuated diphtheria toxin gene driven by the ITF promoter were protected against experimental colitis, and this paradoxical result has been attributed to the increased production of trefoil factor peptides by remaining goblet cells (Itoh et al., 1999).

Thus, animal studies demonstrate key roles of intestinal epithelial cell types in maintenance of barrier properties and health. However, redundancy exists and there are compensatory mechanisms that develop to maintain homeostasis in the absence of a specific intestinal epithelial cell lineage.

7.2.2. Intestinal barrier architecture, gut homeostasis and inflammation.

The integrity and structure of the epithelial cell is largely modulated by microtubules and actin. Microtubules play a central role in maintaining cellular integrity, directing intracellular transport and secretion, and coordinating organelle movements. On the other hand, actin regulates epithelial permeability through interactions with apical junctional proteins, which are composed of tight junctions, intermediate or adherens junctions, desmosomes or macula adherens, and gap junctions (Figure 7.3) (Mooseker, 1985; Turner, 2009). Apical junctional proteins play an important role in the maintenance of epithelial polarity. Both adherens junctions and desmosomes are responsible in binding

epithelial cells together. Adherens junctions also have a crucial role in cell renewal and are necessary for proper assembly of the tight junctions (Sancho et al., 2004; Turner, 2009).

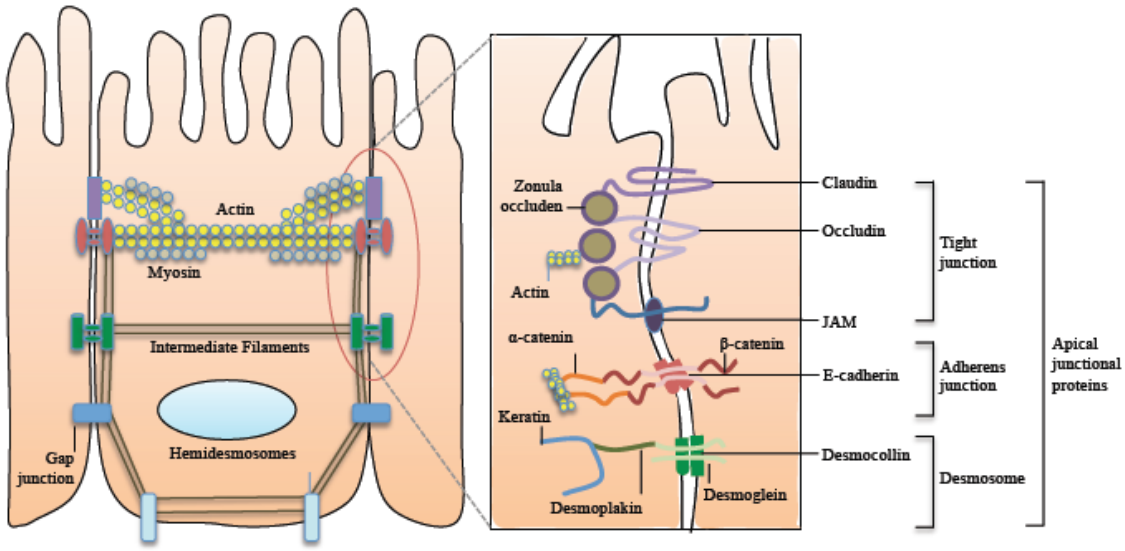

Figure 7.3. Intestinal epithelial barrier structure.
(A) The integrity and structure of epithelial cell is largely modulated by the cytoskeleton. Within the cell, two types of cytoskeletal proteins are present: (1) α-integrin, which polymerizes to form microtubules; (2) actin, that polymerizes to form filamentous actin. Actin filaments form a dense cross-linked actin cortex at the apical region of the epithelial cells, and play a role in regulating epithelial permeability through interactions with apical junctional proteins. (B) Apical junctional complex composed of tight junctions, intermediate or adherens junctions, desmosomes or macula adherens, and gap junctions bound epithelial cells together and maintain epithelial polarity. Adherens junction is composed of cadherins, such as E-cadherin (also called cadherin-1), and is bound to n eand ancatenins. Moreover, adherens junctions are necessary for proper assembly of the tight junctions, which are junctional proteins that are most apically located. The tight junctions are composed of transmembrane proteins occludins, claudins, and junctional adhesion molecule (JAM) that are linked to the actin cytoskeleton through zonula occludens (ZO) proteins.

Paracellular transport across the barrier is controlled by apical junction proteins, which dynamically respond to different stimuli including pathogens, commensal bacteria

and bacterial products. Epithelial cells allow a small amount of luminal antigens to pass transcellularly across the epithelium either through receptor-mediated endocytosis or non-selectively via fluid-phase endocytosis. This process is called translocation. A small degree of bacterial mucosal translocation is normal and contributes to the physiological sampling of luminal content by the host's immune system (Slack et al., 2009). However, when the host cannot effectively contain gut bacteria to the mucosal compartment, increased immune activation and inflammation may occur (Natividad et al., 2009).

Animal models lacking specific components of apical junctional proteins highlight the importance of these molecules in the maintenance of intestinal homeostasis. Expression of dominant negative N-cadherin in the mouse intestinal epithelium led to spontaneous development of transmural enteritis, similar to Crohn's disease (Hermiston and Gordon, 1995). Also, mice lacking intracellular microbial sensors, Nod1 and Nod2, displayed decreased colonic E-cadherin expression, paralleled by increased paracellular permeability and decreased antimicrobial protein production (Natividad et al., 2011). The barrier defect in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice was insufficient to cause spontaneous inflammation, however it increased susceptibility to subsequent acute experimental colitis (Natividad et al., 2011). Similarly, mice that lacked JAM, one of the components of tight junction, did not spontaneously develop intestinal inflammation, but expressed increased sensitivity to colitis (Laukoetter et al., 2007; Vetrano et al., 2008). On the other hand, SAMP1/YitFc mice, which exhibited increased permeability in ileum and aberrant expression of the tight junction proteins Claudin-2 and occludin, spontaneously developed ileitis similar to Crohn's disease (Kosiewicz et al., 2001; Olson et al., 2006; Vidrich et al., 2005). When

raised under germ-free conditions, SAMP1/YitFc mice still displayed altered ileal permeability suggesting that the barrier phenotype is independent of the presence of gut bacteria (Bamias et al., 2007; Reuter and Pizarro, 2009). Taken together, these studies suggest that a *de-novo* defect in barrier integrity may be insufficient to disrupt intestinal homeostasis in a way that leads to disease but it may increase the risk or severity of inflammation, particularly in the presence of other triggers.

Compromised barrier integrity has also been observed during active inflammation (Berkes et al., 2003; Spiller et al., 2000; Wirtz et al., 2007). The exact contribution of barrier dysfunction at this stage of disease remains uncertain. Inflammatory markers such as cytokines can disrupt *per se* barrier integrity and function (Madara and Stafford, 1989; Turner, 2009). A defect in barrier function during active inflammation may be a consequence of disease, and although an inflammation-amplifying role cannot be ruled out, a primary role in the development of inflammation is difficult to ascertain.

7.2.3. Antimicrobial peptides, gut homeostasis and inflammation.

Epithelial cells secrete a wide variety of molecules, which are natural antibiotics with broad antimicrobial activity. Paneth cells, primarily located at the base of small intestinal crypts, are the main source of antimicrobials in the intestine. About 70% of Paneth cells' granules contain α-defensins and the rest is a mixture of lysozymes, secretory phospholipase A_2, C-type lectins such as RegIII-γ and RegIII-β, calprotectins and ribonucleases such as angiogenin-4 (Ang-4) (Mukherjee et al., 2009). Enterocytes also have the ability to secrete antimicrobials including RegIII-γ, cathelicidins, small

amounts of α-defensins, and β-defensins (Lehrer, 2004; Natividad et al., 2011). Antimicrobials are present in both the small and large intestine, and can either be constitutively expressed or inducible (Table 7.1).

Studies in animals have revealed that epithelial-derived antimicrobial peptides are important in the prevention and clearance of intestinal pathogens. This is evident in mice lacking cryptidins, which is a murine counterpart of human α-defensins, and RegIII-γ proteins (Brandl et al., 2007; Salzman et al., 2003; Wilson et al., 1999; Zheng et al., 2008). Similarly, the contribution of α-defensins to host defense against enteric pathogens has been demonstrated in a study showing that transgenic mice expressing human α-defensin *HD5* are fully resistant to oral lethal infection with *Salmonella typhimurium.* Impaired α-defensin expression may lead to a reduction of antibacterial activity, making susceptible hosts more vulnerable to intestinal bacterial invasion and attachment. Indeed, antimicrobial peptides are strategically located at the epithelial surface along the mucus layer (Vaishnava et al., 2011). Their location hints to their role in keeping intestinal bacteria at bay, and in preventing epithelial cell stress and immune activation. It has been shown that a lack of RegIII-γ was associated with an increased number of bacteria in close contact with the epithelium (Vaishnava et al., 2011). Increased microbial-epithelial contact in RegIII-$\gamma^{-/-}$ mice was paralleled with heightened adaptive immune response, implying that a loss of bacterial segregation induces unnecessary immune activation (Vaishnava et al., 2011). It is possible that the increased immune activation in RegIII-$\gamma^{-/-}$ mice reflects a compensatory mechanism mounted by the host to compensate for the lack of RegIII-γ expression. It remains to be determined whether this adaptation could

contribute to disease if overwhelmed, particularly in the presence of intestinal injury or inflammatory triggers.

Table 7.1. Expression pattern of epithelial-derived antimicrobials.

Family	Members	Localization	Transcriptionally Induced
α-defensins	HD-5 (Human)	Paneth cells; small intestinal and colonic enterocytes	No
α-defensins	HD-6 (Human)	Paneth cells; small intestinal and colonic enterocytes	No
α-defensins	Cryptidins (Mouse)	Paneth cells	No
α-defensins	Cryptidins related peptides (Mouse)	Paneth cells	No
β-defensin	HBD-1 (Human)	Colonic enterocytes	No
β-defensin	HBD-2 (Human) mBD-3 (Mouse)	Colonic enterocytes	Yes
β-defensin	HBD-3 (Human)	Colonic enterocytes	Yes
β-1,4-glycosidase	Lysozyme C	Paneth cells	No
Phospholipid sn-2 enterase	$sPAL_2$	Paneth cells	No
C-type Lectin	RegIII- α or HIP/PAP (Human) RegIII-γ (Mouse)	Paneth cells; small intestinal and colonic enterocytes	Yes
C-type Lectin	RegIII-β (Mouse)	Paneth cells; small intestinal and colonic enterocytes	Yes
Ribonuclease	ANG-4	Paneth cells	Yes
Cahelicidins	LL37 (Human)	Paneth cells; small intestinal and colonic enterocytes	No

Using transgenic mice that express HD5, it has been suggested that α-defensin may be involved in preventing mucosa-associated bacteria such as segmented filamentous bacteria (SFB) to colonize in close proximity with the epithelium, leading to Th17 activation. *HD5* transgenic mice, however, still express mouse cryptidins. As a result, the observations found in the *HD5* transgenic mice may not necessarily reproduce

what is happening under physiological conditions (Menendez et al., 2010; Salzman et al., 2010). Overall, antimicrobial peptides likely contribute to intestinal homeostasis by acting as natural host antibiotics that protect the vulnerable mucosal surface from microbial invasion and penetration and inappropriate immune activation.

In addition to its role as natural host antibiotics (Hooper, 2004), antimicrobial peptides have immuno-modulatory properties (Bowdish et al., 2005; Steinstraesser et al., 2011). For instance, cathelicidin has been shown to modulate macrophages *in-vitro* into an anti-inflammatory phenotype (Brown et al., 2011). Antimicrobial peptides may also be essential in regulating intestinal microbiota composition (Salzman et al., 2007). In particular, secretion of Ang-4, which preferentially targets gram-positive bacteria while sparing gram-negative bacteria, has been suggested to shape the composition of the intestinal microbiota at the time of weaning when the proportion of gram-positive bacteria decrease and gram-negative anaerobic organisms become established (Hooper, 2004). Likewise, HD5 may also influence the composition of gut microbiota (Menendez et al., 2010; Salzman et al., 2010). Altogether, these studies highlight the role of epithelial derived antimicrobials as active innate immune mediators and regulators of intestinal microbiota composition.

In both animal models and clinical studies, antimicrobial peptides seem to be up-regulated during active inflammation or at the site of inflammation (Ogawa et al., 2003; Rahman et al., 2011; Wehkamp et al., 2008; Wehkamp and Stange, 2010). This may reflect a defense mechanism against invading bacteria during mucosal breaks or permeability increases. On the other hand, increased antimicrobial peptide production

during active inflammation could be potentially detrimental and affect beneficial bacteria with anti-inflammatory effects (Sokol et al., 2008). Thus far, the brunt of evidence supports the concept that a balanced expression of antimicrobial peptides is crucial to maintain intestinal homeostasis and prevent inflammation.

7.2.4. Mucus layer, gut homeostasis and inflammation.

Two mucus layers can be found within the intestine (Figure 1) (McGuckin et al., 2009). The unstirred (inner) mucus layer, is approximately 100 μm thick, is firmly adherent, rich in epithelial-derived antimicrobials and mucin, and has low bacterial density. The stirred (outer) mucus layer is composed of mucin and diluted antimicrobials, and some bacteria are able to penetrate this layer. Mucin constitutes a viscoelastic gel that lines the intestinal epithelial barrier. The thickness and composition of the mucus layer varies depending on the location within the intestinal tract (Swidsinski et al., 2007). The highest mucus viscosity is found in the distal colon (Swidsinski et al., 2007). Interestingly, microbial density increases along the proximal to distal parts of the intestinal tract, reaching up to 10^{12} bacteria per ml of luminal content in the colon (Dethlefsen et al., 2007; Garrett et al., 2010). It is unclear whether the increased viscosity at the distal colon reflects a defense mechanism of epithelial cells to protect against potentially invading bacteria, or whether it is the direct result of higher microbial load.

Goblet cells are the primary source of mucins in the intestine. There are 20 different mucins that have been identified, and nine of those are expressed by both murine and human intestine and are either secreted or membrane bound (Table 7.2). Mucins are

divided into neutral and acidic subtypes. Acidic subtypes are further classified as sulfated (sulfomucins) or nonsulfated (sialomucins) groups. MUC(in) 2 is the predominant mucin found in the large intestine. Interestingly, mice knockout for the MUC2 gene spontaneously develop colitis (Van der Sluis et al., 2006). On the other hand, mice null for MUC13 genes do not spontaneously develop intestinal inflammation but have shown to be more susceptible to experimental colitis (Sheng et al., 2011). In contrast, IL-$10^{-/-}$ mice crossed to human MUC1-transgenic mice develop more severe spontaneous colitis with a higher incidence of colon cancer development as compared to IL-$10^{-/-}$ mice (Beatty et al., 2007). These studies highlight that subsets of mucins have distinct functions in the maintenance of intestinal homeostasis and in their role in disease progression.

Table 7.2. Regional expression of intestinal mucins.

MUC Type	Membrane bound / Secreted	Localization
MUC1 (Human, Mouse)	Membrane bound	Large intestine
MUC2 (Human, Mouse)	Secreted	Small and large intestine
MUC3A (Human, Mouse)	Membrane bound / Secreted	Small and large intestine
MUC3B (Human, Mouse)	Membrane bound / Secreted	Small and large intestine
MUC4 (Human, Mouse)	Membrane bound	Small intestine
MUC11 (Human, Mouse)	Membrane bound	Small and large intestine
MUC12 (Human, Mouse)	Membrane bound	Large intestine
MUC13 (Human, Mouse)	Membrane bound / Secreted	Small and large intestine
MUC15 (Human, Mouse)	Membrane bound	Small and large intestine
MUC17 (Human, Mouse)	Membrane bound	Large intestine
MU19 (Human, Mouse)	Secreted	Large intestine
MUC 21 (Human, Mouse)	Membrane bound	Large intestine

The mucus layer is also composed of diverse proteins such as glycoproteins and peptides of the trefoil factor family (TFF). These peptides are secreted by goblet cells and enterocytes and have been shown to protect the epithelium from a variety of insults

(Taupin and Podolsky, 2003). Mice lacking TFF3, one type of TFF peptide, showed increased susceptibility to experimental colitis. The underlying mechanism could be related to the impaired ability of *TFF3*$^{-/-}$ mice to heal the mucosa and promote regeneration. Therefore, TFF peptides are important initiators of mucosal healing contributing to intestinal homeostasis.

The role of the mucus layer in influencing intestinal microbiota composition has not yet been fully defined, but it is possible that differential expression of mucin both in composition and location across the gastrointestinal tract has a role in intestinal colonization as well as in shaping the composition of intestinal microbiota.

7.2.5. Microbial recognition, gut homeostasis and inflammation.

Intestinal epithelial cells express a wide variety of pattern recognition receptors such as Toll-like receptors (TLR) and NOD-like receptors. Expression of these receptors, however, is tightly controlled to prevent inappropriate immune activation while still allowing constant vigilance. For example, TLR5, which recognize flagellin, has been found to be exclusively expressed at the basolateral side of epithelial cells while some TLRs such as TLR9 and TLR7 as well as NOD-like receptors are intracellular, ensuring that these receptors can only be activated by invading antigens or microorganisms (Artis, 2008). The importance of innate immune activation at the level of intestinal epithelium is highlighted by the development of spontaneous inflammation in specific pathogen free (SPF) mice with intestinal epithelial cell deletion of the components of the IKK complex, which is a downstream signaling pathway of both PRR and upstream kinase required for

NF-κβ activation (Nenci et al., 2007). Basal recognition of commensal bacteria by intestinal epithelial cells influences the secretion of the immuno-regulatory cytokines such as TSLP and TGF-β, which have the ability to dampen pro-inflammatory responses (Artis, 2008; Dignass and Podolsky, 1993; Taylor et al., 2009). Moreover, the anaerobic commensal bacterium *Bacteroides thetaiotaomicron* has been shown to attenuate intestinal inflammation. The anti-inflammatory capability of *B. thetaiotaomicron* may be attributed to its ability to activate PPAR-γ dependent pathway in intestinal epithelial cells (Kelly et al., 2004). This data suggest that activation of PPAR-γ pathway in intestinal epithelial cells, perhaps using PPAR-γ agonist, may be beneficial in ameliorating intestinal inflammation.

7.3. Regulation of the Intestinal Barrier by Gut Microbiota

Compositional changes in intestinal microbiota (dysbiosis) have been found in patients with celiac disease, IBS and IBD (Cerf-Bensussan and Gaboriau-Routhiau, 2010). It has been proposed that dysbiosis may play a role in the pathogenesis of these diseases. So far the evidence of a role of dysbiosis in gastrointestinal disease is limited to association. It is possible that dysbiosis contributes to intestinal inflammation by disturbing the signals that maintain barrier function. A better understanding of how the intestinal microbiota modulate these processes will help develop therapeutic targets to prevent or treat gastrointestinal disease.

7.3.1. Microbial effects on intestinal epithelial cell renewal.

A balance between cell proliferation and cell death is required for normal intestinal barrier function. A number of studies comparing germ-free and conventionally raised animals have shown that lack of intestinal microbiota greatly impairs intestinal morphology, architecture and cell renewal properties (Hooper et al., 2001; Smith et al., 2007). A striking difference between mice reared under germ-free and conventional conditions is that the total intestinal surface area is significantly decreased in the absence of gut bacteria (Gordon and Bruckner-Kardoss, 1961; Meslin et al., 1999). This is paralleled by aberrant intestinal morphology with shorter ileal villi and smaller intestinal crypts in germ-free mice (Abrams et al., 1963). Moreover, the rate of turnover of ileal and Peyer's patches in germ-free mice was found to be lower than in mice raised in conventionalized conditions (Abrams et al., 1963). Eight days after colonization, cell renewal reverted to the same degree as that of conventional mice (Khoury et al., 1969). Decreased cell renewal may have detrimental consequences on epithelial proliferation and regenerative ability. Indeed, germ-free mice have been shown to be more susceptible to dextran sulfate sodium (DSS)-colitis (Maslowski et al., 2009), underscoring that intestinal bacteria confer protection against intestinal injury by regulating epithelial cell turnover and restitution.

The exact molecular and cellular signaling pathway by which intestinal microbiota regulate cell renewal is still unclear. However, indirect evidence suggests that microbial associated molecular patterns (MAMP) induce relevant signals for barrier fortification. Mice lacking bacterial sensing such as Toll-like receptors and Nod-like receptors are

more susceptible to colitis compared to wild-type controls (Chen et al., 2008; Natividad et al., 2011; Rakoff-Nahoum et al., 2004). Increased susceptibility to colitis has been associated with altered cell renewal and restitution (Chen et al., 2008; Rakoff-Nahoum et al., 2004). Depletion of gut microbiota using broad-spectrum antibiotics in both wild-type and mice deficient in either Nod2 or MyD88, an adaptor protein for TLR receptors, did not ameliorate DSS induced intestinal injury but rather promoted more severe morbidity (Chen et al., 2008; Rakoff-Nahoum et al., 2004). Oral ingestion of bacterial products such as LPS or muramyl dipeptide (MDP), a ligand for Nod2 receptors, prior or during DSS challenge, protected wild-type mice that received oral antibiotics and/or mice lacking innate bacterial sensors (Rakoff-Nahoum et al., 2004; Watanabe et al., 2008). One limitation in most of these studies is the fact that microbial recognition is also defective in other cells, particularly hematopoietic cells. Thus, the specific contribution of microbial recognition in colitis at the level of intestinal epithelium is still unclear. Nevertheless, these studies suggest that microbial signals are important for protection against intestinal injury, likely through cell renewal and promotion of wound healing. IL-$10^{-/-}$ mice spontaneously develop intestinal inflammation in SPF conditions (Chen et al., 2010; Kuhn et al., 1993; Madsen et al., 1999; Sellon et al., 1998). Colitis onset however, is delayed if IL-$10^{-/-}$ mice are re-derived germ-free, highlighting that intestinal bacteria are necessary for the induction of intestinal inflammation in this model (Madsen et al., 1999; Sellon et al., 1998).

A recent study has indicated that the probiotic bacterium *Lactobacilli rhamnosus* GG promotes cell renewal and augments mucosal repair following DSS induced colitis

via reactive oxygen species generation in epithelial cells (Swanson et al., 2011). It is however unknown whether commensal bacteria directly or indirectly signal to the epithelial cells to promote cell renewal and wound healing. Certain components of the microbiota generate a variety of short chain fatty acids (SCFA) such as butyrate, succinate and propionate, which have a crucial role in regulating colonic epithelial cell growth and differentiation and are important energy sources for colonocytes (O'Keefe, 2008). Overall, maintenance of intestinal barrier homeostasis requires complex interactions between the microbiota, host genes, and other environmental factors such as diet.

7.3.2. Microbial effects on intestinal permeability.

The ability of the intestinal microbiota to affect intestinal permeability has been mostly supported by the use of probiotics, exogenously administered bacteria that are thought to have beneficial effects on the host. Acute or psychological stress induces barrier dysfunction characterized by increased macromolecular flux and changes in ion secretion (Gareau et al., 2007). Oral administration of probiotics containing *Lactobacillus spp*. prevented stress-induced intestinal barrier changes (Zareie et al., 2006). Some studies have reported increased permeability in DSS colitis accompanied by changes in apical junction expression and re-organization, before the appearance of notable inflammation, suggesting that intestinal barrier dysfunction may precede the onset of colitis (Kitajima et al., 1999; Poritz et al., 2007). Administration of either *Escherichia coli* Nissle 1917 or *Lactobacillus rhamnosus* during DSS colitis prevented colitis-associated intestinal

barrier dysfunction in mice (Mennigen et al., 2009; Ukena et al., 2007). Treatment with VSL#3, which is composed of eight probiotic bacteria, protected the epithelial barrier in mice after DSS colitis induction (Yu et al., 2012). These effects have not been described with all probiotic bacteria, and we have shown that administration of a defined probiotic, *Bifidobacterium breve* NCC2950, did not modulate the altered permeability observed in naive *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice (Natividad et al., 2011). Interestingly, *B. breve* was able to prevent increased severity of DSS colitis, but the mechanism of protection was unrelated to a correction of the baseline permeability defect observed in these mice (Natividad et al., 2011). Most of the studies show that the effect of probiotics on intestinal permeability cannot be observed in control animals (Natividad et al., 2011; Yu et al., 2012), suggesting that probiotics may be more effective for the prevention of barrier changes and inflammation.

It has been suggested that intestinal barrier dysfunction may precede the onset of spontaneous intestinal inflammation in mice deficient for IL-10 gene (Chen et al., 2010; Madsen et al., 1999). The primary defect in *IL-10*$^{-/-}$ mice was dependent on the presence of intestinal microbiota as germ-free *IL-10*$^{-/-}$ mice did not display increased intestinal permeability (Madsen et al., 1999). *Lactobacillus* have been shown to be reduced in *IL-10*$^{-/-}$ mice (Madsen et al., 1999). Specifically, administration of *Lactobacillus reuteri* during neonatal period attenuated the development of colonic histological injury in *IL-10*$^{-/-}$ mice (Madsen et al., 1999). It is, however, unknown whether reduced inflammation was accompanied by improvement in intestinal permeability. Administration of *Lactobacillus plantarum* was shown to be effective in ameliorating the increased colonic permeability

and changes in tight junctional expression during active disease in *IL-10*$^{-/-}$ mice (Chen et al., 2010). Moreover, administration of *L. plantarum* to germ-free mice before colonization with SPF and continued probiotic therapy thereafter decreased histological injury in *IL-10*$^{-/-}$ mice. Similarly, *Mdr1a*$^{-/-}$ mice, another animal model of spontaneous colitis, displayed intestinal barrier defect before the onset of histological injury (Resta-Lenert et al., 2005). Administration of *Streptococcus thermophilus* and *Lactobacillus acidophilus* prevented increased permeability, which was accompanied by general improvement in the health of mice (Resta-Lenert and Barrett, 2009). Overall, these studies show that specific bacteria can regulate of intestinal barrier function, and this can positively influence the development of intestinal inflammation. More work is needed on specific mechanisms of action by which these specific probiotic strains modulate the intestinal barrier.

Components of the resident intestinal microbiota may also influence intestinal barrier function. This is evident in a recent study showing that *MyD88*$^{-/-}$*;Ticam*$^{-/-}$ mice, which lack Toll-like receptors signaling, raised in gnotobiotic environment and colonized with defined microbiota do not display altered barrier function (Slack et al., 2009). This observation contrasts with other studies using *MyD88*$^{-/-}$ mice reared and maintained under SPF condition (Gibson et al., 2008; Rakoff-Nahoum et al., 2004; Slack et al., 2009). It is possible that mice colonized with a microbiota of limited diversity, lack organisms capable of adversely modulating intestinal permeability.

The maintenance of epithelial barrier has also been suggested to be TLR2 mediated and associated with restoration and increased expression of ZO-1 (Cario, 2005;

Cario et al., 2004). Mono-colonization of germ-free mice with *Bacteroides thetaiotaomicron* modulated expression of genes necessary for intestinal barrier fortification (Hooper et al., 2001). These changes include up-regulation of *s*mall proline-rich protein-2, which acts as cross-bridging proteins linked to desmoplakin, a component of the apical junctional protein desmosomes (Figure 2) (Hooper et al., 2001). It is unclear whether and how the expression of microbial induced barrier-related genes translates into protein expression to modulate intestinal homeostasis. Similarly, mono-colonization of germ-free with *Escherichia coli* Nissle 1917 resulted in up-regulation of ZO-1 but not ZO-2 in intestinal epithelial cells (Ukena et al., 2007). The increased expression of ZO-1 was not observed in germ-free mice mono-colonized with other strains of *E. coli*. Specific probiotics and other commensal bacteria are able to secrete unique molecules such as SCFA, which can have beneficial effects on the intestinal barrier (Peng et al., 2009; Segawa et al., 2011; Suzuki et al., 2008). Lysates of the probiotic *Lactobacillus casei* DN-114, but not *Lactobacilli plantarum,* ameliorated DSS colitis by preventing the increase in permeability and preserving ZO-1 expression in mice. These changes, however, were accompanied by an increase in butyrate producing bacteria (Zakostelska et al., 2011). Thus, it is unclear whether the improvement in intestinal permeability and epithelial integrity was directly mediated by the lysate or were secondary to the change in gut microenvironment. Nevertheless, the data collectively suggest that certain bacterial species produce metabolites that can influence intestinal permeability and integrity.

Although the brunt of data suggest a role of probiotics in modulating barrier function and integrity, most studies have focused on preventative effects before

administration of an infectious or inflammatory challenge. There are still considerable gaps in knowledge on the mechanisms by which probiotics and other commensal bacteria affect intestinal permeability. More importantly, information is lacking on dosage, timing and formulation of exogenously administered microorganisms to prevent and treat disease (Rolfe et al., 2006; Shanahan and Collins, 2010).

7.3.3. Microbial effects on antimicrobial protein expression.

Antimicrobial proteins have a dual role in limiting the invasion of pathogens as well as modulating the intestinal microbiota composition. Certain subsets of intestinal epithelial cells-derived antimicrobial proteins are independent and others are influenced by the gut microbiota, emphasizing the bi-directional interaction between intestinal microbiota and intestinal barrier.

Gnotobiotic studies have revealed that Paneth cell-derived antimicrobials such as lysozyme and $sPLA_2$ are expressed under germ-free conditions. Similarly, cryptidins are expressed in the absence of gut microbiota, suggesting that baseline expression of these molecules do not require signals from gut microbiota (Ayabe et al., 2002; Putsep et al., 2000). However, cryptidin-related sequences (CRS) have been shown to be significantly higher in conventionally raised mice compared to mice raised in germ-free conditions suggesting that gut microbiota may influence the level of expression of crytpidins (Putsep et al., 2000). Thus, it seems the quantity, and quality, of cryptidins can be affected by the microbiota. Expression of α-defensins and cryptidins is significantly lower in human neonates and un-weaned mice and rats, but increase dramatically in adulthood (Mallow et

al., 1996; Ouellette and Cordell, 1988; Ouellette et al., 1989). During the first few years of life, each individual undergoes a transitional intestinal colonization, characterized by dynamic and random colonization with a distinctive microbial combination (Kurokawa et al., 2007; Palmer et al., 2007). The colonization patterns stabilize in time, with each individual eventually possessing a unique fingerprint of diverse intestinal microbiota (Kurokawa et al., 2007; Palmer et al., 2007). The increase in expression of α-defensins occurs concomitantly with colonization and development of a stable gut microbiota, implying that components of microbiota may have a role in the regulation of intestinal α-defensins (Kurokawa et al., 2007; Palmer et al., 2007; Stark and Lee, 1982). It will be interesting to know whether the α-defensins from neonates have the same biological activity as adult α-defensins. Indeed, α-defensins are post-translationally modified into mature defensins by matrilysin (MAT) in mice and trypsin in humans upon secretion (Ghosh et al., 2002; Wilson et al., 1999). Interestingly, MAT expression is undetectable in germ-free mice, implying that microbial stimuli are required for expression of functional cryptidins (Lopez-Boado et al., 2000).

Paneth cell-derived antimicrobials, including α-defensins, are released upon exposure to live bacteria and their antigens, such as lipopolysaccharide (LPS) and muramyl dipeptide (MDP) (Ayabe et al., 2000). In contrast, antimicrobial secretion by Paneth cells is not influenced by fungal or protozoal signals (Ayabe et al., 2000; Mukherjee et al., 2008). The exact signaling pathway that leads to microbial α-defensin expression is unclear but clinical and experimental evidence suggests that innate microbial sensors, particularly Nod2 receptors, may be involved (Kobayashi et al., 2005a;

Petnicki-Ocwieja et al., 2009; Wehkamp et al., 2008). Likewise, microbial signaling through Nod2 receptors is essential for the expression of other kinds of Paneth cells' antimicrobials, namely Defcr4 and Defcr10 (Eckmann and Karin, 2005; Kobayashi et al., 2005b).

Human β-defensin is constitutively expressed in colonic epithelial cells. Mouse β-defensin (mBD)1, mBD3, and mBD4 have been detected in the tongue and stomach of germ-free mice but full comparison between expression of β-defensin in other parts of intestinal tract between germ-free and conventional mice have not yet been fully studied (Schofield et al., 2004). However, upon mono-colonization of germ-free mice with *Candida albicans*, up-regulation of mBD1, mBD3, and mBD4 expression has been observed. Expression of human β-defensin (hBD)2, but not hBD1, requires bacterial or inflammatory stimuli (Eckmann, 2004; O'Neil et al., 1999) (Ramasundara et al., 2009). The probiotic VSL #3, several *Lactobacillus* species, *E. coli* Nissle 1917 and *E. coli* strain DSM 17252 (Symbioflor 2) have been shown to induce expression and secretion of hBD2 in human colonic epithelial cell lines (Mondel et al., 2009; Ohland and Macnaughton, 2010; Sharp et al., 2009; Wehkamp et al., 2004). Furthermore, increased fecal levels of hBD2 have been observed in healthy humans who received Symbioflor 2 twice daily for 3 weeks compared to placebo treated individuals (Mondel et al., 2009). NF-kβ pathway and TLR2 dependent signaling have been shown to be involved in the induction of hBD2 in epithelial cells (Chung and Dale, 2004; Wehkamp et al., 2004). Similarly, bacterial flagellin derived from *E. coli* Nissle 1917 was shown to induce hBD2. Flagellin is

recognized by TLR5, hence it is possible that this receptor plays a role in the induction of human hBD2 in intestinal epithelial cells.

Expression of the antimicrobial ribonucleases Ang-4 and the C-type lectin RegIII-γ and RegIII-β is significantly lower or undetectable in the small intestine of germ-free mice compared to conventionally raised mice, inferring that their expression is under the control of the gut microbiota (Cash et al., 2006; Hooper et al., 2003; Vaishnava et al., 2008). Upon colonization of germ-free mice, RegIII-γ expression increased (Cash et al., 2006; Hooper et al., 2003; Vaishnava et al., 2008). The importance of microbial regulation of RegIII protein expression is also supported by studies showing that mice lacking the MyD88 gene, but not mice lacking Nod2 signaling, have significantly lower RegIII-γ in the small intestine compared to controls (Vaishnava et al., 2008). Specific pathogen-free *Nod1*$^{-/-}$*; Nod2*$^{-/-}$ mice have significantly lower RegIII-γ expression in the colon compared to heterozygote littermates, suggesting that Nod receptors, perhaps Nod1, also influence colonic RegIII-γ expression (Natividad et al., 2011). The probiotic *Bifidobacterium breve* NCC2950 was able to up-regulate RegIIIγ in *Nod1*$^{-/-}$*; Nod2*$^{-/-}$ mice. It remains to be determined whether *B. breve* is able to up-regulate HIP/PAP, which is the human counterpart of RegIII-γ. Mono-colonization of germ-free mice with *Bacteroides thetaiotaomicron* induced a five-fold increase in RegIII-γ expression while mice colonized with *Bifidibacterium longum* repressed RegIII-γ expression (Sonnenburg et al., 2006). Suppression of RegIII-γ by *B. longum*, however, was reverted upon simultaneous colonization of germ-free mice with both *B. longum* and *B. thetaiotaomicron* (Sonnenburg et al., 2006). RegIII-γ has broad antimicrobial activity against gram-positive

bacteria. As *B. longum* is a gram-positive bacterium, it is possible that its ability to down-regulate RegIII-γ may represent its coping mechanism to survive in the gut (Sonnenburg et al., 2006). Overall, these studies indicate that specific bacteria have the ability to differentially regulate antimicrobial expression.

7.3.4. Microbial effects on mucus layer.

Germ-free mice have lower numbers of mucin-secreting goblet cells in the cecum compared to conventionally raised animals (Kandori et al., 1996). Moreover, germ-free mice displayed thinner, less stable and compact mucus layer compared to colonized animals (De Ponti et al., 1990; Deplancke and Gaskins, 2001; Enss et al., 1992; Meslin et al., 1999; Petersson et al., 2011; Reymann et al., 1985; Szentkuti et al., 1990). Exposure to bacterial products LPS and peptidoglycan increased the mucus layer thickness of germ-free mice to the level similar to conventionally raised mice (Petersson et al., 2011). Taken together, these findings suggest that the composition of the gut endogenous microbiota may regulate the mucus layer.

Administration of VSL#3 to rats induced increased MUC2 expression and secretion (Caballero-Franco et al., 2007). Among the bacteria in VSL#3, the expression seemed to be strongly mediated by the *Lactobacilli* bacteria (Caballero-Franco et al., 2007). It remains to be determined the exact mechanism by which *Lactobacilli* induced expression of mucin. Moreover, VSL#3 up-regulated the expression and secretion of MUC1, 2 and 3 in rat but not in mice (Caballero-Franco et al., 2007; Gaudier et al., 2005). Similarly, mucin expression or secretion was not changed upon inoculation of

Bifidobacterium breve to mice, emphasizing the differential effects of probiotics in regulating mucus layer composition (Natividad et al., 2011).

Mucins are essential in preventing luminal bacteria from adhering or penetrating the intestinal epithelium. This property relies on mucins' carbohydrate components. Comparison between germ-free and conventionally raised animal revealed that germ-free mice have a different glycosylation profile (Comelli et al., 2008). It is still unclear to what extent the resident commensals engage in the control of mucin glycosylation. Exposure to LPS from *Escherichia coli* O55:B5 stimulated the release of mucins in germ-free mice (Enss et al., 1996). Furthermore, mono-colonization of germ-free mice with *B. thetaiotaomicron* stimulated the expression of components of mucus layer including α,2-fucosyltransferase mRNA and synthesis of fucosylated glycoconjugates on small intestinal epithelial cells (Nanthakumar et al., 2003). The increase in mucin fucosylation, allowed *B. thetaiotaomicron* to better interact with intestinal epithelial cells. Thus, there seems to be a bi-directional intestinal barrier-microbiota interaction, which is crucial for the maintenance of health.

7.4. Conclusion

The complex community of microorganisms residing within the lumen of the intestinal tract has a mutualistic relationship with the host. Disruption of its composition and host-microbial interactions can alter host functions and has been proposed to contribute to a number of gastrointestinal and inflammatory diseases. It is now recognized that the microbiota influences intestinal physiology, including the intestinal barrier, and

that this relationship is bi-directional. However, the specific molecular and cellular mechanisms remain to be determined. Understanding the interaction between intestinal epithelial barrier and gut microbiota will be pivotal for the development of new prophylactic and therapeutic agents for chronic inflammation. These strategies may be based on the use of specific bacterial species with barrier modulating capacity to prevent intestinal inflammation. Important questions regarding mode and time of delivery of potentially beneficial bacteria, as well as the influence of host genotype in the therapeutic response achieved, need to be addressed in the future.

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