Understanding the Effects of Antibiotic Exposure on Host Immune-Microbiota Crosstalk and the Development of Colitis

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Immunology
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Abstract

The etiology and pathogenesis of inflammatory bowel diseases (IBD) remains unclear but involves a complex interplay between host genetics, the environment, the gut microbiota and the mucosal immune system. Antibiotic use in infants and adults is associated with increased risk of IBD development; however, the mechanism by which this occurs is unknown. This thesis project focused on understanding how altered immune-microbe interactions at the gut mucosa contribute to the development of IBD. I hypothesized that perturbation of the gut microbiota following antibiotic exposure would alter the composition of the intestinal microbial community structure, the status of mucosal lymphocytes and subsequently influence susceptibility to colitis. The timing of the antibiotic exposure could have significant effects; therefore, I assessed adult and neonatal treatments separately.

Herein, I identified that adult antibiotic treatment of adult mice transiently altered the gut microbiota of both WT and Nod2-deficient animals and that Nod2 was involved in microbial resilience. While antibiotic treatment did not influence the severity of DSS-induced colitis, Nod2−/− mice displayed reduced IL-17A and myeloperoxidase levels and improved mucosal healing following anti-CD3 mAb injection. An altered gut microbiota was associated with increased
fecal IgA levels in the absence of Nod2, and transfer of fecal IgA into untreated Nod2−/− mice ameliorated the response to anti-CD3, thereby highlighting the use of IgA as a potential therapeutic mechanism for altering the gut microbiota.

In addition, neonatal antibiotic treatment strongly influenced the gut microbial community at weaning and was maintained until day 35 in Nod2-deficient mice. Antibiotic perturbation resulted in worsened DSS colitis in Nod2−/− mice, associated with an increase in colonic Gata3+ regulatory T cells.

Understanding how altered immune-microbiota crosstalk increases susceptibility to colitis in a genetically susceptible host (i.e., Nod2−/− mice) will help define such alterations in humans and set the stage for development of preventative and therapeutic manipulation of the microbiota to reduce mucosal inflammation.
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Dissemination of Findings

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Oral Presentations:

Mucosal Immunology Course and Symposium, Toronto, ON, July 26-29 2016
- Presentation entitled “Adult Nod2−/− mice show a prolonged microbial disruption following antibiotic perturbation of the microbiota and altered small intestinal mucosal damage”
- Second presentation entitled “Mucosal plasma cells and gut IgA rapidly increase in response to changing microbiota following antibiotic perturbation”

Poster Presentations:

Canadian Student Health Research Forum, Winnipeg, MB, June 6-9, 2017
- Poster entitled “Neonatal antibiotic exposure skews the developing microbiota and mucosal T cell populations resulting in worsened colitis in Nod2−/− mice” *Silver Award for presentation and CIHR Travel Award*

Canadian Institute for Health Research Inflammation in Chronic Disease Networking Workshop, Quebec City, QC, February 2-3, 2017
- Poster entitled “Neonatal antibiotic exposure skews the developing microbiota and mucosal T cell populations” *Best Poster Award*

Mucosal Immunology Course and Symposium, Toronto, ON, July 26-29 2016
- Poster entitled “Adult Nod2−/− mice show a prolonged microbial disruption following antibiotic perturbation of the microbiota and altered small intestinal mucosal damage”
- Second poster entitled “Mucosal plasma cells and gut IgA rapidly increase in response to changing microbiota following antibiotic perturbation”
- Third poster entitled “Neonatal antibiotic exposure distorts the developing microbiota and mucosal T cell populations”

Canadian Digestive Diseases Week (CDDW), Montreal, QC, February 26-29 2015
- Poster entitled “Adult Nod2−/− mice show an altered microbial resilience and an increased fecal IgA response following antibiotic perturbation of the microbiota” *Poster of Distinction*
- Second poster entitled “Nod2-/- mice show a unique response in microbiota and mucosal T cell responses after neonatal exposure to antibiotics”

**Cell Symposia: Human Immunity and the Microbiome in Health and Disease**, Montreal, QC, September 27-29 2015
- Poster entitled “Antibiotic manipulation of the adult murine microbiota has long-term effects on the mucosal immune response in Nod2-/- Mice”

**17th International Congress on Mucosal Immunology**, Berlin, Germany, July 14-18 2015
- Poster entitled “Antibiotic Manipulation of the Adult Murine Microbiota Has Long-Term Effects on the Mucosal Immune Response in Nod2-/- Mice”
- Second poster entitled “Early Life Antibiotics Alters the Development of the Gut Microbiota and Mucosal Immune T cell Populations”

**Canadian Digestive Diseases Week (CDDW)**, Toronto, ON, February 8-11 2014
- Poster entitled “Role of Innate Receptors, Nod1 or Nod2 on Effector and Regulatory T Cell Function In Colitis”

**16th International Congress on Mucosal Immunology**, Vancouver, BC, July 17-20 2013
- Poster entitled “Effector and Regulatory T cell Subsets Deficient in Nod1 or Nod2 Retain the Ability to Induce and Prevent Colitis”

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**Additional Manuscripts during PhD:**


Chapter 1

Introduction

1.1 Overview of Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) encompass illnesses characterized by chronic, relapsing inflammation of the gastrointestinal tract. Canada has one of the highest rates of IBD in the world, with 1 in every 150 Canadians living with either Crohn’s disease (CD) or ulcerative colitis (UC), the two main types of IBD\(^1\). CD can cause inflammation at any point along the alimentary canal, from mouth to anus, whereas inflammation is localized to the colon and rectum in UC. Disease onset typically begins in late teens to early adulthood, and symptoms include severe diarrhoea, abdominal pain, bloating, fatigue, and weight loss. With disease induction occurring during the most productive years and symptoms severely impacting the patient’s quality of life, IBD is a huge burden to the patient, their families, society and the health care system. The prevalence of IBD has risen exponentially over the last 65 years, coinciding with a decline in infectious diseases in the Western World\(^2\). Of the 10,000 new patients diagnosed each year in Canada, the sharpest increase has been in children under the age of ten\(^3\). Paediatric IBD cases doubled in Canada\(^4\) and the United Kingdom\(^5\) in the last 10 years, suggesting that environmental triggers are involved in driving disease development.

While the etiology of IBD remains unclear, it is currently hypothesized to be a multi-hit, multifactorial auto-inflammatory disease (Figure 1.1). The most widely accepted hypothesis is that a genetically susceptible person experiences an environmental trigger that leads to an inappropriate immune response against the commensal gut microbiota, resulting in chronic immune activation, inflammation, epithelial damage, bacterial translocation and further inflammation\(^6\). Despite significant efforts, the environmental trigger(s) leading to this chronic inflammatory cycle remains unknown. The search for a “cure” for IBD requires understanding the fundamental principles governing the interaction between the intestinal immune system and gut microbes and in particular, mechanisms that control responses to “normal” commensal bacteria. Indeed, the challenge to the mucosal immune system is to maintain a controlled and appropriate response to the trillions of gut microbes that make up the commensal gut microbiota. One can imagine how
loss of this regulation could lead to chronic intestinal inflammation and play a key role in the pathogenesis of IBD.

Current therapeutic strategies involve immunomodulators and anti-inflammatory drugs such as corticosteroids or methotrexate. Other options include antibiotics, exclusive enteral nutrition (EEN) therapy and biologic therapy such as anti-tumor necrosis factor (TNF) monoclonal antibody. In all cases, the goal of treatment is to induce remission by limiting and controlling ongoing inflammation and to maintain remission through immune regulation.

Figure 1.1.1: The multi-factorial etiology of IBD. Genetically susceptible people may experience some form of environmental trigger that induces an immune response against commensal gut microbiota. The uncontrolled immune activation is thought to initiate disease or result in a flare. Adapted from R.B. Sartor Nat Clin Pract Gastroenterol Hepatol. (2006).
1.1.1 Genetic risk in IBD

The genetic influence on IBD development has been well studied. Some of the first studies looking at IBD occurrence in twins identified a higher concordance in monozygotic twins than dizygotic twins, 58.3% versus 0% respectively, for CD\textsuperscript{7}. Moreover, a study of Swedish monozygotic twins identified a concordance of 18% in UC and 50% in CD\textsuperscript{8}. This indicated that IBD development, particularly CD, is influenced by genetics. To date over 200 genetic loci have been identified that are associated with an increased risk of developing IBD, 30 of which are specific to CD\textsuperscript{9-11}. The majority of IBD susceptibility genes are linked to pathways involved in immune-microbe interactions, for example: microbial detection, immune activation and suppression, and fucosylation of the mucosal epithelium. Some of these genes include the interleukin-23 receptor (IL-23R), the autophagy-related protein 16-1 (ATG16L1) and the IL-10 receptor (IL-10R)\textsuperscript{9}. The strongest genetic association with CD is nucleotide-binding oligomerization domain-containing protein 2 (NOD2) mutations, with the largest odds ratio of 3.1\textsuperscript{9,12,13}.

NOD2 is a cytosolic pattern recognition receptor that senses muramyl dipeptide (MDP), a component of peptidoglycan found in the cell wall of Gram-positive and –negative bacteria\textsuperscript{14}. Located within the leucine rich repeat (LRR) domain responsible for microbial sensing are the three main NOD2 mutations: Arg702Trp and Gly908Arg, which result in amino-acid substitutions, and the 1007fs frameshift, which results in a premature stop codon and a truncated protein\textsuperscript{12,13} (Figure 1.2). These mutations are thought to result in “loss of function” and cause defective bacterial sensing. Up to 40% of CD patients have at least one allele mutated in NOD2, while mutations in both NOD2 alleles are found in ~10% of CD patients. Upon activation, NOD2 signalling is mediated by Rip2 kinase, which activates nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) leading to increased immune gene expression and inflammation. These observations suggest that innate immune responses to bacteria are a key element in the pathogenesis of CD; however, the mechanism by which this occurs is still unclear\textsuperscript{14-17}.

NOD2 is expressed in numerous cell types, including T and B cells\textsuperscript{18,19}, macrophages and dendritic cells\textsuperscript{20,21}, plus epithelial cells, goblet cells and Paneth cells\textsuperscript{22-24}. Each of these cell subsets are located within the intestine and are involved in either maintaining the epithelial
barrier thus limiting bacterial translocation into the tissue, or bacterial sensing and clearance. Therefore, it is clear that defects in the bacterial sensor NOD2 could impair any or all of the mechanisms available for protecting the host from bacterial-induced inflammation in the gut. Indeed, polymorphisms in NOD2 lead to defective NF-κB activation resulting in inefficient epithelial and macrophage clearance of invasive bacteria. Furthermore, patients with NOD2 mutations have reduced defensin production and secretion by Paneth cells, increased T cell and humoral immune responses and a proposed loss of tolerance to the commensal gut microbiota.

Figure 1.2: NOD2 gene and IBD associated mutations. NOD2 has three functioning domains: CARD, responsible for NFκB activation through interactions with RIP2; NBD, responsible for oligomerization; and the LRR domain, responsible for bacterial sensing via binding of muramyl dipeptide (MDP). The three main mutations in NOD2 are all located near or in the LRR domain and their locations are indicated with the red arrows. Adapted from M. Bhullar World J Gastroenterol. (2014).

1.1.2 Environmental risks in IBD

IBD, along with several other autoimmune diseases, has seen a significant increase over the last 50 years, coinciding with a decrease in infectious diseases. The ‘hygiene hypothesis’ states that this increase in allergy and autoimmunity is due to the loss of immune education during infections, particularly during childhood. Indeed, Canada has seen a significant decrease in childhood parasitic and viral infection compared to countries in Africa, where these infectious diseases remain common. However, a reduction in infectious disease is not sufficient to explain the steady growth in IBD cases. Several other environmental factors that may be contributing include: increased sanitation, changes in socio-economics and dietary transformations, vaccine
development and antibiotic use. Likely it is not just one of these but a combination of environmental factors that can lead to increased IBD incidence.

Epidemiological observations have historically deemed IBD a disease of the Western world, with the highest prevalence in northern Europe and North America. However, IBD is rising as historically low-incidence countries having implemented a more Westernized lifestyle, as seen in India and parts of Asia\textsuperscript{28}. Most recently, migration has further suggested a significant role for the environment in the development of IBD. A study looking at the incidence of IBD in immigrants to Canada (those who were born elsewhere) compared to their offspring (born in Canada to immigrant parents) showed that while immigrants had lower incidence risk, their offspring had risk equivalent to that of nonimmigrant children\textsuperscript{29}. This finding indicates that the Canadian environment and lifestyle can increase the risk of IBD development, highlighting the need to understand these pressures and how they are involved in IBD etiology.

Early life is a critical time for development and early life exposures have been associated with increased IBD risk. Caesarean birth\textsuperscript{30}, farm exposure\textsuperscript{31} and antibiotic use\textsuperscript{32,33} in early life have each been associated with an increased risk of developing IBD. The mechanism of this increased susceptibility is unknown, although likely involves alterations of the gut microbiota and its influence on host immune responses.

Diet and food antigens were once thought to be involved in IBD development however no study has sufficiently identified a causal role of diet. Instead, it is thought that diet may be involved through its influence on the gut microbiota. Diet has long been known to alter the composition of the microbiome, favouring those microbes that can use the food being consumed as substrate\textsuperscript{34}. The Western diet, high in carbohydrates and fat and low in dietary fiber, favours the expansion of \textit{Bacteroides}\textsuperscript{35}. Plant-based diets, such as those consumed by Amerindians or Malawians, favour the fermenters such as \textit{Prevotella} and generally result in increased microbial diversity\textsuperscript{36}. An effective therapeutic option for pediatric IBD patients is the use of an elemental diet, which consists of amino acids, oligosaccharides and medium-chain triglycerides that require minimal digestion in order to be absorbed\textsuperscript{37}. Although the exact mechanism of action is unclear, studies have shown a decrease in pro-inflammatory cytokines and improved mucosal healing with an elemental diet\textsuperscript{38}, likely due to an altered microbiota\textsuperscript{39}. This provides further evidence for the
important role of host immune-microbe interactions on both the development and treatment of IBD.

1.2 Involvement of the Immune System in IBD

Inflammatory bowel diseases are considered autoimmune diseases since the majority of the damage and destruction is directed against host tissues and commensal microbes. Although the exact mechanism remains unknown, multiple components of the immune system are involved in the pathogenesis of IBD, from innate sensing of bacteria to adaptive anti-commensal responses. The gut is home to the largest number of immune cells in the body. Only a single layer of columnar epithelial cells physically separates the numerous luminal antigens from the primed mucosal immune system. The mucosal immune system exists to protect against invading pathogens, while at the same time it must remain tolerant to food antigens and commensal microbes.

1.2.1 Structure and function of the intestine

In order to understand the role of the gut microbiota on mucosal immune development and disease, the structure of the intestine must be considered. The alimentary tract runs from mouth to anus, and while colonization does exist in the mouth and stomach, the majority of the gut microbiota live in the small and large intestine. The small intestine can be considered as three functionally distinct sections: the proximal duodenum, responsible for enzymatic breakdown of ingested food, the medial jejunum and the distal ileum, which are responsible for nutrient absorption. Physiology dictates the structure of the epithelial barrier along the small intestine, from long villi and crypts in the duodenum and jejunum to increase surface area necessary for absorption, to shorter villi in the ileum. Multipotent stem cells inhabit the base of the crypt alongside Paneth cells, and give rise to new epithelial cells to replace the constant turnover of the barrier every 4-5 days. As the epithelial cells migrate up the crypt and villi, they mature into one of the various cell types within the barrier: absorptive enterocytes (the most common), enteroendocrine cells, or mucus-producing goblet cells. Together, these cell subsets maintain a secure barrier along the small intestine, a barrier between the microbes in the lumen and the immune cells in the mucosa. Some of the mechanisms used to protect the barrier include expression of tight junction proteins to reduce the permeability of the epithelium, release of anti-
microbial peptides including defensins from Paneth cells, and mucus production by goblet cells. Furthermore, expression of pattern recognition receptors, such as NOD2, by epithelial cells provides another protective checkpoint. Indeed, NOD2 expression is particularly high in Paneth cells within the ileum, and NOD2 mutations have been associated with ileal inflammation in Crohn’s disease patients.

In contrast, the large intestine has no villi, only crypts, since its main physiological function is the reabsorption of water. The crypts still maintain a base of regenerating stem cells (but lack Paneth cells), which populate the colonic epithelium with enterocytes, enteroendocrine cells and goblet cells. These goblet cells produce a protective mucus composed of two layers: a thin inner layer that is firmly attached to the colonic epithelium and impervious to bacterial translocation during homeostasis, and an outer layer which is significantly thicker but is looser, allowing bacterial habitation. Mucins, such as Muc2, are the structural components of the mucus layer, which can be quantified and used as a measure of the host’s response to microbes. Moreover, immune mediators, such as IL-9 and IL-13, stimulate mucus production.

1.2.2 The gut mucosal immune system

Cells of the mucosal immune system primarily inhabit the area directly below the gut epithelium, which is termed the lamina propria. The exact population of cells varies between mice and humans and along the length of the gut corresponding with the function and immunological needs of the tissue. The main cell types and their functions are briefly described below and schematically represented in Figure 1.3.

Intestinal intra-epithelial lymphocytes (IELs), chiefly of the CD8α+ phenotype and either T cell receptor (TCR) −αβ+ or −γδ+, live directly below and in contact with gut epithelial cells. They have many functions, dictated primarily by whether they are naturally antigen-primed in the thymus or induced by antigen in the gut. In either case, these cells function to protect and maintain the epithelial barrier in a regulatory role, through IL-10 and TGFβ production, and in a protective role, by secretion of cytotoxic mediators and IFNγ release. However, IELs can also have pathogenic effects by exacerbating intestinal damage through inflammatory cytokine production and have been associated with IBD pathogenesis.
Dendritic cells and macrophages are classic antigen-presenting cells (APCs) capable of phagocytizing microbes, degrading them and presenting various antigens to T and B cells. Phagocytes are also responsible for scavenging dead cells within the tissue and producing various cytokines to drive epithelial regeneration. In the gut, inducing and maintaining tolerance through the induction of various T cell subsets, particularly regulatory T cells, is the main function of dendritic cells\textsuperscript{49}. Capable of reaching into the lumen to capture microbes, inducing class switching to drive immunoglobulin-A (IgA) production and producing cytokines to maintain the epithelial barrier, dendritic cells perform multiple critical functions necessary to maintain homeostasis and induce tolerance to both commensal microbes and food antigens\textsuperscript{50}.

**Figure 1.3: Gut immune organization.** A layer of epithelial cells separates the lumen from the mucosa, protected by a mucus layer. Immune cells including dendritic cells, T and B cells are located in the sub-epithelial lamina propria.
B cell-derived plasma cells are found along the length of the intestine and are typically IgA producers\(^{51}\). IgA production is dependent on microbes and its secretion into the intestinal lumen is one of the most potent ways the host immune system regulates the gut microbiota\(^{52,53}\). Classically, presentation of antigen by a dendritic cell to a B cell in the germinal center of a Peyer’s patch, in the context of various mediators including B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), results in class-switching, migration to the lamina propria and plasma cells differentiation\(^{54}\). More recently, evidence for Toll-like receptor (TLR) mediated IgA induction and microbially driven T cell-dependent IgA responses have shed light onto the variety of ways that IgA is involved in microbial interactions\(^{55,56}\).

Conventional T cells, expressing CD4 or CD8, are readily found in the intestinal lamina propria of both the small intestine and colon. The type and function of a T cell is characterized by the transcription factors they express and the cytokine profile they dominantly produce (described below).

### 1.2.3 T cell differentiation

Naïve T cells that undergo positive and negative selection to generate appropriate immune responses leave the thymus for the periphery. Within the tissue, these cells are presented with antigen, which in the context of varying cytokines determines the type of helper T cell it will become (Figure 1.4)\(^{57}\). Classically, during an intracellular infection, Tbet expressing Th1 cells are induced by interleukin-12 (IL-12) and interferon-γ (IFNγ), while parasitic and extracellular infections are known to drive Th2 differentiation through IL-4 and IL-13 induction of Gata3. In order to control and limit the inflammatory response, IL-10 and transforming growth factor-β (TGFβ) induce naïve T cells to express Foxp3 and become regulatory T cells. Together, these three arms of the adaptive T cell response explain the majority of T cell activity. More recently, the presence of a single bacterial species, segmented filamentous bacteria (SFB), was shown to induce the generation of retinoic acid receptor-related orphan receptor-γ (RORγt) expressing Th17 cells in the small intestine of mice via a balance of IL-6, TGFβ and IL-23\(^{58}\). These cells play a unique role in mucosal immunity in that they can act in either a pro-inflammatory or anti-inflammatory manner depending on the mode of stimulation\(^{59}\). Th17 cells producing high amounts of IL-17A perpetuate inflammation; on the other hand, in the context of barrier disruption, RORγt\(^+\) cells can increase the production of IL-23 to enhance epithelial regeneration. Moreover, the presence of RORγt\(^+\) Foxp3\(^-\) double positive cells suggests that RORγt\(^+\) Th17 cells
can transform into regulatory T cells, capable of limiting an ongoing inflammatory response\textsuperscript{60,61}. Recent work indicates that ROR\textgamma t expression in Treg cells represents a subclass of Treg critical for controlling inflammation in the intestine\textsuperscript{62,63}. Indeed, these studies showed that ROR\textgamma t\textsuperscript{+} Treg are microbially-induced in the colon and are required to prevent chemically-induced colitis\textsuperscript{62,63}. Furthermore, ROR\textgamma t\textsuperscript{+} Foxp3\textsuperscript{+} double positive cells were equally capable of preventing colitis in the T cell transfer model compared to single positive Foxp3\textsuperscript{+} Treg\textsuperscript{64}. One study indicated that these cells maintain their ability to produce the cytokine IL-17A, however others have indicated that these cells do not produce IL-17A but instead make IL-10\textsuperscript{64,65}. This difference is likely due to the conditions during which they are generated and likely represents a spectrum of functions and capabilities of these ROR\textgamma t\textsuperscript{+} Foxp3\textsuperscript{+} double positive T cells.

![Diagram of T cell subsets and function](image)

**Figure 1.4: T cell subsets and function.** Dendritic cells present antigen to naïve T cells in the periphery and the surrounding cytokine milieu dictates the differentiation and subsequent function of the T cell. Adapted from AM Jetten *Nucl Recept Signal.* (2009).

Other non-classical regulatory T cells have been described in recent years. These include the Foxp3\textsuperscript{−} high IL-10 producing Tr1 cells, involved in tolerance and controlling autoimmunity\textsuperscript{66}, and the latency-associated peptide (LAP)\textsuperscript{+} Foxp3\textsuperscript{+} Treg, which are suppressive through their significant production of TGF\beta\textsuperscript{67}. In parallel with the ROR\textgamma t expressing regulatory T cells, several teams have described Gata3\textsuperscript{+} Foxp3\textsuperscript{+} Treg\textsuperscript{68}. Like their ROR\textgamma t\textsuperscript{+} cousins, these cells are
likely induced through host-microbe interactions in the gut, and deletion of Gata3 within Treg results in reduced suppressive function of the Treg giving rise to an inflammatory disorder in mice\textsuperscript{69}. Similar to Gata3\textsuperscript{+} helper T cells, IL-4 can induce the expression of Gata3 in Foxp3 Treg\textsuperscript{70}. Interestingly, Gata3 expression can also be induced by co-stimulation with TGFβ and the alarmin IL-33\textsuperscript{71}. IL-33 is produced by gut epithelial cells and released during epithelial barrier disruption, acting as a chemo-attractant for neutrophils that also produce IL-33, resulting in a positive feedback loop. During inflammatory conditions with high levels of IL-33, Gata3 induction in Treg and accumulation of Gata3\textsuperscript{+} Foxp3\textsuperscript{+} Treg in the tissue supports the idea of these cells being involved in limiting barrier damage and stimulating mucosal healing. Intriguingly, Gata3 expression was shown to promote and maintain Foxp3 expression, ensuring continued suppressive activity of the Treg, while also blocking ROR\textgamma t expression\textsuperscript{68,69}. While the exact mechanistic value of having various Treg subsets is not fully understood, each seems to play a unique role in controlling inflammation and restitution of tissue homeostasis.

1.2.4 Immune response in IBD

The gut represents the largest and most diverse site of interaction between the host and the environment. As such, patients with IBD can experience a flare in response to numerous triggers of inflammation. As described above, the lumen is physically separated from the immune cells by highly specialized epithelial cells. IBD patients have altered intestinal permeability (i.e. leaky gut due to reduced intercellular adhesion), possibly allowing for bacterial translocation into the lamina propria\textsuperscript{72-74} although it remains to be determined if this is due to the inflammation or represents an underlying genetic defect. Recent work from our laboratory failed to show a genetic association with abnormal intestinal permeability in healthy subjects\textsuperscript{75}.

Paneth cells, located in the base of the crypts, are secretory cells that specialize in defense by secreting potent anti-microbial defensins. NOD2 is highly expressed in Paneth cells, suggesting that polymorphisms in NOD2 could result in defective anti-microbial defence\textsuperscript{24}. Indeed, CD patients have a reduction in anti-microbial peptide production and morphologically abnormal Paneth cells\textsuperscript{76,77}. A thick layer of mucus lines the gut epithelium acting as a chemo-physical barrier limiting bacterial adherence and translocation\textsuperscript{78}. IBD is associated with a thinner mucus layer that may enhance bacterial invasion of the mucus\textsuperscript{79}. Collectively, epithelial barrier defects are a characteristic feature in IBD patients and may represent a key feature in the breakdown of
host-microbe interactions, as it is the main contact point between luminal microbes and mucosal immune cells.

IBD patients have elevated levels of many pro-inflammatory cytokines such as IL-1β, TNFα, IL-6, and IL-12 in serum and mucosal tissue compared to healthy controls. It has been postulated that this elevation is due primarily to the inability to control the immune response to commensal bacterial antigens. Indeed, several groups have identified increased anti-microbial responses in IBD patients. Specifically, one team identified an increase in highly IgA-coated microbes in the stool of IBD patients, indicating an increased adaptive immune response against commensals. Another group investigated the reactivity of serum antibodies to microbial antigens and found that CD patients have elevated serum IgG reactive to bacterial flagellin. Tissue specific responses also indicate an inability to control inflammation in IBD patients. For example, the alarmin IL-33 is increased in inflamed tissue of IBD patients. Moreover, increases in neutrophils and monocytes within the tissue result in increased reactive oxygen species (ROS) and further tissue damage. Mucosal T cells in IBD patients survive longer than normal T cells and remain within the mucosa. This finding has resulted in a new avenue of therapeutics targeted against gut homing receptors, including the integrin α4β7. Another effective treatment of CD is anti-TNFα therapy, which suppresses immune activation by binding to and neutralizing TNFα produced by immune cells. Limiting the inflammatory response in this way allows for the epithelial barrier to heal thus preventing further microbial translocation into the lamina propria and continuous immune stimulation. Alternatively, some patients respond to oral antibiotic therapy, which reduces the total microbial load in the gut lumen and limits bacterial invasion into the tissue; however, the mechanism by which antibiotics mediate remission are likely due to alterations of the gut microbiota.

1.3 Microbiota

The human body is truly an ecosystem, colonized by a wide variety of microbes, including Archaea, bacteria, protists, viruses, and bacteriophages, and some not so “micro” organisms, such as worms and fungi. The ‘microbiota’ collectively refers to all of these components but more specifically relates to the bacterial community both on and in the body, whereas the ‘microbiome’ denotes the genes and genetics of this community. While the existence and
influence of the ‘virome’ and ‘mycobiome’ have been established, much less is known about their role in health and disease, or the co-evolution with gut bacteria\(^{88-90}\). Symbiosis between the host and its resident microbiota has important consequences for human health and physiology. These interactions may have beneficial nutritional, immunological, and developmental effects or pathogenic effects for the host\(^{91}\). Research characterizing which bacteria are present at various anatomical locations, their abundance and function has exploded over the last decade as we have come to appreciate their essential role in both health and disease.

1.3.1 Quantification and assessment of the microbiota

The community of microbes varies by anatomical location, from skin to vagina, mouth to gut\(^ {92,93}\). While many microbes are found on the skin, the highest concentration of bacteria is located within the gastrointestinal tract. Bacteria live in the mouth \((10^9)\), stomach \((10^3)\), and small intestine \((10^4-10^8)\) but the majority are located in the colon, with nearly \(10^{11}\) bacteria per gram of intestinal content or \(3.8 \times 10^{13}\) bacteria in total\(^ {40}\). The complexity of the community makes it difficult to ascertain exactly how many bacterial species are represented but it is estimated that 500-1000 species colonize the average human colon, the majority of which are obligate anaerobes\(^ {94,95}\). Actual biodiversity and community structure, particularly how the microbes co-habitat and interact with each other, is difficult to ascertain since many organisms cannot yet be cultured \textit{ex vivo}. Some researchers are developing new ways to culture commensal bacteria in physiologically relevant conditions and in ways that attempt to address the community structure, including invention of the “robogut” and other novel culturing techniques\(^ {96,97}\). Advancement of these methodologies will allow for significant advancement of microbial manipulation experiments and testing of potential therapeutics that could alter the microbiota to prevent or eliminate disease. However, until then, molecular methods for investigating the microbial population primarily involve genetic analysis via sequencing variable regions of the 16S ribosomal RNA (rRNA) genes. Carl Woese first described the phylogenetic role of the 16S rRNA gene when he compared the 16S rRNA sequence among living cells and discovered that its high conservation between species allowed for its use in classification of life into Archaea, Bacteria and Eukaryota\(^ {98}\). The gene has highly conserved regions that are nearly identical throughout the Bacteria kingdom, and interspersed are nine highly variable regions (V1-9). High throughput sequencing of selected variable regions of the 16S rRNA gene is currently the most common method for taxonomic identification and assessing community
The standard workflow proceeds where bacterial DNA is isolated from a sample, typically stool, and DNA is sequenced for the 16S rRNA gene, using universal primers for the conserved regions to amplify a segment of the 16S rRNA gene and sequencing the variable regions within that segment for identification. Sequencing reads are formatted into a table of operational taxonomic units (OTUs), aligned with known 16S sequences in public databases and assigned a phylogeny. In this way, sequencing data provides insight into the presence or absence of bacterial taxa and their abundance relative to the entire population, and as such is used to discern the community structure of the microbiota at a given time. Specific measures of the community include alpha diversity, which evaluates species richness or the presence of taxa (e.g., Chao1 or Shannon diversity index), and beta diversity, which determines the presence and abundance of taxa and how it relates to the community (e.g., Unifrac, Bray-Curtis dissimilarity).

Longitudinal sampling of the same subjects allows for both inter- and intra-individual comparisons of the microbial composition, providing insight into how environmental cues (e.g., diet, drugs) can influence the community over time within an individual and if the same patterns can be observed across individuals.

While sequencing the variable regions within the 16S rRNA gene allows for fairly accurate classification, it is important to note that the gene can be found in multiple copies within a single bacterium and the copy number can vary between strains within the same species. Thus it is a useful tool to assign bacteria to a phylogeny, but it is less accurate to use it as a measure of total bacterial load. Instead, use of the RNA polymerase beta-subunit (rpoB) gene, which is present in a single gene copy per bacterium, is a more accurate genetic identifier for quantifying bacterial load. Alternatively, shotgun metagenomic sequencing allows researchers to identify all genes from all organisms present within a sample. This technique involves shearing the DNA within a sample, sequencing many short reads and then combining them into longer, more cohesive sequences. Shotgun sequencing quantifies the abundance of microbial species and also provides insight into their functional and metabolic capabilities. Furthermore, the randomness of the sequencing allows species present at lower abundance to be detected and included within community analysis.

As we understand more about which microbes inhabit which anatomical locations under various conditions, we can begin to extrapolate the functional role of certain taxa or communities of taxa. One method for performing this is called linear discriminant analysis (LDA) effect size.
(LEfSe)\textsuperscript{107}. Developed in the lab of Dr. Curtis Huttenhower, this algorithm analyzes sequencing data in two steps; first, it identifies statistically significant differences in taxa abundance between two groups, and then tests these differences to determine if they are consistent with predicted biological behaviour\textsuperscript{107}. In this way, LEfSe identifies taxonomic differences that could explain a biologically relevant phenotype, making it a unique tool for identifying potential biomarkers of microbial health that could become therapeutic targets.

Alternative methods for gaining insight into the functional profile of the gut microbiota involve other high-throughput techniques, including metabolomics\textsuperscript{108}, metaproteomics\textsuperscript{109} and metatranscriptomics\textsuperscript{110,111}. These systems can provide incredible details into the functional capacity of the microbiome, however the issue of replication of results and the ability to accurately analyze and extrapolate from these types of data are still being developed. When the processing power catches up with the current rate of data generation, we will truly begin to understand the vast ecosystem that comprises the gut microbiota.

1.3.2 Microbial colonization

Evolutionarily, microbes have resided in animals for over 500 million years providing a selection advantage for the host due to contributions of microbes toward metabolism and defence\textsuperscript{112}. The transfer of microbes between generations occurs primarily with birth, where maternal microbes are directly transferred to the offspring. Recently, there has been evidence of a placental microbiota and transfer of microbial components to the offspring in utero, which possibly indicates a role of gestational education of immune cells\textsuperscript{113-115}. However birth itself still provides the vast majority of the foundational colonization, and the gut ecosystem develops rapidly as microbes successively colonize vacant niches. In humans, vaginal and fecal microbes, including \textit{Lactobacillus} and \textit{Prevotella} species, coat and colonize infants born vaginally, whereas skin microbes, such as \textit{Staphylococcus} and \textit{Propionibacterium} species, colonize infants born via Caesarean section\textsuperscript{116,117}. Colonization by vaginal microbes, predominantly lactobacilli, is critical for the function of the foundational microbiota: primarily digestion of milk and human milk oligosaccharides\textsuperscript{116,118}. One study by Palmer \textit{et al.} elegantly described the colonization patterns experienced by 14 infants after birth\textsuperscript{118}. They found that bacterial load varied significantly during the first 14 days of life but subsequently stabilized at \textasciitilde10^9 bacteria per gram of stool; moreover, C-section infants had a reduced bacterial load compared to vaginally born infants.
Bifidobacterium species, in particular, are transferred to vaginally born, but not C-section infants\textsuperscript{119}. The variation between infants was striking and comparisons between infants and maternal vaginal and milk samples indicated that colonization was indeed most strongly influenced by exposure. However, after one year of life, the composition of the infant gut microbiota began to converge and adopt a more “adult-like” composition. Indeed, others have also observed this and likely it is due to a combination of developmental changes in gut structure, dietary changes (with a switch to mainly solid foods) and competition between bacterial taxa\textsuperscript{91,120}.

Evolution of the gut microbiota continues until roughly 3 years of age in humans\textsuperscript{118,120,121}. Recently, Yassour et al. longitudinally sampled 39 Finnish children for the first three years of their lives, collecting 28 samples per child on average and assessing the microbial community structure down to the strain level\textsuperscript{122}. Similar to what was observed previously, samples from the same child were more similar to each other than to samples from age-matched, unrelated children. Additionally, C-section infants and a proportion of vaginally born infants displayed reduced Bacteroides colonization during the first 6 months of life, which was associated with reduced bacterial diversity that persisted to the study endpoint at 36 months of age. Collectively, these data suggest that birth mode and maternal transfer of microbes have significant influence on the gut microbiota of offspring during the first weeks and months of life.

Besides mode of delivery, several postnatal influences including breast- vs. formula-feeding, infant hospitalization, presence of a pet in the home, siblings and birth order all factor into the colonization pattern and succession of bacterial diversity\textsuperscript{91,123,124}. Comparison of colonization patterns during early life between North America, Africa and South America highlight the powerful influence of age, the environment and diet\textsuperscript{36}. Specifically, infants have greater variability and reduced diversity compared to adults. Moreover, children born in the United States had reduced microbial diversity compared to Malawian or Amerindian children\textsuperscript{36}. These differences were also observed when adults from the three distinct geographical communities were compared, and this was associated with differences in diet since the functional metabolic profiles of the microbiomes also differed significantly.

Genetics, along with environment, are also involved in forming the adult microbial community, such that inter-individual variation is extremely high\textsuperscript{125,126}. Indeed, monozygotic twins have
microbiomes that are more similar to one another than to unrelated individuals or married partners. However, it is difficult to tease apart environmental influences from genetics in these cases. A more recent study identified the “heritability” of taxa, where the gut microbiota was more similar between monozygotic than dizygotic twin pairs. More recently, our team identified that nearly one-third of bacterial taxa are heritable, and that host genetics influenced the relative abundance of certain taxa, including Lachnospira, Rikenellaceae, and Faecalibacterium.

The variations observed between individuals is less relevant when one considers that the function of the gut microbiota is fairly stable and consistent across populations. The ability to perform various functions including fermentation, vitamin synthesis and carbohydrate catabolism is maintained not only among humans but also across Mammalia, suggesting a functional cohesion or microbial redundancy.

1.3.3 Composition and function of the gut microbiota

Colonic bacteria typically belong to one of eight Phyla found in the mammalian gut, with Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria being the main four Phyla. Established by the National Institutes of Health, the Human Microbiome Project (HMP) was initiated to develop the tools and framework necessary to investigate the composition of a ‘normal’ microbiota by sampling 242 individuals at 15 or 18 body sites. However, the idea of a ‘normal’ microbiota representative of the average human was quickly dismissed as a possibility due to the enormous variation between individuals. Moreover, the concept of ‘enterotypes’, the ability to cluster microbiomes based on a certain abundance profile, has also proven to be inaccurate. Analysis of the microbial metagenome by the MetaHIT consortium identified only 18 bacterial species common among the 124 individuals sampled. Likewise, 57 species were found in >90% of the cohort, but their relative abundance varied widely. Instead, wide variation in the gut microbiota must be embraced and controlled for in studies investigating any form of genetic or environmental influence on the microbiota, or when performing microbial manipulation experiments in animal models.

Typically, normal members of the gut microbiota are referred to as “commensals” or “symbionts”. Commensalism occurs when one organism benefits from a relationship without a beneficial or detrimental effect on the other organism, whereas symbiosis occurs when both
organisms reap a beneficial effect from the relationship\textsuperscript{142}. While some members of the microbiota surely are commensals, the microbiota as a whole lives in symbiosis with its host. Indeed, these terms most notably refer to the metabolic function of the gut microbiota.

Metabolically, the gut microbiota is essential since mammals have a limited ability to digest complex polysaccharides, including cellulose, pectin, starches and xylan, found in the diet\textsuperscript{143}. \textit{Bacteroides} species are particularly adapted for this, which possibly explains why they are such predominant colonizers across mammalian species. Bacteria ferment dietary carbohydrates, primarily starches, fibre and other polysaccharides, resulting in the generation of short-chain fatty acids (SCFA) that contribute to host well being\textsuperscript{144}. The main SCFA produced are acetic acid, butyric acid, and propionic acid, all of which are small enough to pass through or be absorbed by intestinal epithelial cells\textsuperscript{145}. It has been estimated that nearly 70\% of the energy needed by the colonic epithelium is derived from butyrate\textsuperscript{146}. Indeed, diets richer in plant materials and fibre result in increased SCFA production. Beyond providing energy to the epithelial barrier, SCFA also induce mucus production and decrease permeability. Colonization of germ-free mice with the butyrate producer \textit{Faecalibacterium prausnitzii} induced mucus production and \textit{Bifidobacterium longum} increased tight junction protein expression, limiting intestinal permeability\textsuperscript{147,148}. SCFA also contribute to colonic homeostasis by increasing both the frequency and suppressive activity of colonic Treg through epigenetic modification\textsuperscript{149}. Indeed, giving mice SCFA supplementation or a high-fibre diet increases the proportion of Treg in the gut and limited colitis in multiple models\textsuperscript{150,151}. Microbially derived metabolites also feed other microbes, known as cross-feeding\textsuperscript{152}.

Glycans derived from the host, such as mucins, are also digested by gut microbes providing a unique source of energy for those capable of its degradation, including \textit{Bifidobacterium} and \textit{Bacteroides} species\textsuperscript{153}. Systemic TLR activation results in increased α(1,2)fucosylation of small intestinal epithelial cells, which can be metabolized by the microbiota\textsuperscript{154}. Genome-wide association studies (GWAS) suggest that individuals with mutations in fucosyltransferase 2 (\textit{FUT2}), the enzyme responsible for fucosylation of intestinal epithelial cells, are at increased risk for Crohn’s disease\textsuperscript{9,155}. Moreover, analysis of the microbiome of individuals with \textit{FUT2} mutations and Fut2-deficient mice identified an altered microbiota, at both the compositional and functional levels\textsuperscript{156}. 
The microbiota also protects the host from invading pathogens via competition for nutrients and niches. Symbiotic microbes induce the expression of a variety of host-derived factors that limit pathogen colonization, including secretory IgA, anti-microbial peptides such as regenerating islet-derived protein 3γ (REGIIIγ), and mucus. Antimicrobial protein lipocalin-2 is released during inflammation and limits iron availability during a bacterial infection. Toll-like receptor (TLR) activation drives IL-22 signalling from innate lymphoid cells to induce fucosylation of the epithelium; metabolized by commensal microbes such as Bacteroides thetaiotaomicron, fucose downregulates pathogen virulence factors during a bacterial infection. Moreover, the presence of microbes results in decreased gut permeability by increasing the expression of tight junction proteins, such as occludin and claudins.

Together, it is clear that microbial metabolism not only provides nutritional benefits to the host but also increases mucosal immunity and the maintenance of a healthy microbial community.

1.3.4 Dysbiosis

The term ‘dysbiosis’ refers to a state of imbalance or altered composition of the microbiota. It has been proposed that a state of dysbiosis occurs when harmful microbes overtake the beneficial ones, which is particularly observed during diseased states, such as IBD, obesity, metabolic disorders and infections. A classic example occurs during Clostridium difficile (C. difficile) infection, where antibiotic-induced alterations of the gut microbiota allow C. difficile to exploit the available niche. Treatment of C. difficile infection involves additional antibiotic treatment, further reducing microbial diversity and resulting in relapsing infections in nearly 65% of patients. It has been postulated that antibiotic depletion of the commensal microbiota results in a build up of nutrients in the lumen, and with reduced competition, C. difficile takes advantage to establish infection within the intestine. Recently, fecal microbial transplantation (FMT) has been used successfully to treat patients with ongoing C. difficile infections. Treatment involves the transfer of fecal microbes from a healthy or unaffected individual to a patient, typically without fully knowing which microbes are being transferred. The effectiveness of FMT is thought to act through direct microbial competition for nutrients and space between the ‘healthy’ population and the pathogen. Similar to the concept of probiotics (live microbes that are beneficial when ingested), further understanding of the gut microbial ecology is required.
in order to design and develop safe and efficient microbial therapeutics to treat *C. difficile* infections.

Dysbiosis in IBD has been characterized as increased abundance of Bacteroidetes and Proteobacteria, with loss of Firmicutes. Moreover, IBD patients exhibit a reduction in total bacterial diversity. Loss of certain beneficial microbes in IBD patients, such as *F. prausnitzii*, has also been associated with dysbiosis. The issue with dysbiosis in IBD is that it is nearly impossible to ascribe causation since microbial community shifts observed during disease are likely linked to treatment regimens and/or the on-going inflammatory state within the gut. Indeed, few studies have identified that an altered microbiome precedes disease onset or causes inflammation. One study attempted to more accurately address this issue by looking at the microbiome of newly diagnosed, treatment naïve paediatric patients. They found CD patients exhibited increased abundance of Enterobacteriaceae, Pasteurellaceae and Fusobacteriaceae, and decreased abundance of Erysipelotrichaceae, Bacteroidales and Clostridiales compared with unaffected controls. These differences were observed in mucosal samples (from the terminal ileum and rectum), but were not well reflected in the stool. Moreover, antibiotic treatment further exacerbated this phenotype with further loss of Erysipelotrichaceae and Clostridiales. Others have also identified a reduction in Clostridiales in IBD, particularly members of Clostridium clusters XIVa and IV. Another study investigated the microbial and metabolic profiles of healthy first-degree relatives of paediatric IBD patients; the authors identified that some healthy relatives displayed microbial dysbiosis, which in some cases was also associated with a perturbed metabolome and increased fecal calprotectin indicating a possible pre-disease, sub-clinical state of inflammation. Clearly, there is a need for prospective studies in healthy susceptible first-degree relatives of IBD patients, such as the Genetics, Environment, Microbial (GEM) project, to help resolve whether changes in the microbial communities precede disease onset in IBD.

In summary, IBD implicates a significant role for the gut microbiota, either in driving or perpetuating chronic relapsing inflammation. While an altered microbiota certainly exists in patients with active IBD, whether a ‘dysbiotic’ microbiome is a cause or an effect of IBD is yet to be determined.
1.3.5 Microbiota stability and resilience to perturbations

The adult gut microbiota is relatively stable over time\textsuperscript{133,175}. However, the microbiota is also dynamic, even within an individual, when responding to perturbations. Changes in diet, exposure to antibiotics, or other environmental stressors can cause a shift in the community structure\textsuperscript{176}. Perturbations of the gut microbiota result in a transient state of dysbiosis, where some community members are lost and others bloom beyond their usual abundance, filling an emptied niche or due to a change in the availability of nutrients. The ability of the microbiota to recover from or withstand such perturbations is referred to as ‘resilience’ of the microbiota and lack of resilience, or failure to fully recover, results in a new steady state for the microbiota\textsuperscript{177,178}.

While the microbiome in adults is generally resilient, the microbiome in children, particularly during the first years of life, is less so and thus more sensitive to stressors affecting the community structure\textsuperscript{120,179}. Multiple factors can influence the gut microbiota in childhood, such as diet, infections, urban vs. rural environment, and antibiotics\textsuperscript{180}. The exact effect of some of these exposures is difficult to ascertain due to their wide variety (i.e. infection or home environment), however the effect of antibiotic exposure on the microbiota in early life has been studied in some detail. One study found that Finnish children exposed to either a course of penicillin or macrolide showed reduced microbial diversity for 1 or 2 years post treatment, respectively\textsuperscript{181}. Another study also identified that antibiotic-treated infants had reduced microbial diversity compared to untreated infants; specifically, the authors found that antibiotic-treated infants had more species where a single strain dominated, whereas untreated infants had a wider variety of strains within each species\textsuperscript{122}. Moreover, community stability over time was higher in infants who never received antibiotics compared to treated infants, in whom short-term stability was particularly decreased around the time of antibiotic treatment. Overall, these findings indicate that antibiotic perturbation of the microbiota in early life can have long-lasting effects on microbial diversity and composition. Several consequences of early life antibiotic perturbation have been proposed for diseases such as asthma\textsuperscript{182,183}, obesity\textsuperscript{184} and IBD\textsuperscript{33}. Indeed, understanding what effect antibiotic treatment has on both the development of the gut microbiota and the mucosal immune system could provide valuable insights into host immune-microbe interactions during early life. Moreover, it may identify potential therapeutic targets for correcting the perturbation.
1.4 Immune-Microbe Interactions in IBD

The mucosal immune system develops in response to the presence of a gut microbiome\textsuperscript{78,185,186}. From birth, the newly colonizing gut microbiota interacts with the gut epithelium and host immune system, influencing development, maturation and regulation, which, in turn, influence the development of the microbiota\textsuperscript{187,188}. Indeed, IBD can be viewed as an imbalance in the bidirectional interactions between immune responses and the gut microbiome in genetically susceptible individuals; however, it is not clear if this is due to an abnormal gut microbiome or an abnormal immune response or both. A better understanding of the mechanisms involved in this bidirectional relationship is essential.

1.4.1 Role of microbes in host immune development

The mucosal immune system functions to distinguish between friend (non-threatening symbiotic bacteria) and foe (pathogenic microbe). The latter requires a protective, often inflammatory response while the former, either no response or a controlled response with minimal collateral damage from inflammation. A major challenge to the mucosal immune system is ensuring an “appropriate” response to the large and diverse population of the commensal gut microbiota\textsuperscript{189}. From birth, the mucosal immune system develops alongside microbial colonization of the gut, influencing its development and regulation, which, in turn, influences the development of the microbiota. The result is a healthy immune response that helps to modulate community structure in the developing gut microbiome.

The critical window for immune development is immediately following birth and during the first year of life. From birth, microorganisms including bacteria, viruses and fungi, colonize humans and animals. These microorganisms stimulate the development of the local and systemic immune system. Lymphoid structures are regulated through microbial colonization, as shown through the use of germ-free mice that possess smaller Peyer’s patches, and reduced or absent isolated lymphoid follicles and cryptopatches\textsuperscript{190-192}. As described, colonization is significantly influenced by both maternal and environmental factors. Pioneering work by Drs. Andrew MacPherson and Kathy McCoy identified that the maternal microbiota influences the immune system of the offspring, possibly through transfer of maternal antibodies loaded with microbial antigens\textsuperscript{115}. 
Moreover, viral colonization and infections can influence the developing immune system\textsuperscript{193}. Work by Kernbauer \textit{et al.} demonstrated that infecting germ-free mice with murine norovirus was sufficient to induce the maturation of mucosal lymphocytes and influence intestinal morphology comparable to that of a mouse colonized with a full microbiota, indicating that the virome is also critically involved in mucosal immune development and homeostasis\textsuperscript{194}.

Innate receptors detect microbe-associated molecular patterns (MAMPs) by germline-encoded pattern-recognition receptors (PRRs) e.g. Toll-like receptors (TLRs)\textsuperscript{195} and NOD-like receptors (NLRs)\textsuperscript{16}. These PRRs regulate first-line host responses but also influence the antigen-specific or adaptive immune responses. Tolerance developed in early life during immune and microbiota co-development is essential for maintaining a homeostatic mucosal environment throughout life\textsuperscript{25}. The concept of a mucosal ‘firewall’ elegantly describes the multiple layers of protection involved in maintaining tolerance and limiting inflammation within the mucosa\textsuperscript{196}. Physically, the epithelial barrier and the mucus layer(s), along with secretory IgA and anti-microbial peptides, limit contact of bacteria to underlying immune cells. If bacteria are able to get close to or through the epithelial layer, intestinal macrophages engulf and kill them; alternatively, dendritic cells phagocytose and transport live bacteria to mesenteric lymph nodes to initiate a local, targeted immune response\textsuperscript{197}. Thus, it is not surprising that perturbation of the gut microbiota can affect immune health. Bacteria interact with the immune system via numerous ligands, including: capsular polysaccharide (PSA), lipopolysaccharide (LPS), peptidoglycan, muramic acid, flagellin and unmethylated CpG motifs of bacterial DNA\textsuperscript{198}. In response to these bacterial ligands, cytokines are produced that shape the differentiation of the adaptive immune system, including T cells.

The mucosal lamina propria and associated lymphoid tissue contains the largest proportion of T cells in the body. Human and animal studies show that T cells are involved in both the induction and regulation of mucosal inflammation\textsuperscript{199,200}. T cells discriminate foreign from self-Ag via the T cell receptor (TCR)\textsuperscript{201}. Elimination of self-reactive T cells in the thymus during development is essential for prevention of autoimmune disease. Extra-thymic T cell development contributes to the T cell repertoire, particularly in the intestine\textsuperscript{202}. Indeed, gut immune maturation depends on colonization, as germ-free mice possess severely depleted mucosal immune development\textsuperscript{185,203}. Studies of germ-free mice have highlighted the essential roles microbes play in immune, epithelial and metabolic development of the host\textsuperscript{204-206}. 
Using gnotobiotic mice, researchers have begun to understand the profound effects of microbial stimulation on immune development\textsuperscript{186}. Specifically, studies have determined that IgA and germinal center formation are strongly linked to gut colonization with a diverse microbiota\textsuperscript{54,207-209}. Phenotypic differentiation of T cells is also coordinated through microbial sensing, demonstrated, for example, by development of Th17 cells in response to segmented filamentous bacteria (SFB) in mice\textsuperscript{58}. Moreover, microbial components, such as polysaccharide A (PSA) or cocktails of Clostridia species are potent inducers of regulatory T cells\textsuperscript{210-213}. Chung et al. colonized germ-free mice with either human- or mouse-derived microbiota\textsuperscript{214}. Human vs. mouse colonization resulted in different recipient microbial community profiles, which induced different numbers and transcriptomic profiles of mucosal T cells\textsuperscript{214}. Furthermore, gut bacteria heavily influence Treg cell development and may be of particular importance in preventing bacterial driven mouse models of colitis\textsuperscript{215}. We are just beginning to appreciate the influence of microbial colonization on the modulation of mucosal B and T-cell development and function. Understanding the mechanisms involved and the impact of host genetics on this process will provide opportunities for personalized treatment of IBD patients.

1.4.2 Animal models of IBD

The constant exposure of the intestinal tissue to gut microorganisms maintains the mucosa in a state of minimal "physiological inflammation", which balances tolerogenic and pro-inflammatory responses to maintain homeostasis. IBD is thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host. Mouse models of intestinal inflammation have facilitated investigations of the role of host–microorganism interactions on the development and regulation of disease. These include pathways involved in the maintenance of intestinal epithelial barrier integrity, the promotion of protective and tolerant immune responses within the intestinal mucosa and regulation of the microbiota\textsuperscript{216}.

Over one hundred animal models of colitis exist\textsuperscript{217,218}, including those that are genetically driven, chemically-induced or immune mediated, all of which are represented in mouse models of colitis. Genetically driven models include the interleukin-10 (IL-10) deficient mouse, whose inability to produce IL-10 results in uncontrolled inflammation in the gut\textsuperscript{219,220}, the SAMP1/YitFc mouse, which develops spontaneous ileitis\textsuperscript{221}, the TRUC mouse, whose
deficiency in both T-bet and Rag2 results in exacerbated TNFα responses and a colitogenic microbiota, and the Mdr1a<sup>−/−</sup> mouse, which lacks P-glycoprotein 170, resulting in increased gut permeability, microbial translocation and colitis development. Chemically induced models involve oral or rectal administration of a compound that induces epithelial damage, allowing for microbial translocation and immune activation. These include administration of dextran sulfate sodium (DSS), Peroxicam, or 2,4,6-trinitrobenzene sulfonic acid (TNBS). Models that are driven primarily by immune activation include the T cell transfer model, where transfer of naïve T cells into a lymphopenic recipient results in wasting disease that can be prevented by co-transfer of regulatory T cells and anti-CD3ε monoclonal antibody model, which induces acute small intestinal mucosal damage through T cell activation and a cytokine storm. Infections that drive intestinal inflammation, including Citrobacter rodentium or rotavirus, are also models used to study the inflammatory response in the gut. Interestingly, many mouse models of colitis fail to develop disease under germ-free conditions, indicating that the inflammatory response or perpetuation of the inflammation is driven in large part by the microbes.

Although mutations in the NOD2 gene represent the strongest genetic link to Crohn’s disease, Nod2-deficient mice do not spontaneously develop colitis. Work in our laboratory has shown that Nod2<sup>−/−</sup> T cells show no overt functional defect in terms of proliferative and suppressive function and cytokine production. However, there are conflicting results on whether Nod2<sup>−/−</sup> mice are more or less susceptible to various models of intestinal inflammation (Table 2.1). Ramanan et al. suggested that Nod2<sup>−/−</sup> mice harboring B. vulgatus had increased Piroxicam-induced small intestinal damage. This correlates with our laboratory’s recent finding that Nod2<sup>−/−</sup> mice had delayed epithelial recovery and prolonged small intestinal mucosal damage following intraperitoneal injection of anti-CD3ε mAb. Conversely, we were unable to identify a difference in susceptibility to T cell transfer colitis using Nod2<sup>−/−</sup> T cells, and Amendola et al. identified a protective effect of Nod2 deficiency in 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis. These findings suggest that Nod2 may play a more significant role in modulating small intestinal rather than colonic inflammation. On the other hand, Natividad et al. showed that Nod1<sup>−/−;Nod2<sup>−/−</sup></sup> (NOD-DKO) mice have increased epithelial permeability, which leads to an exacerbated response to DSS colitis. The response to infection by bacteria, viruses, and parasites also varies significantly in Nod2-deficient mice. Therefore, under the appropriate conditions, there could be a perpetuated inflammatory response in the gut related to the Nod2...
mutation. Proper control of environmental variables, including the use of heterozygous derived littermate mice to equalize the microbiota and early life environmental exposures between WT and Nod2-deficient mice, will help to better elucidate the role of Nod2 in intestinal homeostasis and inflammation.

**Table 1.1: Susceptibility of Nod2-deficient mice to models of intestinal inflammation.**

<table>
<thead>
<tr>
<th>Model of Intestinal Inflammation</th>
<th>Susceptibility in Nod2-deficient mice</th>
<th>Inflammatory Response</th>
<th>Location of Inflammation</th>
<th>Use of Littermates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3ε mAb</td>
<td>Increased</td>
<td>Increased IL-17A and MPO</td>
<td>Small Intestine</td>
<td>Yes</td>
<td>235</td>
</tr>
<tr>
<td>Peroxicam</td>
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<td>Increased IFN-γ</td>
<td>Small Intestine</td>
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<tr>
<td><em>Citrobacter rodentium</em></td>
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<td>Decreased IL-17A</td>
<td>Cecum</td>
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<td>239</td>
</tr>
<tr>
<td>T cell Transfer</td>
<td>No difference or Reduced</td>
<td>None or Decreased IFN-γ</td>
<td>Colon</td>
<td>No</td>
<td>18, 234</td>
</tr>
<tr>
<td>Dextran sulfate sodium (DSS)</td>
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<td>Increased IL-6</td>
<td>Colon</td>
<td>No</td>
<td>237, 240</td>
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<tr>
<td>2,4,6-trinitrobenzene sulfonic acid (TNBS)</td>
<td>Reduced</td>
<td>Decreased IL-17A</td>
<td>Colon</td>
<td>No</td>
<td>236</td>
</tr>
</tbody>
</table>

1.5 **Antibiotic Influence on the Microbiota and Chronic Disease Development**

The invention of antibiotic therapy revolutionized medicine. First discovered by Dr. Alexander Fleming in 1929, penicillin and its derivatives have become the most prescribed antibiotic treatments worldwide. Amoxicillin, in particular, is the most prescribed antibiotic to infants and young children, with over 18 million children in the United States taking at least one course in 2010. Like penicillin, the mechanism of action of amoxicillin involves inhibiting bacterial cell wall synthesis during replication. As such, it is effective against Gram-positive and negative species and is classified as a broad-spectrum antibiotic. Amoxicillin is readily absorbed, reaching peak concentrations in the serum within 1-2 hours and passes into breast milk. While there is no doubt that antibiotics have saved millions of lives since their creation, the impact that they have on the symbiotic relationship between a host and its microbiota must be investigated. A better understanding of how antibiotic treatment alters the composition and function of the microbiota is essential, particularly for the purposes of elucidating just how microbial perturbations alter disease susceptibility.
1.5.1 Effect of antibiotic treatment on the gut microbiota

By definition, antibiotics are “against life”, particularly microbial life, and there is substantial evidence describing the effect of antibiotic treatment on the microbiota in humans and animal models (reviewed in 246). The numerous classes of antibiotic, each with differing mechanisms of action, each affecting different classes of bacteria, combined with the vast variation of microbial composition prior to treatment means that specific effects of antibiotics on the bacterial ecosystem of a microbiome are complex and may be difficult to ascertain. One study characterized the variation in response to a short course of amoxicillin in six healthy individuals and found that one of the six subjects maintained an altered microbiome two months after perturbation 247; comparatively, another study found that a short course of ciprofloxacin resulted in long-term changes in all three subjects, but each subject displayed a unique recovery response 177. As described, the gut microbiota has significant influence over the activity and status of the mucosal immune system, and thus alterations in these microbial signals likely influences the immune system. Indeed, Morgun et al. described how antibiotic depletion of the murine microbiota resulted in drastic changes in the ileal transcriptome, specifically due to three factors: reduction of microbes, expansion of antibiotic-resistant microbes and a direct effect of the antibiotic on the host tissue 248. Reduction of microbes most notably altered gene expression within the lamina propria and these genes were predominantly associated with T and B cell immunity, as well as antigen presentation. Together this indicates that signals from the gut microbiota to the mucosal immune system are modulated by the presence and composition of the microbiota. Thus, antibiotic-induced alterations of these signals could have profound effects on the development and function of immune cells within the lamina propria.

1.5.2 Antibiotic use and chronic disease development

Currently, we are experiencing antibiotic overuse in our society. In the United States, antibiotics were prescribed in more than 20% of all pediatric ambulatory visits 249. A Canadian study revealed that 43% of all children born in British Columbia received antibiotics during their first year of life 250. Exposure to antibiotics during the ‘critical window’ of early life has been associated with increased risk in developing asthma, obesity and IBD.
1.5.2.1 Asthma

Asthma is a chronic inflammatory disease in the lung, where the airways narrow making it difficult to breathe. Like many autoimmune diseases, asthma incidence has increased dramatically over the last 50 years\textsuperscript{251}. In 2011, two epidemiological reviews identified an association between early life antibiotic use and subsequent asthma development\textsuperscript{183,252}. In an attempt to model this in mice, researchers at the University of British Columbia treated mice from birth until weaning with vancomycin and subsequently induced asthma. Vancomycin-treated mice displayed exacerbated allergic asthma with increased eosinophils and elevated systemic IgE compared to untreated controls\textsuperscript{253}. The effect of the antibiotic was specific to early life exposure, as exclusive \textit{in utero} exposure did not cause exacerbated asthma\textsuperscript{254}. More recently, one study emphasized that antibiotic exposure in association with respiratory infections in early life resulted in childhood asthma\textsuperscript{182}, and another found that infants born by C-section were at increased risk of developing asthma, especially if one of the parents has allergies\textsuperscript{255}. Furthermore, analysis of the Canadian Health Infant Longitudinal Development (CHILD) study identified 4 bacterial genera, including \textit{Faecalibacterium}, \textit{Lachnospira}, \textit{Veillonella} and \textit{Rothia}, which were decreased during the first four months of age in children at-risk of developing asthma\textsuperscript{256}. Therefore, early life microbial composition is important for ‘educating’ the immune system within the lung and antibiotic treatment during this ‘critical window’ seems to cause heightened, pro-inflammatory status of the lung leading to asthma in genetically susceptible individuals.

1.5.2.2 Obesity

Obesity is a widespread epidemic, with nearly 2 billion adults and 41 million children being overweight or obese (World Health Organization, 2014). Obesity is the excessive accumulation of fat in the body and has been associated with both genetic and microbial factors\textsuperscript{257}. The use of low-dose antibiotic therapy to increase mass gain in livestock has been used for more the 50 years by the agricultural industry\textsuperscript{258}. Mechanistic insight into this phenotype was recently described in mice that received sub-therapeutic antibiotic treatment for 7 weeks, starting from weaning\textsuperscript{259}. Antibiotic-treated mice displayed increased adiposity, altered metabolism and an altered gut microbiota compared with untreated controls. Follow-up studies have further identified that the altered microbial community in early life is responsible for enhanced growth and obesity development\textsuperscript{260,261}. Analysis in human populations has also identified a link between
early life antibiotic exposure and increase body mass index in children\textsuperscript{184,262-264}. Together, this indicates that antibiotic alterations of the microbiota during early life can have profound, lifelong effects on metabolism, increasing the risk for development of metabolic disorders such as obesity and type 2 diabetes\textsuperscript{265}.

1.5.2.3 **Inflammatory Bowel Diseases**

The roles of environmental factors that influence the microbiota are becoming increasingly relevant for understanding the pathogenesis of IBD. The effects of antibiotic treatment on the microbiota are significant and long lasting\textsuperscript{177}. Several studies have investigated the influence of antibiotic exposure on IBD development. Pediatric IBD patients were more likely to have been exposed to antibiotics in their first year of life than controls\textsuperscript{33}. Indeed, antibiotic exposure during the first year of life led to a 5.5 fold increased risk for IBD development\textsuperscript{266}. Furthermore, the effects of the antibiotics were found to be cumulative and age-dependent, where initial antibiotic exposure at an older age (>5 years old) was associated with decreased risk\textsuperscript{32}. Additionally, two groups have retrospectively identified a comparable trend in adult IBD patients, where antibiotic exposure 2-5 years prior to diagnosis was associated with increased risk\textsuperscript{267,268}. The mechanism of this increased susceptibility related to antibiotic exposure is unknown. Since the commensal microbiota is significantly involved with the inflammatory flares in IBD patients, and an environmental trigger is thought to initiate disease progression, one can imagine that alteration of the gut microbiota, particularly during the ‘critical window’ of early life, could have substantial effects on the susceptibility to developing IBD.
1.6 Goals of the Thesis Project

Antibiotic use in both early life and adulthood has been correlated with subsequent IBD development, but the underlying mechanism is unknown. I hypothesize that the effect of antibiotic perturbation is two-fold: firstly, antibiotic treatment will alter the community structure of the gut microbiota, and secondly, the altered gut microbiota will influence the mucosal immune system resulting in increased susceptibility or severity of colitis. A greater understanding of just how the host mucosal immune system responds to antibiotic-induced perturbations of the microbiota is required. Moreover, the influence of host genetics, particularly those associated with host-microbe interactions and tagged as ‘high risk’ for IBD, on the resilience of the microbiota is poorly understood.

The main goals of this thesis project were to:

1. Determine the effect of a course of broad-spectrum antibiotic on the adult murine microbiota, the resilience of the microbiota, and the influence of an altered gut microbiota on colitis susceptibility, while delineating the role for Nod2 in this response.

2. Investigate the effect of antibiotic exposure during early life on the development of the gut microbiota, the adaptive mucosal immune system and susceptibility to colitis, while elucidating a role for the IBD susceptibility gene, *Nod2*. 
Chapter 2

Nod2 modulates the gut microbiota and immune responses following antibiotic exposure in adult mice in association with increased intestinal IgA levels

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2.1 ABSTRACT

The cause of inflammatory bowel disease (IBD) is unknown but appears to involve genetic susceptibility, environmental triggers and alterations of the gut microbiota leading to altered local immune responses. The strongest genetic association with Crohn’s disease is with the cytosolic microbial receptor Nod2. Since environmental perturbations such as antibiotics induce transient shifts in microbiota composition, we sought to determine whether Nod2 plays a role in antibiotic-induced dysbiosis and resilience of the microbiota, and whether this leads to an altered immune response. Antibiotics altered the microbiota of adult WT and Nod2−/− mice, with delayed microbial recovery observed in Nod2−/− and Nod2+/− derived littermates. Antibiotics increased fecal IgA concentration in Nod2−/− but not WT mice, which was correlated with several bacterial taxa including Erysipelotrichaceae. While antibiotic treatment did not alter colitis severity using the DSS model, prior antibiotic exposure significantly reduced IL-17 responses in Nod2−/− mice following T cell-induced small intestinal mucosal damage. Transfer of fecal supernatant containing IgA also ameliorated the anti-CD3 induced IL-17 response in water-treated Nod2−/− mice. Together, our data suggest that Nod2 plays a role in the recovery from (and immune response to) perturbations of the gut microbiota and this altered composition influences subsequent immune responses.
2.2 INTRODUCTION

Symbiosis of a host and their microbiota has become a key focus in understanding the fine balance between health and disease. The mammalian gut typically harbours \( \sim 4 \times 10^{13} \) bacteria\(^{40} \), along with viruses, fungi, bacteriophages and other micro-eukaryotes, in a dynamic community whose structure varies depending on the physical and chemical environment and host genetics\(^{36,93,131,133,175} \). Inflammatory bowel disease (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), is a multifactorial disease for which the etiology remains unclear. Currently, the most widely accepted hypothesis is that genetically susceptible individuals experience an environmental perturbation resulting in an inappropriate immune response directed against the commensal gut microbiota\(^{6,269} \). Patients with IBD possess an altered microbial community compared to healthy controls\(^{171,172,270,271} \); however, whether an altered microbiota induces inflammation or vice versa, remains a “chicken and egg” conundrum. Interestingly, several of the strongest genetic risk associations with IBD are involved in host-microbe interactions, including nucleotide-binding oligomerization domain-containing protein 2 (NOD2), autophagy related 16 like 1 (ATG16L1), and interleukin 23 receptor (IL23R)\(^{9,272,273} \).

Nod2 is a cytosolic pattern recognition receptor that recognizes muramyl dipeptide, a component of the bacterial cell wall\(^{14} \). NOD2 mutations are associated with ileal Crohn’s disease location\(^{13,274} \). Recently, work from our laboratory has identified that Nod2 knockout mice show exacerbated small intestinal mucosal damage and delayed epithelial recovery following T cell driven small intestinal damage induced by anti-CD3 injection\(^{235} \). Together, this suggests a role for Nod2 in small intestinal homeostasis.

Genetic risk alone is not sufficient to explain the rapid increase in IBD over the last five decades. There is strong evidence suggesting that the Western lifestyle and environment also plays an important role, indicated by the significant increase in IBD incidence in offspring of immigrants from low-incidence countries born in high-incidence areas\(^{29} \). Among environmental factors one might consider diet, altered hygiene and antibiotic (over)use. In the past decade, antibiotic exposure has been associated with increased risk of developing asthma, obesity, and multiple sclerosis\(^{183,184,252,262,264,275,276} \). Retrospective epidemiological studies have also identified a correlation between antibiotic use (both in early life and adulthood) and IBD.
development. The mechanism by which antibiotic exposure alters susceptibility to colitis remains unknown.

Here, we investigated the effect of antibiotic treatment of adult mice on the gut microbiota composition and susceptibility to colitis, and whether Nod2 plays a role in these changes including microbial resilience. We found that adult mice deficient in Nod2 had prolonged microbial perturbation following antibiotic exposure and this was associated with elevated stool IgA levels. The altered microbiota had no effect on DSS-induced colitis but significantly influenced mucosal IL-17 responses and mucosal healing responses in the small intestine after anti-CD3 induced enteropathy. Moreover, transfer of fecal IgA was sufficient to ameliorate the IL-17 response in Nod2−/− mice following anti-CD3. Together, these data highlight how antibiotic treatment can alter the gut microbiota and influence intestinal homeostasis, and provides further evidence for a regulatory role of Nod2 in the small intestine on this process. These results also highlight the important therapeutic potential of IgA for IBD patients.
2.3 METHODS AND MATERIALS

Mice
Wild-type (WT), Nod2\(^{-/-}\), Nod2\(^{floox/floox}\) and Nod2\(^{ΔIEC}\) (all on a C57BL/6 background) were bred in-house under specific pathogen-free conditions. All mice were housed on the same rack within the facility and received normal chow (Teklad\textsuperscript{©} irradiated Rodent Chow #2918) and autoclaved drinking water (pH~7) \textit{ad libitum}. Nod2\(^{-/-}\) mice were originally obtained from Dr. J.P. Hugot (Hôpital Robert Debré, Université Paris Diderot, Paris, France)\textsuperscript{278} and Nod2\(^{floox/floox}\) mice were obtained from Dr. P. Rosentiel (Institute for Clinical Molecular Biology, Christian-Albrechts-University, Kiel, Germany)\textsuperscript{279}. WT and Nod2\(^{-/-}\) mice were maintained separately (referred to as separate strains) and then crossed to generate Nod2\(^{+/−}\) littermates (depicted in Fig 2.3A). For littermate experiments, mice were genotyped for Nod2 expression at weaning (3 weeks old) and then caged by sex and genotype. Experiments began when mice were 8 weeks old. All experiments were performed in accordance with University of Toronto’s Department of Comparative Medicine approved animal use protocols.

Antibiotic treatment
Antibiotic (Abx) treated mice received amoxicillin (BioShop) at 200 mg/L in autoclaved drinking water (pH~7) from day 0-7, followed by autoclaved drinking water from day 8 to endpoint. Abx water was refreshed every other day. Water treated mice received autoclaved drinking water for the duration of all experiments. Water consumption was monitored and all mice drank \textasciitilde4-5 mL/day, irrespective of treatment.

Fecal sampling and bacterial DNA isolation
Fecal samples were collected weekly from each mouse at the same time of day. Samples were stored at -80\(^{°}\)C until DNA isolation. Bacterial DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen). The following modifications were performed: samples were first homogenized in 1.2 mL RLT buffer and 0.7 g of 0.7 mm garnet beads (MoBio) for 3 min on the Disruptor Genie\textsuperscript{®} (Scientific Industries, Inc.). Homogenate was then transferred into a new O-ring tube with 1 g of 0.1 mm glass beads (MoBio), and further homogenized for 8 min on the Disruptor Genie\textsuperscript{®} to physically disrupt the bacterial cell wall. The resulting suspension was further processed as per the manufacturer’s recommended protocol.
Bacterial DNA was used to assess stool bacterial load by quantitative PCR for the gene encoding RNA polymerase beta subunit (\textit{rpoB}) as previously described\textsuperscript{99}. Since \textit{rpoB} is expressed in a single gene copy per bacterium, it is a more accurate measure of total bacterial load than 16S ribosomal gene, which exhibits differences in gene copy number between bacterial species and strains.

\textbf{16S sequencing and analysis}

The V4 hypervariable region of bacterial 16S ribosomal DNA (16S rDNA) was sequenced in paired-end modus (2 x 150 base pair) on the MiSeq platform (Illumina) using primers 515F/806R\textsuperscript{280}. The resulting paired reads were assembled using PANDAseq v 2.7 to generate an amplicon size of 250 base pairs\textsuperscript{281}. Sequences were processed by the quantitative insights into microbial ecology (QIIME v1.8.0) pipeline using default parameters\textsuperscript{100}. The mean number of quality-controlled reads was $72760 \pm 33893$ (mean $\pm$ SD) per mouse. Assembled reads were demultiplexed and processed by QIIME v1.8.0 pipeline using the default parameters\textsuperscript{100}. Chimeric sequences were identified \textit{de novo}, reference based, and then removed using usearch\textsuperscript{61}\textsuperscript{282}. The non-chimeric sequences were then clustered into operational taxonomic units (OTUs) at 97.0\% sequence similarity using a closed reference-based picking approach with UCLUST software against Greengenes database 13\_8 of bacterial 16S rRNA sequences\textsuperscript{102}. After rarefaction at 10,000 reads per sample, bacterial alpha diversity was estimated using the Chao1 diversity index. Samples with fewer than 10,000 reads after quality filtering were removed from the analysis. OTUs with a prevalence <5\% were removed from the analysis. Analyses using R software v2.14.1 were restricted to merged OTUs with the same taxonomic assignment. A paired Student’s t-test was applied to compare the relative abundance of taxa and alpha diversity differences in microbial profiles in WT and \textit{Nod2}\textsuperscript{−/−} mice, and between antibiotic and untreated groups. Associations between bacterial taxa and groups were considered to be significant after a false-discovery rate (FDR) correction of the p-value (q < 0.05). Beta diversity was assessed using Bray-Curtis dissimilarity distances. Procrustes analyses based on Bray-Curtis distance was applied to compare microbial orientation differences following antibiotic treatment and a Monte Carlo simulation was performed using 10,000 permutations on the first five dimensions of the PCoA. Microbial community similarity between the different groups of mice was addressed by performing an ANOSIM test with 10,000 permutations on the beta diversity metrics as described above.
**Concentration of IgA and lipocalin-2 in stool**

Stool samples were collected and stored at -80 °C until processing. Briefly, samples were homogenized in PBS with 0.01% Tween-20 at 100 mg/mL, normalized to stool weight. Samples were homogenized for 8 minutes in the Disruptor Genie®, and spun at 10,000 xg for 8 minutes. Supernatants were harvested immediately and stored at -80 °C. Total IgA (Bethyl Laboratories) and lipocalin-2 (R&D Systems) concentration in the stool supernatant was measured by ELISA following manufacturer's recommended protocol.

**Dextran sulfate sodium (DSS) colitis**

3.5% (w/v) of DSS (MW: 36,000-50,000; MP Biomedical) was dissolved in autoclaved drinking water (pH~7) and given *ad libitum* for 5 days, followed by 3 days of water. Body weight was recorded and stool was collected on day 0, 3 and 5. Tissue was collected on day 8. Mouse colonic tissue was fixed with 10% formalin and embedded in paraffin. Four-micrometer sections were stained with Hematoxylin and eosin (H&E). Histopathology was scored by a blinded pathologist as previously described283,284. Briefly, a combined score of tissue damage, degree of inflammation and inflammatory cell depth was determined as follows: Tissue damage: score 0, no mucosal damage; 1, minor architectural changes and/or small erosions; 2, moderate damage, with loss of crypts in large areas; 3, severe or extensive ulceration and loss of mucosa; Degree of inflammation: 0, none; 1, mild; 2, moderate; 3, severe inflammation; Inflammatory cell depth: score 0, occasional inflammatory cells in the lamina propria (LP); 1, increased infiltrate in the LP predominantly at the base of crypts; 2, confluence of inflammatory infiltrate extending into the mucosa; 3, transmural extension of infiltrate.

**Anti-CD3 model of small intestinal mucosal damage**

On day 35, mice received an intraperitoneal injection of 50 µg of anti-CD3ε monoclonal antibody (clone: 145-2C11, Biolegend). Mice were monitored for clinical signs for enteropathy, including appearance of coat, activity level, and signs of diarrhea or dehydration. Tissues were collected 72 hours post injection. A 1 cm piece of medial small intestine was used for tissue cytokines and myeloperoxidase quantification, as described below. An adjacent 1 cm piece was stored in RNAlater® (Thermo Fisher Scientific) at -80 °C and used for mRNA analysis. The
remaining small intestinal tissue was fixed in 10% formalin and embedded in paraffin. Four-micrometer sections were stained with Hematoxylin and eosin (H&E). Histopathology was scored as previously described\textsuperscript{227,285}.

**Tissue cytokines and myeloperoxidase**

Quantification of the cytokines interleukin 17 (IL-17) and interferon-gamma (IFN\(\gamma\)) and myeloperoxidase (MPO) in small intestinal tissue was assessed using ELISA kits (R&D Systems). Briefly, a 1 cm piece of medial small intestine was harvested into 500 \(\mu\)L of PBS with protease inhibitors in a O-ring tube with ceramic beads (MoBio) and stored at 4 \(^\circ\)C until homogenization. The tissue was homogenized for 60 seconds at 5.0 m/s using a FastPrep-24\textsuperscript{®} (MP Biomedical) homogenizer. Resulting suspension was spun at 10,000 xg for 5 minutes at 4 \(^\circ\)C, supernatants were harvested immediately and stored at -80 \(^\circ\)C.

**Host mRNA analysis and qPCR**

RNA was extracted from a 1 cm piece of medial small intestine using AllPrep DNA/RNA Mini kit (Qiagen). Synthesis of cDNA was performed using qScript cDNA SuperMix (Quanta Biosciences), as recommended by the manufacturer. cDNA was then used at a 1/10 dilution in qPCR reactions.

Samples were normalized internally using the average cycle quantification (Cq) of TBP, GUSB, and rpl19, simultaneously. Real time assays were run on a Bio-Rad C1000 Touch Thermal Cycler. Expression data are expressed as relative values after Genex macro analysis (Bio-Rad).

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
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<td>CAA-CGG-TGC-AGT-GGT-CAG-AG</td>
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<tr>
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<td>TFG(\beta)</td>
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<td>AGT-AGA-CGA-TGG-GCA-GTG-GCT</td>
</tr>
<tr>
<td>IL-1(\beta)</td>
<td>TGT-CTT-TCC-CGT-GGA-CCT-TC</td>
<td>TCA-TCT-CGG-AGC-CTG-TAG-TG</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACA-AAG-CCA-GAG-TCC-TTC-AG</td>
<td>TGG-ATG-GTC-TTG-GTC-CTT-AG</td>
</tr>
</tbody>
</table>
**Bacterial Transfer and qPCR**

Bacteria were isolated from mouse intestines on modified reinforced clostridial media under anaerobic conditions. Four species in the Clostridia XVI group (*Erysipelotrichaceae* family, *Allobaculum* spp. see table below) were sub-cultured for 48 h in modified tryptone yeast glucose broth, and then combined. Sodium bicarbonate (1%) was added to the combined inoculum and 500 µL was loaded into syringes with disposable gavage needles. Loaded syringes were individually sealed in sterile bags, and then sealed into double-bags. Gavage was within 30 min of mixing cultures and within 20 min of leaving the anaerobic chamber.

Each 48-h culture looked similarly turbid (by eye). A 1:5 dilution of the gavage mixture gave an OD A600 of 0.264 (1.32 at full concentration).

**Table 2.1: Bacterial species used in transfer experiments**

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Read length (bp)</th>
<th>% Similarity</th>
<th>Gut compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Catenisphaera adipatacculans</em></td>
<td>815</td>
<td>91</td>
<td>Colonic mucosa/contents</td>
</tr>
<tr>
<td><em>Faecalitalea cylindroides</em></td>
<td>904</td>
<td>99</td>
<td>Cecum</td>
</tr>
<tr>
<td><em>Clostridium innocuum</em></td>
<td>962</td>
<td>99</td>
<td>Distal colon</td>
</tr>
<tr>
<td><em>Eubacterium dolichum</em></td>
<td>899</td>
<td>96</td>
<td>Colonic mucosa/contents</td>
</tr>
</tbody>
</table>

Confirmation of transfer was performed by qPCR on bacterial DNA isolated from stool on day 22 (24 h post gavage), compared to the relative abundance on day 0 and 7, using universal and group-specific 16S rRNA primers. The primer sequences used were: *Eubacteria* sense: ACT-CCT-ACG-GGA-GGC-AGC-AGT, *Eubacteria* antisense: ATT-ACC-GCG-GCT-GCT-GGC, *Clostridium XIVa* sense: ACT-CCT-ACG-GGA-GGC-AGC, *Clostridium XIVa* antisense: GCT-TCT-TAG-TCA-GGT-ACC-GTC-AT, *Erysipelotrichaceae cluster XVI* sense: ACC-TGC-GGT-GCA-TTA-GYT-GG, *Erysipelotrichaceae cluster XVI* antisense: GCA-TYG-CTC-GTT-CAG-GCT-TGC. Relative abundance values were determined by normalizing the ΔCt for each target group to the total 16S rRNA gene copy number (using the universal Eubacteria housekeeper).

**Statistical analysis**
Results are depicted as mean ± SEM, unless otherwise indicated. Statistical analysis was performed using two-way ANOVA with post hoc Tukey or Holm-Šídák test, or Mann-Whitney $U$ test, as specified. Differences between groups were considered significant when $p < 0.05$. For all statistical comparisons, *$P<0.05$, **$P<0.01$ ***$P<0.001$, ****$P<0.0001$, and ns means no significance. Calculations were performed using GraphPad Prism 7.0 software (GraphPad, La Jolla, CA).
2.4 RESULTS

2.4.1 Nod2 plays a role in microbiota resilience after antibiotic exposure

The microbiota begins to develop in utero\textsuperscript{113,114}, with birth providing the maternally derived foundation\textsuperscript{118}. From birth, environmental exposures drive the development of intestinal microbial composition (reviewed in \textsuperscript{287}). In humans, the microbiota is highly malleable until about 3 years of age\textsuperscript{36,122}, at which point it becomes more stable and resembles the adult. The adult microbiota is relatively stable and extremely resilient to short term perturbations\textsuperscript{133,177}. In mice, the foundational microbiota is set at birth with an adult-like composition achieved by 3-4 weeks of age\textsuperscript{288}. Mice are considered to have a fully mature microbiota by 6-8 weeks of age. As such, we sought to investigate how a single course of a broad-spectrum antibiotic at 8 weeks of age would influence the adult murine microbiota, and whether \textit{Nod2} genotype would influence this perturbation. Amoxicillin is the most prescribed antibiotic worldwide, particularly to paediatric patients\textsuperscript{243}. As a broad-spectrum beta-lactam, it is effective against both Gram-positive and -negative species. We extrapolated that a typical human paediatric dose given during infection (e.g. otitis media) of 50 mg/kg/day, would be equivalent to 200 mg/L for an average adult mouse weighing 20 g and drinking \textasciitilde 5 mL/day.

Separate strain wild-type (WT) and \textit{Nod2}\textsuperscript{-/-} mice were given either water or antibiotic treatment (Fig 2.1A). Pre-treatment stool samples were collected on day 0. While stool bacterial load, quantified by copies of the gene encoding the beta subunit of RNA polymerase (\textit{rpoB}) per gram of stool, did not differ between mouse strains at day 0 (Fig 2.1B), microbial composition was significantly different (Fig 2.1C,D). Since the starting composition between the separate strain WT and \textit{Nod2}\textsuperscript{-/-} mice differed significantly, microbial changes after treatment were only compared within the same genotype, as strain differences at day 0 could account for differences in response to the antibiotic treatment.

Stool samples at day 7 were collected to assess the maximal perturbation of the microbiota due to antibiotic exposure, and recovery from the perturbation was assessed at day 21. Each mouse was compared to its pre-treatment composition (day 0) and water-treated controls.
Figure 2.1: Separate strain *Nod2*<sup>−/−</sup> mice have a significantly different microbiota than WT mice at homeostasis.

A. Experimental strategy. WT and *Nod2*<sup>−/−</sup> mice received either water or antibiotic (Abx) treatment for 7 days followed by 4 weeks of water to allow for microbial recovery. Arrows indicate time points of stool collection for sequencing (black) and induction of mucosal damage (red).
B. Copies of RNA polymerase B (rpoB) per gram of stool (n=5 per genotype, water; n=10-12 per genotype, Abx).
C. Microbial composition at the Phylum level on day 0.
D. Relative abundance of the five main phyla at day 0 in separate strain WT (gray) and Nod2⁻/⁻ (black) mice.
Data are presented as mean ± SEM (B, D) or mean (C). *P<0.05, **P<0.01; by two-way ANOVA and post hoc Tukey test (B), Kruskal-Wallis (C), or Mann-Whitney U test (D). (WT n=15, Nod2⁻/⁻ n=16. Data are from five independent experiments).
Using 16S rDNA sequencing, microbial diversity was evaluated for species richness (as measured by the Chao1 rarefaction index) and beta-diversity (as measured by Bray-Curtis dissimilarity, which factors in presence/absence and relative abundance of operational taxonomic units (OTUs)). Antibiotic exposure resulted in a significant decrease in bacterial load (Fig 2.1B) and species richness at day 7 (Fig 2.2A), while water treatment had no effect on either measure in WT or *Nod2*<sup>−/−</sup> mice. Moreover, antibiotic exposure resulted in a significant shift in bacterial composition from water-treated controls (Fig 2.2B, middle panel ‘Day 7’), with a bloom in Bacteroidaceae and Enterobacteriaceae (Gammaproteobacteria) and a loss of Clostridiales and Erysipelotrichaceae in both WT and *Nod2*<sup>−/−</sup> mice (Fig 2.2C). Thus, the murine adult microbiota are significantly altered by a seven-day course of amoxicillin treatment.

We were also interested in the resilience, or recovery, of the microbiota following antibiotic exposure, and whether Nod2 influenced this resilience. To allow sufficient recovery time, we collected stool on day 21 (Fig 2.1A), 14 days after removal of the antibiotic-supplemented water, for 16S sequencing. At day 21, the microbiota of Abx-treated WT mice resembled that of water-treated WT controls, in terms of bacterial load (Fig 2.1B), species richness and composition (Fig 2.2A-C). Conversely, stool microbial composition of Abx-treated *Nod2*<sup>−/−</sup> mice at day 21 remained significantly different from the water-treated controls and from day 0 pre-treatment composition (Fig 2.2A-C). Specifically, *Nod2*<sup>−/−</sup> mice failed to recover to pre-treatment species richness, due in large part by the failure of Clostridiales and Erysipelotrichaceae to recover (Fig 2.2C). In order to determine which bacterial taxa contributed to the differences both statistically and biologically, we utilized linear discriminant analysis (LDA) effect size (LEfSe) analysis<sup>107</sup>. Comparison of the composition of Abx-treated *Nod2*<sup>−/−</sup> stool at day 0 vs. 21 revealed that several taxa were lost after antibiotic exposure, including Erysipelotrichaceae, Clostridium and Desulfovibrionaceae, and these failed to recover by day 21 (Fig 2.2D). Furthermore, there was a maintained elevation in the abundance of Bacteroidaceae following its bloom at day 7 (Fig 2.2C,D). Together, this suggests that Nod2 plays a role in the recovery from perturbations of the microbiota in response to a course of antibiotics.
Figure 2.2: Nod2<sup>−/−</sup> mice show prolonged microbial disruption following antibiotic perturbation.

A. Species richness at day 0, 7, and 21 as measured by the Chao1 rarefaction index.
B. Bray-Curtis Dissimilarity principal coordinate plots for each time point. Each dot represents one mouse. (ANOSIM, WT + Abx d0 vs. 7: R=0.7617 p=0.001; Nod2<sup>−/−</sup> + Abx d0 vs. 7: R=0.5875 p=0.002; WT + Abx d0 vs. 21: R=0.0823 p=0.121; Nod2<sup>−/−</sup> + Abx d0 vs. 21: R=0.2891 p=0.004).
C. Bacterial composition at the Family level of bacterial taxonomy in Abx-treated mice over time. Statistical differences in taxa abundance between days, as identified by Kruskal-Wallis with a false-discovery rate (FDR) corrected p<0.05, are indicated in the table. Differences in WT composition are marked with a *, and Nod2<sup>−/−</sup> differences are marked with an #.
D. Bar plot of the taxonomic differences identified by linear discriminant analysis (LDA) coupled with effect size (LEfSe) between Abx-treated Nod2<sup>−/−</sup> mice on day 0 (taxa enriched on day 0, green) and 21 (taxa enriched on day 21, red).

Data are presented as mean ± SD (A) and mean (C). Statistical analysis by paired Student’s t test (A), ANOSIM (B), and Kruskal-Wallis (C). (WT: water n=7, Abx n=8; Nod2<sup>−/−</sup>: water n=10, Abx n=7. Data are from five independent experiments).
2.4.2 Nod2-deficient mice show prolonged microbial disruption following antibiotics as compared to littermates

Due to significant differences in microbial composition between the separate strain WT and Nod2$^{-/-}$ mice at day 0, we assessed the response and recovery from antibiotics in littermates where the starting stool microbial composition is the same. We generated Nod2$^{+/+}$ littermates by crossing a Nod2$^{-/-}$ dam with a WT sire (Fig 2.3A). Using the F1 generation of Nod2$^{+/+}$ littermates as breeders, litters were composed of Nod2$^{+/+}$ (WT), Nod2$^{+-}$ (HET) and Nod2$^{-/-}$ mice; all receiving the same maternally derived microbiota. At weaning, pups were caged by sex and Nod2 genotype. All mice received autoclaved drinking water (pH~7) and normal chow ad libitum. Mice were housed in identical conditions on the same rack in the same room of the animal facility to minimize differences in environmental influence on the microbiota. At 8 weeks of age, stool samples were collected for 16S rDNA sequencing analysis. The analysis showed that Nod2 genotype was not associated with any difference in the microbiota in terms of beta-diversity, species richness or community composition (Fig 2.3B-D).

WT and Nod2$^{-/-}$ littermates were then given either water or antibiotic treatment, as previously described (Fig 2.1A), and stool samples were collected at day 0, 7, and 21. Antibiotic treatment shifted the microbial community away from water-treated controls, and had significant influence on the species richness and composition (Fig 2.4A-C) in both WT and Nod2$^{-/-}$ littermates at day 7. This was characterized by loss of Erysipelotrichaceae, Clostridiales and Lactobacillaceae, with corresponding increases in Enterobacteraceae and Bacteroidaceae (Fig 2.4C); similar to what was observed in the separate strains. LEfSe analysis identified differences in the microbial composition between Abx-treated WT and Nod2$^{-/-}$ littermates at day 7 (Fig 2.5A). Specifically, Abx-treated Nod2$^{-/-}$ mice had an increased abundance of the Epsilon-Proteobacteria, Helicobacteraceae, compared to Abx-treated WT mice (Fig 2.5A,B). Furthermore, Abx-treated Nod2$^{-/-}$ mice had a higher abundance of Bacteroidaceae but less Enterobacteriaceae on day 7 compared to Abx-treated WT littermates (Fig 2.5C). Since there were no significant differences between WT and Nod2$^{-/-}$ mice at day 0, the differences in the microbial response to the antibiotic treatment suggest that Nod2 plays a role in regulating the changes within the microbiota during perturbation with a broad-spectrum antibiotic.
Figure 2.3: Nod2 genotype does not influence the murine gut microbiota at homeostasis.

A. Generation of Nod2+/− littermate colony to normalize the microbiota.

B. PCoA of Bray-Curtis Dissimilarity between Nod2+/− derived littermates at 8 weeks of age by genotype (ANOSIM, WT vs. Nod2−/−: R= -0.058 p=0.918).

C. Species richness of 8-week-old littermates as measured by the Chao1 rarefaction index.

D. Microbial composition at the Family level of Nod2+/− derived littermates.

Data are presented as mean ± SD (C) or mean (D). Statistical analysis by ANOSIM (B). (WT n=22, HET n=18, Nod2−/− n=22. Data are from five litters).
Figure 2.4: *Nod2*−/− derived littermates also show prolonged disruption following antibiotic treatment.

A. PCoA of Bray-Curtis Dissimilarity for each time point. Each dot represents one mouse. (ANOSIM, WT + Abx d0 vs. 7: R=0.6778 p=0.001; Nod2−/− + Abx d0 vs. 7: R=0.7922)
p=0.001; *WT + Abx d0 vs. 21* R=0.1494 p=0.013; **Nod2−/− + Abx d0 vs. 21** R=0.2144 p=0.001).

B. Species richness at day 0, 7, and 21 as measured by the Chao1 rarefaction index.

C. Changes in bacterial composition, at the Family level, over time in antibiotic-treated WT and Nod2−/− mice. Statistical differences in taxa abundance between days are indicated in the table. Differences in WT composition are marked with a *, and Nod2−/− differences are marked with an #.

Data are presented as mean ± SD (B) and mean (C). *P<0.05, **P<0.01 ***P<0.001; by ANOSIM (A), paired Student’s t test (B) and Kruskal-Wallis (C). (WT n=7, Nod2−/− n=7, WT + Abx n=11, Nod2−/− + Abx n=13. Data are from three independent experiments).
Recovery of the microbiota following antibiotic treatment was then assessed on day 21. Unlike the separate strain mice, both Abx-treated WT and $Nod2^{-/-}$ mice maintained a significantly different microbiota composition from genotype-matched water-treated controls (Fig 2.4A, right panel). Moreover, species richness at day 21 was significantly lower than pre-treatment richness for both genotypes (Fig 2.4B). LEfSe analysis comparing the microbiota at day 0 vs. 21 of Abx-treated $Nod2^{-/-}$ mice further identified several taxa that differed between the time points (Fig 2.5D, right). Taxa that were enriched at day 0 (and reduced or absent at day 21) included: Erysipelotrichaceae and Rikenellaceae, Odoribacteraceae and Alphaproteobacteria (Fig 2.5D, right panel, green bars). Taxa enriched at day 21 (which were in significantly lower abundance at day 0) included: Gamma-Proteobacteria, Desulfovibrionaceae, and Bacteroidaceae (Fig 2.5D, right panel, red bars). While many of these taxa changes were also observed in Abx-treated WT mice (Fig 2.5D, left), there were differences between the genotypes (Fig 2.5D, right panel, bolded taxa). Specifically, at day 21, Abx-treated $Nod2^{-/-}$ mice were enriched for the Gammaproteobacteria Enterobacteriaceae and had lost the family of Mogibacteriaceae compared to WT mice. These data suggest that Nod2 influences both the changes in microbes in response to antibiotic treatment, and the resilience, or recovery, of the microbiota.
Figure 2.5: Differential microbiota perturbations in Nod2<sup>+/−</sup> mice compared to WT littermates.
A. Bar plot of the taxonomic differences identified by linear discriminant analysis (LDA) coupled with effect size (LEfSe) between Abx-treated WT (taxa enriched in WT, green) and Nod2−/− (taxa enriched in Nod2−/−, red) mice on day 7.

B. Relative abundance of Helicobacteriaceae in Abx-treated WT and Nod2−/− mice on day 7.

C. Relative abundance of Bacteroidaceae and Enterobacteriaceae families in Abx-treated WT and Nod2−/− mice over time.

D. Bar plot of the taxonomic differences identified by LEfSe between Abx-treated WT (left) and Nod2−/− (right) mice on day 0 (taxa enriched on day 0, green) and 21 (taxa enriched on day 21, red). Bolded taxa are statistically different between Abx-treated WT and Nod2−/−. Data are presented as mean ± SEM (B, C). *P<0.05, **P<0.01 ***P<0.001; by Mann-Whitney U test (B) or two-way ANOVA and post hoc Tukey test (C). (WT + Abx n=11, Nod2−/− + Abx n=13. Data are from three independent experiments).
2.4.3 Nod2 plays a role in the anti-commensal IgA response

The composition of the intestinal microbiota is influenced by many host-derived factors, including mucus, anti-microbial peptides and innate and adaptive immune responses. Antibiotic exposure had no effect on the expression of the mucin Muc2, or the anti-microbial peptide RegIIIγ in small intestinal tissue on day 7 in either WT or Nod2−/− mice (Fig 2.6A,B). Furthermore, expression of cytokines TGFβ, IL1β, IL4, and IL6, all associated with immune activation, were not significantly altered by antibiotic treatment in either genotype (Fig 2.6C-E). Together, this suggested that there were no overt changes in mucus or major cytokine responses due to antibiotic treatment.

Since IgA plays a critical role in limiting bacteria from interacting with the epithelial barrier, and the antibiotic treatment changed the community structure of the microbes in the lumen, we therefore investigated the effect of antibiotic exposure on intestinal IgA responses. As such, we measured total IgA concentration in stool on day 0, 7, and 21. Baseline levels of total IgA in stool (on day 0) were not different between separate strain WT and Nod2−/− mice, or between Nod2−/− derived littermates (Fig 2.7A,B). However, Nod2−/− mice (both separate strain and littermates) had a significant increase in IgA concentration on day 7 of antibiotic treatment (Fig 2.7A,B). WT mice (separate strain and littermates) and water-treated mice showed no increase in IgA at day 7 (Fig 2.7A,B). By day 21, antibiotic treated Nod2−/− mice no longer showed any difference in IgA compared to water treated Nod2−/− or WT mice (Fig 2.7A,B), indicating that the increase was short lived.

Both WT and Nod2-deficient mice experienced significant perturbations of their microbiota with antibiotic exposure, but only Nod2-deficient mice displayed the strong IgA response at day 7. Antibiotic exposure did not increase intestinal permeability as measured by FITC-dextran concentration in serum (Fig 2.7C) or induce an increase in lipocalin-2 (Fig 2.7D), a neutrophil-derived anti-microbial peptide and marker of intestinal inflammation, at day 7. Moreover, no overt changes in stool frequency or consistency were observed with the antibiotic treatment. To investigate whether the IgA response was due to the absence of Nod2 signalling within the epithelial barrier, we used a conditional knockout mouse specific for the intestinal epithelium (Nod2flx/flx x Villin-cre)235,279. Nod2flx/flx and Nod2ΔIEC littermate mice were treated with antibiotic or control water.
Figure 2.6: Antibiotic treatment has no effect on mucus, anti-microbial peptide or cytokine mRNAs within the small intestine of littermates. A 1 cm piece of medial small intestine was harvested on day 7 of the antibiotic treatment. RNA was isolated and mRNA transcript expression was quantified using quantitative RT-PCR. Expression was normalized using three reference genes simultaneously: TBP, GUSB, and rpl19. No differences in expression were observed between genotypes or treatments for:

A. Muc2
B. RegIIIγ
C. TGFβ
D. IL1β
E. IL4
F. IL6 mRNA in the small intestine at day 7.

Data are presented as mean ± SEM. (WT n=5, WT + Abx n=8, Nod2⁻/⁻ n=5, Nod2⁻/⁻ + Abx n=10. Data are from 2-3 independent experiments).
Stool samples were collected on day 0, 7 and 21 from separate strain and littermate mice. Concentration of IgA and/or lipocalin-2 within the stool supernatant was quantified.

A. Stool IgA response over time in water and antibiotic-treated separate strain WT and Nod2\(^{-/-}\) mice. (WT n=10, WT + Abx n=16, Nod2\(^{-/-}\) n=13, Nod2\(^{-/-}\) + Abx n=16).

B. Stool IgA response over time in water and antibiotic-treated Nod2\(^{+/-}\) derived littermates. (WT n=8, WT + Abx n=13, Nod2\(^{+/-}\) n=8, Nod2\(^{-/-}\) + Abx n=16).

C. No difference in intestinal permeability measured by levels of FITC-dextran in the serum at day 7. Each dot represents a mouse. (Nod2\(^{-/-}\) n=5, Nod2\(^{-/-}\) + Abx n=5)

D. No increase in lipocalin-2 concentration in stool at day 7. Each dot represents a mouse. (WT n=9, Nod2\(^{-/-}\) n=8, WT + Abx n=10, Nod2\(^{-/-}\) + Abx n=12.)
E. Stool IgA response in water and antibiotic-treated $Nod2^{flox/flox}$, $Nod2^{ΔIEC}$ and $Nod$-DKO on day 7. Each dot represents a mouse. ($Nod2^{flox/flox}$ $n=6$, $Nod2^{flox/flox}$ + Abx $n=7$, $Nod2^{ΔIEC}$ $n=12$, $Nod2^{ΔIEC}$ + Abx $n=7$, $Nod$-DKO $n=8$, $Nod$-DKO + Abx $n=12$.)

Data are presented as mean ± SEM. **P<0.01 ****P<0.0001; by two-way ANOVA and post hoc Tukey test (A, B) or Mann-Whitney $U$ test (C, E). (Data are from 2 (C, D), 3 (E) or 5 (A,B) independent experiments).
Neither $\text{Nod}2^{\text{flox}}/\text{flox}$ nor $\text{Nod}2^{\Delta\text{IEC}}$ displayed increased IgA in response to antibiotic exposure, although the variation between samples could suggest that the data is underpowered (Fig 2.7E). However, antibiotic treatment of $\text{Nod}1^{+/+};\text{Nod}2^{-/-}$ double knockout ($\text{Nod-DKO}$) mice phenocopied the IgA response of the Nod2-deficient mice (Fig 2.7E). Together, this suggests that Nod2 signalling within non-epithelial cells may regulate the IgA response observed following antibiotic perturbation.

We next investigated the relationship between the changes in bacterial taxa and the changes in stool IgA concentration. Each mouse had microbial sequencing data for three time points (day 0, 7 and 21) and corresponding stool IgA concentrations; therefore, the relative abundance of individual taxa were compared against stool IgA concentration. In order to identify associations between bacterial taxa and IgA concentration a Spearman correlation was performed; both WT and $\text{Nod}2^{-/-}$ mice were included in the correlation, as were all three time points for each mouse, since IgA was only increased at day 7 in Abx-treated $\text{Nod}2^{-/-}$ mice. Analysis of separate strain WT and $\text{Nod}2^{-/-}$ mice identified nine taxa that were significantly associated with fecal IgA concentration (Table 2.1). In analyzing the WT and $\text{Nod}2^{-/-}$ littermates, 17 taxa were identified as significantly associated with IgA levels (Table 2.2). Interestingly, of the two taxa that were positively associated with increased IgA concentration in littermates, one was Helicobacteraceae (Fig 2.8A), which was significantly increased in abundance in $\text{Nod}2^{-/-}$ mice compared to WT littermates (Fig 2.5A,B). Comparing the significant IgA-associated taxa between the separate strain and littermate mice, five taxa associations were replicated in both sets of experiments: $\text{Prevotella}$, $\text{Akkermansia}$, Erysipelotrichaceae, $\text{Sutterella}$, and Christensenellaceae (Table 2.1,2.2, Fig 2.8B). The five taxa all decreased in abundance as IgA concentration increased, resulting in a negative Spearman correlation coefficient, r (Fig 2.8C). A bi-plot of the littermate microbial composition as it related to the IgA concentration was generated in order to visualize the association of the various taxa with the IgA response (Fig 2.8D). Each data point represents the microbial composition of a mouse and its corresponding IgA concentration; the data are coloured by treatment (water (black) vs. Abx (pink)). Superimposed are the bacterial taxa at the family level (gray spheres) as they associate with the IgA response. The size of the sphere represents the mean relative abundance of the taxa and location indicates which taxa are influencing the clustering pattern between samples. The clustering pattern observed in the bi-plot corroborated the individual taxa-IgA associations identified by Spearman correlation.
Table 2.2: Correlated bacterial taxa to IgA level in separate strain WT and Nod2^{-/-} mice

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Spearman Correlation r</th>
<th>p value</th>
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Table 2.3: Correlated bacterial taxa to IgA level in littermate WT and Nod2^{-/-} mice

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Spearman Correlation r</th>
<th>p value</th>
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Figure 2.8: Increased IgA due to antibiotic treatment is associated with changes in bacterial taxa.

A. Helicobacteraceae and Enterobacteriaceae were positively associated with increased fecal IgA concentration by Spearman correlation in littermates. Each dot represents a mouse at a single time point.
B. Venn diagram of IgA-taxa correlation similarities between littermates and separate strains.

C. Genera that were negatively associated with fecal IgA concentration by Spearman correlation. Each dot represents a mouse at a single time point.

D. Bi-plot displaying littermate microbial composition as compared to fecal IgA concentration. Each dot represents a mouse at a single time point; dots are coloured by treatment (water in black; Abx in pink). Bacterial families are shown in grey spheres and labeled. Size of the sphere represents the mean relative abundance and location indicates which taxa are influencing the clustering pattern between samples.

Data are presented as mean ± 95% confidence intervals (A, C). Spearman correlation coefficients, r, and corrected p-values are displayed on each plot. (n=128 XY pairs, where 1 pair is microbiota data and corresponding fecal IgA concentration from one mouse at a single time point).
Together, our data strongly suggest that antibiotic-induced perturbations and recovery of the microbiota are associated with the day 7 IgA responses observed in *Nod2*−/− mice.

2.4.4 Antibiotic exposure in adults fails to increase susceptibility to DSS colitis

Antibiotic exposure has been associated with IBD development; however, the mechanism remains unclear. Given that a seven-day course of antibiotics had long-lasting impact on the gut microbiota of Nod2-deficient mice, we assessed whether this perturbed microbiota altered susceptibility to and/or severity of experimental colitis. Colitis was induced on day 35, 4 weeks after removal of antibiotic supplemented water (Fig 2.1A); both WT and *Nod2*−/− antibiotic treated mice showed no difference in microbiota composition between day 21 and 35 (Fig 2.9). Water and antibiotic-treated WT and *Nod2*−/− littermates were subjected to dextran sulphate sodium (DSS) supplemented drinking water for 5 days to induce colitis. DSS was removed on day 5 and mice returned to normal drinking water until experimental endpoint on day 8. Individual body weight was recorded and level of lipocalin-2 (Lcn-2) in stool was measured on day 0, 3 and 5 of DSS treatment. DSS exposure resulted in significant weight loss, which was not altered by *Nod2* genotype or antibiotic exposure (Fig 2.10A). Lipocalin-2 concentration in stool was also significantly increased in DSS-treated mice, but no differences were identified between genotypes or antibiotic exposure (Fig 2.10B). Colon length and pathological assessment of colitis score further identified no effect of antibiotic exposure on susceptibility or severity of DSS colitis in WT or *Nod2*−/− littermate mice (Fig 2.10C-E).

2.4.5 Antibiotic perturbation alters T-cell induced small intestinal mucosal damage in *Nod2*−/− mice

Recently, we identified that mice deficient in Nod2 displayed prolonged mucosal damage and delayed epithelial regeneration following T-cell induced small intestinal enteropathy. We therefore investigated whether the *Nod2*−/− mice previously exposed to antibiotics would show an altered response to T cell induced small intestinal mucosal damage. On day 35, water and antibiotic-treated WT and *Nod2*−/− mice were given a single intra-peritoneal injection of 50 µg of anti-CD3 monoclonal antibody (mAb), and mucosal damage was assessed 72 hours post injection.
Figure 2.9: Antibiotic-treated mice show no difference in microbiota composition between day 21 and 35.

A. PCoA of Bray-Curtis Dissimilarity of antibiotic-treated littermate mice at day 21 vs. 35 for WT + Abx (left) and Nod2\(^{-/-}\) + Abx (right). Each dot represents one mouse. (ANOSIM, WT + Abx d21 vs. 35: R=0.0507 p=0.324; Nod2\(^{-/-}\) + Abx d21 vs. 35: R=0.0557 p=0.246).
Figure 2.10: Antibiotic exposure fails to increase susceptibility to DSS colitis.

On day 35, colitis was induced using DSS. Water and antibiotic-treated WT and Nod2<sup>−/−</sup> littermates were weighed and stool collected on day 0 (day 35 from start of experiment), 3 and 5 of DSS exposure.

A. Percent of initial weight over time.
B. Lipocalin-2 concentration in stool over time.
C. Colon length, measured on day 8.
D. Colitis score based on pathology.
E. Representative photographs at 50x magnification of H&E stained sections of the colon on day 8. Data are presented as mean ± SEM (A-D). (Control (Nod2−/− mice that did not receive Abx or DSS treatment) n=9, WT n=5, Nod2−/− n=5, WT + Abx n=6, Nod2−/− + Abx n=7. Data are from two independent experiments).
As observed previously, *Nod2*-/- mice displayed exacerbated mucosal damage compared to WT mice, characterized by heightened interleukin-17A (IL-17A), interferon gamma (IFNγ) and myeloperoxidase (MPO) levels in the small intestine as measured by ELISA (Fig 2.11C). Additionally, *Nod2*-/- mice had significantly elevated expression of pro-inflammatory cytokine mRNAs *IL1β* and *IL6* compared to WT at 72 hours post i.p. injection of anti-CD3 (Fig 2.11D). While previous antibiotic exposure had no effect on the pathology that developed in WT mice, antibiotic-treated *Nod2*-/- mice displayed a significant decrease in T cell induced enteropathy as compared to water-treated *Nod2*-/- controls (Fig 2.11A-B). Moreover, antibiotic-treated *Nod2*-/- mice produced significantly less IL-17A, IFN-γ and MPO than water-treated *Nod2*-/- mice (Fig 2.11C). Previous antibiotic treatment decreased these responses in *Nod2*-/- mice to levels indistinguishable from that of WT mice. Analysis of the mRNA profile of the small intestine identified significantly reduced levels of *IL1β* and *IL6* expression in antibiotic-treated *Nod2*-/- mice, compared to water-treated *Nod2*-/- mice (Fig 2.11D), suggesting an overall reduction in the inflammatory response and improvement in mucosal healing. This difference was also observed in the *Nod2*+/- derived littermates. Water-treated *Nod2*+/- mice had exacerbated levels of IL-17 and MPO, which was significantly reduced if the mice had received antibiotics four weeks prior (Fig 2.11E). Expression of *IL1β* and *IL6* was also significantly reduced in antibiotic-treated *Nod2*+/- mice compared to water-treated controls (Fig 2.11F). WT mice showed no difference in small intestinal damage in response to anti-CD3 mAb between water or antibiotic treatment. Collectively, these data emphasize the important role of Nod2 in microbial sensing and regulation of the immune response in the small intestine.

### 2.4.6 Erysipelotrichaceae failed to increase the IL17a response to anti-CD3

Antibiotic exposure transiently alters the gut microbiota in humans\footnote{\textsuperscript{177}}, however, the microbiota is resilient, returning to pre-treatment composition shortly after removal of the antibiotic\footnote{\textsuperscript{177,178}}. Analysis of the resilience of the microbiota in both separate strain and littermate *Nod2*-/- mice revealed a common pattern of loss of the family Erysipelotrichaceae following antibiotic exposure. This Gram-positive anaerobic family of Firmicutes is a common member of the mammalian gut microbiota, but is relatively unknown functionally\footnote{\textsuperscript{293}}. Recently, Palm et al. identified a member of the Erysipelotrichaceae family to be highly IgA-coated in both mice and IBD patients, as determined by IgA-SEQ\footnote{\textsuperscript{53}}.
Figure 2.11: Antibiotic perturbation decreases the response to anti-CD3 induced mucosal damage in Nod2−/− mice.
On day 35, mice received a single intraperitoneal injection with 50µg of anti-CD3 mAb to induce T cell driven small intestinal mucosal damage.

A. Pathology scoring at 72 h post injection of anti-CD3 mAb.

B. Representative photographs at 10x magnification of H&E stained sections of the small intestine 72 hours post-injection.

C. Cytokine response in small intestinal tissue: IL-17A, IFN-γ and MPO.

D. *IL1b* and *IL6* mRNA expression in small intestinal tissue. (WT n=9, *Nod2*−/− n=13, WT + Abx n=12, *Nod2*−/− + Abx n=11. Data are from three independent experiments.)

E. IL-17 and MPO response in small intestinal tissue of littermates.

F. *IL1b* and *IL6* mRNA expression in small intestinal tissue of littermates. (WT n=4, *Nod2*−/− n=6, WT + Abx n=6, *Nod2*−/− + Abx n=7. Data are from two independent experiments.)

Data are presented as mean ± SEM. *P<0.05, **P<0.01 ***P<0.001; by two-way ANOVA with post hoc Holm-Šídák test (A, C-F).
Moreover, transfer of these bacteria (along with nine other highly IgA-coated bacterial species) into germ-free recipients exacerbated the response to DSS-induced colitis. Since Erysipelotrichaceae was significantly reduced in relative abundance in antibiotic-treated Nod2\(^{-/-}\) mice, which were then protected from the prolonged mucosal damage following anti-CD3 injection, we hypothesized that returning the bacteria to pre-treatment concentration would ‘rescue’ the heightened IL17A response to anti-CD3. As such, Nod2\(^{-/-}\) mice received control or antibiotic-treated water for seven days as previously. On day 21, mice were gavaged twice, six hours apart, with 150 \(\mu\)L of a cocktail of four bacterial species, all members of the Erysipelotrichaceae family cluster XVI (Fig 2.12A). After 24 hours, stool was collected to look for the bacteria and mice were injected with 50 \(\mu\)g of anti-CD3 mAb i.p. At day 0, no differences were observed in Erysipelotrichaceae abundance between water and antibiotic-treated Nod2\(^{-/-}\) littermates (Fig 2.12B), as previously observed. Abundance of Clostridium cluster XIVa was measured as a control since the Erysipelotrichaceae family includes the closely related Clostridium cluster XVI. On day 7 of antibiotic treatment abundance of both Clostridium XIVa and Erysipelotrichaceae declined significantly (Fig 2.12B). Additionally, fecal IgA concentration was significantly increased (Fig 2.12C), and correlation between fecal IgA concentration and Erysipelotrichaceae abundance was strongly negative (Fig 2.12D). On day 22, 24 hours following the second bacterial gavage, abundance of Erysipelotrichaceae was not different between water and antibiotic-treated mice (Fig 2.12B), however, fecal IgA concentration was increased in mice who received the bacterial gavage (Fig 2.12C). Following anti-CD3 injection, antibiotic-treated Nod2\(^{-/-}\) mice had a significant reduction in IL17A and MPO levels in the small intestine compared to water-treated littermates, however the bacterial gavage of Erysipelotrichaceae had no effect on either measure (Fig 2.12E). While Erysipelotrichaceae is significantly decreased in antibiotic-treated Nod2\(^{-/-}\) mice and could play a role in the IgA response, adding it back to the microbiota alone and in the timeline described was not sufficient to ‘rescue’ the IL17A response in Nod2\(^{-/-}\) mice with previous antibiotic exposure.
Figure 2.12: Erysipelotrichaceae failed to increase the IL17a response in antibiotic-treated Nod2−/− mice.

A. Experimental outline. Mice were treated as described in Fig 2.1A. On day 21, a subset of Abx-treated Nod2−/− mice received 2 oral gavages of bacteria (indicated by the black arrow). The next day (day 22) all mice were injected with anti-CD3 mAb. Tissues were harvest on 72 hrs post injection.
B. Relative abundance of Clostridium XIVa and Erysipelotrichaceae as measured by quantitative PCR on bacterial DNA isolated from stool.

C. IgA concentration in stool supernatant over time.

D. Spearman correlation between Erysipelotrichaceae and IgA concentration in stool. Each dot represents one mouse at one time point. Spearman correlation coefficient, r, and corrected p-value are displayed on the plot.

E. IL17A and MPO response in small intestinal tissue 72 hrs post i.p. anti-CD3 mAb. Data are presented as mean ± SEM (B, C, E) and mean ± 95% confidence interval (D). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; by two-way ANOVA and post hoc Tukey test (B, C) or Holm-Šidák test (C), or Mann-Whitney U test (E). (Water n=7, Abx n=9, Abx + Bac Gav. n=21. Data are from three independent experiments).
2.5.1 IgA transfer into water-treated \textit{Nod2}^{-/-} mice reduces IL-17A response to anti-CD3

Bacterial adherent IgA has previously been reported to identify “colitogenic” bacteria based on IgA-coating\textsuperscript{53}. Since antibiotic-treated \textit{Nod2}^{-/-} mice had a significant increase in stool IgA concentration at day 7, we sought to determine whether transferring the IgA could protect water-treated \textit{Nod2}^{-/-} mice from T cell induced mucosal damage. \textit{Nod2}^{-/-} mice were treated with water or antibiotic supplemented water as described above, and stool harvested on days 3 to 7 (Fig 2.13A). Stool was homogenized and supernatants containing IgA isolated. Supernatants from individual mice were combined and then orally gavaged into water-treated \textit{Nod2}^{-/-} littermates daily for five days. In order to exclude the possibility of amoxicillin being transferred along with the IgA, supernatants were assessed using an agar diffusion test, which found no anti-microbial activity of water or antibiotic-treated supernatants against \textit{Escherichia coli}, Nissle strain. Similar to our previous observations, stool supernatant from antibiotic-treated \textit{Nod2}^{-/-} mice had significantly increased IgA concentration, compared to water-treated \textit{Nod2}^{-/-} mice (Fig 2.13B). On average, each daily gavage of 100 µL of stool supernatant from antibiotic-treated mice contained approximately 30 µg of IgA, whereas water-treated supernatant contained ~5 µg of IgA. Three days after the last oral gavage, recipient mice were injected i.p. with anti-CD3 mAb and tissues were assessed 72 hours post injection. Transfer of high IgA-containing supernatant from antibiotic-treated \textit{Nod2}^{-/-} mice resulted in a significant reduction in IL-17A in the small intestine of recipient mice (Fig 2.13C). Transfer of supernatant from water-treated \textit{Nod2}^{-/-} mice had no effect on the IL-17A response, compared to untreated \textit{Nod2}^{-/-} mice. These data suggest that the IgA produced in \textit{Nod2}^{-/-} mice in response to antibiotic perturbation of the microbiota is involved in neutralizing bacteria that could later exacerbate an inflammatory response in the small intestine.
Figure 2.13: IgA ameliorates response to anti-CD3 in water-treated Nod2⁻/⁻ mice.

A. Experimental outline. Nod2⁻/⁻ donor mice were treated with control or antibiotic water as described. Stool was collected daily from day 3-7. Supernatant containing IgA was isolated and then gavaged into water-treated Nod2⁻/⁻ littermates. Arrows indicate gavage of stool supernatant containing IgA from water or Abx-treated donors. Three days after the last gavage of SN, recipients received an i.p. injection of anti-CD3 mAb.

B. IgA concentration in stool supernatant from water and antibiotic-treated donors (Donors: Water Nod2⁻/⁻ n=5, Abx Nod2⁻/⁻ n=12). Pre-treatment fecal IgA concentration in recipients was determined on day 3, immediately prior to gavage.

C. IL17A and MPO response in small intestinal tissue after anti-CD3 mAb injection. Data are presented as mean ± SEM (B-C). *P<0.05, ****P<0.0001; by two-way ANOVA with post hoc Holm-Šidák test (B) or Mann-Whitney U test (C). (Recipients: Water-IgA Tx n=5, Abx-IgA Tx n=11. Data are from two independent experiments).
2.6 DISCUSSION

We sought to elucidate the effect of an antibiotic treatment on the composition of the microbiota and whether Nod2, the strongest IBD genetic risk association and a microbial receptor, would influence the response. Our study demonstrates a role for Nod2 in the resilience of the microbiota following antibiotic perturbation and subsequent immune responses.

Our separate strain \textit{Nod2}⁻/⁻ mice had a microbiota congruent to that described for IBD patients, with reduced Firmicutes and elevated Bacteroidetes abundance\textsuperscript{167}. When these mice were treated with a seven-day course of a broad-spectrum antibiotic, Firmicutes were further reduced and Proteobacteria bloomed, a phenotype also seen in IBD\textsuperscript{294}. Others have also suggested that the murine \textit{Nod2}⁻/⁻ microbiota is ‘dysbiotic’, favouring more Bacteroidetes and fewer Firmicutes\textsuperscript{23,240,295}. In some of these studies, an antibiotic treatment was used to reduce the total microbial load in the gut; this required multiple antibiotics (i.e. metronidazole, vancomycin etc.) and a higher dose (1 g/L compared to our 200 mg/L), whereas our study was interested in the effect of the most commonly prescribed antibiotic to paediatric patients, amoxicillin, and used a typical paediatric dose.

Antibiotic exposure transiently alters the gut microbiota in humans\textsuperscript{177}; however, the microbiota is resilient, returning to pre-treatment composition shortly after removal of the antibiotic for most subjects\textsuperscript{177,178}. The altered microbiota in antibiotic-treated \textit{Nod2}⁻/⁻ mice was maintained 14 days after the removal of antibiotic treatment suggesting a lack of resilience when compared to WT mice, which fully recovered to pre-treatment composition. Moreover, microbial resilience was not investigated in the previous studies making this paper the first to elucidate the role of Nod2 in microbial resilience following an antibiotic perturbation.

To further support these findings, we generated a new line of \textit{Nod2}⁺/⁺ derived littermates in order to investigate the specific genetic impact of Nod2 on the microbial response to antibiotic treatment and resilience. Corroborating previous work by our team, there was no effect of \textit{Nod2} genotype on the stool microbial composition between littermates\textsuperscript{296}. Furthermore, despite \textit{Nod2} mutations being the strongest genetic risk association with IBD, no group, including ours, has been able to identify a role for Nod2 in directing the microbiota in healthy (non-inflamed) subjects\textsuperscript{131}.
Similar to what was observed in the separate strain mice, antibiotic-treated WT and $Nod2^{−/−}$ littermates experienced a bloom of Enterobacteriaceae and Bacteroidaceae and a loss of Firmicutes on day 7 of treatment. This is in keeping with previous studies showing opportunistic expansions of these bacterial taxa during antibiotic treatment suggesting this is a typical niche filling response$^{297,298}$. Furthermore, we showed that the abundance of these taxa along with Helicobacteraceae differed between WT and $Nod2^{−/−}$ littermates, suggesting a role for Nod2 in controlling their expansion. A further role for Nod2 was apparent during the recovery from the antibiotic exposure, on day 21, where $Nod2^{−/−}$ mice maintained elevated abundance of Enterobacteriaceae and Desulfovibrionaceae, and reduced Mogibacteriaceae compared to WT littermates and pre-treatment composition. The lack of full recovery in the WT littermates compared to the separate strain WT mice highlights the adaptability of the microbiota, such that responses to perturbations are dynamic and flexible depending on the initial microbiota and other environmental cues. As such, this work reinforces the critical importance of using littermates and controlling for cage effects in microbiota experiments$^{141}$.

We chose to use a clinically relevant antibiotic and dose and found that antibiotic treatment did not alter the epithelial barrier or induce overt inflammation at day 7. Surprisingly, the only immune-mediated difference observed at day 7 between water and antibiotic-treated $Nod2^{−/−}$ mice was a significant increase in fecal IgA concentration. The gut mucosal immune system and the microbiota have an essential symbiotic relationship. Alterations in the community of microbes can influence the development and status of immune cells within the intestine, including plasma cells$^{299}$. IgA, secreted by long-lived plasma cells in the lamina propria, is generated in response to microbial sensing by dendritic cells, which present microbial antigens to germinal centre B cells$^{300}$. IgA levels were not different between WT and $Nod2^{−/−}$ littermates at day 0 or across all time points in water-treated mice and antibiotic-treated WT mice, suggesting the antibiotic-induced changes in the microbiota specifically increased the IgA response. As such, we investigated the relationship between the changes in the relative abundance of bacterial taxa and fecal IgA levels using Spearman correlation and identified five taxa-IgA associations that were replicated between our two $Nod2^{−/−}$ mice colonies (separate strain and littermate). Some of the taxa associated with the IgA response have been identified previously as being highly IgA-coated in mice and IBD patients, including $Prevotella$, Enterobacteriaceae, and Erysipelotrichaceae$^{53}$. Our study is the first to identify an association between Nod2 and mucosal
IgA, providing a new direction for host genetic-microbe interactions involved in IBD that warrants further investigation.

Next, we examined the effects of prior antibiotic exposure on colitis susceptibility and severity. First, the dextran sulphate sodium (DSS) model was utilized to induce colitis but no effect of genotype or treatment was observed. DSS induces colitis through epithelial damage, and it remains possible that the damage induced was too significant for us to detect any small differences between treatment groups. Subsequently, we used the anti-CD3 mAb model of acute T-cell induced small intestinal mucosal damage. *Nod2*<sup>−/−</sup> mice that had received antibiotic treatment four weeks prior to the anti-CD3 injection showed a reduction in the inflammatory response compared to water-treated controls. This phenotype, observed in both our separate strain and littermate *Nod2*<sup>−/−</sup> mouse colonies, indicates that microbial sensing by Nod2 is involved in mucosal healing within the small intestine. Moreover, antibiotic-induced alterations of the microbiota can have long-lasting influence on subsequent immune responses in the gut, particularly in genetically susceptible hosts.

We next attempted to pinpoint which microbe might be involved in the decreased inflammatory response to anti-CD3 mAb. Erysipelotrichaceae, which failed to recover in *Nod2*<sup>−/−</sup> mice, was previously shown to be colitogenic<sup>53</sup>; thus, its absence in the antibiotic-treated *Nod2*<sup>−/−</sup> mice might explain the ameliorated response to anti-CD3-induced inflammation. While the bacterial gavage failed to increase the IL-17A or MPO response to anti-CD3 mAb, the bacterial transfer seemed to induce an IgA response on day 22 (24 hours after the bacterial gavage), and we again detected a strong negative association between Erysipelotrichaceae abundance and fecal IgA concentration in *Nod2*<sup>−/−</sup> mice. This supports previous work identifying Erysipelotrichaceae as a target of luminal IgA.

Knowing that fecal IgA specificity should be predominantly directed toward commensal microbial antigens, we transferred fecal supernatants containing IgA to water-treated *Nod2*<sup>−/−</sup> recipients and then induced small intestinal mucosal damage with anti-CD3 mAb. While the recipients of water-treated fecal supernatants containing low/normal levels of IgA were not different from water-treated *Nod2*<sup>−/−</sup> mice, recipients of the antibiotic induced high-IgA containing supernatant had a decreased IL-17A response, indicating that IgA transfer can alter
the outcome of immune-mediated mucosal damage in the gut. These data emphasize the crucial role of anti-commensal IgA on altering the microbiota, or at least changing the way the immune system senses commensal microbes during an inflammatory response. Together, this hints at an avenue for future investigation between the commensal Erysipelotrichaceae family, sensing by Nod2 and IgA. Indeed, it would be interesting to determine whether IBD patients who have a \textit{NOD2} mutation have an altered IgA response against this (or any other) bacterial family.

Overall, our data identifies a role for Nod2 in the resilience of the microbiota in response to an antibiotic treatment. Antibiotic induced alterations of the microbiota can have long-lasting effects on subsequent immune responses in the small intestine, particularly in genetically susceptible hosts. Moreover, microbiota manipulation via targeted IgA transfer may be a potential future therapeutic for IBD.
Neonatal Antibiotic Exposure Skews The Developing Gut Microbiota And Mucosal T Cell Populations Resulting In Worsened Colitis In Nod2−/− Mice

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3.1 ABSTRACT

Early life represents a ‘critical window’ of development for the gut immune system and the microbiota. From birth, we are colonized with trillions of bacteria and other microorganisms, which influence and co-develop with the mucosal immune system. Antibiotic exposure during early life is associated with development of auto-inflammatory diseases, including Crohn’s disease, but the underlying mechanism remains unclear. We hypothesized that early life antibiotic exposure would have a prolonged impact on both microbiota composition and T cell function within the gut, and that this would influence risk for colitis and possibly severity, particularly in a genetically susceptible host (Nod2−/− mice).

Heterozygous Nod2+/− breeders were used to generate littermates. Dams and littermates received water or amoxicillin [200mg/L] in the drinking water until weaning. Amoxicillin passes through the milk from dam to pups. Fecal samples collected at weaning and at 5 weeks old were analyzed by 16S rRNA sequencing. At 8 weeks old, mucosal lymphocyte populations of the small and large intestine were analyzed via flow cytometry. Colitis was induced at 8 weeks of age by five days of 3.5% DSS in the drinking water.

Neonatal amoxicillin treatment resulted in shifts of the gut microbial community structure at weaning, with a significant reduction of Bifidobacterium and Erysipelotrichaceae and a bloom of Enterobacteriaceae, which was irrespective of Nod2 genotype or sex. At 5 weeks old, antibiotic-treated Nod2−/− mice had reduced microbial diversity and maintained a loss of Bifidobacterium and Erysipelotrichaceae compared to both antibiotic-treated WT mice and water-treated controls. Antibiotic-treated Nod2−/− mice had an increase of Foxp3+, RORγt+, and Foxp3+RORγt+ T cells in the gut lamina propria. These mice also showed worsened DSS colitis compared to WT mice and water treated controls, with increased weight loss, increased levels of fecal lipocalin-2 and greater colonic shortening. Analysis of colonic lymphocytes after DSS identified a significant increase in Gata3+ Tregs and increased IL4, IL6 and IL33 expression.

These results suggest that neonatal antibiotic exposure has lasting effects on microbiota composition and alters microbe-driven immune cell development via Nod2 signalling. Moreover, these cells may be involved in the regulation of colitis and could be potential targets for therapeutics.
3.2 INTRODUCTION

Symbiosis between a host and its microbiota is a complex bidirectional relationship in the gut, one that remains poorly understood but is essential for proper development. Environmental perturbations during the critical window of early life can alter the ‘normal’ development of the gut microbiota, the mucosal immune system or both, leading to an altered state that leaves or makes the host more susceptible to disease.

Mouse and human studies suggest that antibiotic exposure during early life is associated with an increased risk of inflammatory diseases, including asthma, obesity and inflammatory bowel disease (IBD). IBD, including Crohn’s disease (CD) and ulcerative colitis (UC), is thought to arise when genetically susceptible individuals experience an environmental trigger resulting in an altered homeostatic balance between the immune response and the gut microbiota. Indeed, epidemiological observations indicate that early and prolonged antibiotic exposure significantly increases the risk for developing IBD. Although the mechanism by which this occurs is unclear, it is possible that antibiotics induce an imbalance in the gut microbiome that does not fully recover in a genetically susceptible individual. A potentially related idea is that the altered microbiome in early life influences the mucosal immune development, skewing it to a less balanced, more pro-inflammatory state.

Here, we investigated whether early life antibiotic exposure would have a prolonged impact on both microbiota composition and T cell development within the gut, particularly in a genetically susceptible host (Nod2−/− mice), and if this would influence susceptibility to colitis. We found that early life antibiotic treatment significantly shifted the gut microbiota composition away from water-treated controls and that Nod2 plays a role in shaping the microbiota in early life. Moreover, perturbation of the gut microbiota during this critical window altered the microbe-associated mucosal immune cells with increased proportions of Foxp3+ regulatory T cells (Treg), RORγt+ T cells, and RORγt+ Foxp3+ Treg in the small intestine. Antibiotic-treated Nod2−/− mice were more susceptible to DSS colitis, with an altered cytokine milieu including increased expression of the alarmin, IL-33, and favouring expansion of Gata3-expressing T regulatory cells. Together, these data illustrate the broad and long-lasting effects of antibiotic perturbation of the microbiota in early life, and how this can lead to increased susceptibility to colitis in a genetically susceptible host.
3.3 MATERIALS AND METHODS

Mice

Nod2+/– littermates (on a C57BL/6 background) were bred in-house under specific pathogen-free conditions. Breeders were maintained in Heterozygous (Het) x Het crosses, using sister dams whenever possible. Mice received breeder chow (Teklad irradiated Rodent Chow #2919) and autoclaved drinking water (pH~7) *ad libitum*. Mice were genotyped for Nod2 expression at weaning (3 weeks old) and then were caged by sex and genotype. Briefly, genomic DNA was isolated from ear notches using the HotSHOT protocol. PCR amplification of Nod2 was performed using the following primers: Nod2oriA2: AAC-CGC-ATT-ATT-CGA-TGG-GGC, Nod2oriS: GTC-ATT-TCC-TGA-CCT-CTG-ACC, PKGpolyA: GCC-TGC-TCT-TTA-CTG-AAG-GCT-AAG.

All mice were housed on the same rack within the facility and received normal chow (Teklad irradiated Rodent Chow #2918) and autoclaved drinking water (pH~7) *ad libitum* from weaning to endpoint. Mice were weighed weekly to monitor growth. All experiments were performed in accordance with University of Toronto’s Department of Comparative Medicine approved animal use protocols.

Antibiotic treatment

Antibiotic (Abx) treated mice received amoxicillin (BioShop) at 200 mg/L in autoclaved drinking water (pH~7) from one day after birth until weaning (day 21), followed by autoclaved drinking water from weaning to endpoint (Fig 3.1A). Abx water was started once the litter was born so as to not interfere with the colonization birth provides. Bottles were refreshed every other day. Amoxicillin was chosen because it is the most commonly prescribed antibiotic to paediatrics and it passes through the breast milk from the dam to the pups. Water treated mice received autoclaved drinking water for the duration of all experiments. Antibiotic treatment was given in the water to minimize the handling and stressing of the dams and litters. To control for any stress due to changing the Abx water, water treated cages were handled identically, such that the bottle was removed briefly and then replaced.

Fecal sampling and bacterial DNA isolation

Fecal samples were collected weekly from each mouse at the same time of day. Samples were stored at -80 °C until DNA isolation was performed. Bacterial DNA was isolated using the
QIAamp DNA Stool Mini Kit (Qiagen). The following modifications were performed: samples were first homogenized in 1.2 mL RLT buffer and 0.7 g of 0.7 mm garnet beads (MoBio) for 3 min on the Disruptor Genie® (Scientific Industries, Inc.). Homogenate was then transferred into a new O-ring tube with 1 g of 0.1 mm glass beads (MoBio), and further homogenized for 8 min on the Disruptor Genie® to physically disrupt the bacterial cell wall. The resulting suspension was further processed as per the manufacturer’s recommended protocol.

16S sequencing and analysis
The V4 hypervariable region of bacterial 16S ribosomal DNA (16S rDNA) was sequenced in paired-end modus (2 x 150 base pair) on the MiSeq platform (Illumina) using primers 515F/806R. The resulting paired reads were assembled using PANDAseq v 2.7 to generate an amplicon size of 250 base pairs. Sequences were processed by the quantitative insights into microbial ecology (QIIME v1.8.0) pipeline using default parameters. The mean number of quality-controlled reads was 72760 ± 33893 (mean ± SD) per mouse. Assembled reads were demultiplexed and processed by QIIME v1.8.0 pipeline using the default parameters. Chimeric sequences were identified de novo, reference based and then removed using usearch61. The non-chimeric sequences were then clustered into operational taxonomic units (OTUs) at 97.0% sequence similarity using a closed reference-based picking approach with UCLUST software against Greengenes database 13_8 of bacterial 16S rRNA sequences. After rarefaction at 10,000 reads per sample, bacterial alpha diversity was estimated using the Chao1 and Shannon diversity indices. Samples with fewer than 10,000 reads after quality filtering were removed from the analysis. OTUs with a prevalence of <5% were removed from the analysis. Analyses using R software v2.14.1 were restricted to merged OTUs with the same taxonomic assignment. A paired Student’s t-test was applied to compare the relative abundance of taxa and alpha diversity differences in microbial profiles in WT and Nod2−/− mice, and between antibiotic and untreated groups. Associations between bacterial taxa and groups were considered to be significant after a false-discovery rate (FDR) correction of the p-value (q < 0.05). Beta diversity was assessed using Bray-Curtis dissimilarity distances. Procrustes analyses based on Bray-Curtis distance was applied to compare microbial orientation differences following antibiotic treatment and a Monte Carlo simulation was performed using 10,000 permutations on the first five dimensions of the PCoA. Microbial community similarity between the different groups of mice
was addressed by performing an ANOSIM test with 10,000 permutations on the beta diversity metrics described above.

**Concentration of serum immunoglobulins and fecal IgA and lipocalin-2**

Serum was isolated from blood collected by tail vein draw or cardiac puncture on day 56 and stored at -80°C. Concentration of total IgG, IgG1, IgG2a, IgG3 and IgA (Bethyl Laboratories) in sera was measured by ELISA as per the manufacturer’s recommended protocol.

Stool samples were collected and stored at -80°C until processing. Briefly, samples were homogenized in PBS with 0.01% Tween-20 at 100 mg/mL, normalized to stool weight. Samples were homogenized for 8 minutes in the Disruptor Genie®, and spun at 10,000 xg for 8 minutes. Supernatants were harvested immediately and stored at -80°C. Total IgA (Bethyl Laboratories) and lipocalin-2 (R&D Systems) concentrations in the stool supernatant were measured by ELISA following the manufacturer's recommended protocol.

**Lymphocyte isolation and flow cytometry**

Spleens were physically disrupted through a 40 µm cell strainer into RPMI and cells were isolated by centrifugation (380g x 5 min). Red blood cells were lysed via incubation with Ammonium-Chloride-Potassium (ACK) lysis buffer for 5 min.

Isolation of gut lamina propria lymphocytes was performed as previously described\(^{235}\). In brief, the small and large intestine were extracted, opened longitudinally, washed in PBS, and incubated in stripping buffer (PBS, 5 mM EDTA) for 15 min at 37°C on a shaking rotator. Cloudy stripping buffer (containing epithelial cells) was removed and replaced with fresh buffer and incubated for another 15 min. After stripping, the tissue was washed in 1x PBS twice, cut in 0.5-cm pieces, and digested in buffer containing RPMI 1640 medium, 10% FBS, 1% Penicillin/Streptomycin, 2 µg/mL DNase and 2 mg/mL collagenase D (Roche) for 1 h at 37°C on a shaking rotator. Digested material was passed through 100- and 70-µm cell strainers, and the cells were then collected by centrifugation (380 xg x 5 min).

For flow cytometry, cell viability was determined using the LIVE/DEAD Fixable Violet Dead Cell Stain kit (ThermoFisher). Surface staining was performed using anti-mouse: CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), and CD19 (1D3). Cells were stained for 30 min at 4°C.
For detection of intracellular transcription factors, cells were permeabilized with the Foxp3 Fixation/Permeabilization Kit (eBioscience), and Fc receptors were blocked using mouse CD16/CD32 antibody (eBioscience). Cells were then incubated with fluorescently labeled anti–mouse Foxp3 (FJK-16s), RORγt (B2D), and Gata3 (TWAJ) for 30 min. Samples were acquired on a FACSFortessa X20 (BD Biosciences) and analyzed using FlowJo Software (Tree Star).

**Dextran sulfate sodium (DSS) colitis**

3.5% (w/v) of DSS (MW: 36,000-50,000; MP Biomedical) was dissolved in autoclaved drinking water (pH~7) and given *ad libitum* for 5 days to induce colitis starting on day 56 post birth. This treatment was followed by 3 days of water. Body weight data and stool were collected on day 56, 59 and 61; tissue was collected on day 64. Mouse colonic tissue was fixed with 10% formalin and embedded in paraffin. Four-micrometer sections were stained with Hematoxylin and eosin (H&E). Histopathology was scored by a blinded pathologist as previously described\textsuperscript{283,284}. Briefly, a combined score of tissue damage, degree of inflammation and inflammatory cell depth was determined as follows: Tissue damage: score 0, no mucosal damage; 1, minor architectural changes and/or small erosions; 2, moderate damage, with loss of crypts in large areas; 3, severe or extensive ulceration and loss of mucosa; Degree of inflammation: 0, none; 1, mild; 2, moderate; 3, severe inflammation; Inflammatory cell depth: score 0, occasional inflammatory cells in the lamina propria (LP); 1, increased infiltrate in the LP predominantly at the base of crypts; 2, confluence of inflammatory infiltrate extending into the mucosa; 3, transmural extension of infiltrate.

**Host mRNA analysis and qPCR**

RNA was extracted from a 1 cm piece of proximal colon using AllPrep DNA/RNA Mini kit (Qiagen). Synthesis of cDNA was performed using qScript cDNA SuperMix (Quanta Biosciences), as per manufacturer’s recommended protocol. cDNA was then used at a 1/10 dilution in qPCR reactions. Samples were normalized internally using the average cycle quantification (Cq) of *TBP* and *rpl19*, simultaneously. The primer sequences are listed below. Real-time assays were run on a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad). Expression data are expressed as relative values after Genex macro analysis (Bio-Rad).
<table>
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<tr>
<th>Target Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>TBP</td>
<td>GCA-ACA-GCA-GCA-GCA-ACA-AC</td>
<td>CAA-CGG-TGC-AGT-GGT-CAG-AG</td>
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<tr>
<td>rpl19</td>
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<td>CTG-GTC-AGC-CAG-GAG-CTT</td>
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<tr>
<td>IL1β</td>
<td>TGT-CTT-TCC-CGT-GGA-CCT-TC</td>
<td>TCA-TCT-CGG-AGC-CTG-TAG-TG</td>
</tr>
<tr>
<td>IL4</td>
<td>CGG-AGA-TGG-ATG-TGC-CAA-AC</td>
<td>AAG-CCC-GAA-AGA-GTC-TCT-GC</td>
</tr>
<tr>
<td>IL6</td>
<td>ACA-AAG-CCA-GAG-TCC-TTC-AG</td>
<td>TGG-ATG-GTC-TTG-GTC-CTT-AG</td>
</tr>
<tr>
<td>IL10</td>
<td>ACA-GCC-GGG-AAG-ACA-ATA-AC</td>
<td>GGC-TTG-GCA-ACC-CAA-GTA-AC</td>
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<tr>
<td>IL17a</td>
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<td>ACG-TGG-AAC-GGT-TGA-GGT-AG</td>
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<tr>
<td>IL33</td>
<td>TGC-CAT-GTC-TAC-TGC-ATG-AG</td>
<td>CCT-AGA-ATC-CCG-TGG-ATA-GG</td>
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**Statistical analysis**

Results are depicted as mean ± SEM, unless otherwise indicated. Statistical analysis was performed using two-way ANOVA with post hoc Tukey or Holm-Šidák test, or Mann-Whitney U test, as specified. Differences between groups were considered significant when p < 0.05. For all statistical comparisons, *P*<0.05, **P*<0.01 ***P*<0.001, ****P*<0.0001, and ns means no significance. Calculations were performed using GraphPad Prism 7.0 software (GraphPad, La Jolla, CA).
3.4 RESULTS

3.4.1 Early life antibiotic exposure alters the gut microbiota

Beginning with birth, environmental exposures drive development of the intestinal microbial composition\(^{287}\). We sought to investigate the influence of a broad-spectrum antibiotic given from birth to weaning (day 21 after birth) on the development of the neonatal murine microbiota, and whether Nod2 genotype would have an effect on this. Amoxicillin is the most commonly prescribed antibiotic worldwide, particularly within paediatric patients\(^{243}\). The broad-spectrum beta-lactam also passes through breast milk, making it an ideal candidate for studies of antibiotic exposure to pups as well as also avoids the stress and potential concomitant alterations of the microbiome due to handling\(^{302,303}\).

Breeders were set up in Nod2 Het crosses and sister dams were used, whenever possible. After the pups were born, dams and litters were treated with either antibiotic or water only (Fig 3.1A). Amoxicillin-supplemented water was provided to antibiotic-treated cages \textit{ad libitum} and refreshed every other day; water-treated cages were handled identically. Antibiotic treatment was continuous from the day of (or day after) birth until weaning on day 21 (Fig 3.1A). At weaning, pups were separated by sex and genotyped for Nod2. Body weight and stool samples were collected to assess the microbiota composition at weaning. All pups received normal drinking water (pH ~7) from weaning until endpoint. In order to monitor the maturation of the microbiota, stool samples were also collected on day 35 (14 days after weaning and cessation of antibiotic treatment). Mice are considered fully mature by 8 weeks of age (day 56), which was when mucosal immunological responses were measured and/or colitis was induced (Fig 3.1A, red arrow).

Stool samples collected at weaning from all littermates were used to assess the microbiota composition via 16S rDNA sequencing. Neonatal antibiotic exposure resulted in a significant shift of the microbial community structure away from water-treated litters at day 21 (Fig 3.1B).
Figure 3.1: Early life antibiotic exposure alters the gut microbiota.

A. Experimental outline. WT and Nod2−/− littermates were divided into two treatment groups:
   i. Litters received only non-acidified control water from day 0 throughout the study.
   ii. Litters received treatment with non-acidified water supplemented with amoxicillin
       (Abx) at 200 mg/L from the day of birth (day 0) until weaning (day 21). Weaned pups
       then received control water until endpoint.

   Arrows indicate stool collection time points. Immunological assessment and/or colitis
   induction occurred on day 56 (red arrow).

   PCoA of Bray-Curtis Dissimilarity comparing:

B. Treatment: Water (blue circle) and antibiotic-treated (Abx, red square),
C. Litter (chronology and treatment),
D. Bray-Curtis Dissimilarity distances between litters by treatment; boxes represent median with upper and lower quartiles, whiskers calculated using the Tukey method.

E. *Nod2* genotype: WT (orange circle), HET (red square), *Nod2*−/− (blue triangle), and

F. Sex: female (red circle), male (blue square).

*P*<0.05, ****P<0.0001; by one-way ANOVA and post hoc Tukey test (D). Each dot represents one mouse. (n=8-14 mice per genotype per treatment. Data are from 4 untreated litters and 5 antibiotic-treated litters).
A strong clustering pattern within litters was observed (Fig 3.1C); however, clustering between litters was treatment dependent, with greater variability between antibiotic-treated litters than water-treated litters, as measured by Bray-Curtis dissimilarity distance (Fig 3.1D). Nod2 genotype did not influence microbiota composition at weaning (Fig 3.1E), nor did sex (Fig 3.1F). Since Nod2 protein expression in cells and tissues of the heterozygous littermates is variable and not easily quantified, only homozygous WT and Nod2−/− littermates were used in the subsequent experiments and analysis.

3.4.2 Nod2 is involved in shaping the microbial composition at weaning following antibiotic treatment

Since antibiotic treatment from birth until weaning significantly altered the gut microbiota at weaning, we next explored the influence of Nod2 on this response. The microbial communities of antibiotic-treated WT and Nod2−/− mice were significantly different from that of their water-treated genotype-matched controls (Fig 3.2A-C). Unexpectedly, we identified a difference between the water-treated WT and Nod2−/− litters at weaning (Fig 3.2A). Community richness, as measured by the Chao1 (Fig 3.2B) and Shannon diversity (Fig 3.2C) indices, were not different between water-treated WT and Nod2−/− mice, but was significantly diminished in antibiotic-treated WT and Nod2−/− mice. Indeed, antibiotic-treated Nod2−/− mice exhibited further reduced diversity compared to antibiotic-treated WT littermates (Fig 3.2C). These differences were reflected in the microbial composition. The only significant difference between water-treated WT and Nod2−/− litters was an increased abundance of the Bacteroidetes family Paraprevotellaceae in Nod2−/− mice (Fig 3.2D). Antibiotic treatment reduced the abundance of Bifidobacteriaceae, Lactobacillaceae, Lachnospiraceae, and Erysipelotrichaceae, and induced a bloom of Bacteroidaceae in both WT and Nod2−/− mice (Fig 3.2D). Interestingly, several differences in the response to antibiotic treatment were identified between WT and Nod2−/− littermates. Antibiotic-treated Nod2−/− mice also experienced a loss of Coriobacteriaceae, Rikenellaceae, S24-7, Paraprevotellaceae, Ruminococcaceae, and a bloom on Enterobacteriaceae compared to Nod2−/− water-treated controls (Fig 3.2D); whereas, antibiotic-treated WT mice only had an elevated abundance of Verrucomicrobiaceae compared to their water-treated controls (a change not observed in the antibiotic-treated Nod2−/− mice).
**Figure 3.2:** Nod2 influences the microbial response to antibiotic exposure in early life.

A. Principal coordinates plot of Bray-Curtis dissimilarity identified separation of microbial communities by treatment, irrespective of Nod2 genotype, at day 21. Each dot represents one mouse. (ANOSIM, WT vs. Nod2\(^{-/-}\): R=0.1796 p=0.019, WT vs. WT + Abx: R=0.5229 p=0.001; Nod2\(^{-/-}\) vs. Nod2\(^{-/-}\) + Abx: R=0.4929 p=0.001; WT + Abx vs. Nod2\(^{-/-}\) + Abx: R=0.0906 p=0.084).

B. Species richness as measured by the Chao1 rarefaction index.

C. Richness measured by the Shannon diversity index.

D. Bacterial composition at the Family level at weaning (day 21). Taxa that differed significantly between untreated WT and Nod2\(^{-/-}\) mice (§), or between untreated and antibiotic-treated WT (*), or Nod2\(^{-/-}\) (§) mice are indicated (false discovery rate corrected p-value<0.05).

Data are presented as mean ± SD (B, C) or mean (D). *P<0.05, **P<0.01, ***P<0.001; by paired Student’s T test (B, C) or Kruskal-Wallis (D). (n=9-14 mice per genotype per treatment. Data are from 4 untreated litters and 5 antibiotic-treated litters).
In order to determine the statistical and biological significance of these taxonomic differences, linear discriminant analysis (LDA) coupled with effect size (LEfSe) analysis was performed. All of the antibiotic-induced changes between water and antibiotic-treated WT mice were also observed in \( \text{Nod2}^{-/-} \) mice (Fig 3.3A, non-bolded taxa); yet \( \text{Nod2}^{-/-} \) mice displayed additional changes (Fig 3.3A, bolded taxa), suggesting a role for Nod2 in shaping the microbial response to antibiotic treatment. Comparison between antibiotic-treated WT and \( \text{Nod2}^{-/-} \) mice further highlighted microbial differences due to host genetics (Fig 3.3B). Together, these data indicate a role for Nod2 in shaping the microbiota in early life and in response to antibiotic perturbation.

### 3.4.3 Nod2-deficient mice experience sustained effects of early life antibiotic treatment

The gut microbiota in early life is highly susceptible to environmental perturbations. In mice, weaning pups from their mother and a predominantly milk diet, to separate housing and rodent chow have significant effects on the microbiota.\(^{304}\) We sought to determine if the antibiotic-induced changes in the microbiota would still be evident 14 days after weaning and cessation of the antibiotic treatment. Stool samples from water and antibiotic-treated littermate mice were collected on day 35 after birth (14 days post weaning) for 16S rRNA sequencing. No differences were observed between water-treated WT and \( \text{Nod2}^{-/-} \) littermates, or between water and antibiotic-treated WT mice (Fig 3.4A). In contrast, antibiotic-treated \( \text{Nod2}^{-/-} \) mice remained significantly different from water-treated \( \text{Nod2}^{-/-} \) controls (Fig 3.4A), and this was echoed in the species richness data with antibiotic-treated \( \text{Nod2}^{-/-} \) mice having lower community richness (Fig 3.4B) and diversity (Fig 3.4C) than their water-treated controls. Analysis of the bacterial composition did not identify any bacterial taxa that were significantly different between water-treated WT and \( \text{Nod2}^{-/-} \) littermates, nor between water and antibiotic-treated WT mice (Fig 3.4D). In contrast, \( \text{Nod2}^{-/-} \) mice that had received antibiotic treatment had increased abundance of Porphyromonadaceae, and decreased levels of Bifidobacteriaceae, Rikenellaceae and Erysipelotrichaceae compared to their water-treated controls (Fig 3.4D). LEfSe analysis further identified several taxa that differed due to antibiotic treatment of Nod2-deficient mice (Fig 3.5A), including increased Prevotellaceae and Lactobacillaceae, and decreased Clostridaceae. Comparison between antibiotic-treated WT and \( \text{Nod2}^{-/-} \) littermates emphasized the loss of Erysipelotrichaceae and overabundance of Coriobacteriaceae as the strongest differences between the genotypes (Fig 3.5B).
Figure 3.3: Differential response to early life antibiotic treatment in \( \textit{Nod2}^{-/-} \) mice compared to WT littermates at weaning.

A. Taxonomic differences were identified by linear discriminant analysis (LDA) coupled with effect size (LEfSe). Bar plot of the differences between water vs. antibiotic-treated \( \textit{Nod2}^{-/-} \).
mice at day 21. Taxa enriched in water-treated are shown in green; taxa enriched in antibiotic-treated are red. Bolded taxa differed between WT and *Nod2*<sup>−/−</sup> mice.

B. Bar plot of the differences between antibiotic-treated WT vs. antibiotic-treated *Nod2*<sup>−/−</sup> mice at day 21. Taxa enriched in antibiotic-treated WT are shown in green; taxa enriched in antibiotic-treated *Nod2*<sup>−/−</sup> are red.
Figure 3.4: *Nod2*−/− mice maintain a different microbiota 14 days after cessation of early life antibiotic treatment.

A. Principal coordinates plot of Bray-Curtis dissimilarity identified maintained separation of microbial communities by treatment at day 35. (ANOSIM, *WT* vs. *Nod2*−/−: R=0.0199 p=0.250; *WT* vs. *WT + Abx*: R=0.1322 p=0.091; *Nod2*−/− vs. *Nod2*−/− + *Abx*: R=0.3726 p=0.001; *WT + Abx* vs. *Nod2*−/− + *Abx*: R=0.2377 p=0.046)

B. Species richness measured by the Chao1 rarefaction index.

C. Richness measured by Shannon diversity index.

D. Bacterial composition at the Family level at day 35. Significantly different taxa between antibiotic-treated and untreated *Nod2*−/− mice are indicated with an # (false discovery rate corrected p-value<0.05).

Data are presented as mean ± SD (B, C) and mean (D). *P<0.05, ***P<0.001; by paired Student’s T test (B-C) or Kruskal-Wallis (D). (n=9-14 mice per genotype per treatment. Data are from 4 untreated litters and 5 antibiotic-treated litters).
Figure 3.5: Absence of Nod2 signalling impairs the ability of the microbiota to recover from antibiotic perturbation.

A. Taxonomic differences were identified by linear discriminant analysis (LDA) coupled with effect size (LEfSe). Bar plot of the differences between water vs. antibiotic-treated Nod2^-/- mice at day 35. Taxa enriched in water-treated are shown in green; taxa enriched in antibiotic-treated are red). Bolded taxa differed between WT and Nod2^-/- mice.

B. Bar plot of the differences between antibiotic-treated WT vs. antibiotic-treated Nod2^-/- mice at day 35. Taxa enriched in antibiotic-treated WT are shown in green; taxa enriched in antibiotic-treated Nod2^-/- are red).
These data further highlight a role for Nod2 in shaping the microbiota in early life, with loss of Nod2 signalling resulting in a failure to fully recover from antibiotic perturbation.

As described, major environmental changes occur in the first 10 days following weaning, specifically housing and diet. At weaning, all pups received identical housing, water bottles and rodent chow in order to minimize environmental differences that could alter the microbiota. Nevertheless, we examined the microbial changes between weaning (day 21) and day 35 in water and antibiotic-treated littermates in order to evaluate changes due to the new environment and changes due to the maturation of the microbiota after removal of the antibiotic. As expected, the microbiota shifted significantly between weaning and day 35 in water-treated WT or Nod2\(^{-/-}\) mice (Fig 3.6A), likely due to housing and diet changes. Both antibiotic-treated WT and Nod2\(^{-/-}\) mice saw large shifts in community structure relative to water-treated controls (Fig 3.6A). In order to determine if the recovery from antibiotics involved greater changes in microbial composition than those induced by the new environment, the distance from day 21 to day 35 composition was determined for each mouse. Comparison between day 21 and 35 within treatment groups indicated greater dissimilarity in antibiotic-treated littermates compared to water-treated controls, suggesting that the shifts observed in antibiotic-treated mice were greater than those due to the new housing and diet seen in the water-treated controls (Fig 3.6B). Indeed, by day 35, antibiotic-treated mice had reduced Enterobacteriaceae and Bacteroidaceae and increased relative abundance of Lactobacillaceae and S24-7 compared to their composition at day 21 (Fig 3.6C). Together, these data reiterate the importance of controlling the environment during microbial analysis. Moreover, it suggests that antibiotic-induced changes in the gut microbiota are transient once the treatment has stopped, enabling the microbiota to recover to a composition similar to untreated mice.

### 3.4.4 Early life antibiotic treatment alters immune development

Early life antibiotic treatment has been shown to increase weight gain in mice\(^{260}\); as such water and antibiotic-treated pups were weighed at weaning and weekly afterwards until day 56 (8 weeks of age). Although antibiotic-treated WT mice were significantly smaller than their water-treated controls at weaning (Fig 3.7A), this difference was abrogated by day 28 and no differences were observed between genotypes or treatments over time (Fig 3.7B).
Figure 3.6: Microbial changes between day 21 and 35 demonstrate the resilience of the gut microbiota.

A. PCoA of Bray-Curtis dissimilarity identified a substantial shift in the microbial community from weaning to day 35. Dashed lines encircle day 21 data points, and solid lines with shaded circles identify day 35 data points. (ANOSIM, WT d21 vs. d35: R=0.2521 p=0.007; Nod2-/- d21 vs. d35: R=0.4468 p=0.001; WT + Abx d21 vs. d35: R=0.4306 p=0.008; Nod2-/- + Abx d21 vs. d35: R=0.4721 p=0.001)

B. Bray-Curtis dissimilarity distances between day 21 and 35 by genotype and treatment; boxes represent median with upper and lower quartiles, whiskers calculated using the Tukey method.

C. Bacterial composition at the Family level at days 21 and 35 in antibiotic-treated mice.
Data are presented as mean (C). ***P<0.001, ****P<0.0001; by one-way ANOVA and post hoc Tukey test (B). (n=9-14 mice per genotype per treatment. Data are from 4 untreated litters and 5 antibiotic-treated litters).

**Figure 3.7: Early life antibiotic exposure does not affect weight gain through life.**

A. Weight of pups at weaning.
B. Weight gain over time was not affected by treatment or genotype.

Data are presented as mean ± SEM. *P<0.05; by Mann-Whitney U test (A). (n=12-15 mice per genotype, per treatment. Data are from 4 untreated litters and 5 antibiotic-treated litters).
The murine immune system begins development in utero, with environmental and microbial exposures continuing to shape development and maturation for weeks after birth. As described, mice are fully developed by 8 weeks of age, day 56 post birth. We first examined whether Nod2 deficiency alone altered T cell populations in the spleen. Water-treated WT and Nod2−/− littermates did not differ in the proportion of splenic B or T cells (Fig 3.8A). Furthermore, the percent of CD4+ and CD8α+ cells within the CD3+ T cell population were similar, as were the percent of Foxp3+ Treg and RORγt+ T cells (Fig 3.8B-C). This suggested that Nod2 deficiency had no effect on homeostatic immune development, corroborating previous work from our laboratory describing no functional differences between non-littermate WT and Nod2−/− T cells234.

The gut microbiota influences mucosal immune development, therefore we investigated whether the antibiotic-perturbed microbiota would alter immune development. On day 56, five weeks after cessation of the antibiotic treatment, serum was collected and systemic antibody concentration was quantified. No differences in concentration of serum IgG, including total IgG, IgG1, IgG2a or IgG3 subclasses, existed between water-treated WT and Nod2−/− mice (Fig 3.9A). Early life antibiotic treatment resulted in a significant decrease in total IgG and IgG2a, but not IgG1 in both WT and Nod2−/− littermates (Fig 3.9A). Intriguingly, IgG3, which is thought to be involved in microbial regulation305, was significantly different between antibiotic-treated WT and Nod2−/− littermates but not in water-treated controls. Serum IgA was higher in water-treated Nod2−/− mice compared to WT littermates, but antibiotic treatment significantly reduced the concentration of IgA in Nod2−/− mice to that of WT mice (Fig 3.9B). The main function of IgA in the gut is to prevent microbes from coming into contact with the epithelial barrier; accordingly the majority of IgA produced in the lamina propria is secreted into the gut lumen and thus can be measured in stool. At weaning, very low levels of IgA were detected in the stool of mice and did not differ by genotype or treatment, and it is probable that this was maternally derived IgA (Fig 3.9C). On day 35, fecal IgA concentration was significantly lower in antibiotic-treated mice than their water-treated controls, although by day 56 no differences were apparent (Fig 3.9C). Along with the diminished serum IgA levels in antibiotic-treated Nod2−/− mice, this alluded to a possible reduction in the number of IgA-producing plasma cells after antibiotic exposure. Therefore, the proportion of B cells and IgA+ plasma cells in the spleen, small intestine and colon were compared between water and antibiotic-treated Nod2−/− mice.
Figure 3.8: Nod2 deficiency does not influence splenic immune populations at homeostasis.

A. Percent of CD3$^+$ and CD19$^+$ cells within live lymphocytes.
B. Percent of CD4$^+$ and CD8$^\alpha$ cells within CD3$^+$ lymphocytes.
C. Percent of Foxp3$^+$ and ROR$\gamma$t$^+$ cells within CD3$^+$CD4$^+$ T cells.

Data are presented as mean ± SEM. (WT n=5, Nod2$^{-/-}$ n=9. Data are from one experiment).
Figure 3.9: Early life antibiotic exposure is associated with decreased systemic and fecal IgA.

A. Systemic total IgG, as well as individual subclasses IgG1, IgG2a and IgG3, concentrations in serum.

B. IgA concentration in serum.
C. IgA concentration in stool, normalized to stool weight, over time.
D. B220⁺ B cell and IgA⁺ plasma cell populations in the spleen, small intestine (S.I.) and colon were determined by flow cytometry. (Nod2⁺/⁻ n=4, Nod2⁻/⁻ + Abx n=6. Data are from two independent experiments).

Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; by Mann-Whitney U test (A-B, D) and two-way ANOVA and post hoc Tukey test (C). (n=7-16 mice per genotype, per treatment from 6 untreated litters and 8 antibiotic-treated litters).

**Figure 3.10: Early life antibiotic exposure alters microbiologically driven T cell development.**

A. Number of total lymphocytes and CD3⁺ T cells in the small intestinal lamina propria.
B. Percent of Foxp3⁺, RORγt⁺, or RORγt⁺Foxp3⁺ double positive T cells within the small intestinal lamina propria.

Data are presented as mean ± SEM. **P<0.01, ***P<0.001; by Mann-Whitney U test (A-B). (n=6-13 mice per genotype, per treatment from 4 untreated and 4 antibiotic-treated litters).
There were no differences in B220+ populations within lymphocytes in any of the tissues, however antibiotic-treated mice had a reduction in splenic but an expansion of colonic IgA-producing plasma cells (Fig 3.9D). Together this suggests that antibiotic exposure has long-lasting effects on the systemic humoral responses, possibly through altering the maintenance of plasma cells.

Next, we examined the small intestinal immune populations in antibiotic-treated mice compared to water-treated controls. No differences existed in total lymphocytes isolated from the tissue or in the total number of CD3+ T cells between genotypes and treatments (Fig 3.10A). Interestingly, T cell populations known to be induced via host-microbe interactions were significantly increased in antibiotic-treated mice. These cell populations included Foxp3+ regulatory T cells (Treg), RORγT+ T cells, and RORγT+Foxp3+ double positive Treg (Fig 3.10B). Overall, these data suggest that the altered microbiota due to antibiotic treatment was associated with alterations in the development of adaptive immunity, including systemic and mucosal antibodies and plasma cells, as well as enhancement of gut T cells known to regulate microbial interactions.

3.4.5 Antibiotic treatment worsens DSS colitis in Nod2−/− mice

Antibiotic exposure has been associated with IBD development, thus we assessed whether the antibiotic-treated mice had an increased susceptibility to chemically-induced colitis. Mice were allowed to recover from antibiotic treatment for 5 weeks and colitis was initiated on day 56. On day 56 (day 0 of disease induction), mice were challenged with dextran sulfate sodium (DSS) supplemented drinking water for five days followed by a two-day rest on regular drinking water and tissues were analyzed on day 8 (Fig 3.11A). Water-treated WT and Nod2−/− mice and antibiotic-treated WT mice lost ~10% of their initial body weight by day 8, whereas antibiotic-treated Nod2−/− mice lost significantly more weight, nearly 25% (Fig 3.11B). Stool collected on days 0, 3, 5 and 8 was used to quantify fecal lipocalin-2 concentration, an anti-microbial peptide produced by neutrophils and epithelial cells that is released during intestinal inflammation. Corresponding with the increased weight loss, antibiotic-treated Nod2−/− had elevated lipocalin-2 concentration at days 5 and 8, signifying worsened inflammation (Fig 3.11C). Furthermore, antibiotic-treated Nod2−/− mice had greater colonic shortening compared to water-treated Nod2−/− mice and antibiotic-treated WT littermates (Fig 3.11D). Moreover, pathological scoring indicated increased tissue damage and inflammatory cell depth in antibiotic-treated Nod2−/− mice compared to WT littermates (Fig 3.11E).
Figure 3.11: Early life antibiotic exposure results in increased susceptibility to colitis in Nod2−/− mice.

A. Experimental timeline. Mice were given antibiotic-supplemented water or untreated water from the day of birth until weaning on day 21. Colitis was induced on day 56 by providing 3.5% DSS water for five days. Tissues were analyzed on day 8 from DSS induction.

B. Percent of initial weight over time.

C. Fecal lipocalin-2 concentration was measured by ELISA and normalized to stool weight.

D. Colon length, measured on day 8.

E. Colonic tissue was fixed, H&E stained and scored for colitis severity.

Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; by two-way ANOVA and post hoc Holm-Šidák test (B-C) and Mann-Whitney U test (D-E). (n= 6-16 mice per genotype, per treatment from 4 untreated litters and 5 antibiotic-treated litters).
No differences in colitis susceptibility or severity were observed between water- and antibiotic-treated WT mice, nor were differences observed between water-treated WT and Nod2-deficient littermates indicating that the antibiotic perturbation combined with a Nod2 genetic defect resulted in worsened disease.

Regulatory T cells are involved in controlling and limiting inflammation. We previously identified antibiotic-induced differences in Foxp3$^+$ and RORγ$^+$Foxp3$^+$ double positive Treg in the small intestine at homeostasis. We postulated that the differences in Treg could be involved in the exacerbated response to DSS observed in the antibiotic-treated Nod2$^{-/-}$ mice. In order to address this, we performed flow cytometry on splenic and colonic lymphocytes from water and antibiotic-treated Nod2$^{-/-}$ mice and their DSS-exposed littermates. In accordance to our previous observations, early life antibiotic treatment resulted in increased percentages of Foxp3$^+$ and RORγ$^+$Foxp3$^+$ double positive Treg in the colonic lamina propria (Fig 3.12A). Indeed, RORγ$^+$Foxp3$^+$ double positive Treg were also increased in the spleen (although far fewer than those in the gut); however, no difference in splenic Foxp3$^+$ Treg was observed (Fig 3.12A). DSS exposure induced an increase in Foxp3$^+$ Treg in the colon of water-treated Nod2$^{-/-}$ mice but not a significant increase in the already elevated population in antibiotic-treated mice (Fig 3.12A). DSS had no effect on the proportion of RORγ$^+$Foxp3$^+$ double positive Treg in water-treated Nod2$^{-/-}$ mice; conversely, these double positive Treg were significantly diminished in both the spleen and colon of DSS-exposed, antibiotic-treated Nod2$^{-/-}$ mice (Fig 3.12A). Coinciding with the reduction in RORγ$^+$Foxp3$^+$ double positive Treg, we detected a significant increase in Gata3-expressing Foxp3$^+$ Treg in antibiotic-treated Nod2$^{-/-}$ mice following DSS exposure (Fig 3.12A). These cells are thought to be involved with controlling inflammation and are induced through TCR activation and cytokines$^{68,69}$. In order to examine the cytokines involved in the DSS response and possibly influencing the change in Treg response, mRNA was extracted from a 1 cm piece of proximal colon from each mouse. DSS-induced colitis resulted in increased expression of the pro-inflammatory cytokines IL1β and IL6 in both water and antibiotic-treated Nod2$^{-/-}$ mice, with a further increase in IL6 expression in antibiotic-treated mice (Fig 3.12B), complementary to the exacerbated weight loss and pathology observed. Expression of the anti-inflammatory cytokine IL10 was also induced with DSS exposure, but no difference was detected between water and antibiotic-treated mice (Fig 3.12B). Expression of IFNγ, TNFα, and IL17 were not different between treatments, irrespective of DSS treatment (Fig 3.12B).
Figure 3.12: DSS exposure alters the regulatory T cell populations and cytokine milieu in the colon of antibiotic-treated *Nod2*−/− mice.
A. Spleen and colonic lamina propria lymphocytes were analyzed for Foxp3, RORγt, and Gata3 expression by intracellular flow cytometry. (Nod2−/− n=4, Nod2−/− + Abx n=6, Nod2−/− + DSS n=4, Nod2−/− + Abx + DSS n=5)

B. Cytokine expression in the proximal colon was assessed by quantitative PCR. Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; by one-way ANOVA and post hoc Tukey test (A) or Mann-Whitney U test (B). (n=4-10 mice per genotype, per treatment. Data are from two independent experiments).
*Gata3* expression in Foxp3+ Treg can be induced through the cytokines IL-4, TGFβ and IL-33. Indeed, their expression was significantly increased in antibiotic-treated *Nod2−/−* mice exposed to DSS (Fig 3.12B). Both *IL10* and *TGFβ* contribute to Treg maintenance, but enhanced *IL4* and *IL33* expression along with minimal *IL17* expression suggests that the cytokine milieu favoured the generation and support of Gata3+Foxp3+ Treg during DSS colitis.

Together, our data indicate that early life antibiotic treatment in a genetically susceptible host can alter the colonic Treg populations. During inflammation, this results in exacerbated disease characterized by a skewed cytokine milieu and expansion of Gata3+Foxp3+ Treg.
3.5 DISCUSSION

This study demonstrated that early life antibiotic treatment significantly alters the gut microbiota and intestinal immune development, and results in exacerbated colitis in Nod2-deficient mice. We found that early life antibiotic treatment significantly shifted the gut microbiota composition away from water-treated controls and that Nod2 plays a role in shaping the microbiota in early life. Moreover, microbiota perturbation early in life altered the microbe-associated mucosal immune cells with increased proportions of Foxp3\(^+\) regulatory T cells (Treg), ROR\(\gamma\)t\(^+\) T cells, and ROR\(\gamma\)t\(^+\) Foxp3\(^+\) Treg in the small intestine. Antibiotic-treated \(\text{Nod}2^{-/-}\) mice were more susceptible to DSS colitis, with an altered cytokine milieu and expansion of Gata3-expressing T regulatory cells. Together, these data illustrate the long-lasting effects of antibiotic perturbation of the microbiota in early life, and how this can lead to increased susceptibility to colitis in a genetically susceptible host.

Early life antibiotic exposure has been associated with several auto-inflammatory diseases including asthma, obesity and IBD\(^{33,184,250}\). In humans, the microbiota is highly malleable until 2-3 years of age\(^{36,122}\); in mice, an adult-like composition is achieved by ~4 weeks of age\(^{288}\). In order to model the effects of repeated antibiotic exposure during the first years of an infant’s life, we developed an antibiotic treatment regime, from birth to weaning, using the antibiotic most commonly prescribed to pediatric patients, amoxicillin\(^{243}\). We found that amoxicillin treatment caused a significant shift in the community structure of the gut microbiota in mice at weaning. Antibiotic treatment significantly reduced the microbial richness (the number of unique bacterial taxa present in the community) and the diversity (the presence and abundance of the taxa). Moreover, antibiotic treatment resulted in loss of Bifidobacteriaceae, Lactobacillaceae, Lachnospiraceae, and Erysipelotrichaceae, whereas Bacteroidaceae and Enterobacteriaceae bloomed. These changes are in agreement with antibiotic-induced alterations observed previously in both humans and mice\(^{122,179,253,260}\). Specifically, the maintained loss of Lachnospiraceae and expansion of Porphyromonadaceae following antibiotic treatment was also observed in a longitudinal study in children followed from birth until 2 years of age\(^{179}\).

Interestingly, we identified several differences in the microbiota community between antibiotic-treated WT and \(\text{Nod}2^{-/-}\) littermates. Furthermore, the influence of Nod2 on the gut microbiota was still apparent two weeks after stopping the antibiotic treatment during the microbial recovery. Indeed, antibiotic-treated \(\text{Nod}2^{-/-}\) mice displayed a persistent loss of
Erysipelotrichaceae and increased levels of Prevotellaceae compared to water-treated Nod2<sup>−/−</sup> mice. Although direct comparisons to human data cannot be made, this study emphasizes the role of host genetics on shaping the gut microbiota in early life and in response to environmental perturbations. Indeed, it would be interesting to investigate if genetics alter the microbial response in children who are exposed to antibiotics in the first years of life. Some studies have successfully described the effects of different classes of antibiotics on the gut microbiota in children<sup>122,181</sup>, and others have gone on to implicate these changes with disease development later in life<sup>263</sup>. However, no study has looked at the influence of host genetics, particularly genes involved in host-microbe interactions or associated with chronic inflammatory diseases, on the response to antibiotic exposure or the resilience of the microbiota. This information could help distinguish the causation from correlation in microbiome-disease association studies.

Long-term antibiotic treatment has been shown to alter the murine transcriptome, particularly immune pathways in the lamina propria<sup>248</sup>. However, the influence of early life antibiotic therapy on immune development and maturation has not been examined. In accordance with previous work in the lab, Nod2 deficiency had no effect on the splenic T cell populations at homeostasis<sup>234</sup>. Early life antibiotic exposure reduced systemic IgG and IgA levels in Nod2<sup>−/−</sup> mice, and altered the IgA-producing plasma cells in the colon. Production of IgA in the gut is required to protect the epithelial barrier, thus an altered microbiota could skew the generation of plasma cells, particularly in a host deficient for a microbial sensor like Nod2. Indeed, sensing of the gut microbiota by TLR5-mediated detection of flagellin is required to induce plasma cell generation and maintenance<sup>306</sup>. Fecal IgA concentration was not different between genotypes or treatment groups at day 56, but antibiotic-treated Nod2<sup>−/−</sup> mice had more colonic IgA<sup>+</sup> plasma cells than water-treated littermates, thus it is possible that the influence of the perturbed microbiota on IgA production was through alterations in plasma cell induction and maintenance in the gut<sup>52</sup>. The link between microbially driven plasma cells in the gut and systemic humoral immunity is being investigated; indeed, antibodies reactive to commensal antigens can be detected in serum<sup>307</sup>. Moreover, this response was found to develop between 6-12 months of age, further implicating the composition of early life microbiota on host immune development.

Antibiotic treatment also altered intestinal T cell populations involved in host-microbe interactions, including Treg, Th17 and RORγt<sup>+</sup>Foxp3<sup>+</sup> Treg. In contrast to a study that found that
neonatal vancomycin treatment reduced colonic Foxp3⁺ Treg²⁵⁴, our neonatal amoxicillin treatment increased intestinal (but not splenic) Foxp3⁺ Treg populations. Typically it is thought that increased antigen load results in increased Treg, however the antibiotic treatment reduced microbial diversity in our mice, which would suggest reduced antigen load. Our findings point to the idea that the development of intestinal Treg may be more complex, such that the type of antigen and the context in which it is presented is more important than the overall antigen load. The induction of non-classical Treg, including RORγt⁺Foxp3⁺ and Gata3⁺Foxp3⁺ Treg, in our antibiotic-treated mice indicates the important influence of microbes on the maturation of these cells as well. Indeed, previous studies have suggested that these cells are generated in response to the gut microbiota⁶⁰,³⁰⁸. RORγt⁺Foxp3⁺ Treg develop from Foxp3⁺ Treg³⁰⁹, and are functionally equivalent, if not superior, to Foxp3⁺ Treg in protecting against inflammation in the T cell transfer model of colitis⁶⁴. By contrast, Gata3⁺Foxp3⁺ Treg do not differ in abundance between germ-free and SPF mice⁶², instead these cells are driven by IL-33 released during barrier disruption and mediate tissue damage⁷¹. Interestingly, one study demonstrated that Gata3 expression blocked Rorc expression in Foxp3⁺ Treg⁶⁸. This could explain why the increase in Gata3⁺Foxp3⁺ Treg coincided with a decrease in RORγt⁺Foxp3⁺ Treg observed in antibiotic-treated Nod2⁻/⁻ mice following DSS induced colitis. Together, this suggests that the altered intestinal Treg populations following early life antibiotic treatment can result in dysregulated immune responses and exacerbated colitis in a genetically susceptible host.

Our study identified a role for Nod2 in shaping the gut microbiota in mice in early life and in response to antibiotic-induced perturbations of gut microbial communities. It also highlights the long-lasting influence of an altered microbiota on mucosal immune development, maturation, and development of disease. One can imagine how IBD patients with NOD2 mutations (or others also involved in host-microbe interactions, such as ATG16L1 or IL23R) could experience incomplete resilience following microbial perturbation, which in turn could increase their susceptibility to intestinal inflammation. Overall, our data indicates that further investigation into the intestinal immune development as it relates to the gut microbiota is necessary and required to better understand this bidirectional relationship. In particular, identifying how microbes influence the development of various immune cell populations, including Treg, will provide new therapeutic targets to treat or prevent the development of IBD.
Chapter 4

DISCUSSION and FUTURE DIRECTIONS
4.1 Cumulative Discussion

This thesis project was undertaken to investigate the role of host immune-microbe interactions on the development of colitis. The goals of this thesis were to investigate: 1) the effect of antibiotic treatment on the adult gut microbiota and susceptibility to colitis, and 2) the influence of early life antibiotic treatment on the development of the neonatal microbiota and mucosal immune system and whether these changes altered susceptibility to colitis. In both instances, the response and recovery of the microbiota from the perturbation was investigated. Moreover, I explored whether Nod2 played a role in the microbial and immune responses.

In both cases, I found that antibiotic exposure altered the murine gut microbiota, that Nod2 influenced the recovery from the perturbation and that antibiotic exposure had long-term effects resulting in an altered response to small intestinal mucosal damage and chemically induced colitis in genetically susceptible hosts. Together, the findings presented in this thesis add to the growing understanding of the complex host-microbe relationship, with a focus on how IBD genetic susceptibility can influence this fine balance.

4.1.1 Not all mice are created equal – the need for littermate controls

When studying host interactions with the microbiota, it is essential to know if the starting microbes are the same. I found that separate strain WT and Nod2\(^{-/-}\) mice had significantly different microbial community structures at baseline. Others have also described a ‘dysbiotic’ phenotype in Nod2\(^{-/-}\) mice.\(^{23,240,295,310-312}\) Interestingly, this phenotype occurs in the two most common Nod2 knockout mouse strains, which were developed separately and involve different mutations to disrupt the Nod2 gene.\(^{15,278}\) Together, these findings indicate that in the absence of Nod2 signalling the gut microbiota may be skewed to a state favouring the expansion of Bacteroidetes and loss of Firmicutes. Indeed, a separate strain of Nod2-deficient mice harboured a microbiota similar to that described in IBD patients, with reduced Firmicutes and increased abundance of Bacteroidetes.\(^{167,171,172}\)

To accurately assess the impact of Nod2 genotype on the gut microbiota, I generated littermates by breeding mice that were heterozygous for Nod2. In this way, litters were composed of pups that were either homozygous wild-type, heterozygous or deficient for Nod2. In this way, all pups...
in a litter received the same maternally-derived foundational microbiota at birth and experienced the same environment until weaning. Although I cannot exclude the possibility that different cages experienced slightly differing environments, this was controlled for as much as possible by maintaining the mice on the same chow and water (non-acidified) and on the same rack in the same room of the animal facility, and by handling the mice in the same order throughout experiments.\textsuperscript{141}

At weaning, mice were separated by sex and \textit{Nod2} genotype. Microbial composition was not significantly influenced by sex in these studies, but it is possible that my experiments did not extend long enough to see an effect (beyond 13 weeks of age). Indeed, sex has been shown to alter the gut microbiota and microbially-induced autoimmune diseases, such as type 1 diabetes\textsuperscript{303,313}. To ensure a sex difference was not overlooked, both male and female mice were used in all experiments and data were analyzed separately to confirm no differences before being combined. Similar to previous findings by our team, no influence of \textit{Nod2} genotype on the microbiota was apparent at homeostasis in 8-week-old littermate mice\textsuperscript{296}. However, assessment of the microbiota at weaning identified a small, but statistically significant, difference in untreated \textit{Nod2$^{-/-}$} mice compared to WT littermates. This was characterized by increased abundance of the Bacteroidales family Paraprevotellaceae, again highlighting the expansion of Bacteroidetes in the absence of \textit{Nod2}. This difference was not apparent at day 35 (two weeks later), indicating that the influence of the environment could pre-dominate over that of host genetics\textsuperscript{287,314}. Indeed, another study revealed that shaping of the microbiota is dominated by diet rather than host genotype by comparing the effects of a dietary intervention on >200 outbred and five inbred mouse strains, including \textit{Nod2$^{+/+}$} mice\textsuperscript{315}. Together, these data emphasize the need for littermate controls in experimental models seeking to understand the genetic influence on disease models.

4.1.2 \textbf{Nod2 plays a role in microbial resilience}

My data identify a role for \textit{Nod2} in both the response to antibiotic exposure and the recovery from it. Indeed, our separate strain \textit{Nod2$^{+/+}$} mice displayed a prolonged microbial perturbation two weeks after removal of the antibiotics in adulthood, and this phenotype was confirmed in the \textit{Nod2$^{+/+}$}-derived littermates. Some bacterial taxa changed in response to antibiotic treatment
irrespective of host genotype, suggesting a direct antibiotic-microbe effect. However, several taxa differed between antibiotic-treated WT and \( \text{Nod2}^{+/–} \) littermates, indicating a role for Nod2 in this response. The bacterial taxa that differed between antibiotic-treated WT and \( \text{Nod2}^{+/–} \) mice were a mix of Clostridia (Ruminococcaceae and Lachnospiraceae) and Bacteroidales (S24-7). Moreover, \( \text{Nod2}^{+/–} \) mice maintained a significant increase in Enterobacteriaceae abundance, which was also identified in neonatal antibiotic-treated \( \text{Nod2}^{+/–} \) mice at weaning, compared to their WT littermates. Similarly, decreased Clostridia and increased Enterobacteriaceae have been observed several times in IBD patients\(^{170,171,316} \). In fact, elevated abundance of Proteobacteria has been associated with an altered microbiome observed in IBD\(^{204,317,318} \). Furthermore, \( \text{NOD2} \) mutations have been linked with these differences in IBD patients\(^{319} \). Indeed, one study detected increased bacteria in the mucosa of IBD patients with \( \text{NOD2} \) mutations compared to healthy controls\(^{320} \), while another found a significant effect of \( \text{NOD2} \) genotype on the microbial composition of both IBD patients and healthy subjects, which was apparent in an allele-dosage effect\(^{172} \). Overall, mutations in Nod2 result in an altered gut microbial composition in both mice and humans. Although it remains difficult to tease apart which occurs first in humans, the microbial changes or the inflammation, the data presented in this thesis indicate that environmental influences can alter the gut microbiota in a Nod2-dependent manner, which can subsequently result in increased susceptibility to mucosal damage and colitis.

Neonatal antibiotic treatment significantly altered the gut microbiota at weaning. Bacterial families within each of the five represented phyla were significantly different compared to water-treated mice, with a significant reduction in Firmicutes and Actinobacteria, and increased abundance of Bacteroidetes and Proteobacteria. As described, this matches what has been observed in IBD, and correlates with microbiome data from early life antibiotic exposure in human infants\(^{181,321} \). Similar to the study performed by Yassour et al., the Canadian Healthy Infant Longitudinal Development (CHILD) study is a cohort of 3500 children who are being sampled longitudinally from birth until 5 years of age; the study is investigating the influences of environmental exposures and genetics on development and health\(^{322,323} \). As a part of this study, investigators will determine if the gut microbiota in early life is a predictor of asthma development\(^{256} \). Since the children are also being genotyped, it will be interesting to explore the impact of host genetics on the gut microbiota during early life and how this could be altered through environmental exposures, particularly antibiotics. In this way, we could assess whether
NOD2 genotype alters early life microbiota in a large cohort of healthy Canadian infants. Moreover, whether microbial resilience following antibiotic treatment differs by NOD2 genotype could also be determined. These data could lead to the identification of a microbial signature, or biomarker, that could be used to assess children genetically at risk for developing IBD, thus providing the opportunity for intervention and theoretically, prevention.

4.1.3 Nod2 modulates mucosal damage through sensing of gut microbes

Antibiotic exposure in early life and in adulthood have both been associated with developing IBD. I was interested in determining whether the antibiotic exposure experienced by the mice altered susceptibility to colitis. Indeed, I showed that an antibiotic altered microbiota in adult Nod2−/− mice remained significantly different and influenced the response to anti-CD3 mAb induced small intestinal mucosal damage. Previous work by our laboratory had identified a difference between WT and Nod2−/− mice in response to anti-CD3 mAb induced inflammation, with Nod2−/− mice displaying a delay in mucosal healing 72 hours post injection. This response was dependent on the gut microbiota, since germ-free mice failed to develop significant pathology and treatment with a cocktail of broad-spectrum antibiotics for 10 days prior to and during the response to anti-CD3 mAb resulted in an improvement in mucosal healing. Similarly, I found that antibiotic exposure four weeks prior to the anti-CD3 mAb injection resulted in a decrease in both IL-17A and MPO responses, indicating improved mucosal healing compared to water-treated Nod2−/− mice. Together, these data suggest that certain microbes could mediate exacerbated mucosal damage in a genetically susceptible host. Indeed, one of the key taxa reduced after antibiotic treatment was Erysipelotrichaceae, which is known to be highly IgA-coated in IBD patients and was found to worsen colitis when transferred into germ-free recipients. Moreover, exclusive enteral nutrition (EEN) therapy (i.e. an elemental diet) decreased Erysipelotrichaceae abundance, which correlated with improved disease activity in five paediatric CD patients. Erysipelotrichaceae has also been associated with colorectal cancer in humans and animal models, further implicating its involvement during inflammation. Although transfer of Erysipelotrichaceae into antibiotic-treated Nod2−/− mice failed to alter IL-17A responses, it would be interesting to colonize both WT and Nod2−/− germ-free mice with a limited microbial consortium (such as altered Schaedler flora or one created using the robogut) and add Erysipelotrichaceae species in to assess their influence on the
mucosal immune system and susceptibility to colitis. Similar to EEN, antibiotic treatment is used with some success in treating CD patients, likely through the manipulation of the gut microbiota and limiting microbial translocation into tissues\textsuperscript{327}. It would be interesting to collect samples from these CD patients before, during and after antibiotic treatment to see which microbes change and if this can be correlated back to patient outcomes and responsiveness to EEN therapy. Whether such responses could subsequently be associated with patient genetics would also be valuable and would further demonstrate a link between host genetics, microbes and mucosal inflammation.

Time of environmental exposures can significantly influence microbiota development. The first year of life is thought to be critical for development of both the gut bacterial community and the mucosal immune system\textsuperscript{120,314,328}. Perturbations during this time can result in lasting effects and influence susceptibility to various diseases, including asthma, obesity, multiple sclerosis and IBD\textsuperscript{180,276}. Indeed, I found that neonatal antibiotic exposure in mice significantly changed the gut microbiota, mucosal T cell populations and increased susceptibility to colitis. Effector and regulatory T cells are significantly involved in the inflammatory response in IBD; moreover, IBD patients have mucosal T cells directed against commensal microbes\textsuperscript{329}. Antibiotic treatment increased the proportion of Treg and Th17 cells in the small intestine and colon, irrespective of Nod2 genotype. However, the severity of colitis was worsened in antibiotic-treated Nod2\textsuperscript{-/-} mice associated with an increase in Gata3\textsuperscript{+} Treg. Indeed, IL33 expression, involved in induction of Gata3\textsuperscript{+} Treg during mucosal damage, is increased in the inflamed mucosa of IBD patients\textsuperscript{330}. It would be of interest to use another model of mucosal barrier disruption, such as TNBS colitis or infection with \textit{Citrobacter rodentium}, to determine if this is a common phenotype in antibiotic-treated Nod2\textsuperscript{-/-} mice. Since antibiotic treatment dramatically altered mucosal T cells, it would also be interesting to determine if Nod2 deletion specifically in T cells, through the use of CD4\textsuperscript{Cre} mice\textsuperscript{331}, would reproduce the phenotype. In this way, I could determine if Nod2 within T cells is sensing and responding to the microbial perturbation.

As described, timing is key for environmental manipulation; therefore, using different schemes of antibiotic treatment in an animal model will further elucidate which time is most strongly influenced by the treatment. In my studies, treatment began after birth and lasted until weaning; alternatively, treatment could begin on day 10 post birth and last until weaning, which would allow pups some time to begin developing with an unperturbed microbiota. In general, infants
receiving antibiotic treatment in early life receive it in doses lasting an average of 5-10 days\textsuperscript{249}. To model this style of pulsed antibiotic exposure, mice could be treated at three separate times, for 2-3 days each during the first 21 days of life; this would allow for perturbation and recovery such that the gut microbiota would settle at a new steady state. The influence of these other exposure timelines on the bacterial community and T cell populations would provide further insight into the mechanisms involved and key time points during development.

4.1.4 Nod2 - IgA link

Immune modulation of the luminal microbiota heavily relies on the production and secretion of IgA\textsuperscript{208}. In fact, more than 80\% of all plasma cells are IgA producers and reside in the lamina propria in humans\textsuperscript{332}. Several groups have shown that IgA shapes the microbiota, from neutralizing potential pathogens to maintaining the sterility of the inner mucus layer\textsuperscript{52,207,333,334}. In both components of this project, antibiotic exposure altered the IgA response and this was influenced by the absence of Nod2.

Neonatal antibiotic treatment resulted in a significant decrease in fecal IgA at day 35 and in systemic IgA at day 56. While fecal IgA concentration did not differ by genotype or treatment at day 56, there was a significant increase in colonic IgA\textsuperscript{+} plasma cells in antibiotic-treated Nod2\textsuperscript{-/-} mice compared to water-treated controls. Induction of intestinal IgA producing plasma cells occurs postnatally and is heavily influenced by the microbiota\textsuperscript{52,333}, thus it is not surprising that changes in the microbiota in early life altered both systemic IgA and colonic plasma cells. It would be of interest to test whether maturation of naïve intestinal B cells into plasma cells and induction of IgA production in vitro differs between of water vs. antibiotic-treated mice, and determine if Nod2 is involved. Alternatively, transfer of the microbiota at weaning from water vs. antibiotic-treated pups into germ-free recipients could help determine which bacteria are involved in the IgA phenotype. Using a combination of WT and Nod2\textsuperscript{-/-} donor samples and WT and Nod2\textsuperscript{-/-} germ-free recipients could elucidate whether Nod2 signalling is important for shaping the microbiota or in responding to it, or both. To determine a specific role for Nod2 signalling in B and plasma cells, researchers could utilize the CD19\textsuperscript{Cre} mouse, which expresses Cre-recombinase under the CD19 promoter and results in excision of a LoxP-flanked region following expression of CD19, which is found on B cells during development\textsuperscript{335}. Crossing this
mouse to the \textit{Nod2}\textsuperscript{flox} mice would result in littermates that are Nod2 sufficient and deficient in their B cell and plasma cell populations. Indeed, associating the IgA response observed in antibiotic-treated neonates with functional data highlighting the role of Nod2 in plasma cells would be of great interest and could further understanding of pattern recognition receptor activity in regulating intestinal immune homeostasis.

Antibiotic treatment of adult mice resulted in a significant increase in fecal IgA concentration in \textit{Nod2}\textsuperscript{-/-} mice that was detected by day 3 and maintained through day 7 of treatment. I correlated this response to changes in bacterial abundance and identified several taxa of interest, including \textit{Prevotella} and Erysipelotrichaceae, which were previously identified as IgA coated in IBD patients\textsuperscript{53}. Coating of commensal microbes with IgA has been described in both humans and mice\textsuperscript{336}, and recently shown to be directed predominantly against small intestinal microbes in a T cell-independent manner\textsuperscript{337}. Additionally, the specificity of T cell independent luminal IgA is thought to be polyreactive and of low affinity, suggesting that it is functional against a range of microbes\textsuperscript{56,338}. In keeping with this concept, I found that transfer of fecal IgA from antibiotic-treated \textit{Nod2}\textsuperscript{-/-} mice into water-treated \textit{Nod2}\textsuperscript{-/-} mice reduced subsequent IL-17A responses to i.p. anti-CD3 mAb, indicating that IgA transfer modulated either the bacteria directly or immune sensing of the microbes. The increase in luminal IgA observed during antibiotic exposure, and its ability to neutralize microbes in transfer experiments indicate that the immunoglobulin is likely targeting commensal microbial antigens. However, this does not shed light on mechanisms underlying this response. It is possible that increased IgA is not ‘additional’, but instead is the unbound IgA left over when the bacterial target is ablated by antibiotic treatment. Alternatively, the IgA detected could be in response to the immune system sensing ‘new’ bacteria, those now in closer proximity to the epithelial barrier due to niche availability\textsuperscript{332}.

To know which microbial antigens the IgA is reactive to would be important. This could be assessed in a few ways, each are associated with benefits and pitfalls. Firstly, fecal IgA could be used as a primary antibody in a western blot against a cocktail of commensal microbes, as described by Macpherson \textit{et al}\textsuperscript{209}. While this method would indicate whether the IgA is reactive against commensal antigens, the strength and specificity (which microbes are targeted) would remain unknown. Another possibility would be to sort fecal bacteria based on IgA coating, using a technique called IgA-Seq\textsuperscript{53,339}. Briefly, bacteria are isolated from stool and stained with an anti-
IgA fluorescent antibody, which is used during flow cytometry to sort bacteria by level of IgA coating, since highly bound bacteria will stain brighter. These sorted bacterial populations (IgA⁺ vs. IgA⁻) can then be sequenced to determine their identity. While these data would shed light on which bacterial taxa are being targeted, the specificity of the IgA for an antigen would still remain unknown. A third possibility is to use a microarray of bacterial antigens to determine what the IgA binds to, as described by Christmann et al\(^{307}\). In this way, the specificity of the IgA for known bacterial antigens would be elucidated. Indeed, a combination of these three techniques would provide the most insight into the reactivity of the IgA response. Moreover, such knowledge could open new avenues for IgA-microbe interactions that could be governed by host genetics, including Nod2 status.

4.2 Significance

Numerous studies in human and mice have described microbial, immunological and disease phenotypes based on Nod2 genotype. However, until now, very few studies have investigated a role for Nod2 on microbial composition and resilience, the immune response and susceptibility to colitis. This thesis provides a foundational assessment of how antibiotic exposure at different times in life influence both the gut microbiota and mucosal immune responses, and how mutations in Nod2 can alter these responses. By approaching experimental questions from both a microbial- and immune-focused point of view, this work addressed the hypotheses in a cohesive way, allowing for correlations between host and microbial responses. Furthermore, this thesis project developed a model to assess mechanisms involved in the epidemiological association of antibiotic exposure and IBD development through the use of a clinically relevant antibiotic, amoxicillin, at a clinically relevant dose. Animal models of IBD are not perfect, but the use of “multi-hit” models involving two or more of the four factors involved in IBD (host genetics, environment, immune response and the gut microbiota) improve the translatability of the observations.

The work arising from this project opens several new avenues of investigation for the future, including:

1. Identifying the mechanism by which Nod2 is involved in microbial resilience:
   a. Which cells are responsible for controlling microbial resilience?
b. Do NOD2 mutations in humans lead to altered resilience following antibiotics?
c. Does loss of Nod2 regulation of the gut microbiota lead to the induction of anti-commensal immune responses observed in IBD?

2. Translating the effects of early life antibiotic exposure to children at risk of developing IBD:
   a. Does NOD2 genotype in children alter the microbial response to antibiotic exposure?
   b. Can a biomarker be used to identify a microbial “risk” phenotype associated with NOD2 genotype?

3. Further elucidating the role of Nod2 microbial sensing on mucosal healing:
   a. Does Nod2 sensing of bacteria lead to increased regulatory T cell production or activity?
   b. Can certain microbes improve or worsen mucosal healing in a Nod2-dependent manner?

4. Understanding a link between Nod2 and mucosal IgA responses:
   a. Does microbial sensing by Nod2 play a role in IgA⁺ plasma cell development?
   b. Is there a regulatory role for Nod2 signalling on anti-commensal IgA responses?
   c. The protective effect of IgA transfer – a new therapeutic for IBD?

Collectively, the data presented in this thesis contribute to current understanding of the complex interplay between a host and the gut microbiota.
REFERENCES


