Fall 12-21-2011

# Microbial Regulation of Allergic Inflammation

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**Abstract**

Allergic diseases have reached pandemic levels and represent a significant source of morbidity, mortality and healthcare cost. These chronic inflammatory diseases are characterized by interleukin (IL)-4, IL-5, IL-9 and IL-13 production by CD4$^+$ T helper type 2 (T$_H$2) cells, immunoglobulin E (IgE) production by B cells, and the recruitment of effector cells to sites of tissue inflammation. In addition to host genetic polymorphisms and environmental triggers, studies in patients and model systems suggest that commensal bacterial-derived signals influence susceptibility to T$_H$2 cytokine-mediated allergic inflammation. For example, patients with allergies display altered commensal bacterial populations and antibiotic treatment increases allergy susceptibility. However, the influence of antibiotic treatment on intestinal bacterial communities, and subsequent effects on innate immune cells that influence the development of allergic inflammation, are poorly defined. **Chapter 2** describes the development and characterization of a new murine antibiotic treatment model using 454 deep sequencing techniques and details antibiotic-induced temporal and spatial alterations to bacterial communities colonizing the murine intestine. Having characterized the effects of antibiotic treatment on commensal populations, **Chapter 3** and **Chapter 4** examine the cellular and molecular mechanisms through which innate immune cells respond to commensal-derived signals and regulate T$_H$2 cytokine-mediated allergic responses. Data in **Chapter 3** describe antibiotic-induced steady-state elevations in serum IgE levels and circulating basophil populations, an innate granulocyte implicated in contributing to allergic T$_H$2 cell responses, as well as exaggerated basophil-mediated inflammation in models of allergic disease. Data in **Chapter 4** indicate that circulating basophil populations correlate with serum IgE levels in mice and patients with hyper-IgE syndrome, and that B cell-intrinsic MyD88 expression limits murine IgE levels and basophil responses. These results provide novel mechanistic insights into how commensal bacterial-derived signals influence T$_H$2 cytokine-mediated allergic inflammation, and have implications for the development of new preventative or therapeutic interventions for allergic disease.

**Degree Type**
Dissertation

**Degree Name**
Doctor of Philosophy (PhD)

**Graduate Group**
Cell & Molecular Biology

**First Advisor**
David Artis

**Keywords**
immunology, antibiotic, allergy, microbiology, basophil, commensal bacteria

**Subject Categories**
Allergy and Immunology

# MICROBIAL REGULATION OF ALLERGIC INFLAMMATION

David Andrew Hill

A DISSERTATION

In

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

2011

_____

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# Acknowledgements

# ABSTRACT

## MICROBIAL REGULATION OF ALLERGIC INFLAMMATION

David Andrew Hill

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Allergic diseases have reached pandemic levels and represent a significant source of morbidity, mortality and healthcare cost. These chronic inflammatory diseases are characterized by interleukin (IL)-4, IL-5, IL-9 and IL-13 production by CD4$^+$ T helper type 2 (T$_H$2) cells, immunoglobulin E (IgE) production by B cells, and the recruitment of effector cells to sites of tissue inflammation. In addition to host genetic polymorphisms and environmental triggers, studies in patients and model systems suggest that commensal bacterial-derived signals influence susceptibility to T$_H$2 cytokine-mediated allergic inflammation. For example, patients with allergies display altered commensal bacterial populations and antibiotic treatment increases allergy susceptibility. However, the influence of antibiotic treatment on intestinal bacterial communities, and subsequent effects on innate immune cells that influence the development of allergic inflammation, are poorly defined. **Chapter 2** describes the development and characterization of a new murine antibiotic treatment model using 454 deep sequencing techniques and details antibiotic-induced temporal and spatial alterations to bacterial

communities colonizing the murine intestine. Having characterized the effects of antibiotic treatment on commensal populations, **Chapter 3** and **Chapter 4** examine the cellular and molecular mechanisms through which innate immune cells respond to commensal-derived signals and regulate $T_H2$ cytokine-mediated allergic responses. Data in **Chapter 3** describe antibiotic-induced steady-state elevations in serum IgE levels and circulating basophil populations, an innate granulocyte implicated in contributing to allergic $T_H2$ cell responses, as well as exaggerated basophil-mediated inflammation in models of allergic disease. Data in **Chapter 4** indicate that circulating basophil populations correlate with serum IgE levels in mice and patients with hyper-IgE syndrome, and that B cell-intrinsic MyD88 expression limits murine IgE levels and basophil responses. These results provide novel mechanistic insights into how commensal bacterial-derived signals influence $T_H2$ cytokine-mediated allergic inflammation, and have implications for the development of new preventative or therapeutic interventions for allergic disease.

# Table of Contents

# Table of figures

## Publications

The contents of this thesis include portions of modified text and/or figures from some of the following published or submitted manuscripts.

**Hill, D.A.**, Siracusa, M.C., Abt, M.C., Kim, B.K., Kobuley, D., Kambayashi, T., Kubo, M., LaRosa, D.F., Renner, E.D., Orange, J.S., Bushman, F.D., Artis, D. Commensal bacterial-derived signals limit basophil development and basophil-mediated allergic inflammation. (2011) (Submitted).

Siracusa, M.C., Saenz, S.A., **Hill, D.A.**, Kim, B.S., Headley, M.B., Doering, T.A., Wherry E.J., Jessup, H.K., Siegel, L.A., Kambayashi, T., Dudek, E.C., Kubo, M., Cianferoni, A., Spergel, J.M., Ziegler, S.F., Comeau, M.R., Artis, D. TSLP promotes IL-3-independent basophil hematopoiesis and type 2 inflammation. (2011). *Nature.* (In Press).

**Hill, D.A.**, Artis, D. Intestinal bacteria and the regulation of immune cell homeostasis. (2010). *Annual Reviews of Immunology.* Apr;28:623-67. PMID: 20192812.

**Hill, D.A.**, Hoffmann, C., Abt, M.C., Du, Y., Kobuley, D., Kirn, T.J., Bushman, F.D., Artis, D. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. (2010). *Mucosal Immunology.* Mar;3(2):148-58. PMID: 19940845.

**Hill, D.A.**, Artis, D. Maintaining diplomatic relations between mammals and beneficial microbial communities. (2009). *Science Signaling.* Nov;2(98):77-79. PMID: 19934432.

Hoffmann, C., **Hill, D.A.**, Minkah, N., Kirn, T., Troy, A., Artis, D., Bushman, F. Community-Wide Response of the Gut Microbiota to Enteropathogenic Citrobacter rodentium Infection Revealed by Deep Sequencing. (2009). *Infection and Immunity.* Oct;77(10):4668-78. PMID: 19635824.

In some cases, collaborations with other researchers were undertaken to develop and perform the experiments included in this thesis. In such cases, collaborators have been appropriately acknowledged in the figure legend. In all collaborations, the author was the primary researcher responsible for developing and performing the experiments with the exception of Figure 17, some of the data for which was contributed by Michael Abt.

# Chapter 1: An introduction to commensal bacteria and the immune system

Microbes are the most abundant life form on earth. While many are free living, some have evolved to participate in close and often long-lasting interactions with multicellular hosts. Though some of these microbes have evolved as pathogens, others have evolved commensal relationships that are essential to normal host development and physiology. The existence of such symbiotic relationships with microbes is a conserved feature of multicellular organisms including plants (Paszkowski 2006), insects (Muyskens, Guillemin 2008), nematodes (Rae et al. 2008), fish (Bates et al. 2007), birds (Brisbin, Gong & Sharif 2008), and mammals (Hill, Artis 2010). The mammalian intestine is colonized by both the largest and most complex symbiotic microbial community comprised of approximately $10^{14}$ bacteria and numerous viral and parasitic microbes. In mammals, these microbes supply essential nutrients, aid in the digestion of otherwise indigestible compounds, promote angiogenesis and enteric nerve function, defend against opportunistic pathogens, and influence the development and homeostasis of the immune system (Hill, Artis 2010, Hooper, Gordon 2001, Macpherson, Harris 2004).

To maintain immunologic homeostasis, the mammalian immune system has evolved complex immunosurveillance networks able to respond to pathogens while maintaining tolerance to commensals and environmental antigens. When these mechanisms fail, host immune responses to commensal-derived signals can contribute to the development of multiple human diseases including inflammatory bowel disease (IBD) (Manichanh et al. 2006, Hanauer 2006), obesity (Ley et al. 2005), cancer (Moore, Moore 1995), diabetes (Bollyky et al. 2009), and allergy (Penders et al. 2007, Marra et al. 2009). This thesis focuses on commensal bacteria that colonize the mammalian intestine, and the role that commensal bacterial-derived signals play in limiting $T_H2$ cytokine-dependent allergic responses. Understanding the mechanisms by which

2

commensal-derived signals influence the mammalian immune system is essential to both fully understand mammalian physiology, and to better define the etiology of human inflammatory diseases in an effort to develop new preventative or therapeutic approaches.

Potentially detrimental alterations to commensal bacterial communities that result from antibiotic exposure are of particular medical relevance given recent epidemiological associations between antibiotic treatment and the development of allergic disease (Marra et al. 2009, Kummeling et al. 2007). However, the influence of oral antibiotic treatment on qualitative and quantitative measures of commensal bacterial communities is poorly defined. Therefore, **Chapter 2** of this thesis describes the development of a new murine oral antibiotic treatment model and the characterization of the subsequent effects on commensal bacterial communities that colonize the murine intestine. Having characterized the effects of antibiotic treatment on intestinal bacterial communities, the remainder of this thesis utilizes oral antibiotic treatment and germ-free mouse models to investigate the influence of commensal bacterial-derived signals on innate granulocyte populations known to contribute to the initiation or propagation of $T_H2$ cytokine-dependent allergic responses. Data in **Chapter 3** indicate that antibiotic treatment results in steady-state increases in serum IgE levels and circulating basophil populations, an innate granulocyte implicated in contributing to the development or propagation of $T_H2$ cell responses. Consistently, antibiotic-treatment resulted in exaggerated basophil-mediated inflammation in three murine models of allergic disease. Finally, the molecular and cellular mechanisms by which the mammalian immune system recognizes commensal bacterial-derived signals and limits circulating basophil populations are the focus of **Chapter 4**. Circulating basophil populations correlated with

serum IgE levels in mice and DOCK8- humans, and B cell-intrinsic MyD88 expression was identified as a regulatory pathway that limits murine IgE levels and basophil responses. Together, these results provide novel mechanistic insights into how commensal bacterial-derived signals influence $T_H2$ cytokine-mediated allergic inflammation, and have broad implications for the development of new preventative or therapeutic interventions for allergic disease.

In the remainder of **Chapter 1**, I first review previously published data supporting a role for commensal bacteria in influencing the development or progression of human disease. I then give an overview of commensal bacterial acquisition and detection of commensal bacterial-derived signals by the host immune system. Next, I discuss data from germ-free or antibiotic-treated mouse models that indicate a role for commensal-derived signals in influencing the development and/or homeostasis of specific host immune cell populations. Finally, I review basophil physiology and what is known of how basophils cooperate with other innate immune cell types to influence the development and/or propagation of $T_H2$ cytokine-dependent inflammatory responses.

## 1.1 Intestinal bacteria in health and disease

Over the past century, studies of humans and animal models have identified important roles for commensal bacterial communities in promoting the optimal digestion of food (Hooper, Midtvedt & Gordon 2002), maintenance of epithelial homeostasis (Artis 2008), modulating fat metabolism (Backhed et al. 2004), promoting angiogenesis (Stappenbeck, Hooper & Gordon 2002) and enteric nerve function (Husebye, Hellstrom & Midtvedt 1994), supporting resistance to infection (Sekirov et al. 2008) and promoting

normal development and homeostasis of the host immune system (Hill, Artis 2010, Cebra 1999) (**Figure 1**). Given these important roles for commensal-derived signals in maintaining normal host physiology, it is not surprising that perturbations to commensal communities have been implicated as both a bio-marker for, and a potential contributing factor to multiple human diseases. In this section, what is known of the role that commensal bacterial-derived signals play in the pathophysiology of inflammatory bowel disease and allergy are discussed as models of intestinal and systemic human disease states, respectively.
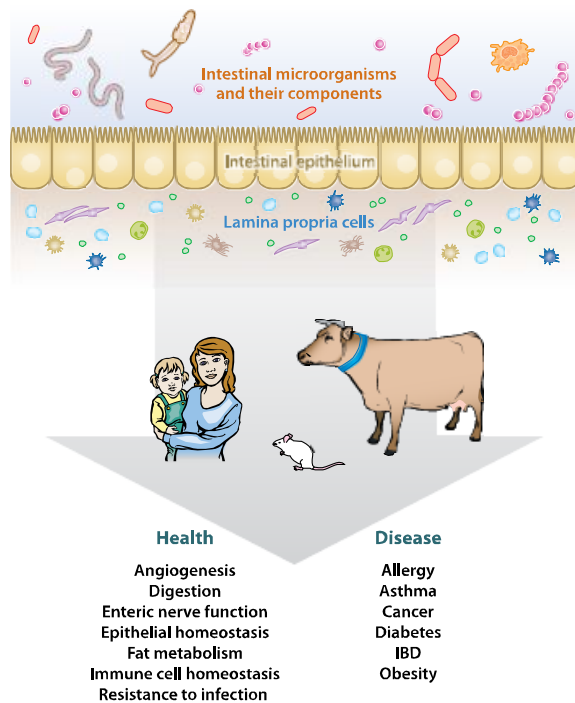


**Figure 1: Intestinal bacteria in mammalian health and disease**

Schematic of the known influences of intestinal bacteria on normal mammalian physiology and disease states. Adapted from (Hill, Artis 2010).

Inflammatory bowel disease (IBD; Crohn's Disease and Ulcerative Colitis) is thought to result in part from inappropriate and ongoing mucosal immune responses to commensal intestinal bacteria (Podolsky 2002). IBD patients have altered commensal bacterial communities compared to healthy individuals and tolerance to intestinal bacteria is broken in IBD leading to inappropriate local and systemic immune responses to commensal-derived signals (Macpherson et al. 1996)(Manichanh et al. 2006, Macpherson et al. 1996, Duchmann et al. 1995, Lodes et al. 2004, Adams et al. 2008). For example, IBD patients display aberrant cytokine production, T cell activation, and mucosal and systemic antibody responses to intestinal bacterial-derived antigens (Macpherson et al. 1996, Duchmann et al. 1995). Consistent with commensal-bacterial derived signals being a precipitating factor in IBD, antibiotic therapy designed to deplete the commensal bacterial load is a component of IBD therapeutic regimens (Talley et al. 2011). Despite these findings, the molecular and cellular causes of the dysregulated immune responses to commensal bacteria observed in IBD patients are not fully understood, however, polymorphisms in innate pattern recognition receptors are associated with susceptibility to IBD. Most notably, mutations in NOD2, a component of the innate immune system that is important for immune recognition and responses to intracellular bacteria, are associated with increased risk of IBD development (Ogura et al. 2001, Hugot et al. 2001, Hisamatsu et al. 2003). These findings implicate inappropriate recognition of commensal-derived signals, and subsequent priming and activation of the adaptive immune system, as central to IBD pathogenesis.

Animal models have supported a role for inappropriate immune responses to commensal bacterial-derived signals in contributing to IBD pathogenesis. For example, akin to the antibiotic treatment in human IBD patients, reducing commensal stimulation

in several murine models of IBD, achieved by rearing animals under germ-free conditions or via oral antibiotic treatment, ameliorates intestinal disease. This is the case with IL-2-deficient mice that spontaneously develop intestinal inflammation when raised under conventional conditions, but have a delayed and more mild disease course when raised under germ-free conditions (Schultz et al. 1999). Similarly, IL-10-deficient mice also develop spontaneous colitis associated with inappropriate T cell responses when maintained under conventional conditions, but are protected against disease when maintained under germ-free conditions or when treated with oral antibiotics (Dianda et al. 1997, Sellon et al. 1998, Madsen et al. 2000, Hoentjen et al. 2003). These findings support the view that inappropriate immune responses to commensal-derived signals contribute to the development or progression of disease in these immunologically impaired hosts.

Pathogenic commensal communities can also initiate disease states in hosts with an intact immune system. For example, immunodeficient mice deficient in the inflammatory transcription factor T-bet and the recombinase-activating gene RAG2 (TRUC mice) develop spontaneous colitis that is ameliorated by antibiotic treatment (Garrett et al. 2007). Importantly, when wild-type mice are fostered by or co-housed with TRUC mice they develop colitis, indicating that vertical or horizontal transmission of colitogenic bacterial communities can cause disease in immunocompetent animals (Garrett et al. 2007). This observation indicates that alterations in commensal bacterial communities might not only be a consequence of disease, but that normal commensal communities may protect against the development of colitis in healthy individuals. Consistent with this, disruption of commensal communities via antibiotic treatment increases patient risk of developing *Clostridium difficile* colitis, and alteration of

commensal populations has been implicated in precipitating IBD in select cases (De La Cochetiere et al. 2008, Hviid, Svanstrom & Frisch 2011).

In addition to influencing intestinal inflammatory responses, commensal-derived signals have also been implicated in influencing susceptibility to asthma and other systemic atopic disorders in humans (Penders et al. 2007). Allergic diseases have reached pandemic levels and represent a significant source of morbidity, mortality, and healthcare cost (Eder, Ege & von Mutius 2006, Bahadori et al. 2009). The development of allergies is determined by interactions between genetic and environmental factors. For example, allergy susceptibility is associated with genetic polymorphisms in more than 33 genes including HLA alleles and components of allergic cytokine signaling pathways (Vercelli 2008). Additionally, environmental stimuli including pollutants, diet, and infection history can influence susceptibility to allergic disease (Gilliland 2009, Zeiger 2003, Harnett, Harnett 2010). More recently, alterations in the composition or quantity of intestinal commensal bacterial communities have been implicated in contributing to allergy susceptibility (Hill, Artis 2010, Round, Mazmanian 2009). However, the cellular and molecular mechanisms by which commensal bacteria influence allergic inflammation are poorly understood.

Allergic patients display altered commensal bacterial community structure in the intestine, and infants who go on to develop allergies display altered commensal bacterial community structure early in life (Penders et al. 2007, Kalliomaki et al. 2001). Additionally, antibiotic exposure modifies commensal bacterial community structure of the intestine and significantly correlates with increased risk of allergy development in children, implicating commensal bacterial-derived signals in influencing susceptibility to allergic diseases (Marra et al. 2009, Kummeling et al. 2007, Hill et al. 2009). In some

8

cases, specific bacterial groups have been identified as potential contributors to allergy susceptibility. For example, associations have been found between colonization with *Bifidobacterium*, *C. difficile* or *Escherichia coli*, and the development of eczema in humans (Penders et al. 2007, Gore et al. 2008, Penders et al. 2008).

Animal models have provided additional insights into defined species of commensal bacteria, and specific commensal-derived signals, which influence allergic responses. Antibiotic treatment of mice results in exaggerated food allergy-like symptoms characterized by increased levels of allergen-specific IgE in the serum compared to conventionally-reared mice (Sudo et al. 1997). TLR4 signaling is important for commensal bacterial-derived signals to promote oral tolerance as TLR4-deficient mice display similar levels of food allergy compared to mice treated with oral antibiotics (Bashir et al. 2004). Similarly, commensal bacterial-derived signals also seem to limit disease in models of allergic asthma. Antibiotic treatment resulted in the selective outgrowth of fungal microbes in the intestine and worse disease outcomes in an IL-13-dependent mechanism (Noverr et al. 2005). Despite these strong epidemiologic and experimental associations between alterations in commensal bacterial populations and allergic inflammation, relatively little is known about the molecular and cellular mechanisms by which the innate immune system recognizes commensal bacterial-derived signals and influences the development $T_H2$ cytokine-dependent allergic inflammation.

## 1.2 Commensal bacteria and the mammalian host

### 1.2.1 The bacterial composition and colonization dynamics of the mammalian intestine

After birth, the epithelial surfaces of mammals are colonized with viruses, fungi, bacteria, protozoa, and helminth parasites creating complex microbial communities in multiple environmental niches. Several terms have been used to describe relationships between microbes that colonize multicellular organisms, but for the purposes of this thesis "commensal" is used to describe a mutualistic relationship within which the microbe provides some benefit to the host. The mammalian gastrointestinal (GI) tract is the best studied of these microbial environments. By adulthood, the GI tract is colonized by members of all three domains of life; though intestinal bacterial communities are both the most abundant and best characterized of microbial community (Whitman, Coleman & Wiebe 1998), and as a result are the primary focus of this thesis.

Though exposed to bacteria throughout life, mammals are thought to undergo two dominant phases of intestinal colonization; the first during breast milk or formula feeding and the second upon weaning to solid foods. Mammalian breast milk is both a source of defined microbes and of passive immunity in the form of immunoglobulins and other maternal-derived molecules that shape developing intestinal communities (Diaz et al. 2004, Martin et al. 2007b, Martin et al. 2007a). The temporal and spatial patterns of intestinal colonization in infants are variable between individuals (Palmer et al. 2007) and dependent upon multiple factors including country of birth (Benno et al. 1986), length of gestation (Penders et al. 2006, Stark, Lee 1982a, Blakey et al. 1982), mode of delivery (Penders et al. 2006, Gronlund et al. 1999), history of hospitalization (Penders

et al. 2006), antibiotic use (Penders et al. 2006) and feeding practices (Penders et al. 2006, Hayashi, Sakamoto & Benno 2002a, Harmsen et al. 2000, Lundequist, Nord & Winberg 1985, Stark, Lee 1982b, Yoshioka, Iseki & Fujita 1983, Penders et al. 2005). For example, vaginally born and breast-fed infants have a predominance of *Bifidobacteria* in their intestine, with lower contributions of *E. coli*, Bacteroides, and Clostridia species while infants delivered by cesarean section have delayed colonization kinetics compared to vaginally born infants and persistent changes to bacterial communities including lower burdens of *Bifidobacteria* and Bacteroides groups, and higher burdens of *C. difficile* (Penders et al. 2006, Gronlund et al. 1999, Bullen, Tearle & Willis 1976). Though these individuals likely acquire normal adult bacterial communities upon transition to solid foods, these differences may not be without consequence as associations between early alterations to intestinal bacterial communities and increased risk of allergic disease have been identified (Renz-Polster et al. 2005, Salam et al. 2006).

At maturity, the bacterial burden of the mammalian GI tract is estimated at over 100 trillion individual organisms at a density of $10^{11}$ to $10^{14}$ cells per gram of luminal contents (Whitman, Coleman & Wiebe 1998, Eckburg, Lepp & Relman 2003). Studies carried out as early as the 1960's using culture-based and microbiological identification methods identified Lactobacilli, Streptococci and members of the Bacteroides genus as residents of the normal adult human intestine (Dubos et al. 1965). However, a large percentage of intestinal bacteria are anaerobes that lack the enzymes necessary for the detoxification of oxygen. As such, even under ideal conditions it is estimated that only half of intestinal bacteria are culturable indicating that these early studies were limited in their sensitivity (Adlerberth, Wold 2009).

11

More recently, molecular advances in DNA bar coding and 454 pyrosequencing of 16S ribosomal RNA gene segments are allowing previously unattainable insights into both culturable and non-culturable bacterial communities of the mammalian intestine (Hayashi, Sakamoto & Benno 2002b, Hold et al. 2002, Wang et al. 2003, Eckburg et al. 2005). These studies have identified the Firmicutes and Bacteroidetes phyla as the major bacterial groups present in the mammalian intestine (**Figure 2**) but are placing total species estimates from conservative numbers of 1000-2000 to numbers as high as 15,000-40,000 individual members (Hayashi, Sakamoto & Benno 2002b, Hold et al. 2002, Wang et al. 2003, Eckburg et al. 2005, Frank, Pace 2008). Of the Firmicutes, 95% belong to the Clostridia class, while there are large variations in the Bacteroidetes phylotypes among individuals (Hayashi, Sakamoto & Benno 2002b, Hold et al. 2002, Eckburg et al. 2005, Finegold, Sutter & Mathisen 1983). Other phyla present in relatively low abundance include the Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia (Hayashi, Sakamoto & Benno 2002b, Wang et al. 2003, Eckburg et al. 2005, Finegold, Sutter & Mathisen 1983, Suau et al. 1999). The complexity of intestinal bacterial communities suggests intimate coevolution between commensal communities and mammalian hosts, and supposes that alterations in commensal bacterial communities could impact numerous facets of mammalian physiology.
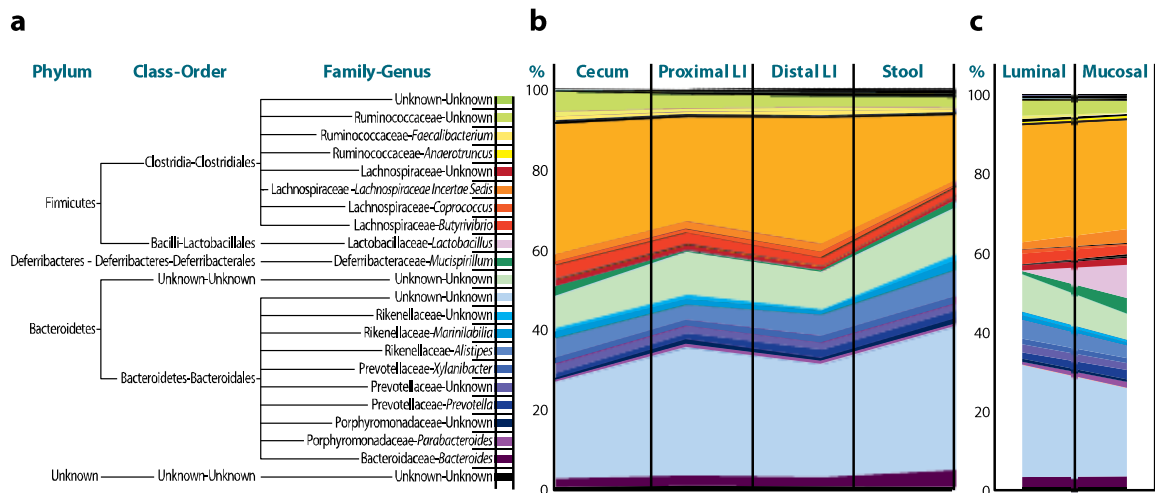
**Figure 2: The composition of bacterial communities along the length and between luminal and mucosal compartments of the mammalian intestine**

Stool pellet, luminal content, or mucosal-associated communities were sterilely collected. Total sample DNA was extracted and bacterial 16S rDNA fragments were PCR amplified with bar code-tagged primers, subjected to pyrosequencing, and taxonomic assignments for each sequence were determined using RDP Classifier. (**a**) Commonly found bacteria in the murine colon. (**b**) Relative frequencies and distribution of bacteria along the length of the murine colon. (**c**) Relative frequencies and distribution of bacteria between luminal and mucosal-associated compartments of the murine colon. Adapted from (Hill, Artis 2010).

Intestinal bacterial communities exhibit differences along the length of the colon and differ significantly between the luminal and tissue attached microenvironments suggesting that defined microbial communities at different anatomical locations may be important for distinct aspects of normal mammalian physiology (**Figure 2b,c**) (Eckburg et al. 2005, Zoetendal et al. 2002, McKenna et al. 2008). For example, while Lactobacilli have been cited for potential probiotic effects, and can be isolated from approximately 80% of adults, they represent a relatively low proportion of luminal bacteria (Finegold, Sutter & Mathisen 1983, Kuitunen et al. 2009). However, this group represents a significant proportion (up to 13%) of mucosal-associated bacteria (**Figure 2**) indicating

that colonization of this anatomical niche may be important for Lactobacilli's potentially beneficial physiologic effects for the host (Hill et al. 2009). To maintain homeostasis with colonizing bacteria, mammals have evolved complex compensatory responses to commensal-derived signals (Hill, Artis 2009). The host immune response to microbial colonization will be discussed in the next section.

### 1.2.2 The host immune response to microbial colonization

The initial immune response to bacterial colonization by the mammalian host peaks within a week of birth and subsequently stabilizes over the first year of life (Shroff, Meslin & Cebra 1995). Initially, the host mounts an innate immune response to bacterial colonization that is important for maintenance of the mucosal barrier (reviewed in (Dann, Eckmann 2007)). This includes direct detection of commensal-derived signals by intestinal epithelial cells via cell-autonomous MyD88 activation resulting in increased expression of the antimicrobial peptides REGIIIγ and RELMβ (Wang et al. 2005, Vaishnava et al. 2008). Additionally, the host mounts an adaptive immune response characterized by production and secretion of IgA into the intestinal lumen (Macpherson 2006). IgA production occurs in response to small numbers of intestinal bacteria that initially penetrate the intestinal epithelium where they are phagocytosed by DCs that initiate IgA responses through T cell-dependent and -independent mechanisms (Renshaw et al. 1994, Macpherson et al. 2000, Macpherson, Uhr 2004a, MacPherson et al. 2004, Macpherson, Uhr 2004b, Bergqvist et al. 2006). In contrast to disease states such as IBD, these B cell responses are limited to the mesenteric lymph nodes (mLNs) and do not occur in systemic secondary lymphoid structures, indicating that induction of this commensal-specific IgA response is compartmentalized to mucosal tissues under

14

normal physiological conditions (Macpherson, Uhr 2004b, Konrad et al. 2006). Secretion of IgA into the intestinal lumen acts as a homeostatic feedback loop to reduce epithelial penetration by intestinal bacteria, and subsequent host inflammatory responses (Macpherson et al. 2001). The initiation of these homeostatic responses is critically dependent on recognition of commensal-derived signals by immune cells via innate pattern recognition receptors. Innate recognition of commensal bacterial-derived signals by pattern recognition receptors is reviewed in the next section.

## 1.3 Recognition of commensal-derived signals by pattern recognition receptors

As discussed above, the colonization of the mammalian intestine results in a rapid compensatory response by the mucosal immune system that is important for maintaining mucosal integrity and immune homeostasis. A component of this innate response is the recognition of microbial-derived cell wall components, DNA segments, and metabolites by host pattern recognition receptors (PRRs) including Toll-like receptors, Nod-like receptors, and G protein coupled receptors. This section reviews commensal bacterial-derived signals, the innate receptors that recognize them, and focuses on the role that intestinal epithelial cells (IECs) and dendritic cells (DCs) play in responding to these signals and modulating subsequent immune responses.

### 1.3.1 Recognition of commensal-derived signals by Toll-like receptor ligands

Toll-like receptors (TLRs) are innate pattern recognition receptors that bind evolutionarily conserved motifs found in bacteria and other microbes (Medzhitov, Preston-Hurlburt &

Janeway 1997). TLRs have evolved to recognize multiple microbial-derived signals including double-stranded viral RNA (TLR3), gram-negative lipopolysaccharide (LPS) (TLR4), and gram-negative and gram-positive flagellin (TLR5) (West, Koblansky & Ghosh 2006). Most TLRs are expressed on the surface of cells with the exception of TLR3, 7, 8, and 9 which are localized to endolysosomal compartments (Medzhitov 2007).

Studies in humans and animal models have implicated TLRs as important modulators of both physiologic and pathologic host immune responses to commensal bacteria. In some cases, recognition of commensal-derived signals by TLRs contributes to disease states. For example, dominant-negative TLR5 polymorphisms in humans result in reduced responses to flagellin, and protect against the development of Crohn's disease (Gewirtz et al. 2006). Consistent with these findings, studies in animal models have shown that recognition of bacterial flagellin by TLR5 influences proinflammatory gene expression by intestinal epithelial cells (Gewirtz et al. 2001). However, recognition of bacterial-derived signals by TLRs can also be protective in other settings. For example, LPS administration signals through TLRs to protect against chemically-induced experimental colitis while treatment with flagellin (which signals via TLR5) protects against chemical, bacterial, viral, and radiation-induced mortality in multiple animal models (Rakoff-Nahoum et al. 2004, Vijay-Kumar et al. 2008). Finally, administration of the TLR9 ligand CpG reduces severity of food allergy in TLR4-deficient mice (Bashir et al. 2004). The potential protective roles for TLR signaling in disease have spurred interest in TLR ligands as therapeutic agents and TLR ligands are currently being investigated as treatments for both human allergy and as adjuvant therapies for cancer (Racila, Kline 2005, Krieg 2007).

16

### 1.3.2 Recognition of commensal-derived signals by NOD-like receptors

NOD-like receptors (NLRs) detect intracellular ligands and have been identified as key mediators of proinflammatory and immunoregulatory responses in animals and humans (Fritz et al. 2006). NLRs recognize several commensal bacterial-derived signals including peptidoglycan containing meso-diaminopimelic acid (NOD1) and muramyl dipeptide (NOD2) (Carneiro et al. 2008). In the setting of an invading pathogen, NOD2 ligation initiates NF-κB activation and upregulation of proinflammatory cytokines (Fritz et al. 2006). Consistently, NOD-1 dependent recognition of commensal-derived signals has been shown to be important for normal neutrophil-mediated killing of pathogenic bacteria (Clarke et al. 2010). However, NOD-dependent signaling can also play a protective role in various disease states. In humans, approximately 15% of patients with Crohn's disease have homozygous or compound heterozygous mutations in the gene that encodes NOD2 (CARD15) implicating NOD signaling in a protective role (Ogura et al. 2001, Hugot et al. 2001). Consistent with this hypothesis, chronic stimulation of NOD2 has been shown to mediate tolerance to bacterial products and NOD2-deficient mice have an increased susceptibility to experimental colitis (Watanabe et al. 2006, Hedl et al. 2007, Watanabe et al. 2008). The mechanisms by which NOD-dependent signals can have such diverse immunomodulatory outcomes are an active area of research. One likely explanation is that NOD-dependent signals have different immune outcomes depending on the broader immunological context in which they are acting (Watanabe et al. 2006, Watanabe et al. 2004).

### 1.3.3 Recognition of commensal-derived signals by GPCRs and other receptors

In addition to the TLR and NOD ligands, immunoregulatory G protein coupled receptor (GPCR) ligands such as the purine adenosine (Ado) are of growing interest in the fields of IBD and other inflammatory diseases (Jacobson, Gao 2006). Ado may function as an endogenously generated regulator of inflammation with different immune outcomes depending on the receptor it binds. For example, an Ado A2A receptor agonist did not alter the course of chemical colitis while Ado A2B-deficient mice have increased susceptibility to chemical colitis (Selmeczy et al. 2007, Frick et al. 2009). More recently, intestinal bacteria were found to be a source of adenosine triphosphate (ATP) that can drive $T_H17$ cell differentiation in the lamina propria by inducing IL-6 and IL-23p19 production by a population of CD70 (high) CD11c (low) cells (Atarashi et al. 2008). Accordingly, germ-free mice exhibit lower concentrations of luminal ATP, as well as fewer numbers of $T_H17$ cells in the LP, a defect that could be reversed through the systemic or rectal administration of ATP (Atarashi et al. 2008).

Commensal bacteria that colonize the mammalian intestine also produce significant amounts of butyrate, as well as other short chain fatty acids (Pryde et al. 2002). The receptor for butyrate, GPR109A, is expressed on IECs and is down regulated in human colon cancer, in a mouse model of intestinal/colon cancer, and in colon cancer cell lines (Avivi-Green et al. 2000, Hague et al. 1995). Consistent with the hypothesis that GPR109A signaling suppresses tumor development or progression, expressing GPR109A in colon cancer cells induces apoptosis in the presence of butyrate (Thangaraju et al. 2009). Butyrate has been shown to have an immunoregulatory role in the steady-state by signaling through GPR109A on IECs to suppress NF-κB signaling (Thangaraju et al. 2009, Kumar et al. 2009). Consistent with a primarily

immunoregulatory role, butyrate reduces production of TNFα, TNFβ, IL-6, and IL-1β by lamina propria lymphocytes (LPLs) of IBD patients (Luhrs et al. 2002, Segain et al. 2000). Together, these findings indicate that butyrate inhibits tumorigenesis and limits proinflammatory responses in the intestine.

Succinate, a component of the citric acid cycle, is a commensal metabolite that has been shown to modulate dendritic cell function by signaling through the extracellular GPCR GPR91. In one study, succinate signaling triggered intracellular calcium release, induced DC migratory responses, and acted in synergy with TLR ligands to induce the production of proinflammatory cytokines by DCs (Rubic et al. 2008). Succinate also enhanced antigen-specific activation of human and mouse helper T cells, while GPR91-deficient mice had reduced Langerhan cell migration to draining lymph nodes and impaired tetanus toxoid-specific recall T cell responses (Rubic et al. 2008). Together, these results implicate the sensing of succinate through GPR91 as an important signal of immunologic danger.

Finally, bacterial adhesion via surface proteins can directly modulate immune cell function. For example, *Lactobacillus acidophilus* NCFM attaches to DCs resulting in production of IL-10 and IL-12p70 (Konstantinov et al. 2008). The bacterial surface component surface layer A protein (SlpA) was found to be sufficient for this immunomodulatory function as purified SlpA protein bound and activated DCs directly (Konstantinov et al. 2008). Consistently, T cells primed with DCs that were stimulated with *L. acidophilus* NCFM lacking SlpA produced less IL-4 than those stimulated with wild-type *L. acidophilus* NCFM (Konstantinov et al. 2008). In summary, commensal-derived signals can have proinflammatory or immunoregulatory effects depending on the immunologic context in which they act. I will next discuss how recognition of commensal-

derived signals by IECs and DCs via innate PRRs influences the development of local and systemic immune responses.

## 1.4 Recognition of commensal-derived signals by innate immune cells

### 1.4.1 Recognition of commensal-derived signals by intestinal epithelial cells

The intestinal epithelium has a diverse set of physiologic functions including digestion and absorption of nutrients, the maintenance of a physical barrier from the external environment, and immunological surveillance of intestinal bacteria and potential pathogens. Intestinal epithelial cells (IECs) are continually replaced from a pool of Lgr5$^+$ multipotent stem cells that reside in crypts of the intestine (Gordon, Hermiston 1994, Sato et al. 2009). IECs utilize intercellular tight junctions to prevent paracellular traffic and actin-rich microvillar extensions to create an apical brush border that impedes microbial attachment and invasion (Shen, Turner 2006). As a result, selective uptake of macromolecules, particulate antigens, and microorganisms across the intestinal epithelia occurs primarily via active vesicular transport across epithelial cells, and as such is restricted by multiple cellular and molecular mechanisms (reviewed in (Neutra, Pringault & Kraehenbuhl 1996)).

In addition to physical adaptations that control transport across the epithelium, biochemical adaptations have evolved including goblet cells, a cell type that produces a heavily glycosylated, mucin-rich secretion that creates a relatively impermeable apically adhered glycocalyx (Frey et al. 1996). The epithelium also produces antimicrobial peptides including defensins, cathelicidins, and calprotectins that confer broad-spectrum antimicrobial properties through the formation of pores in the bacterial cell wall (Ganz

2003). These adaptations are consistent with the view that IECs, in addition to promoting digestion and absorption of nutrients, perform essential barrier functions that obstruct the entry of beneficial and pathogenic microorganisms into the underlying lamina propria.

IECs are in continuous contact with beneficial and pathogenic bacteria and, as a result, are ideally located for "immunologic surveillance" of the intestinal lumen. IECs express TLRs, NLRs, and GPCRs that recognize microbial components and modulate cellular responses (**Figure 3**) (Hisamatsu et al. 2003, Gewirtz et al. 2001, Cario, Podolsky 2000). For example, IEC expression of innate pattern recognition receptors is important for mounting immune responses to pathogenic microbes (Carneiro et al. 2008, Zilbauer et al. 2007) by mediating the expression of pro-inflammatory cytokines, chemokines, and antimicrobial peptides, as well as the modulating of other immune cell types including the direct induction of IgA class switching by B cells (Macpherson et al. 2000, Medzhitov 2007, Fritz et al. 2006, He et al. 2007, Xu et al. 2007). Responses by IECs to intestinal bacteria are not uniform however. IECs selectively initiate pro-inflammatory response to pathogenic bacteria while promoting tolerance to beneficial bacteria (Shen, Turner 2006, Zilbauer et al. 2007, Jung et al. 1995). For example, the gram-negative bacteria *Bacteroides thetaiotaomicron* which induces IEC expression of the antimicrobial peptide REGIIIγ, while the gram-positive *Bifidobacterium longum*, a common component of intestinal communities, does not (Cash et al. 2006, Sonnenburg, Chen & Gordon 2006).
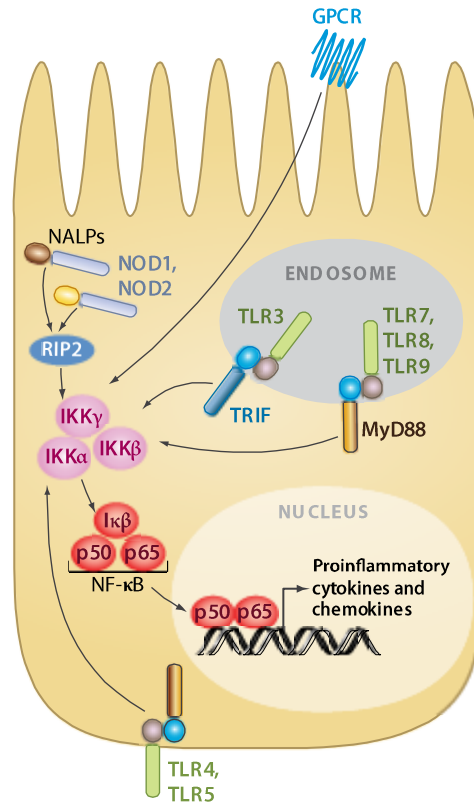
**Figure 3: Innate receptors and signaling cascades of mammalian intestinal epithelial cells**

Schematic shows the location of innate pattern-recognition receptors and their signaling cascades in mammalian IECs. Innate pattern-recognition receptors converge on a common NF-κB signaling cascade to regulate transcription of proinflammatory or immunoregulatory cytokines and chemokines**.** Adapted from (Hill, Artis 2010).

Two mechanisms by which IECs may discriminate between beneficial and pathogenic bacteria are through subcellular sequestering of pattern-recognition receptors away from luminal signals and by differential receptor expression. For example, TLR5 (which recognizes bacterial flagellin) has been reported to be expressed exclusively on the basolateral surfaces of IECs (Gewirtz et al. 2001). Additionally, TLR3, 7, 8 and 9 are expressed exclusively in intracellular endosomal organelles, while NLRs

are localized to the cytoplasm. In all these cases, reducing exposure of these receptors to luminal bacterial-derived signals limits pro-inflammatory responses to primarily pathogenic settings (Medzhitov 2007, Philpott, Girardin 2004, Uehara et al. 2007). In addition, under steady-state conditions, IECs are reported to express little or no TLR2, TLR4, or CD14 further minimizing the recognition of bacterial LPS from beneficial bacteria (Abreu et al. 2001, Melmed et al. 2003). However, subcellular localization and differential expression alone cannot account for all discrimination between beneficial and pathogenic bacteria by IECs as GPCRs that recognize and mount inflammatory responses to bacterial products are continuously expressed on their apical surface. As such, further investigation is necessary to fully understand how IECs operate in the context of the mucosal immune system to discriminate against beneficial and pathogenic bacteria.

The recognition of bacterial signals by IECs is essential to mucosal homeostasis implicating IECs as central modulators of subsequent adaptive responses (Rakoff-Nahoum et al. 2004, Bouskra et al. 2008, Cario, Gerken & Podolsky 2007). One mechanism by which IECs can influence proinflammatory or immunoregulatory responses is by influencing DCs, macrophages, and lymphocytes through the local expression of immunoregulatory cytokines including thymic stromal lymphopoietin (TSLP), IL-10, transforming growth factor β (TGFβ), prostaglandin E2 (PGE2), retinoic acid or IL-25 (Zaph et al. 2008, Brown et al. 2007, Dignass, Podolsky 1993, Rimoldi et al. 2005a, Rimoldi et al. 2005b, Taylor et al. 2009, Zeuthen, Fink & Frokiaer 2008a, Zeuthen, Fink & Frokiaer 2008a). For example, monocyte-derived or circulating DCs conditioned with supernatants from IECs isolated from healthy patients induced Foxp3^{+} Treg differentiation, while IECs from Crohn's disease patients did not suggesting that

IECs may condition DCs to have primarily immunoregulatory phenotypes under physiologic conditions (Iliev et al. 2009b). This effect was dependent on the production of TGFβ and retinoic acid by IECs, but not TSLP, as DCs deficient in the TSLP receptor (TSLPR-/-) and wild-type DCs had a similar ability to convert naïve T cells into Treg cells (Iliev et al. 2009a). Indeed, a population of intestinal DCs induced FoxP3$^+$ Tregs in a TGFβ and retinoic acid-dependent manner *in vitro* further implicating epithelial derived signals in the conditioning of DCs and subsequent adaptive responses, while deletion of NF-κB signaling specifically in IECs resulted in the dysregulated expression of DC-derived proinflammatory cytokines and the development of spontaneous or infection-induced intestinal inflammation (Nenci et al. 2007, Zaph et al. 2007, Sun et al. 2007, Coombes et al. 2007). Together, these findings indicate that IECs play a crucial role in conditioning intestinal DC responses.

Epithelial—derived TSLP has been shown to have important immunomodulatory roles. High levels of *Tslp* mRNA are expressed by epithelial cells at the mucosal surfaces of the skin, airways, and intestine and expression can be upregulated by infection, inflammation or tissue injury in an NF-κB-dependent manner (Rimoldi et al. 2005b, Zaph et al. 2007, Li et al. 2006, Allakhverdi et al. 2007, Bogiatzi et al. 2007, Kato et al. 2007, Lee, Ziegler 2007). *In vitro* studies have shown that TSLP-conditioned human DCs can promote $T_H2$ cell responses through the inhibition of IL-12 production and the induction of OX40L expression (Rimoldi et al. 2005b, Soumelis et al. 2002, Al-Shami et al. 2005, Ito et al. 2005, Liu 2007). *In vivo* studies in the skin and lung have shown that transgenic overexpression of TSLP in cutaneous or pulmonary epithelial cells results in the onset of $T_H2$ cytokine–mediated inflammation resembling atopic dermatitis or asthma, respectively, suggesting that TSLP is both necessary and sufficient for the

initiation of $T_H2$ cytokine–driven inflammation in some settings (Soumelis et al. 2002, Al-Shami et al. 2005, Yoo et al. 2005, Zhou et al. 2005) (reviewed in (Ziegler, Liu 2006)). Consistently, TSLP expression and TSLP-TSLPR interactions are important for immunity to the intestinal nematode *Trichuris muris*, and protection against experimental colitis through the *in vivo* inhibition of IL-12/23p40 production by DCs (Taylor et al. 2009, Zaph et al. 2007). Another mechanism by which TSLP may influence $T_H2$ cell responses is via regulation of basophil development. It has recently been shown that TSLP enhances the development of a bone marrow-resident basophil precursor in an IL-3-independent manner (Siracusa et al. In Press). Compared to IL-3-elicited basophils, TSLP-elicited basophils produce more IL-4 upon stimulation with IL-18 or IL-33, and were sufficient to recover $T_H2$ cell-dependent immunity to *T. muris* infection in a susceptible host model system (Siracusa et al. In Press). Together, these results highlight the role of this important epithelial-derived cytokine in mediating normal $T_H2$ cytokine-dependent allergic responses.

Finally, IECs are in direct contact with intraepithelial lymphocytes and express all the molecular machinery required for antigen processing and presentation including proteolytically active cathepsins, the invariant chain, and MHC class II molecules (Hershberg, Mayer 2000). *In vitro* studies showed that rodent IECs, although less potent than professional antigen-presenting cells, can process and present antigen through the MHC class II pathway (Telega, Baumgart & Carding 2000). Supporting a role in directly promoting T cell responses, IECs isolated from patients with IBD express MHC class II molecules and can localize exogenous antigens to the late endosome on their basolateral surfaces (Buning et al. 2006). However, the lack of costimulatory molecule expression by IECs suggests IECs cannot prime naïve T cells under normal conditions,

though memory T cells exhibit less stringent requirements for co-stimulation and may therefore be influenced by IEC-intrinsic antigen presentation (Sanderson et al. 1993, Arens et al. 2011). Finally, IECs may deliver inhibitory or tolerogenic signals directly to T cells consistent with their known role in controlling B cell responses (Xu et al. 2007). Taken together, these studies describe how IEC-mediated recognition of commensal-derived signals may influence the development and propagation of local or systemic immune responses.

## 1.4.2 Recognition of commensal-derived signals by dendritic cells

Dendritic cells (DCs) are perhaps the most efficient modulators of adaptive immune responses and they represent an important link between the innate and adaptive immune systems (Trombetta, Mellman 2005). DCs that survey mucosal tissues take on specific phenotypic characteristics and perform distinct functions depending on their anatomical location. In the small intestine, DCs reside both in organized lymphoid structures such as Peyer's patches (PP) and are present directly beneath the epithelial layer in the intestinal lamina propria (LP DCs) (**Figure 4**) (Coombes, Powrie 2008).
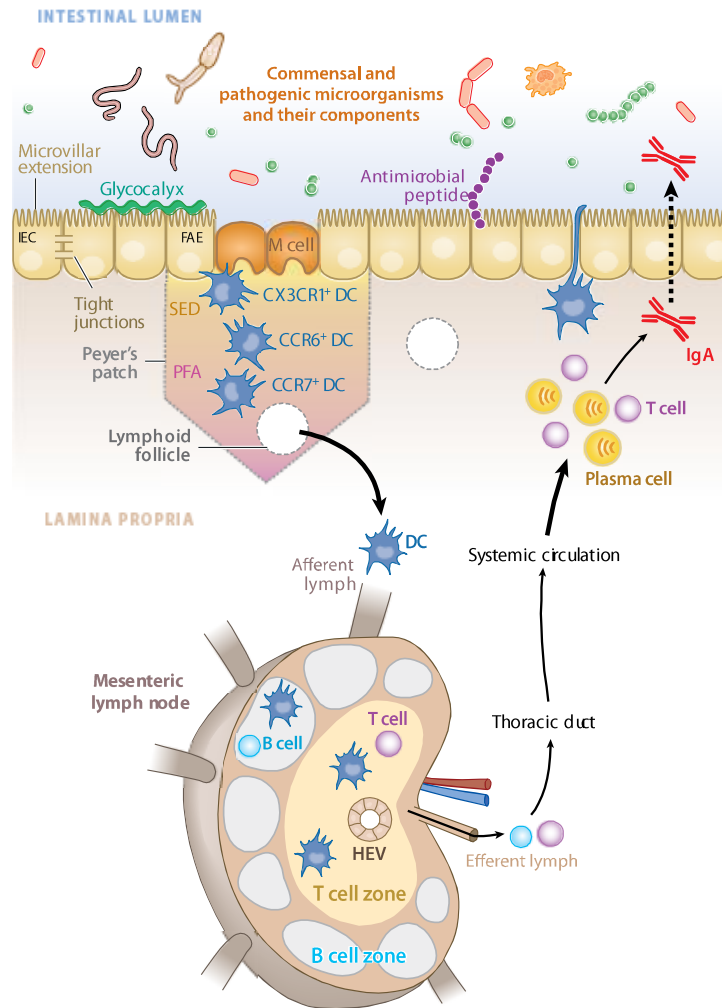
**Figure 4: The mucosal immune system of the mammalian intestine**

Innate recognition of signals from intestinal bacteria takes place at the intestinal epithelium, in the lamina propria and in gut-associated lymphoid tissues such as Peyer's patches and isolated lymphoid follicles. Specialized intestinal epithelial cells known as M (microfold) cells overlie Peyer's patches and lymphoid follicles and facilitate luminal sampling and transport of microbial components to professional antigen-presenting cells present in the subepithelial dome (SED). Dendritic cells (DCs) in the SED and perifollicular area (PFA) acquire antigens and influence adaptive responses. Additionally, specialized DC subsets directly sample luminal antigens. Intestinal DCs transport antigens to mesenteric lymph nodes through the afferent lymphatic system. DCs in the mesenteric lymph node promote differentiation of regulatory and effector T lymphocytes, as well as class switching of B lymphocytes, which then exit through the efferent lymph into the systemic circulation. Some of these cells home back to the intestine where they exert effector functions. Adapted from (Hill, Artis 2010).

Peyer's patch DCs are divided into three groups based on chemokine receptor expression, anatomical location, and functional characteristics. CX3CR1[+] PP DCs are found in close contact with the follicle-associated epithelium (FAE) where they participate in a close functional relationship with the epithelial M cell to sample luminal antigens in a pattern recognition receptor-dependent manner (Milling, Cousins & MacPherson 2005, Tyrer et al. 2006). CCR6^{+} PP DCs are found in the sub-epithelial dome but can quickly migrate to the FAE in response to microbial stimulation, and CCR7^{+} PP DCs are found in T cell areas where they orchestrate helper T cell responses (Iwasaki, Kelsall 1999, Iwasaki, Kelsall 2000, Salazar-Gonzalez et al. 2006), T cell migration (Johansson-Lindbom et al. 2005, Mora et al. 2003), and IgA in response to microbial signals production (Spalding et al. 1984, George, Cebra 1991, Powrie 2004).

While DCs resident in the PP are important mediators of immune function, PPs are relatively rare along the length of the intestinal tract. BrdU pulse-chase experiments suggest that circulating DCs have evolved to migrate directly to the lamina propria (LP) of the small intestine through the expression of CD103 (Jaensson et al. 2008). These LP DCs express tight-junction proteins that allow for direct luminal sampling through the extension of dendrites between IECs, a process that is dependent on the CX3C-chemokine receptor 1 (CX3CR1) and TLR ligation (Rescigno et al. 2001, Niess et al. 2005, Chieppa et al. 2006). Accordingly CX3CR1^{-/-} mice exhibit defective luminal sampling by DCs and impaired resistance to *Salmonella typhimurium* infection, implicating a crucial role for luminal sampling in the development of protective immune responses in the intestine (Niess et al. 2005).

Intestinal DCs recognize bacteria through the expression of innate pattern recognition receptors (Medzhitov 2007) which can modify luminal sampling (Chieppa et al. 2006), migration (Turnbull et al. 2005, Yrlid et al. 2006), and the induction of T cell differentiation (Minns et al. 2006, Uematsu et al. 2006, Uematsu et al. 2008). Intestinal resident DCs are thought to be tolerogenic compared to systemically circulating DCs, a phenotype that may be important for controlling inflammatory immune responses to beneficial intestinal bacteria (Sun et al. 2007, Coombes et al. 2007). For example, stimulation of intestinal DCs with the TLR4 ligand lipopolysaccharide (LPS) resulted in elevated IL-10 production, while stimulation of systemic DCs resulted in pro-inflammatory activation (Monteleone et al. 2008, Takenaka et al. 2007). The mechanisms by which intestinal DCs may be skewed towards a tolerogenic phenotype are still under investigation and include reduced TLR expression (Monteleone et al. 2008, Takenaka et al. 2007), hyporesponsiveness to TLR stimulation (Cerovic et al. 2009), and/or negative regulation of the NF-κB pathway via NOD2 signaling (see section 1.3.2) (Watanabe et al. 2006, Watanabe et al. 2008, Watanabe et al. 2004, Zeuthen, Fink & Frokiaer 2008b, Yang et al. 2007). Finally, as discussed in section 1.4.1, IECs may play an active role in promoting the tolerogenic phenotype of intestinal DCs via direct or indirect mechanisms (Iliev et al. 2009b).

Intestinal resident DC populations regulate local T cell responses in part through the production of IL-12 and IL-23. IL-12 is a key regulatory cytokine that induces $T_H1$ cell differentiation and plays an important role $T_H1$-mediated experimental models of autoimmune diseases including inflammatory bowel disease (Neurath et al. 1995, Simpson et al. 1998, Davidson et al. 1998, Trinchieri 2003, Elson et al. 2005). IL-23 is a heterodimer cytokine composed of a p40 subunit (that is shared with IL-12) and a unique

p19 subunit (Oppmann et al. 2000). While one study has implicated IL-23 to have a protective role in a murine model of colitis (Becker et al. 2006), others have shown that IL-23 drives inflammatory $T_H17$ responses (Lyakh et al. 2008, Yen et al. 2006) (reviewed in (Ahern et al. 2008)) and is a causative agent in a number of inflammatory disorders including joint inflammation (Murphy et al. 2003), intestinal inflammation (Yen et al. 2006, Hue et al. 2006, Izcue et al. 2008, Kullberg et al. 2006, Uhlig et al. 2006), and psoriasis (Chan et al. 2006, Kopp et al. 2003). The recent identification of the IL23R as a gene that is associated with IBD risk has further increased clinical interest in IL-23 inflammatory axis (Duerr et al. 2006).

Importantly, both PP and LP DCs continuously transport self and bacterial antigens, as well as live bacteria, to the mesenteric lymph nodes where they can modify systemic immune responses (Macpherson, Uhr 2004b, Trombetta, Mellman 2005, Huang et al. 2000, Fujita et al. 2006, Jang et al. 2006). Intestinal bacteria have been shown to induce lamina propria and mLN DCs to activate a maturation program that promotes subsequent conversion of naïve $CD4^+$ T cells into regulatory T cells in a retinoic acid and TGF-β dependent manner (Sun et al. 2007, Coombes et al. 2007, Coombes, Powrie 2008, Baba et al. 2008). Intestinal DCs in the mLNs can subsequently target B and T lymphocytes back to the intestine by causing the upregulation of CCR9 and α4β7 (Johansson-Lindbom et al. 2005, Mora et al. 2003, Mora et al. 2006, Stagg, Kamm & Knight 2002, Svensson et al. 2004). In summary, intestinal DCs have a predominantly tolerogenic phenotype that is thought to be mediated in part through signals from IECs or other immune cells as well as direct interactions with intestinal bacteria. This unique characteristic is thought to be important for maintaining tolerance to commensal bacterial-derived signals as well as other luminal antigens.

## 1.5 The influence of commensal-derived signals on immune cell development and homeostasis

As discussed above, signals from intestinal bacteria appear to influence human and murine models of disease by modulating innate and adaptive immune responses. One model used to study the role of microbial signals in immune cell development is the germ-free mouse (reviewed in (Smith, McCoy & Macpherson 2007)). Germ-free mice are born and live in a sterile environment and are therefore free of exposure to live microbial signals (Macpherson, Harris 2004). As such, germ-free mice have been instrumental in dissecting the role of bacterial signals in the development of the immune system. Additionally, animals with altered intestinal communities, primarily achieved through the administration of oral broad-spectrum antibiotics, have provided a complimentary approach to germ-free studies and have identified key roles for bacterial signals in the regulation of immune cell homeostasis (Sekirov et al. 2008, Hill et al. 2009, Rakoff-Nahoum et al. 2004, Zaph et al. 2008, Hall et al. 2008). In this section, we review findings from the germ-free and antibiotic-treatment mouse models that implicate commensal-derived signals in supporting normal physiology of the mammalian immune system.

### 1.5.1 The influence of commensal-derived signals on intestinal morphology and function

Germ-free and antibiotic-treated mice display a number of morphologic defects as compared to conventionally-reared animals. Perhaps most striking is the dramatic

enlargement of the cecum (an intestinal segment located between the distal small intestine and proximal colon) observed in these models. This enlargement is due in part to the accumulation of undegraded mucus glycoproteins that are produced by the intestinal epithelium and are normally degraded by glycoside hydrolases from intestinal bacteria (namely *Peptostreptococcus micros* and members of the genera Ruminococcus and Bifidobacterium) (Lindstedt, Lindstedt & Gustafsson 1965, Gustafsson, Midtvedt & Strandberg 1970, Hoskins et al. 1985, Carlstedt-Duke et al. 1986). Accordingly, cecal enlargement can be rapidly reversed through selective monoassociation with *Peptostreptococcus micros* (Carlstedt-Duke et al. 1986). Germ-free animals also accumulate bile acids in their cecum and large intestine which may contribute to cecal distention by causing osmotic imbalances across the epithelial wall (Eyssen, Parmentier & Mertens 1976).

Characteristic histologic alterations in the architecture of the intestinal epithelium also occur in germ-free and antibiotic-treated animals. The villi of the cecum are longer and wider compared to conventionally-reared mice and morphologic studies in rats suggest that crypts are shorter and contain fewer cells in the germ-free as compared to conventionally-reared animals (Alam, Midtvedt & Uribe 1994). These changes in crypt architecture could also be in part due to decreased turnover of IECs that occurs in germ-free as compared to conventionally-reared animals or due to anatomical defects discussed above (Abrams, Bauer & Sprinz 1963).

In conventionally-reared animals, the intestine undergoes waves of peristalsis that help move luminal contents proximally to distally. Intestinal bacteria modify enteric nerve function and transient manipulations of intestinal communities can lead to persistent neuromuscular dysfunction and enteritis (Barbara, Vallance & Collins 1997).

Consistently, germ-free animals show defects in small intestinal peristalsis characterized by slower and less frequent migrating motor complexes as a result of reduced responsiveness to enteroendocrine cell products (Strandberg et al. 1966). Intestinal motility is important for the maintenance of intestinal bacterial communities as the ablation enteric neurons specifically in the jejunum and ileum resulted in bacterial overgrowth and patchy jejuno-ileitis containing foci of hemorrhagic necrosis (Bush et al. 1998). Peristaltic defects can be reversed through conventionalization of germ-free animals suggesting that bacterial signals dynamically influence intestinal neuromuscular function (Husebye, Hellstrom & Midtvedt 1994).

## 1.5.2 The influence of commensal-derived signals on gut-associated lymphoid tissues

It is estimated that the intestinal mucosa of humans contains more lymphocytes, and produces more antibodies, than any other organ in the body (Mestecky, McGhee 1987). In mice, the majority of immune cells reside in gut-associated lymphoid tissues (GALT) which include the mLN, Peyer's patches (PP), cecal patch and isolated lymphoid follicles (ILF) which occur along the length of the gastrointestinal (GI) tract with increasing frequency in the colon and rectum (Langman, Rowland 1986, Brandtzaeg et al. 1989).

Germ-free mice display reduced intestinal lymphatic tissue and an underdeveloped lymphatic system as compared to conventionally-reared mice implicating commensal bacterial-derived signals in contributing to the development of these structures (Hudson, Luckey 1964, Hooijkaas et al. 1984). Specifically, PP numbers and cellularity are reduced, as are the number of ILFs (Bouskra et al. 2008, Cebra et al. 1998). Defects in lymphoid tissue genesis are not limited to the immediate intestinal

compartment as mLNs are smaller, less cellular, and have fewer germinal centers in germ-free compared to conventionally-reared animals (Glaister 1973). Consistently, antibiotic treatment has been shown to decrease the cellularity of intestinal Peyer's patches, implicating bacterial signals in the maintenance of intestinal lymphoid tissues (Yaguchi et al. 2006). These findings indicate that microbial signals are required for lymphoid tissue development and/or maintenance in the mammalian intestine.

Several studies have begun to identify the bacterial-derived signals that contribute to the maintenance of intestinal lymphoid tissues. For example, the selective colonization of germ-free animals with intestinal bacteria results in the recovery of lymphoid structures in the intestinal compartment and the recovery of mLN size and cellularity (Hudson, Luckey 1964, Glaister 1973). Additionally, peptidoglycan from gram-negative bacteria have been shown to be both necessary and sufficient to induce ILF formation in the mammalian intestine in a NOD1-dependent manner (Bouskra et al. 2008).

### 1.5.3 The influence of commensal-derived signals on dendritic cell and macrophage differentiation and function

Signals from intestinal bacteria have both developmental and regulatory influences on intestinal APCs. Intestinal DCs have been shown to be present in reduced numbers in the intestine of germ-free compared to conventionally-reared animals (Haverson et al. 2007, Williams et al. 2006). These defects appear to be isolated to the intestinal compartment as DCs from the spleen and mLNs of germ-free animals had normal surface marker expression and ability to stimulate T cell proliferation (Walton et al.

2006b). Intestinal DCs were recruited to the lamina propria upon monoassociation of germ-free animals with *E. coli* suggesting that intestinal bacteria influence the anatomical locations of intestinal DC populations (Haverson et al. 2007).

Monocyte/macrophage development is also influenced by signals from intestinal bacteria. Monocyte/macrophage numbers in the intestine were either normal or reduced in germ-free as compared to conventionally-reared animals (Williams et al. 2006, Zhang et al. 2008). In addition, systemic monocyte/macrophage numbers are reduced in germ-free as compared to conventionally-reared animals (Zhang et al. 2008). Again, systemic and intestinal defects in monocytes/macrophage could be recovered upon monoassociation of germ-free animals with *L. acidophilus* and *L. reuteri* implicating signals from intestinal bacteria in the regulation of these cell types (Zhang et al. 2008).

As discussed previously, intestinal APCs are thought to have a uniquely tolerogenic phenotype that is important for maintaining tolerance to oral antigens (reviewed in (Medzhitov 2007, Takenaka et al. 2007)). For example, intestinal CD11c+ lamina propria cells expressed TLR5 and proinflammatory cytokines in response to pathogenic bacteria, but not in response to beneficial bacteria (Uematsu et al. 2006). It is thought that the tolerogenic nature of intestinal APCs may be imparted directly or indirectly by select intestinal bacteria. For example, *Lactobacillus rhamnosus* has been shown to decrease TNFα production in LPS-activated macrophages in a contact-independent manner, a phenomenon that is likely important for controlling pathogenic, proinflammatory immune responses (Iwasaki 2007, Pena, Versalovic 2003). Indeed, Crohn's disease patients were found to have higher numbers of intestinal macrophages that expressed the innate-immune receptor CD14 and produce larger amounts of proinflammatory cytokines as compared to healthy controls (Kamada et al. 2008). These

cells expressed both macrophage (CD14, CD33, CD68) and DC (CD205, CD209) markers and evoked stronger $T_H1$ and $T_H17$ cell differentiation than non-CD14 expressing macrophages suggesting that intestinal APCs that lack tolerogenic properties can contribute to inflammatory disease (Kamada et al. 2009). In summary, maintenance of tolerogenic properties of antigen presenting cells may be in part mediated by interactions with commensal populations.

### 1.5.4 The influence of commensal-derived signals on lymphoid-tissue inducer and natural killer cell differentiation and function

Lymphoid-tissue inducer (LTi) cells are RORγt$^+$ IL-7Rα$^+$ innate leukocytes that induce lymph node development in the embryo through the production of lymphotoxin-β and TNF and the recruitment of circulating LTi cells, their precursors, and more mature lymphocytes (Colonna 2009). In adult mice, clusters of LTi cells are found in the cryptopatches of the small intestine, and in secondary lymphoid organs such as the spleen, where they participate in maintaining local lymphoid tissue anatomy (Colonna 2009). LTi cells are an innate source of IL-17 and IL-22 (an IL-10-family member that contributes to epithelial-cell resistance and repair by inducing the production of antimicrobial proteins such as β-defensins, RegIIIγ, S100 calcium-binding proteins, and regenerating gene family proteins) (Takatori et al. 2009). Recently, CD4$^+$ LTi cells were shown to promote innate immunity to Citrobacter rodentium infection in an IL-23-dependent mechanism and IL-23-responsive innate lymphoid cells were found to be increased in inflammatory bowel disease implicating these cell types as important contributors to human disease (Geremia et al. 2011, Sonnenberg et al. 2011). The finding that lymphoid follicles are underdeveloped in germ-free mice has led some to

speculate that LTi cells might be regulated by bacterial signals; however, one study to date indicated that LTi cell numbers and function were similar in the mucosa of germ-free as compared to conventional mice (Sanos et al. 2008).

Nevertheless, an intriguing connection exists between LTi cells and another cell population known to be regulated by intestinal bacteria; namely, IL-22 producing intestinal Natural Killer (NK)-like (NK-22) cells. Intestinal NK-22 cells are found in the cryptopatches and lamina propria of adult mice where they are an important innate source of IL-22 (Sanos et al. 2008, Cella et al. 2008, Satoh-Takayama et al. 2008, Luci et al. 2009). Production of IL-22 by mucosal NK-22 cells may contribute to defense against the extracellular enteric pathogen *Citrobacter rodentium* as the partial depletion of mucosal NK-22 cells increased the mortality of infected mice (Cella et al. 2008, Satoh-Takayama et al. 2008). In addition, NK-22 cells have been shown to mediate protection from experimental colitis supporting the hypothesis that these cells are involved in defense against assaults to the enteric mucosa (Zenewicz et al. 2008).

LTi cells can differentiate into NK-like cells *in vitro* that express both LTi and NK cell receptors (Mebius, Rennert & Weissman 1997) (reviewed in (Vivier, Spits & Cupedo 2009)). Additionally, human fetal LTi cells gave rise to NKp44^{+} cells that produced IL-17 and IL-22 *in vitro* (Cupedo et al. 2009). However, questions remain as to the relationship between LTi cells and NK-22 cells *in vivo* as LTi cells develop independently of commensal-derived signals but are required for lymphoid tissue development, while lymphoid tissue development and the development of IL-22 producing intestinal NK cells are both dependent on bacterial signals. The dependence of NK-22 cells on RORγt expression further suggests that LTi cells and NK-22 cells may be related either developmentally or functionally (Colonna 2009). Intestinal LTi cells and NK-22 cells both

upregulate IL-22 production shortly after birth suggesting that colonization of the intestine drives IL-22 production by these cell types in humans and mice (Sanos et al. 2008, Satoh-Takayama et al. 2008). Finally, absolute and relative numbers of NK-22 cells were reduced in germ-free as compared to conventionally-reared mice (Sanos et al. 2008) further implicating signals from intestinal bacteria in NK-22 cell development. These findings suggest that signals from intestinal bacteria promote mucosal homeostasis in part by promoting the induction of IL-22 producing innate leukocytes.

## 1.5.5 The influence of commensal-derived signals on B cell differentiation and function

A typical intestinal lymphoid follicle has a germinal center, a B lymphocyte–rich peripheral zone, and a dome-shaped "corona" containing B cells, CD4$^+$ T cells, dendritic cells, and macrophages (Brandtzaeg et al. 1988) (**Figure 4**). APCs immediately under the epithelium are presumably active in uptake and killing of incoming pathogens as well as in processing, presentation, and perhaps storage of antigens. There is an intimate relationship between intestinal communities and B lymphocytes that reside in intestinal lymphoid structures. As discussed previously, the first immunological response to bacterial signals during colonization is IgA production and secretion into the intestinal lumen (Macpherson 2006, Klaasen et al. 1993). In fact, the large majority of B cells in the intestine are immunoglobulin A (IgA)-producing plasma cells which produce and secrete IgA into the intestinal lumen at an estimated rate of 0.8 grams per meter of intestine per day (Brandtzaeg et al. 1989, Goldblum, Hannson & Brandtzaeg 1996). This secretory immune response, which is characterized by class-switching of B cells from IgM to IgA production, is orchestrated through an intimate functional relationship

38

between secretory epithelia and local plasma cells and is mediated by TLRs through T-cell dependent and independent mechanisms (Macpherson et al. 2000, He et al. 2007). For example, intestinal bacteria trigger T cell-independent IgA class switching in B cells through IEC secretion of cytokines including APRIL (He et al. 2007, Xu et al. 2007). In addition, IEC-derived TSLP and IL-10, produced in response to bacterial signals, may orchestrate local B cell responses (He et al. 2007, Xu et al. 2007, Astrakhan et al. 2007). Secretory IgA creates a first-line defense against mucosal compromise that is lost during IBD (Lodes et al. 2004, Brandtzaeg, Carlsen & Halstensen 2006). Together, these findings implicate early recognition of bacterial-derived signals and the subsequent modulation of B cell responses as important processes that promote normal mucosal homeostasis.

There are several findings in germ-free mice that support a role for bacterial signals in B cell development. There are reduced numbers of plasma cells in the germ-free small intestine which correlates with reduced IgA production (Glaister 1973, Crabbe et al. 1970). Systemically, there are also reduced numbers of germinal centers and plasma cells in germ-free mice which correlate with reduced systemic immunoglobulin levels (Hooijkaas et al. 1984, Gustafsson, Laurell 1959, Gordon, Brauckner-Kardoss 1961, Wagner, Wostmann 1961, Ikari 1964, Sell 1964). Germ-free mice also show reduced antigen-specific immunoglobulins to some antigens (DNP-BSA, *E. coli*) but not others (RBC, phosphorylcholine, DNP-lys-Ficoll) (Horowitz et al. 1964, Ohwaki et al. 1977, Etlinger, Heusser 1986). In general, these intestinal and systemic immunoglobulin defects are corrected upon conventionalization of germ-free animals suggesting that these defects are not developmental and that commensal-derived signals actively

maintain these aspects of B cell physiology (Crabbe et al. 1970, Gustafsson, Laurell 1959, Wagner, Wostmann 1961, Moreau et al. 1978).

One exception to the trend of reduced immunoglobulin levels is IgE. There are increased IgE bearing B cells in the Peyer's patches of germ-free mice as well as increased serum IgE responses upon antibiotic treatment of conventionally-reared mice (Bashir et al. 2004, Durkin, Bazin & Waksman 1981). The IgE found in germ-free mice is composed of natural specificities and induced by a mechanism independent of MHC class II (MHC II) cognate help (McCoy et al. 2006). These findings suggest that in general, microbial signals act as an adjuvant to immunoglobulin responses with the exception of allergic IgE responses, where commensal-derived signals may play an immunoregulatory role.

### 1.5.6 The influence of commensal-derived signals on CD8^{+} cytotoxic T cell differentiation and function

The intestinal mucosa of conventionally-reared animals normally contains a large population of resident T lymphocytes.  In the conventionally-reared mouse intestine, CD4^{+} T cells are found primarily in the lamina propria while CD8^{+} T cells dominate in the intraepithelial compartment (Jabri, Ebert 2007). The development or homeostasis of CD8^{+} IELs is dependent on bacterial signals as MyD88-deficient and germ-free mice display reduced CD8^{+} T cell numbers the IEL compartment of the small intestine (Imaoka et al. 1996, Kawaguchi-Miyashita et al. 1996, Yu et al. 2006). Cytotoxic activity of IELs is impaired in both germ-free and antibiotic-treated mice, a phenotype that can be reversed through the administration of the TLR4 ligand LPS (Kawaguchi-Miyashita et al. 1996, Paulos et al. 2007). In germ-free mice, this cytotoxic defect was isolated to αβ

40

TCR-bearing IELs while the number and cytotoxicity of γδ TCR-bearing IELs was comparable between germ-free and conventionally-reared mice (Kawaguchi-Miyashita et al. 1996). These defects may be in part due to impaired clonal expansions of CD8αβ⁺ and CD8αα⁺ IELs (Helgeland et al. 2004).

The systemic distribution of CD8⁺ T cells is also dependent on bacterial signals. Restricted-flora mice have a selective reduction in systemic plasmacytoid dendritic cells as a result of enhanced cytotoxic T lymphocyte activity that is not observed in germ-free mice (Fujiwara et al. 2008). Additionally, the liver is a site of activated CD8⁺ T cell sequestering and subsequent apoptosis during systemic immune responses, a phenomenon that is dependent on TLR4 ligands such as endotoxin from intestinal bacteria (John, Crispe 2005). Finally, animals colonized with nonpathogenic Clostridium sp. have reduced numbers of naïve, splenic CD8⁺ lymphocytes as compared to conventionally-reared controls suggesting that signals from intestinal bacteria play an active role in CD8⁺ lymphocyte regulation. Some of these effects may be a result of a lack of MyD88-dependent signaling as CD8-intrinsic MyD88-dependent signaling is important for CD8⁺ T cell survival and expansion (Huang et al. 2005, Rahman et al. 2008).

Though likely influenced by multiple immune cells in addition to CD8⁺ cytotoxic T cells, microbial stimulation can also result in tumorigenesis under some conditions. The first observation that microbial signals could influence tumor immunity came in the 18th century when Deidier reported a correlation between patient infection and the remission of malignant disease, a finding that eventually led to the use of LPS to treat primarily inoperable sarcoma with a cure rate of better than 10% (Garay et al. 2007). These early findings indicate that microbial signaling through TLRs is important for initiating or

sustaining anti-tumor immune responses (reviewed in (Rakoff-Nahoum, Medzhitov 2009)). This hypothesis has now been supported by several studies in animal models and patients (Krieg 2007, Paulos et al. 2007, Apetoh et al. 2007, Yusuf et al. 2008).

Animal models have provided additional insights into how commensal-derived signals influence tumor immunity. MyD88-deficient mice are protected against the development of spontaneous intestinal tumors in a model of familial associated polyposis (FAP) and germ-free animals have reduced intestinal tumorigenesis as compared to conventionally-reared animals following the same protocol of colorectal cancer induction (Rakoff-Nahoum, Medzhitov 2007, Vannucci et al. 2008). Similarly, hepatocellular carcinoma, the most common liver cancer, occurs mainly in males. This gender disparity is also seen in mice given the chemical carcinogen diethylnitrosamine and was shown to be dependent on MyD88-mediated increases in serum interleukin-6 (IL-6) (Naugler et al. 2007). In humans, associations between intestinal bacteria and cancer risk have been explored and studies were performed to identify fecal bacterial communities associated with high colorectal cancer risk though they failed to provide conclusive results (Moore, Moore 1995, Benno et al. 1986, Finegold et al. 1975, Huycke, Gaskins 2004). In summary, clarifying the influence of bacterial signals in establishing normal cytotoxic immune capacities has led to exciting therapeutic possibilities but more research is needed to fully understand the role that signals from intestinal bacteria play in tumor-genesis and control.

## 1.6 The influence of commensal bacterial-derived signals on CD4^{+} T helper cell responses

### 1.6.1 CD4^{+} T helper cells

Protective adaptive immunity in mammals is dependent on the development and activation of antigen-specific CD4+ T cells by innate accessory cells such as DCs and macrophages. Innate accessory cells signal in *cis* and *trans* via the T cell receptors, co-stimulatory molecules, Notch signaling and autocrine and paracrine cytokine signaling to specify the optimal helper T cell fate to combat the specific pathogen (Zhu, Yamane & Paul 2010). Helper T cells subsequently mobilize distinct innate and adaptive effector cells, as well as induce characteristic changes in non-immune cell types, important for host defense. T cell subsets are defined as T helper type ($T_H$) 1, $T_H$2, $T_H$17 and regulatory T cells (Treg) based on their unique cytokine production profiles, transcription factor expression profiles and effector functions. In this section I discuss the influence of commensal bacterial-derived signals on the development and function of the $T_H$ subsets.

### 1.6.2 The influence of commensal-derived signals on T helper 17 and regulatory T cell responses

The differentiation of $T_H$17 cells is dependent on the retinoic acid-related orphan receptor (RORγt), requires TGF-β and IL-6 or IL-21, and relies on IL-23 for $T_H$17 cell maturation and survival (Korn et al. 2009). In the steady state, IL-17–producing cells are present in high numbers in lamina propria of the small intestine and the $T_H$17-related cytokines IL-22 and IL-17 play a role in host protection against extracellular pathogens (Kastelein, Hunter & Cua 2007, Dong 2008, Ivanov et al. 2008). Conventionally-reared animals

have TLR-independent "spontaneous" IL-17 production in the lamina propria of their small intestine (Ivanov et al. 2008). Spontaneous IL-17 production was absent in germ-free animals, suggesting that intestinal bacteria signal through a TLR-independent mechanism to promote $T_H17$ cell development (Ivanov et al. 2008). One mechanism by which intestinal bacteria may regulate $T_H17$ cell development independent of TLR signaling is through the production of other bioactive molecules such as metabolites (discussed above). Indeed, ATP has been shown to stimulate LP APCs to produce IL-6, IL-23, and TGF-β, resulting in $T_H17$ cell differentiation (Atarashi et al. 2008).

While $T_H17$ cells are reduced in the small intestine of germ-free mice, more $T_H17$ cells have been observed in the large intestine of germ-free as compared to conventionally-reared mice (Zaph et al. 2008). In the large intestine, bacterial signals regulate $T_H17$ cell development through bacterial-dependent production of the IL-17 family cytokine IL-25 (IL-17E), which down regulates IL-17 production through the inhibition of IL-23 production by lamina propria macrophages (Zaph et al. 2008). These findings suggest that regulation of $T_H17$ cell differentiation by commensal-derived signals is dependent on anatomical location along the intestine. Indeed, the small and large intestines are microbiologically and immunologically distinct sites. As discussed previously, there are site specific differences in intestinal bacterial communities along the length of the intestine. In addition, there are more intra-epithelial lymphocytes (IELs) and lamina-propria lymphocytes (LPLs) per epithelial cell in the small intestine, and the lymphocyte composition and migration to the small versus large intestine is differentially regulated (Ivanov et al. 2008, Cheroutre 2004). These fundamental differences may underlie differential regulation of $T_H17$ cells in distinct anatomical sites.

Studies in antibiotic treated mice mirror those from germ-free mice and support a role for bacterial signals in the regulation of $T_H17$ cell homeostasis. For example, mice treated with vancomycin have fewer $T_H17$ CD4$^+$ T cells in the LP of the small intestine (Atarashi et al. 2008, Ivanov et al. 2008, Niess et al. 2008). Additionally, $T_H17$ cell differentiation in the lamina propria of the small intestine has been shown to require specific intestinal bacteria, specifically, cytophaga-flavobacter-bacteroidetes (CFB) bacteria (Ivanov et al. 2008). This induction of $T_H17$ cells was independent of TLR signaling, IL-21, or IL-23, but required TGFβ activation suggesting that specific intestinal bacteria regulates the $T_H17$/Treg balance in the mammalian intestine.

An intimate relationship exists in the intestinal mucosa between proinflammatory $T_H17$ cells and CD4$^+$ regulatory T cells that play an important role in controlling $T_H17$ cell responses (Belkaid, Tarbell 2009). Tregs are characterized as CD4$^+$ CD25$^+$ cells that express the forkhead box P3 transcription factor (FoxP3) and have been shown to suppress proliferation of effector T cells *in vitro* and to protect from autoimmune and other inflammatory diseases in several animal models. For example, mice carrying a loss-of-function mutation of FoxP3 completely lack Tregs and develop lethal autoimmune disease while *Lactobacillus* and *Bifidobacterium* strains have been shown to cause expansion of Tregs in the intestinal intraepithelial compartment and protect against experimental colitis (Brunkow et al. 2001, Roselli et al. 2009). These findings identify Foxp3 as a master regulator of Treg differentiation and function and identify Tregs as an important regulatory cell type that contributes to intestinal and systemic immune homeostasis.

There are differing reports of the influence of bacterial signals on Treg development and function. Consist with studies that showed reduced FoxP3 mRNA in

CD4$^+$ T cells from mLNs of germ-free mice, early studies in germ-free animals showed a selective reduction in the percentage of FoxP3$^+$CD4$^+$CD25$^+$ T cells in the mLN of germ-free mice  (Strauch et al. 2005, Ostman et al. 2006). In addition to reduced frequencies, it has been reported that Tregs from the mLN of germ-free animals did not suppress CD4$^+$ T cell proliferation *in vitro* as well as Tregs from conventionally-reared animals (Ishikawa et al. 2008). Furthermore, Tregs from the mLN of germ-free mice produced less IL-10 and did not protect as well against disease in a transfer model of experimental colitis as compared to Tregs from conventionally-reared animals (Strauch et al. 2005). Recent studies have identified Clostridium species as important inducers of colonic regulatory T cells suggesting that specific commensal-derived signals are important for maintenance of this cell population (Atarashi et al. 2011). Others have shown that local and systemic Treg populations seem to be similar between conventionally-reared mice and mice with reduced commensal stimulation (Zaph et al. 2008, Ichinohe et al. 2011). Together, these results suggest that signals from intestinal bacteria are important for normal development and maintenance of Treg numbers and function in intestinal tissues.

However, there seem to be different effects of bacterial-derived signals on other Treg populations. One study reported no change in the frequency of lamina propria CD4$^+$FoxP3$^+$ T cells from the colon of germ-free animals while another reported increased percentages of CD4$^+$FoxP3$^+$ in the small intestine (Zaph et al. 2008, Ivanov et al. 2008). These differing results could be due sampling Treg cell subsets from different anatomical locations (mLN vs. intestine). Additionally, these differences could be due to experimental methods, animal housing, diet, non-bacterial microbial stimulation, or Treg identification methods employed.

46

### 1.6.3 The influence of commensal-derived signals on the $T_H1/T_H2$ cell balance

In humans, as in mice, two distinct patterns of cytokine secretion have been defined among CD4$^+$ helper T-cell clones; type 1 helper type ($T_H$) 1 cells, but not $T_H2$ cells, produce IL-2, IFNγ, and TNFα, while $T_H2$, but not $T_H1$, cells produce IL-4, IL-5, IL-9 and IL-13 but not IL-2 or IFNγ (reviewed in (Abbas, Murphy & Sher 1996)). These distinct immune responses are important for protective responses to distinct infectious agents; Th1 cell responses are protective against bacterial and protozoan infections while $T_H2$ cell responses act in resistance to helminths and ectoparasites.

The influence of intestinal bacteria on $T_H2$ cytokine-dependent inflammation has been of particular interest of late given recent epidemiological findings that antibiotic use in children results in significantly increased risk of developing allergic disease (Penders et al. 2007, Marra et al. 2009, Kummeling et al. 2007). There is also a strong experimental literature correlating states of reduced commensal stimulation with increased $T_H2$ cytokine-dependent inflammation. For example, germ-free mice have impaired oral tolerance and increased levels of allergen-specific IgE compared to conventionally-reared mice (Sudo et al. 1997). TLR4 signaling is important for commensal bacterial-derived signals to promote oral tolerance as TLR4 deficient mice display similar levels of food allergy compared to mice treated with oral antibiotics (Bashir et al. 2004). Commensal bacterial-derived signals also seem to limit disease in models of allergic asthma. Antibiotic treatment resulted in the selective outgrowth of fungal microbes in the intestine and worse disease outcomes in an IL-13-dependent mechanism (Noverr et al. 2005). In some cases specific bacterial species have been shown to influence the development of $T_H2$ cell responses. For example, treating animals with antibiotics during infancy promoted a shift in the $T_H1/T_H2$ balance towards a

T$_H$2-dominant immunity, and this effect could be reduced by oral inoculation of mice with *Enterococcus faecalis* or *Lactobacillus acidophilus* (Oyama et al. 2001, Sudo et al. 2002). Consistent with commensal-derived signals promoting T$_H$1 cell environments, optimal induction of T$_H$1 cell development *in vitro* by DCs required TLR2 signaling (Wang et al. 2006) and commensal-derived signals acts as adjuvants to promote immunity to the protozoan parasite *Toxoplasma gondii* (Benson et al. 2009). Additionally, there is evidence for DC-dependent CD4+ T$_H$1 cell responses to commensal bacteria in normal human intestinal lamina propria (Howe et al. 2009). Despite these associations, the mechanisms by which the innate immune system recognizes commensal-bacterial derived signals and influences the development of T$_H$2 cytokine-dependent allergic inflammation are not well understood.

As discussed previously, DCs recognize commensal bacterial-derived signals to modulate subsequent adaptive T cell responses (Medzhitov 2007, Takenaka et al. 2007). However, DCs have recently been shown to be insufficient to prime optimal T$_H$2 cell responses in select cases, implicating other innate immune cells in this role (Perrigoue et al. 2009, Sokol et al. 2009, Yoshimoto et al. 2009). In addition to their well-established roles as innate effector cells, basophils have recently been shown to function as accessory cells by providing early sources of IL-4 and contributing to the initiation of CD4$^+$ T$_H$2 cell responses (Gessner, Mohrs & Mohrs 2005, Mohrs et al. 2005, Min et al. 2004). These findings implicate regulation of innate granulocyte populations as a potential mechanism by which commensal-derived signals may limit T$_H$2 cytokine-dependent inflammation. The following section reviews basophil development and effector functions as well as the recently appreciate roles for basophils in contributing to the development of optimal T$_H$2 cell responses.

## 1.7 Basophils and the initiation and propagation of Type 2 cytokine-dependent allergic inflammation

Discovered in 1897, basophils are the least abundant granulocyte population making up less than 1% of peripheral blood leukocytes. Originally, basophils were thought to be redundant to mast cells as they also bind immunoglobulin (Ig)E and release histamine upon activation (Ishizaka et al. 1972). However, subsequent comparisons of basophils and mast cells revealed that these two granulocytes are distinct and differ in many aspects including their morphology, expression of surface markers, factors required for growth and differentiation, activation and signal transduction pathways and release of inflammatory mediators (MacGlashan et al. 1983, Siracusa et al. 2010). This section will discuss classical roles for basophils as key effector cells recruited to sites of tissue inflammation and the newly identified roles for basophils as innate cells that initiate and propagate optimal allergic $T_H2$ cell responses.

### 1.7.1 Basophil development and effector functions

Basophils develop from bone marrow resident granulocyte-monocyte precursor cells (GMPs) which also give rise to the eosinophil and mast cell lineages (Arinobu et al. 2005). Two basophil precursor cell types have been identified that reportedly give rise to mature basophils. The basophil precursor (BaP) is a bone marrow-resident population that exits the bone marrow upon differentiation into a mature basophil while the spleen-resident basophil-mast cell precursor (BMCP) gives rise to both basophil and mast cell populations (Iwasaki, Akashi 2007). Expression of the transcriptions factors GATA-2 and CCATT enhancer-binding protein C/EBPα are important for basophil development

(Iwasaki, Akashi 2007) along with expression of IL-4 and IL-13 transcripts (Gessner, Mohrs & Mohrs 2005). Unlike mast cells, which are not considered mature until they enter a peripheral tissues site, basophils exit the bone marrow with a mature phenotype (Galli 2000) and are only thought to survive between 60-70 hours (Iwasaki, Akashi 2007, Mellblom 1980, Ohnmacht, Voehringer 2009). As such, circulating basophil populations are likely continuously replenished by precursor populations (Ohnmacht, Voehringer 2009).

Classically, basophils are recognized as innate effector cells that are recruited to sites of tissue inflammation upon activation. One of the primary routes of basophil activation is crosslinking of immunoglobulins bound to the cell surface. Basophils express the high affinity IgE receptor (εεR1α), which in turn binds natural and antigen-specific IgE from the serum. Upon cross-linking of FcεRIα, basophils release multiple signaling molecules including histamine, leukotrienes, cytokines and chemokines that contribute, along with mast cells, to the clinical sequelae of immediate hypersensitivity reactions (Schroeder 2009). Basophils also express the IgG receptor FcγR and have been shown to be the major cell type responsible for some immunoglobulin-G-mediated immediate hypersensitivity reactions in some settings (Tsujimura et al. 2008, Ohnmacht et al. 2010). In addition to immediate hypersensitivity, basophils have been shown to contribute to chronic IgE-mediated allergic inflammation in mice (Ohnmacht et al. 2010, Obata et al. 2007). In this model, basophils were not required for the immediate- or late-phase responses to subcutaneous antigen administration, but were necessary for the subsequent edema and recruitment of eosinophilic and neutrophilic infiltrates to the site of antigen injection. These findings suggest that basophils mediate the recruitment of other innate effector cells to inflammatory sites. Finally, basophils have been shown to

be activated by IgD, a class of antibody the function of which remains unclear. Produced in the upper respiratory tract of mammals and implicated in immunity to respiratory pathogens, IgD-mediated activation of basophils resulted in the production of IL-4, anti-microbial peptides, B cell-activating factor and the induction of class switching of B cells to IgM, IgD and IgA isotypes (Chen et al. 2009). Together, these findings identify several pathways of basophil activation by immunoglobulins that result in diverse effector functions.

Other avenues to basophil activation include cytokines, proteases, TLRs, complement proteins and super-antigens. IL-3 is a T cell-derived cytokine and a potent regulator of basophil responses. Though basophils represent a relatively small proportion of circulating immune cells in the steady-state, circulating basophil populations can expand dramatically in response to IL-3 (Lantz et al. 1998, Lantz et al. 2008, Shen et al. 2008, Ohmori et al. 2009). IL-3 has been shown to both increase basophil survival and act on bone marrow-resident basophil precursors to increase the population of circulating basophils (Lantz et al. 1998, Lantz et al. 2008, Ohmori et al. 2009). Additionally, IL-3 augments IL-4 and IL-13 production by purified human basophils following activation by IgE (Gibbs et al. 1996, MacGlashan et al. 1994). However, while these studies identify an important role for IL-3 in mediating basophil responses, IL-3-IL-3R signaling is not absolutely essential for basophil development or survival as IL-3 receptor-deficient mice display near normal circulating basophil frequencies (Lantz et al. 1998). Consistent with other cytokines or molecules supporting steady-state basophil homeostasis, IL-18 and IL-33, which are produced by innate immune cells including macrophages and epithelial cells in response to allergic and parasitic stimuli (Kroeger, Sullivan & Locksley 2009, Pecaric-Petkovic et al. 2009), have

51

been shown to enhance basophil survival and augment basophil cytokine production (Kroeger, Sullivan & Locksley 2009, Yoshimoto et al. 1999).

Another mechanism of basophil activation is via proteases that are thought to directly activate basophils independently of immunoglobulins or cytokines. This mechanism of basophil activation presumably evolved to respond to proteases secreted from helminth parasites, but plays an active role in basophil responses to allergic stimuli. For example, papain, a cysteine protease and potent allergen associated with occupational allergy in humans (Novey et al. 1979) and $T_H2$ cell responses in mice (Sokol et al. 2008), is capable of directly inducing IL-4 and IL-6 production from bone marrow-derived murine basophils (Sokol et al. 2008). Additionally, the house dust mite (HDM) protease Derp1 induces production of IL-4, IL-5 and IL-13 from a human basophil cell line (Phillips et al. 2003). In both the case of papain and Derp1, activation of basophils is dependent on protease activity (Sokol et al. 2008, Phillips et al. 2003), and in the case of Derp1, may result from functional mimicry of the TLR4 receptor complex (Trompette et al. 2009). Consistent with basophils contributing to HDM-induced allergic inflammation, depletion of basophils via treatment with a monoclonal antibody targeting CD200R resulted in reduced HDM-elicited $T_H2$ cytokine-responses (Hammad et al. 2010). One mechanism by which basophils may sense protease activity is via protease-activated receptors (PARs), a family of receptors known to activate innate and adaptive immune cells in response to infectious or allergic stimuli (Shpacovitch et al. 2008). However, PAR expression has not been detected in murine basophils implicating other receptors or molecular mechanisms in the activation of basophils by proteases (Falcone, Morroll & Gibbs 2005).

Finally, additional molecular signals known to influence innate basophil response include TLR ligands, complement proteins and super-antigens. While basophils are reported to express TLR1, TLR2, TLR4, TLR6 and TLR9, only TLR2 ligands have been shown to activate basophils directly (Siracusa et al. 2010, Sabroe et al. 2002, Bieneman et al. 2005, Komiya et al. 2006). Specifically, lipopolysaccharide treatment of purified human basophils resulted in their production of IL-4 and IL-13 (Sabroe et al. 2002, Bieneman et al. 2005). Human basophils also express receptors to the complement proteins CR1, CR3, CR4 and CD88 and produce histamine in response to C5a (Hook, Siraganian & Wahl 1975, Siraganian, Hook 1976). Finally, direct, non-specific activation of basophils by super-antigens has been reported. For example, the gp120 glycoprotein of HIV interacts with the VH3 region of IgE to induce IL-4 and IL-13 production from human basophils (Patella et al. 2000) while the IPSE/alpha-1 glycoprotein from *Schistosoma mansoni* eggs induces IgE-dependent, antigen-independent IL-4 production by murine basophils *in vivo* (Schramm et al. 2007). Together, these findings identify important roles for basophils as innate effector cell populations that contribute to immunity to parasitic infections and pathology of inflammatory diseases.

### 1.7.2 Synergistic roles for basophils and dendritic cells in the initiation of $T_H2$ cell responses

As discussed previously, dendritic cells (DCs) are perhaps the most efficient initiators of adaptive immune responses and represent an important link between the innate and adaptive immune systems (Trombetta, Mellman 2005). Consistently, mouse models indicate that DCs contribute to the development of $T_H2$ cell-dependent immunity and inflammation. For example, DCs exposed to *S. mansoni* egg antigen (SEA) preparations

promote T$_H$2 cell differentiation *in vitro* and *in vivo* (Marshall, Pearce 2008), an immunologic outcome that may be mediated by low dose stimulation of the TCR (Steinfelder et al. 2009). Another mechanism by which T$_H$2 responses are initiated may be through direct modulation of DC activity by helminth-derived signals. For example, excretory-secretory products from *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis*, but not *T. muris*, can directly suppress DC production of IL-12p40 (Massacand et al. 2009). Recently, loss-of-function studies have shown DC populations to be critically important for the development of optimal T$_H$2 cell responses to some stimuli. For example, depletion of DCs early in the immune response against *S. mansoni* resulted in impaired T$_H$2 cell development and increased production of IFN-γ by CD4$^+$ T cells (Phythian-Adams et al. 2010). Additionally, DCs are sufficient to induce protective immunity to *T. muris* when interferon γ is blocked with a neutralizing antibody (Perrigoue et al. 2009). Finally, a FcεRIα-expressing inflammatory DC sub-type were found to be necessary and sufficient for the induction of T$_H$2 immunity and allergic airway disease using two murine asthma models, while other innate immune cell types including basophils played a supporting role (Hammad et al. 2010, van Rijt et al. 2005).

Despite findings that indicate that DCs are necessary for T$_H$2 cell induction under some conditions, there is a growing body of evidence suggesting that DCs, when acting independently from other innate cell types, are not sufficient for the induction of optimal T$_H$2 cytokine responses. For example, restriction of MHCII expression to CD11c$^+$ cells was found to be insufficient for optimal induction of protective immunity to *T. muris* (Perrigoue et al. 2009). Similarly, DCs were not required for T$_H$2 cell induction in response to papain while basophils were found to be critically required (Sokol et al. 2009). Together, these results suggest that cognate, MHCII-dependent interactions

54

between T cells and CD11c⁻ cell populations contribute to the development of optimal $T_H2$ cell responses.

In addition to their well-established roles as late phase innate effector cells, basophils have also recently been shown to function as important accessory cells by providing early sources of IL-4 and contributing to the initiation and/or propagation of CD4⁺ $T_H2$ cell responses (Gessner, Mohrs & Mohrs 2005, Mohrs et al. 2005, Min et al. 2004). Human basophils can produce more IL-4 per cell than activated $T_H2$ cells (Mitre et al. 2004) and murine studies indicate that basophil-derived IL-4 contributes to $T_H2$ cell development in the presence of antigen and dendritic cells (Hida et al. 2005, Oh et al. 2007). Together, these findings identify basophils as an innate cell population that can produce IL-4 and augment $T_H2$ cell differentiation *in vitro*. In addition, studies in murine disease models have identified a role for basophils in promoting $T_H2$ cell responses *in vivo*. For example, environments enriched for basophils, via continuous IL-3 treatment or deficiency in  the interferon regulatory factor 2, result in preferential differentiation of $T_H2$ cells (Hida et al. 2005, Oh et al. 2007). Consistent with basophils influencing T cell responses, IL-4 expressing basophils are recruited from the circulation to draining lymph nodes in response to infectious (Perrigoue et al. 2009) or allergic stimuli (Sokol et al. 2008) where they augment $T_H2$ cell differentiation. Critically, $T_H2$ cell responses are reduced upon basophil depletion in multiple allergy models including papain (Sokol et al. 2008) and house dust mite allergen (Hammad et al. 2010), indicating that basophils contribute to the development of optimal $T_H2$ cell responses in these settings.

Finally, in addition to being an innate source of IL-4, murine and human studies have demonstrated that basophils express MHC class II, endocytose soluble antigen, express MHC II and co-stimulatory molecules, and in select cases can act as antigen

presenting cells that promote antigen-specific $T_H2$ cell differentiation (Perrigoue et al. 2009, Sokol et al. 2009, Yoshimoto et al. 2009). Consistently, basophils have been shown to promote $T_H2$ cell differentiation *in vitro* and *in vivo* (Sokol et al. 2009, Yoshimoto et al. 2009). Furthermore, adoptive-transfer of antigen-pulsed basophils into MHC II-deficient hosts indicated that basophils were sufficient to promote antigen-specific $T_H2$ cell differentiation (Yoshimoto et al. 2009). Finally, adoptive-transfer of basophils increased $T_H2$ cell differentiation and immunity to *T. muris*, as well as $T_H2$ cell responses to *S. mansoni* egg preparations *in vivo* (Siracusa et al. In Press, Perrigoue et al. 2009). Together, these data indicate that in addition to providing innate IL-4 that augments DC-dependent $T_H2$ cell development, basophils can support the initiation or propagation of antigen-specific $T_H2$ cell responses in an MHC II-dependent manner. These recently appreciated roles for basophils in augmenting $T_H2$ cell responses and $T_H2$ cytokine-mediated allergic inflammation have led to corollary studies in humans and models of human disease. For example, basophils were found to be activated by self-reactive IgE antibodies and recruited to lymph nodes where they promoted $T_H2$ cell differentiation in a model of lupus nephritis (Charles et al. 2010). Patients with systemic lupus erythematous were found to have elevated self-reactive IgE levels and activated basophils that express MHC II and were associated with disease activity and active lupus nephritis (Charles et al. 2010). Together, these results identify basophils as an innate granulocyte population that contributes to the development of optimal $T_H2$ cytokine-dependent allergic inflammation and is correlated with human $T_H2$ cytokine-mediated diseases. However, the potential influence of commensal bacterial-derived signals on these and other innate granulocytes known to contribute to allergic $T_H2$ cell responses is not characterized.

# Chapter 2: The influence of antibiotic treatment on commensal bacterial communities of the intestine

## 2.1 Abstract

Despite widespread use of antibiotics, few studies have measured their effects on the burden or diversity of bacteria in the mammalian intestine. This chapter describes the development of an oral antibiotic treatment protocol and the characterization of its effects on murine intestinal bacterial communities. Antibiotic administration resulted in a ten-fold reduction in the amount of intestinal bacteria present and sequencing of 16S rDNA segments revealed significant temporal and spatial effects on luminal and mucosal-associated communities including reductions in luminal Firmicutes and mucosal-associated *Lactobacillus* species, and persistence of bacteria belonging to the Bacteroidetes and Proteobacteria phyla. This comprehensive temporal and spatial metagenomic analyses provides a resource and framework to test the influence of bacterial communities in murine models of human disease.

## 2.2 Introduction

The human intestine is colonized by 100 trillion microorganisms belonging to each of the three domains of life (Eckburg, Lepp & Relman 2003). Of these, bacteria are the most abundant—the colon is home to approximately $10^{11}$ to $10^{14}$ bacterial cells per mL of stool (Whitman, Coleman & Wiebe 1998) and has a diversity of at least 1000 species (Ley, Peterson & Gordon 2006). Through recent advances in surveying non-culturable intestinal communities using 16S ribosomal DNA (16S rDNA) sequencing (Pace, Olsen & Woese 1986) and microarray-based methods, it has become clear that the major bacterial phyla in the mammalian intestine include Bacteroidetes *(*Bacteroidetes class*)*, Firmicutes *(*Clostridia class*)*, Proteobacteria *(*Alpha*,* Beta*,* Gamma*,* and Epsilon classes*)*,

58

Actinobacteria, and Fusobacteria (Palmer et al. 2007, Eckburg et al. 2005). Some intestinal bacteria are mutualists that promote normal mammalian physiology including proper digestion (Hooper, Midtvedt & Gordon 2002), metabolism (Backhed et al. 2004), epithelial cell function (Artis 2008), angiogenesis (Stappenbeck, Hooper & Gordon 2002), enteric nerve function (Husebye, Hellstrom & Midtvedt 1994), and immune system development (Hill, Artis 2010). While bacterial communities promote normal immune homeostasis, patients with inflammatory bowel disease (IBD) (Frank et al. 2007) and atopic allergies (Bjorksten et al. 2001) have altered intestinal communities, indicating that microbial communities might influence the pathogenesis of these diseases (Marra et al. 2009, Strober, Fuss & Blumberg 2002).

Antibiotic treatment has the adverse effect of altering intestinal microbial communities (Hoban 2003) and antibiotic exposure is linked to increased risk of *Clostridium difficile* colitis (De La Cochetiere et al. 2008) and asthma (Marra et al. 2009, Kummeling et al. 2007, Wickens et al. 1999) in humans. In keeping with these findings, antibiotic treatment in animal model systems has identified complex pro-inflammatory and immunoregulatory roles for intestinal communities in modulating intestinal cytokine responses (Zaph et al. 2008, Hall et al. 2008, Mazmanian, Round & Kasper 2008), in altering resistance to enteric pathogens (Sekirov et al. 2008, Hall et al. 2008, Stecher et al. 2007, Garner et al. 2009, Croswell et al. 2009), in maintaining mucosal homeostasis (Rakoff-Nahoum et al. 2004, Kang et al. 2008), and in controlling allergic inflammation (Bashir et al. 2004). However, while the use of oral broad-spectrum antibiotics in humans and animal models is common, knowledge of how antibiotics influence intestinal bacterial communities is limited.

Previous studies using culture and fluorescence *in situ* hybridization based methods have provided some insights into antibiotic effects on a limited subset of intestinal bacteria (Sekirov et al. 2008, Miller, Bohnhoff 1963). More recently, molecular based examination of antibiotic treatment in humans showed effects on intestinal communities, but was confounded by inter-individual variability (Dethlefsen et al. 2008). Animal models have proven well-suited for metagenomic studies because they allow for control of host genetic background, feeding practices, and antibiotic administration. In one such study of bacterial recovery after antibiotic perturbation, single-antibiotic effects were characterized at the phylum level at a single time point and anatomic location (Antonopoulos et al. 2009). Other groups have reported the general effects of oral antibiotic treatment on intestinal bacteria, including significant changes to lactobacilli, enterococci/group D streptococci, Firmicutes, Bacteroidales, or segmented filamentous bacteria, depending on the specific antibiotic and treatment protocol (Sekirov et al. 2008, Stecher et al. 2007, Garner et al. 2009, Croswell et al. 2009). However, the interpretation of these studies is limited as in-depth temporal and spatial analysis of the effects of antibiotic treatment on microbial communities was not undertaken.

In this chapter, we characterized the effects of two different antibiotic treatment protocols on murine intestinal bacterial communities and immune cell homeostasis. We found that published protocols involving administration of multiple antibiotics (ampicillin, gentamicin, metronidazole, neomycin, and vancomycin) in drinking water altered intestinal microbiota, but were complicated by the development of severe dehydration in the host. For this reason, we developed an oral gavage-based protocol that facilitated controlled antibiotic dosing without the development of dehydration. Using DNA bar-coding and pyrosequencing of 16S rDNA gene segments, we examined temporal effects

of antibiotics on bacterial communities and found significant reductions in the frequency of bacteria belonging to the Firmicutes phylum and persistence of the Bacteroidetes and Proteobacteria phyla over time. Additionally, we performed spatial analysis of luminal and mucosal-associated bacterial communities following antibiotic treatment and found significant effects in the cecum, proximal colon, and distal colon including reductions in mucosal-associated *Lactobacillus* species.

## 2.3 Methods

### 2.3.1 Animals

Conventionally reared, six to eight week old female C57BL/6 mice were obtained from Charles River Laboratory and maintained in a specific pathogen–free facility. Animals were housed by litter and fed autoclaved LabDiet 5010 mouse chow (LabDiet, Richmond, IN) and autoclaved water. Germ-free animals were maintained in plastic isolator units and fed autoclaved LabDiet 5021 mouse chow (LabDiet, Richmond, IN) and autoclaved water. Isolators were checked weekly and consistently cultured negative for microbial contaminants. All experiments were approved by and performed following the guidelines of the University of Pennsylvania Institutional Animal Care and Use committee.

### 2.3.2 Antibiotic Treatment

For continuous antibiotic treatment, animals were provided with autoclaved drinking water, or autoclaved drinking water supplemented with ampicillin (1 mg/mL), gentamicin (1 mg/mL), metronidazole (1 mg/mL), neomycin (1 mg/mL), and vancomycin (0.5

mg/mL). For antibiotic treatment by oral gavage, animals had access to autoclaved food and water and were subjected to oral gavage daily for ten days with 200 µL of autoclaved water or autoclaved water supplemented with ampicillin (1 mg/mL), gentamicin (1 mg/mL), metronidazole (1 mg/mL), neomycin (1 mg/mL), and vancomycin (0.5 mg/mL).

### 2.3.3 16S rDNA sample acquisition and quantification of 16S rDNA

Stool pellet, luminal content, and washed mucosal-associated tissue samples were collected and total DNA was extracted using the QIAamp® DNA Stool Mini Kit (stool pellet/luminal samples)(Qiagen, Inc., Valencia, CA) or the DNeasy Blood and Tissue Kit (tissue samples)(Qiagen, Inc., Valencia, CA). Quantification of 16S rDNA was performed by real-time RT-PCR using degenerate bacterial 16S rDNA specific primers (5'– AGAGTTTGATCCTGGCTCAG-3';forward), (5'-CTGCTGCCTYCCGTA-3';reverse), (5'- FAM-TA+ACA+CATG+CA+AGTC+GA-BHQ1-3'; probe; + precedes the position of LNA base).

### 2.3.4 DNA manipulations

16S rRNA gene fragments were obtained as previously described (McKenna et al. 2008). Samples were amplified using 8 nucleotide-barcoded primer pairs (BSF8; 5´- AGAGTTTGATCCTGGCTCAG-3´), (BSR357; 5´-CTGCTGCCTYCCGTA-3´). 50 µL PCR reactions were carried out using the AmpliTaq System (Applied Biosystems, Inc., Foster City, CA) (Buffer II, 2 mM MgCl$_2$, 200 µM each dNTP, 10 µM each primer, 0.1 mg/mL BSA, 100 ng (stool) or 500 ng (tissue) of template, 2.5 units polymerase). Cycle

parameters: 1 minute 95° C, 20 cycles (stool pellet and luminal samples) or 25 cycles (tissue samples) of 30 secs at 95° C, 30 secs at 56° C, and 90 secs at 72° C, final extension at 72° C for 8 minutes. PCR products were gel purified using the QIAquick® Gel extraction kit (Qiagen, Inc., Valencia, CA). 100 ng of each amplicon was pooled and subjected to pyrosequencing.

### 2.3.5 Bioinformatic analysis

Sequence quality was accessed and samples with >100 sequences were carried through to subsequent analyses. Sequences were inserted into the 16S rRNA gene tree (Hugenholtz 2002) using parsimony insertion implemented in ARB. The tree constructed using ARB was used to carry out UniFrac analysis to compare global community structure as described previously (Lozupone, Knight 2005, Lozupone, Hamady & Knight 2006, Lozupone et al. 2007). Taxonomic assignments for each sequence were obtained using RDP Classifier (Wang et al. 2007).

### 2.3.6 Histology, stool protein extraction, western blot

At necropsy, cecal tissue sections were removed and fixed in 4% paraformaldehyde (PFA) and paraffin-embedded. 5 μm sections were cut and stained with hematoxylin and eosin (H&E). Crypt morphology was quantified using "NIS Elements BR" imaging software (Nikon, Melville, NY). Fecal protein isolation was performed as previously described (Artis et al. 2004). Samples were equalized by protein content, analyzed by SDS-PAGE, and immunoblotted for RELMβ using a polyclonal rabbit anti-murine RELMβ antibody (PeproTech, Rocky Hill, NJ). Blots were visualized using ECL™ (Amersham,

Piscataway, NJ) and band intensity was quantified using "UN-SCAN-IT" (Silk Scientific, Inc., Orem, UT).

### 2.3.7 Statistics

UniFrac analysis: Regression analysis was performed on day 0-9 stool pellet samples from naïve or antibiotic-treated animals. Pyrosequencing analysis: The frequencies of bacterial group compared using a Mann-Whitney test. Each mouse was treated as a biological replicate.

## 2.4 Results

### 2.4.1 Administration of antibiotics in drinking water alters bacterial communities but results in severe dehydration

Antibiotic treatment has been used extensively to probe the effects of microbial signals in disease, including murine models of intestinal inflammation (Rakoff-Nahoum et al. 2004, Zaph et al. 2008, Hall et al. 2008, Kang et al. 2008) and allergy (Bashir et al. 2004, Noverr et al. 2004). However, to date there has been limited analysis of how oral antibiotics specifically influence intestinal bacterial communities. We sought to characterize the effects of published antibiotic treatment protocols on intestinal bacterial communities through analysis of bacterial 16S rDNA compositions. Following published protocols, conventionally reared animals were given access to autoclaved water or autoclaved water containing ampicillin, gentamicin, metronidazole, neomycin, and vancomycin (Sekirov et al. 2008, Bashir et al. 2004, Rakoff-Nahoum et al. 2004, Zaph et

al. 2008, Hall et al. 2008, Kang et al. 2008). Stool pellets were collected at day 10 post-treatment initiation and total DNA was extracted for quantitative analysis of 16S ribosomal DNA (16S rDNA) genes by real-time RT-PCR. We observed a greater than two log reduction in bacterial 16S rDNA after ten days of antibiotic treatment as compared to control-treated animals (**Figure 5a**).

To examine the effects of antibiotics on the composition of bacterial communities in stool pellets, we used 454/Roche pyrosequencing of 16S rDNA segments and determined taxonomic assignments for each sequence using RDP Classifier. Antibiotic administration resulted in reduced frequencies of bacteria belonging to the Bacteroidetes phylum (Bacteroidaceae family) and Firmicutes phylum (Lachnospiraceae family). Antibiotic administration also resulted in increased frequencies of other members of the Firmicutes phylum (Leuconostocaceae and Streptococcaceae families) as well as members of the Proteobacteria phylum (Enterobacteriaceae and Moraxellaceae families) (**Figure 5b**, H2O vs. ABX).

We next investigated the origin of the bacterial 16S rDNA present in stool pellets after antibiotic treatment. To determine whether these sequences represented a living community that persisted after antibiotic treatment, we analyzed stool pellets from germ-free animals and found bacterial community sequences similar to those present in stool pellets from antibiotic-treated animals (**Figure 5b**). We hypothesized that antibiotic treatment reduced intestinal bacteria to levels at which residual DNA in autoclaved food became the major contributor of 16S rDNA sequences. To test this, DNA was extracted from sterile mouse chow and 16S communities were shown to be indistinguishable from those found in stool pellets from antibiotic-treated or germ-free animals (**Figure 5b**)

indicating that stool pellets from these animals were contaminated with bacterial 16S

rDNA normally present in autoclaved food.
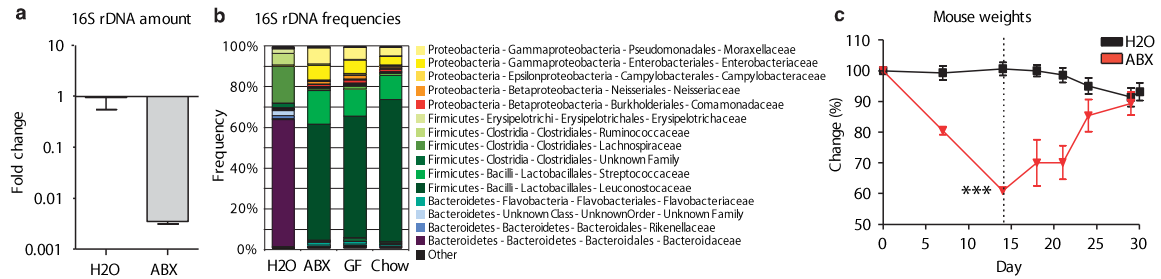


**Figure 5: Antibiotic delivery in drinking water modulates intestinal communities but results in animal dehydration**

(**a**) 16S rDNA gene copies as quantified by real-time RT-PCR from stool pellets collected from naïve (H2O, *n*=5) or antibiotic-treated (ABX; *n*=5) animals (±S.E.M). (**b**) Family-level phylogenetic classification of 16S rDNA frequencies in stool pellets collected from naïve animals (H2O; *n*=5), antibiotic-treated animals (ABX; *n*=5), germ-free animals (GF; *n*=3), or autoclaved animal food (Chow; *n*=3). (**c**) Weights of animals fed unsupplemented (H2O; *n*=4) or antibiotic-supplemented (ABX; *n*=4) water (***$P \leq 0.001$; ±S.E.M.). Antibiotic-treated animals were switched to unsupplemented water at day 14 (dashed line).

We further sought to investigate the effects of antibiotics on intestinal immune homeostasis. Unfortunately, we found that animals fed antibiotics in drinking water rapidly showed signs of dehydration including weight loss, decreased skin turgor, and hard stools while animals fed autoclaved water showed no signs of dehydration (**Figure 5c**). Similar antibiotic treatment protocols without gentamicin or with added sweetener also resulted in dehydration (data not shown). These data reveal an adverse effect of antibiotic treatment that confounds the interpretation of the effects of antibiotics on immune cell homeostasis (Guseinov, Guseinova 2008).

## 2.4.2 Development of a new, broad-spectrum antibiotic treatment regimen that prevents animal dehydration

We developed an alternative protocol in which animals were orally gavaged with autoclaved water or autoclaved water containing ampicillin, gentamicin, metronidazole, neomycin, and vancomycin once daily for ten days. Daily weights and stool pellet samples were taken and animals were sacrificed on day 10 for histologic, microbiologic, and immunologic analyses. Animals treated with antibiotics by oral gavage maintained their body weight (**Figure 6a**) and displayed no signs of dehydration. Treated animals also developed loose stools (data not shown) as seen with antibiotic treatment in human patients (Rohde, Bartolini & Jones 2009).

To examine the effects of this treatment regimen on intestinal bacteria, 16S rDNA copy number was quantified in stool pellet samples using real-time RT-PCR. We observed an initial increase in 16S rDNA copies in stool pellets from antibiotic treated animals at day one post treatment initiation suggesting a flushing of bacterial DNA into the stool, likely representing dead bacteria (**Figure 6b**, day 1). After nine days, we observed a ten-fold reduction in total 16S rDNA in stool pellets of treated animals (**Figure 6b**) consistent with a reduction in total bacterial load.

We next undertook gross anatomic and histologic analyses of antibiotic-treated animals. Treated animals displayed characteristic enlarged ceca, similar to those seen in germ-free animals (**Figure 6c**) (Thompson, Trexler 1971), as well as expansion of the lamina propria and enterocyte hyperplasia resulting in increased intestinal villus width and length (**Figure 6d,e**). Further, examination of RELMβ, a protein secreted from intestinal goblet cells in response to intestinal colonization (Wang et al. 2005), showed a trend towards reduction at day one that reached statistical significance by day five ($P \leq$

67

0.001) and day nine ($P \leq 0.005$) post-treatment initiation (**Figure 6f**). Thus, antibiotic treatment by oral gavage resulted in bacterial depletion without dehydration, and this was accompanied by anatomic, histologic, and immunologic changes characteristic of reduced microbial stimulation.

### 2.4.3 Antibiotic treatment results in two phases of bacterial community restructuring

The temporal effects of antibiotic treatment on bacterial communities were examined using pyrosequencing of bacterial 16S rDNA gene segments. We analyzed 49,994 sequences from 144 samples representing stool pellet, luminal, and mucosal-associated bacterial communities originating from the cecum, proximal large intestine, and distal large intestine of nine animals (four naïve and five antibiotic-treated). To characterize the global effects of antibiotic treatment on the full intestinal microbiome, bacterial communities were compared in control-treated or antibiotic-treated animals by quantifying similarities based on phylogenetic distances using UniFrac as described previously (Lozupone, Knight 2005, Lozupone, Hamady & Knight 2006, Lozupone et al. 2007). UniFrac distances were calculated in two different ways, using only presence/absence information (unweighted) or taking into account the abundance of each bacterial lineage (weighted) and Principal Coordinate Analysis was used to cluster communities along orthogonal axes of maximal variance.
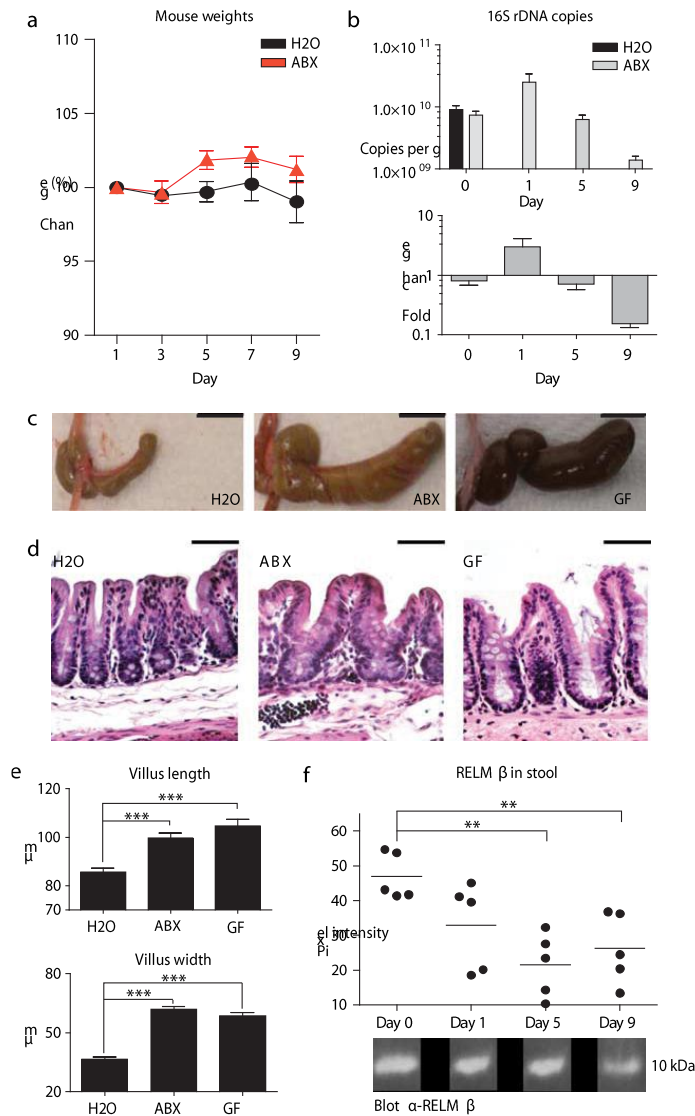
**Figure 6: Antibiotic administration by gavage mimics molecular, anatomic, histologic, and immunologic characteristics of reduced microbial stimulation without animal dehydration**

(**a**) Weights of animals gavaged with unsupplemented (H2O; *n*=4) or antibiotic supplemented (ABX; *n*=5) water (±S.E.M.). (**b**) 16S rDNA gene copies as quantified from stool pellets collected before (day 0) or over the course of antibiotic treatment (days 1, and 9) (*n*=5; (±S.E.M.). (**c**) Cecal images from control-treated (H2O), day 10 antibiotic-treated (ABX), or germ-free (GF) animals (bar, 1 cm). (**d**) Photomicrographs of hematoxylin-and-eosin-stained cecal sections from control-treated (H2O), day 10 antibiotic-treated (ABX), or germ-free (GF) animals showing expansion of the lamina propria and enterocyte hyperplasia in ceca from antibiotic-treated and germ-free animals (bar, 50 µm). (**e**) Quantification of cecal villus length and width from control-treated (H2O; *n*=4), day 10 antibiotic-treated (ABX; *n*=5), or germ-free (GF; *n*=3) animals (***P≤0.001; ±S.E.M). (**f**) Quantification of RELMβ protein in stool pellets by western blot over the course of antibiotic treatment (*n*=5) (**P≤0.01; ±S.E.M).

69

In unweighted UniFrac analysis, the first coordinate separated the stool pellet, luminal, and mucosal-associated samples on the basis of antibiotic treatment, and explained 26.8% of the variance (**Figure 7a**). Weighted UniFrac analysis also separated samples on the basis of antibiotic treatment, with 75.4% of the variance represented by the first principal coordinate (**Figure 7b**). These findings indicated that antibiotic treatment resulted in new luminal and mucosal-associated bacterial communities that were distinct from those seen in control-treated animals both in the proportions of the different groups and in the types of bacteria present. Treated samples grouped tightly in our weighted analysis with respect to the second coordinate axis (**Figure 7b**, 14.1% of group variance) indicating that intestinal communities in treated animals were more similar than those existing prior to treatment.

To examine the temporal effects of antibiotic treatment analysis of stool pellets was utilized. Stool pellet analysis is both non-invasive and representative of luminal community composition (Eckburg et al. 2005), allowing for longitudinal analyses. We found that samples from day zero and day one had distinct graphical locations in both unweighted and weighted UniFrac analyses (**Figure 7a,b**). This finding indicated an initial shift in community structure that occurred quickly after initiation of antibiotic treatment. Furthermore, unweighted and weighted UniFrac analyses revealed that the second and third coordinates, respectively, separated longitudinal pellet samples on the basis of treatment day (**Figure 7c,d**) indicating a second, more gradual change in community structure over time. This trend reached statistical significance in antibiotic-treated ($P \leq 0.0001$, unweighted and weighted) but not control-treated animals. In summary, UniFrac analysis of bacterial communities from control-treated or antibiotic-

70

treated animals revealed a dramatic shift in community structure that occurred quickly after antibiotic initiation, followed by a more gradual alteration in community structure over time.
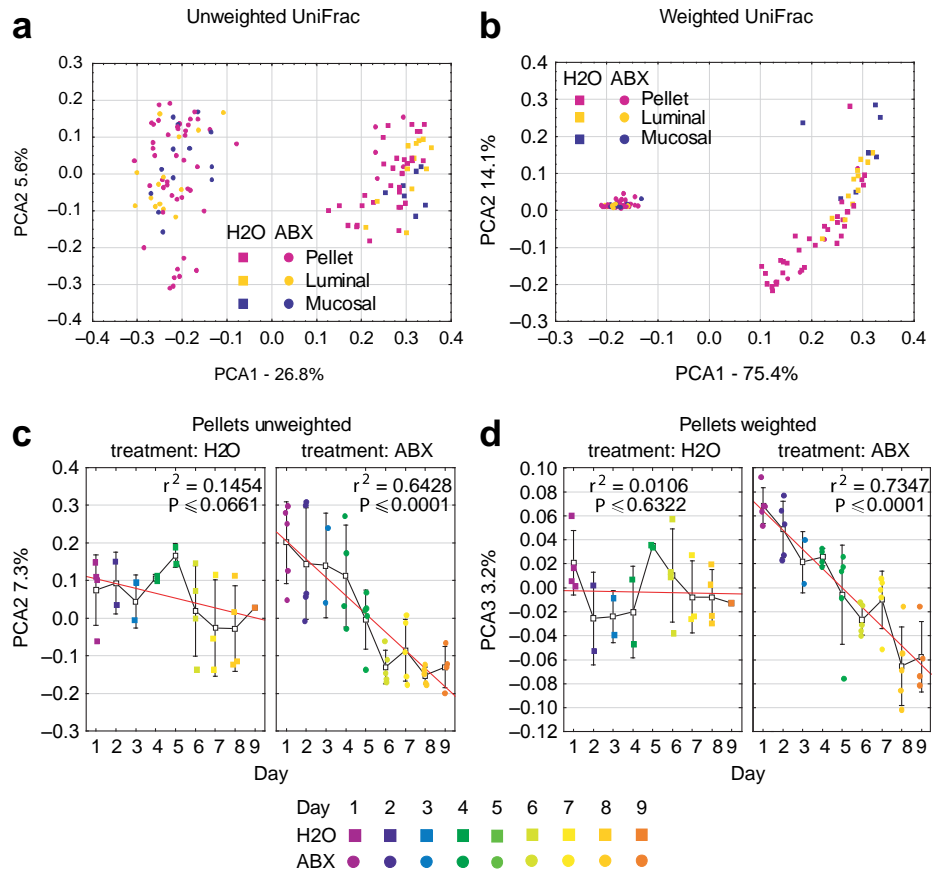


**Figure 7: UniFrac analysis of restructuring of bacterial communities over time**

(**a**) Unweighted or (**b**) weighted UniFrac analysis of stool pellet, luminal content or mucosal-associated samples from control- or antibiotic-treated animals. (**c**) Unweighted or (**d**) weighted UniFrac analysis of stool pellet samples from control-treated (H2O; *n*=4) or antibiotic-treated (ABX; *n*=5) animals from day 1 to day 9 post-treatment initiation. Colored symbols represent treatment day, white squares represent means, red line represents linear regression analysis (±S.E.M., significance determined by regression analysis).

### 2.4.4 Antibiotic treatment causes reductions in Firmicutes with the persistence of Bacteroidetes and Proteobacteria phyla over time

Phylogenetic placements allow the identification of bacterial taxa affected by antibiotic treatment. Temporal analysis of stool pellet samples revealed a significant shift in community composition on day one post-antibiotic treatment initiation (**Figure 8a,b**) that was not observed in control-treated animals (data not shown). The timing of this shift is consistent with the flushing of 16S rDNA into stool observed during quantitative analysis (**Figure 6b**, day 1) and the graphical separation between day zero and day one samples from treated animals in UniFrac analyses (**Figure 7a,b**). These findings indicate that even short antibiotic courses can result in dramatic alterations to intestinal bacterial communities. Temporal analysis of stool pellet samples over the course of treatment further revealed a gradual change in bacterial proportions over time (**Figure 8a**) that was consistent with the gradual alteration in community structure observed by UniFrac (**Figure 7c,d**).

After nine days of antibiotic treatment, there were several statistically significant differences between stool pellets from antibiotic-treated and control-treated animals. These included reductions in the Porphyromonadaceae family, *Marinilabilia* genus, Bacteroidales order, Bacteroidetes phylum, *Weissella* genus, *Butyrivibrio* genus, *Lachnospiraceae incertae sedis* genus, Lachnospiraceae family, *Anaerotruncus* genus, *Ruminococcaceae incertae sedis* genus, Ruminococcaceae family, and *TM7 genera incertae sedis* (*P* values listed in **Figure 8b**; groups significantly reduced shaded in red). Upon antibiotic treatment, there were increases in the frequency of bacteria belonging to the *Bacteroides* genus, *Parabacteroides* genus, *Prevotella* genus, Prevotellaceae family, *Xylanibacter* genus, *Erysipelotrichaceae incertae s*edis, and *Akkermansia* genus (*P*

values listed in **Figure 8b**; groups significantly increased shaded in green). We did not detect any statistically significant differences in bacterial frequencies over the course of treatment in stool pellets from control-treated animals (data not shown). In summary, we found that antibiotic treatment caused significant changes in stool pellet bacterial communities and that these changes occurred in at least two phases, one that occurred immediately after initiating treatment and one that progressed over the course of treatment.
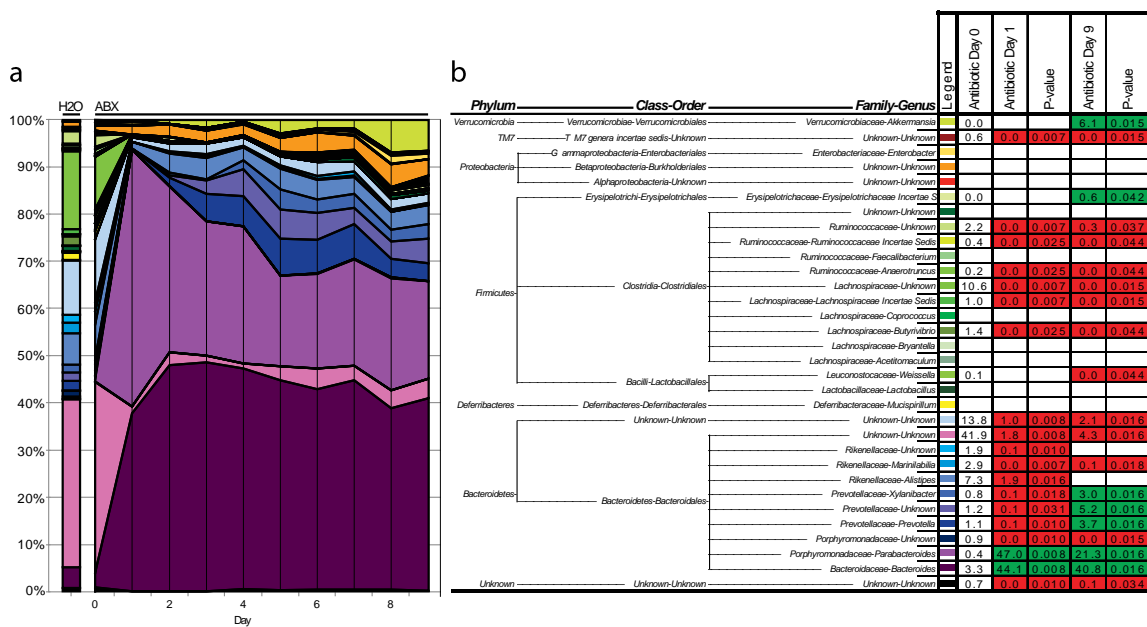


**Figure 8: Metagenomic analysis of restructuring of intestinal bacterial communities over time**

(**a**) Genus-level phylogenetic classification of 16S rDNA frequencies in stool pellets collected from control-treated (H2O; $n$=4) or antibiotic-treated (ABX; $n$=5) animals from day 0 to day 9. (**b**) Average frequency of bacterial groups before (antibiotic day 0) or during (antibiotic day 1 and 9) antibiotic treatment. Mann-Whitney $P$ values of changes in group frequency with antibiotic treatment. Frequency reductions on antibiotic treatment shown in red, increases shown in green, non-significant changes blank.

## 2.4.5 Antibiotic treatment results in significant alterations to luminal and mucosal-associated bacterial communities along the length of the colon

Bacterial communities are distinct along the length of the intestinal tract (Eckburg et al. 2005), and between luminal and mucosal sites (Zoetendal et al. 2002). Therefore, we analyzed the spatial effects of antibiotic treatment on luminal bacterial communities longitudinally along the colon (cecum, proximal colon, distal colon) (**Figure 9a**). In the lumen, antibiotic treatment was associated with lower frequencies of bacteria belonging to the Porphyromonadaceae family, *Alistipes* genus, *Marinilabilia* genus, Rikenellaceae family, Bacteroidales order, Bacteroidetes phylum, *Mucispirillum* genus, *Butyrivibrio* genus, *Coprococcus* genus, *Lachnospiraceae incertae sedis*, Lachnospiraceae family, *Anaerotruncus* genus, *Faecalibacterium* genus, *Ruminococcaceae incertae sedis*, Ruminococcaceae family and Clostridiales order (*P* values listed in **Figure 9b**; groups significantly reduced shaded in red). Antibiotic treatment also resulted in significant increases in the frequency of bacteria belonging to the *Bacteroides* genus, *Parabacteroides* genus, *Prevotella* genus, *Xylanibacter* genus, Burkholderiales order, *Enterobacter* genus, and *Akkermansia* genus (*P* values listed in **Figure 9b**; groups significantly increased shaded in green).
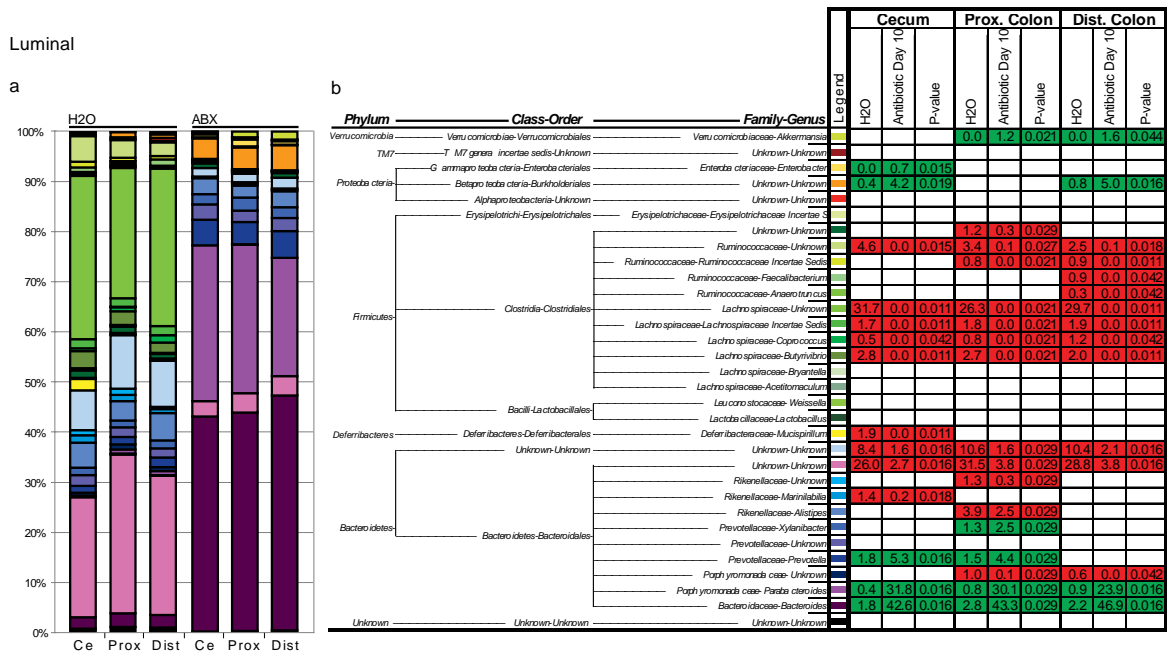
**Figure 9: Antibiotics modify luminal bacterial communities along the length of the colon**

(**a**) Genus- level phylogenetic classification of 16S rDNA frequencies in luminal samples collected from control-treated (H2O; *n*=4) or day 10 antibiotic-treated (ABX; *n*=5) animals from the cecum (Ce), proximal colon (Prox) or distal colon (Dist). (**b**) Average frequency of bacterial groups in samples from control-treated (H2O; *n*=4) or antibiotic-treated (antibiotic day 10; *n*=5) animals. Mann-Whitney *P* values of changes in group frequency with antibiotic treatment. Frequency reductions on antibiotic treatment shown in red, increases shown in green, non-significant changes blank.

Comparison of naïve mucosal-associated bacterial communities to naïve luminal communities revealed statistically higher frequencies of bacteria belonging to the *Mucispirillum* genus (*P* ≤ 0.012) and *Lactobacillus* genus (*P* ≤ 0.001), and lower frequencies of the *Alistipes* genus (*P* ≤ 0.017) (**Figure 9a vs. Figure 10a**). Upon antibiotic treatment, there were significant reductions in the frequency of mucosal-associated bacteria belonging to the *Marinilabilia* genus, Rikenellaceae family, Bacteroidales order, Bacteroidetes phylum, *Mucispirillum* genus, *Lactobacillus* genus, *Acetitomaculum* genus, *Bryantella* genus, *Butyrivibrio* genus, *Coprococcus* genus, *Lachnospiraceae incertae sedis*, Lachnospiraceae family, *Anaerotruncus* genus,

75

Ruminococcaceae family, and the Clostridiales order as compared to samples from control-treated animals (*P* values listed in **Figure 10b**; groups significantly reduced shaded in red). Upon antibiotic treatment, there were also significant increases in the frequency of mucosal-associated bacteria belonging to the *Bacteroides* genus, *Parabacteroides* genus, *Prevotella* genus, Prevotellaceae family, *Xylanibacter* genus, and the Burkholderiales order (*P* values listed in **Figure 10b**; groups significantly increased shaded in green). We were unable to amplify enough sequences from proximal colon mucosal-associated samples for statistical analysis. In summary, phylogenetic analyses revealed significant changes to luminal and mucosal-associated bacterial communities following antibiotic treatment.

**a** Mucosal-Associated

**b**

| Phylum | Class-Order | Family-Genus | Legend | Cecum H2O | Cecum Antibiotic Day 10 | Cecum P-value | Dist. Colon H2O | Dist. Colon Antibiotic Day 10 | Dist. Colon P-value |
|---|---|---|---|---|---|---|---|---|---|
| Verrucomicrobia | Verrucomicrobiae-Verrucomicrobiales | Verrucomicrobiaceae- Akkermansia | | | | | | | |
| TM7 | TM7 genera incertae sedis-Unknown | Unknown-Unknown | | | | | | | |
| Proteobacteria | Gammaproteobacteria-Enterobacteriales | Enterobacteriaceae-Enterobacter | | | | | | | |
| Proteobacteria | Betaproteobacteria-Burkholderiales | Unknown-Unknown | | 0.7 | 3.9 | 0.036 | 0.9 | 5.0 | 0.036 |
| | Alphaproteobacteria-Unknown | Unknown-Unknown | | | | | | | |
| | Erysipelotrichi-Erysipelotrichales | Erysipelotrichaceae- Erysipelotrichaceae Incertae S | | | | | | | |
| Firmicutes | Clostridia-Clostridiales | Unknown-Unknown | | 2.7 | 0.4 | 0.036 | 2.8 | 0.1 | 0.032 |
| | | Ruminococcaceae- Unknown | | | | | | | |
| | | Ruminococcaceae- Ruminococcaceae Incertae Sedis | | | | | | | |
| | | Ruminococcaceae- Faecalibacterium | | | | | | | |
| | | Ruminococcaceae- Anaerotruncus | | 0.6 | 0.0 | 0.017 | 0.5 | 0.0 | 0.017 |
| | | Lachnospiraceae- Unknown | | 29.5 | 0.0 | 0.017 | 25.3 | 0.1 | 0.026 |
| | | Lachnospiraceae-Lachnospiraceae Incertae Sedis | | 3.4 | 0.0 | 0.017 | 3.3 | 0.0 | 0.026 |
| | | Lachnospiraceae- Coprococcus | | 0.3 | 0.0 | 0.017 | | | |
| | | Lachnospiraceae- Butyrivibrio | | 2.8 | 0.0 | 0.017 | 2.2 | 0.1 | 0.026 |
| | | Lachnospiraceae- Bryantella | | | | | 0.7 | 0.0 | 0.017 |
| | | Lachnospiraceae- Acetitomaculum | | | | | 0.3 | 0.0 | 0.017 |
| | Bacilli-Lactobacillales | Leuconostocaceae- Weissella | | | | | | | |
| | | Lactobacillaceae-Lactobacillus | | 13.5 | 0.4 | 0.036 | 5.2 | 0.8 | 0.036 |
| Deferribacteres | Deferribacteres-Deferribacterales | Deferribacteraceae- Mucispirillum | | 8.5 | 0.0 | 0.017 | 0.8 | 0.0 | 0.017 |
| | Unknown-Unknown | Unknown-Unknown | | 5.1 | 2.3 | 0.036 | | | |
| Bacteroidetes | Bacteroidetes-Bacteroidales | Unknown-Unknown | | 19.7 | 4.1 | 0.036 | 22.7 | 4.0 | 0.036 |
| | | Rikenellaceae- Unknown | | | | | 0.8 | 0.2 | 0.036 |
| | | Rikenellaceae- Marinilabilia | | | | | 0.9 | 0.1 | 0.032 |
| | | Rikenellaceae- Alistipes | | | | | | | |
| | | Prevotellaceae- Xylanibacter | | 0.2 | 3.1 | 0.036 | | | |
| | | Prevotellaceae- Unknown | | 0.7 | 4.0 | 0.036 | | | |
| | | Prevotellaceae- Prevotella | | 0.9 | 6.4 | 0.036 | 3.6 | 7.4 | 0.036 |
| | | Porphyromonadaceae- Unknown | | | | | | | |
| | | Porphyromonadaceae- Parabacteroides | | 0.2 | 27.9 | 0.036 | 3.3 | 27.6 | 0.036 |
| | | Bacteroidaceae- Bacteroides | | 1.4 | 39.7 | 0.036 | 4.2 | 37.6 | 0.036 |
| Unknown | Unknown-Unknown | Unknown-Unknown | | | | | | | |

**Figure 10: Antibiotics modify mucosal-associated bacterial communities along the length of the colon**

(**a**) Genus- level phylogenetic classification of 16S rDNA frequencies in mucosal-associated samples collected from control-treated (H2O; *n*=4) or day 10 antibiotic-treated (ABX; *n*=5) animals from the cecum (Ce), proximal colon (Prox) or distal colon (Dist). (**b**) Average frequency of bacterial groups in samples from control-treated (H2O; *n*=4) or antibiotic-treated (antibiotic day 10; *n*=5) animals. Mann-Whitney *P* values of changes in group frequency with antibiotic treatment. Frequency reductions on antibiotic treatment shown in red, increases shown in green, non-significant changes blank.

## 2.5 Discussion

This chapter describes the development of an antibiotic treatment protocol that significantly alters intestinal bacterial communities and immune cell homeostasis without animal dehydration. Deep sequencing was employed to quantify temporal and spatial antibiotic effects on intestinal bacterial communities and show significant changes in several bacterial groups that correlate with altered cytokine production by CD4$^+$ T lymphocytes in intestinal-associated lymphoid tissues.

Animals were initially treated with antibiotics in drinking water in a similar manner to previously published protocols (Sekirov et al. 2008, Bashir et al. 2004, Rakoff-Nahoum et al. 2004, Zaph et al. 2008, Hall et al. 2008, Kang et al. 2008). This treatment protocol reduced intestinal bacteria to the extent that DNA from food composed the majority of recovered 16S rDNA sequences. However, this protocol was associated with animal dehydration that may complicate the interpretation of subsequent immunologic studies (Guseinov, Guseinova 2008). These findings also highlight the presence of sterile microbial-derived signals and emphasize the importance of controlling for DNA contamination from food in future immunologic and metagenomic studies. As an alternative, acute administration of broad-spectrum antibiotics by gavage was employed, while allowing animal access to untreated drinking water; more closely mimicking antibiotic administration in humans. Animals treated by gavage did not show signs of dehydration and developed loose stools similar to known consequences of antibiotic treatment in patients (Rohde, Bartolini & Jones 2009). Antibiotic treatment by oral gavage reduced absolute bacterial numbers and mimicked anatomic, histologic, and immunologic characteristics of reduced bacterial stimulation.

77

Temporal and spatial analyses of antibiotic effects on bacterial communities were carried out using pyrosequencing of bacterial 16S rDNA gene segments. UniFrac analysis revealed that samples from antibiotic treated animals grouped separately from, and clustered more tightly than, samples from control-treated animals indicating that antibiotic treatment promoted a novel community structure that was more similar between antibiotic-treated as compared to control-treated animals. Day one stool pellet samples from treated animals grouped closely with later time points consistent with an initial, fast phase of community alteration. This finding was emphasized in temporal analysis of stool pellet bacterial frequencies during treatment and suggests that even short exposure to oral antibiotics creates distinct intestinal bacterial communities. UniFrac and frequency analyses of stool pellet communities over the course of antibiotic treatment further revealed progressive alterations in community structure, indicating that intestinal bacterial communities are continuously modified over the course of antibiotic treatment.

The influence of antibiotic treatment by oral-gavage on stool bacterial communities was noticeably different from that observed during antibiotic administration in animal drinking water. For example, sequences from the Bacteroidetes group represented a relatively small proportion of sequences recovered from mice treated continuously with antibiotics while a higher proportion of Bacteroidetes sequences were recovered from animals treated with antibiotics by gavage. These differences likely arise due to differences in the duration of treatment (four weeks vs. ten days), in the method of antibiotic administration (continuous vs. gavage), in animal hydration state (physiologic vs. non-physiologic), and in the relative contribution that 16S rDNA from food makes to measured bacterial frequencies (higher with more efficient microbial

depletion). This finding highlights the importance of controlling for 16S rDNA in food in future metagenomic studies.

Previous studies have shown that antibiotic-treated animals develop allergic responses (Bashir et al. 2004) and antibiotic use in children is associated with increased risk of developing asthma later in life (Marra et al. 2009). In addition, early colonization with Bacteroides fragilis has been implicated as a risk factor for asthma development in humans (Vael et al. 2008). As such, the effects of antibiotic treatment on members of the Bacteroidies phylum were examined. Members of the Bacteroidies phylum, namely the Bacteroidales order, represented 60-70% of intestinal bacteria in control-treated animals. After one day, these bacteria represented a combined frequency of greater than 95% in samples from antibiotic-treated but not control-treated animals, and subsequently stabilized at a frequency of approximately 90% in all sampled compartments. Outgrowths of the Bacteroidies phylum were not observed in previous metagenomic studies that examined recovery of intestinal microbiota after antibiotic exposure (Antonopoulos et al. 2009), likely representing differences in antibiotic type and administration and highlighting the utility of temporal analyses in metagenomic studies. It is intriguing to speculate that rapid increases in Bacteroidies frequency after antibiotic initiation may be responsible for associations between early antibiotic exposure in humans and increased susceptibility to allergic inflammation.

Significant reductions in the frequency of bacteria belonging to the Firmicute phylum were also detected in samples from antibiotic-treated as compared to control-treated animals. This bacterial phylum has been shown to be present in an increased frequency in obese patients as compared to healthy individuals (Ley et al. 2006) and may provide the host an enhanced ability to extract energy from otherwise indigestible

dietary polysaccharides (Turnbaugh et al. 2006). As such, antibiotic treatment may result in a reduced capacity for energy harvest by the host which has implications for prolonged antibiotic treatment in patients. In addition, these findings highlight a possible role for antibiotics as a means of modulating intestinal bacterial communities in future treatments for obesity (Ley et al. 2006).

Consistent with previous studies, increased frequencies of *Enterobacteriaceae*, as well as other members of the Proteobacteria phyla were detected in samples from antibiotic-treated as compared to control-treated animals (Antonopoulos et al. 2009). The *Enterobacteriaceae* include a number of nosocomial pathogens, with considerable antibiotic resistance, including *Escherichia*, *Enterobacter*, and *Salmonella*. Given that DNA transfer between intestinal bacteria plays a role in shaping bacterial communities (Ley, Peterson & Gordon 2006, Grasselli et al. 2008) and in the development of pathogenic, antibiotic-resistant organisms (Karami et al. 2007), *Enterobacteriaceae* that survive in an antibiotic exposed intestine may act as a clinically relevant reservoir that could seed subsequent infections with antibiotic resistant bacteria. Outgrowth of *C. difficile*, a common cause of antibiotic-associated colitis that is thought to colonize the intestine when indigenous microbiota are disrupted by antibiotic use (De La Cochetiere et al. 2008), was not observed as a result of our antibiotic treatment protocol. However, this result is not unexpected as metronidazole and vancomycin, two antibiotics commonly used to treat *C. difficile* colitis in humans, were included in the antibiotic cocktails used in this study.

Analyses were extended to spatial examinations of antibiotic effects along the length of the intestine, and between luminal and mucosal-associated bacterial communities. It was found that luminal and stool pellet bacterial communities from naïve

animals were similar, suggesting that examination of stool pellets is an adequate, rough approximation of luminal communities (Eckburg et al. 2005). However, naïve animals displayed significantly higher frequencies of *Lactobacillus* species in mucosal-associated as compared to luminal communities, suggesting that distinct bacterial communities at different anatomical locations may be relevant for mammalian physiology.

Upon antibiotic treatment, there were significant changes to both luminal and mucosal-associated bacterial communities including increases in the Bacteroidies and Proteobacter phyla, and reductions in the Firmicute phylum. In particular, significant reductions in the frequency of mucosal-associated *Lactobacillus* species were observed in antibiotic-treated as compared to control-treated animals. *Lactobacillus* species have been used as "probiotic" bacteria with mixed effectiveness in treating human diseases (Betsi, Papadavid & Falagas 2008, Kozuch, Hanauer 2008). It may therefore be useful to examine whether reductions in this potentially beneficial mucosal-associated bacterial group are responsible for changes in immune homeostasis observed following antibiotic treatment.

In summary, this chapter describes the development and characterization of a new antibiotic treatment protocol that modifies intestinal microbiota without animal dehydration. Deep sequencing analysis showed that antibiotic treatment causes significant temporal and spatial alterations in bacterial groups that have been implicated to have causative or therapeutic roles in human diseases. It is hoped that these findings will provide a resource and framework for analysis and manipulation of intestinal microbial communities in murine models of human infection and disease.

# Chapter 3: Commensal Bacterial-Derived Signals Limit Allergic Inflammation in Mice

## 3.1 Abstract

The prevalence and severity of allergic diseases have been increasing in the U.S. and U.K. for more than fifty years. Recently, experimental and epidemiologic studies have implicated commensal bacteria that colonize mammalian mucosal surfaces in influencing T helper type 2 ($T_H2$) cytokine-mediated inflammation and allergic disease. However, the mechanisms by which the innate immune system recognizes commensal bacterial-derived signals and influences the development of $T_H2$ cell responses are poorly understood. The data presented in this chapter indicate that commensal bacterial-derived signals limit serum immunoglobulin E (IgE) levels and steady-state circulating basophil populations, innate granulocytes implicated in the initiation and propagation of $T_H2$ cell responses. Consistent with dysregulated steady-state basophil responses contributing to exaggerated allergic responses, antibiotic (ABX) treatment of mice resulted in exaggerated basophil-mediated skin and airway inflammation, and the development of enhanced basophil-dependent $T_H2$ cell responses. Together, these results indicate that commensal-derived signals act to limit steady-state circulating basophil responses and the development of basophil-mediated allergic inflammation.

## 3.2 Introduction

Allergic diseases have reached pandemic levels (Eder, Ege & von Mutius 2006) and represent a significant source of morbidity, mortality and healthcare cost (Bahadori et al. 2009). These chronic inflammatory diseases are characterized by interleukin (IL)-4, IL-5, IL-9 and IL-13 production by $CD4^+$ T helper type 2 ($T_H2$) cells, immunoglobulin E (IgE) production by B cells, and the recruitment of effector cells to sites of tissue inflammation

83

(Mowen, Glimcher 2004, Holgate 2008). It is thought that susceptibility to $T_H2$ cytokine-dependent allergic inflammation is determined by polymorphisms in mammalian genes (Vercelli 2008) and environmental factors including diet and exposure to pollutants or infectious agents (Gilliland 2009, Zeiger 2003, Ege et al. 2011). However, the specific genetic and environmental stimuli that enhance allergy susceptibility, and how these factors contribute to the development of allergic disease, are an ongoing field of research.

The human intestine is colonized by 100 trillion microorganisms belonging to each of the three domains of life (Eckburg, Lepp & Relman 2003). Of these, bacteria are the most abundant – the colon is home to trillions of commensal bacteria (Whitman, Coleman & Wiebe 1998) with a diversity of at least 1,000 species (Ley, Peterson & Gordon 2006). Epidemiologic studies have identified associations between alterations in the composition of commensal bacterial communities and the development of allergic disease. For example, infants who develop allergies display altered commensal populations early in life (Kalliomaki et al. 2001), and children who have undergone treatment with broad-spectrum antibiotics are at an increased risk of developing allergic diseases (Marra et al. 2009, Kummeling et al. 2007). Studies in animal model systems have corroborated these findings and shown that commensal bacterial-derived signals limit the development of $T_H2$ cytokine-mediated allergic inflammation (Bashir et al. 2004, Noverr et al. 2005, Noverr et al. 2004, Herbst et al. 2011). However, the innate immune cells that are influenced by commensal bacterial-derived signals and modulate the development of allergic $T_H2$ cytokine-dependent inflammation remain poorly characterized (Lambrecht, Hammad 2010, Paul, Zhu 2010).

Recently, granulocytes including mast cells, eosinophils and basophils have been shown to express MHC class II (Charles et al. 2010, Skokos et al. 2003, Padigel et al. 2007), be potent sources of IL-4 and, depending on the stimulus, contribute to the development of optimal $T_H2$ cytokine responses (Gessner, Mohrs & Mohrs 2005, Mohrs et al. 2005, Min et al. 2004). Given the proposed role for commensal bacteria in influencing allergy susceptibility, and the identification of granulocytes as innate cells that promote $T_H2$ cytokine-mediated inflammation, we sought to test whether commensal-derived signals influence granulocyte homeostasis or allergen-induced $T_H2$ cell responses.

In this chapter, oral delivery of broad-spectrum antibiotics was employed to interrogate the influence of commensal bacterial-derived signals on circulating basophil populations. Both deletion of bacterial communities through the use of germ-free mice, and depletion of bacterial communities using antibiotic treatment, were associated with elevated serum IgE levels, increased circulating basophil populations and exaggerated basophil-mediated $T_H2$ cytokine-dependent allergic inflammation. These findings identify basophils as an important innate immune cell population that is expanded in the circulation of antibiotic-treated or germ-free mice and may contribute to exaggerated $T_H2$ cytokine-mediated allergic inflammation in these models.

## 3.3 Methods

### 3.3.1 Animals

BALB/c or C57BL/6 mice were purchased from Jackson or Charles River Laboratories. Germ-free mice were provided by the Penn Gnotobiotic Mouse Facility. BaS-TRECK

mice were obtained from M. Kubo, IL-4/eGFP reporter mice were obtained from M. Mohrs. All mice used were 8–24 weeks of age. Conventional animals were bred and housed in specific pathogen-free conditions at the University of Pennsylvania. All experiments were performed under Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with the guidelines of the IACUC of the University of Pennsylvania.

### 3.3.2 Reagents and Treatments

For antibiotic treatment, mice were fed autoclaved water or autoclaved water supplemented with ampicillin (0.5 mg/mL), gentamicin (0.5 mg/mL), metronidazole (0.5 mg/mL), neomycin (0.5 mg/mL), and vancomycin (0.25 mg/mL) continuously via water bottle for four weeks. In the occasional event of poor animal oral hydration, both control and antibiotic water was supplemented with artificial sweetener. For MC903, mice were treated daily for 7 days on the ear with 10 μL of ethanol or ethanol containing 2 nMol of MC903 (Tocris Bio. Sci.) as described previously (Li et al. 2006). For house dust mite (HDM) allergen-induced airway inflammatoin, mice were treated on day 0, 7, 14 intranasally with 50 μL of PBS or PBS containing 100μg of *D. pteronyssinus* extract (Greer) and sacrificed on day 17 as described previously (Hammad et al. 2010). For papain, mice were injected subcutaneously in contralateral footpads with 50 ul of PBS or PBS containing 50 μg of papain (Calbiochem) as described previously (Sokol et al. 2008) and were sacrificed on D3 or D4 post injection for basophil or $T_H2$ cell analysis. For basophil depletion by anti-FcεRIα treatment, mice were treated by intraperitoneal injection (i.p.) with 10 μg of MAR1 or isotype antibody (eBioscience) on D-3, -2, -1. For

basophil depletion by diphtheria toxin (DT) treatment, littermate control or BaS-TRECK mice were treated with 750 ng DT i.p. on day -2 and D0 prior to papain exposure.

### 3.3.3 Flow Cytometry

Blood, spleen, lung, bronchoalveolar lavage, ears or lymph nodes were collected at necropsy, lungs and ears were digested with collagenase and dispase, homogenized by passing through a 70 μm nylon mesh filter, purified of red blood cells by histopaque (Sigma-Aldrich) or RBC lysis, and stained with anti-mouse fluorochrome-conjugated monoclonal antibodies against B220, CD3ε, CD4, CD8, CD11c, CD19, CD40, CD45.1, CD49b, CD69, CD80, CD86, CD117, FcεRIα, FcγR, Gr-1, IgE, IL-4, MHC-I or MHC-II (BD Bioscience, BioLegend, eBioscience). Cells were acquired on a FACSCanto II or LSR II with DiVa software (BD Bioscience) and analyzed with FlowJo software (version 8.7.1; Tree Star).

### 3.3.4 Culture, ELISA and Histology

Tissues were harvested and single cell suspensions were made. LNs were cultured for 2-4 days at 200-250,000 cells in 200ul of complete media in the presence or absence of αCD3 and αCD28 (0.5-1.0 μg/mL). For intracellular cytokine analysis, cells were stimulated for 4 hours in the presence of 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 750 ng/mL ionomycin (Sigma-Aldrich) in the presence of 10 μg/mL Brefeldin A (Sigma-Aldrich), treated with Fix/Perm (eBioscience), and stained intracellularly. IgE and IL-4 from serum or cell-free culture supernatants were assayed by sandwich ELISA (BD Bioscience). At necropsy, skin or lung tissue sections were removed and fixed in 4% paraformaldehyde and embedded in paraffin. 5 μM sections

were cut and stained with hematoxylin and eosin. Orthokeratosis was quantified with NIS Elements BR imaging software (Nikon).

### 3.3.5 Statistics

Results shown as mean ± standard deviation for individual animals. Statistical significance was determined by two-tailed Mann-Whitney test or unweighted means analysis two-way ANOVA as indicated. Results were considered significant at $P \leq 0.05$ (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

## 3.4 Results

### 3.4.1 Commensal bacteria limit serum IgE levels and circulating basophils

As reported previously, oral antibiotic treatment (ABX) resulted in significant increases in serum IgE levels (Sudo et al. 1997, McCoy et al. 2006) (**Figure 11a**). As IgE influences granulocyte homeostasis (Kalesnikoff et al. 2001, Kitaura et al. 2003), we investigated whether ABX-induced elevations in IgE were associated with alterations in the frequency or number of circulating eosinophils, mast cells or basophils. ABX-treatment did not alter blood eosinophil (**Figure 11b,c**) or mast cell populations (**Figure 11d,e**). However, frequencies and numbers of basophils (identified as non-B, non-T (NBNT), CD117⁻, CD49b⁺, FcεRIα⁺) were significantly increased in the blood (**Figure 11f,g**) and spleen (**Figure 11h,i**) of ABX-treated compared to conventionally (CNV)-reared mice. Further, basophils from ABX-treated mice displayed increased levels of surface-bound IgE compared to controls (**Figure 12a**), while expression of other surface markers (CD69, CD123, CD200R, FcεRIα, FcγR, Gr1) were unaltered (**Figure 12b**).
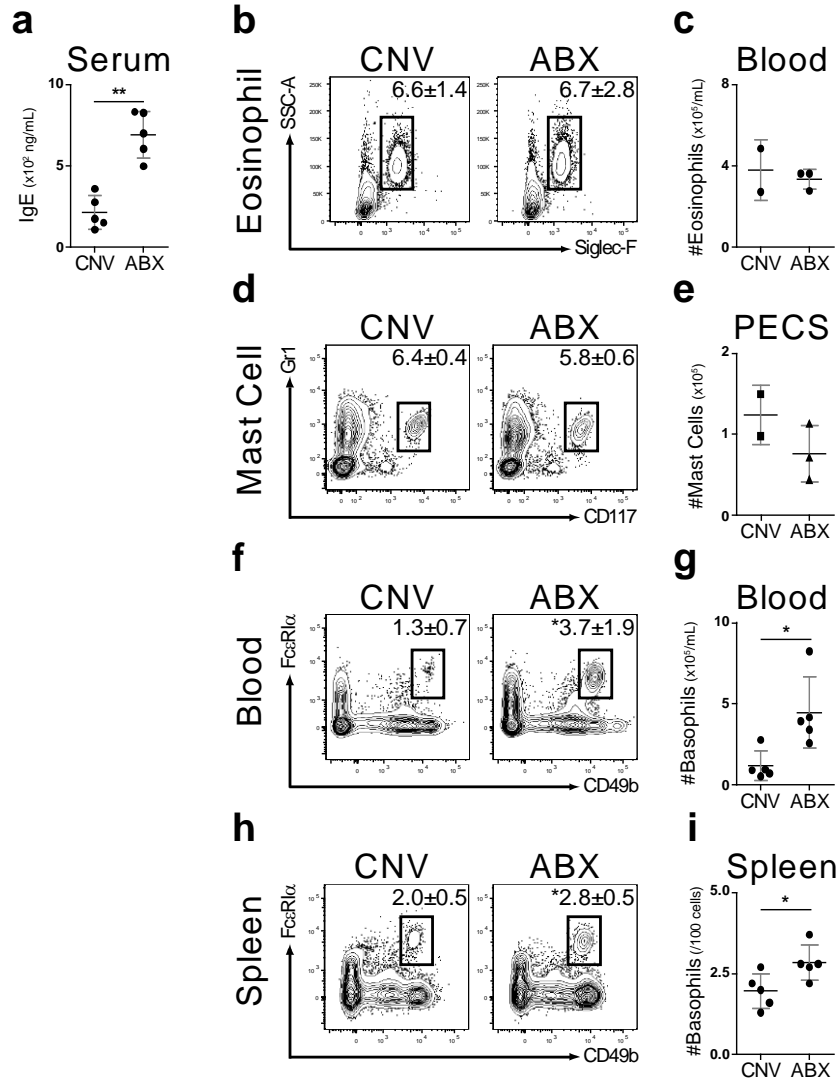
**Figure 11: Elevated steady-state serum IgE levels and circulating basophils in antibiotic-treated mice**

(**a**) Serum IgE from conventionally-reared (CNV) or antibiotic-treated (ABX) mice as measured by ELISA. (**b**) Flow cytometric analysis of blood eosinophils from CNV or ABX mice. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on CD3⁻, CD4⁻, CD8⁻, CD19⁻, CD117⁻ cells. (**c**) Number of eosinophils per mL of blood from CNV or ABX mice. (**d**) Flow cytometric analysis of peritoneal cavity (PECS) mast cells from CNV or ABX mice. Gated on CD3⁻, CD4⁻, CD8⁻, CD19⁻ cells. (**d**) Number of mast cells isolated from the PECS of CNV or ABX mice. (**e**) Flow cytometric analysis of blood basophils from CNV or ABX mice. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on CD3⁻, CD4⁻, CD8⁻, CD19⁻, CD117⁻ cells. (**f**) Number of basophils per mL of blood from CNV or ABX mice. (**g**) Flow cytometric analysis of spleen basophils from CNV or ABX mice. (**h**) Number of spleen basophils from CNV or ABX mice. Data representative of three or more independent experiments (mean ± S.D.; CNV, n=5-18; ABX, n=5; *, P ≤ 0.05).
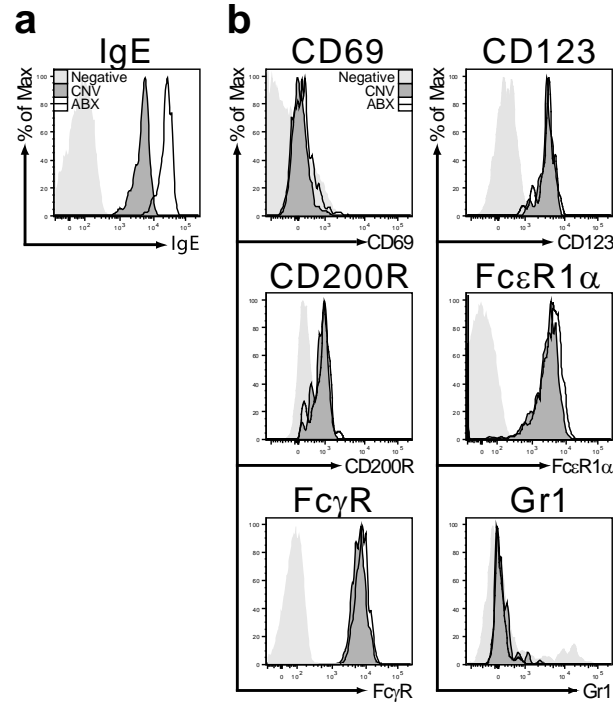
**Figure 12: Increased surface-bound IgE on basophils from antibiotic-treated mice**

(**a**) Mean fluorescence intensity of surface IgE on blood basophils from conventionally-reared (CNV) or antibiotic-treated (ABX) mice as determined by flow cytometry. Gated on CD3⁻, CD4⁻, CD8⁻, CD19⁻, CD117⁻, CD49b⁺, FcεRIα⁺ cells. (**b**) Mean fluorescence intensity of surface CD69, CD123, CD200R, FcεRIα, FcγR and Gr1 on blood basophils from CNV or ABX mice as determined by flow cytometry. Data representative of three or more independent experiments (Negative = negative cell population).

ABX treatment does not eliminate all commensal bacteria (Hill et al. 2009). To investigate whether steady-state levels of IgE or basophil populations were altered in the absence of live microbial stimuli, germ-free (GF) mice were employed (Smith, McCoy & Macpherson 2007). Consistent with the effects of ABX treatment, GF mice exhibited increased serum IgE levels (**Figure 13a**), increased frequencies and numbers of basophils in the blood (**Figure 13b,c**) and spleen (**Figure 13d,e**), and increased basophil-surface-bound IgE levels (**Figure 13f**) compared to CNV-reared mice. Consistent with commensal-derived signals being sufficient to limit serum IgE levels and

90

circulating basophil populations, conventionalization of GF mice resulted in reductions in serum IgE levels (**Figure 13g**), and blood and spleen basophil populations (**Figure 13h,i**). Collectively, these data indicate that commensal bacterial-derived signals limit serum IgE levels and circulating basophil populations in the steady-state.
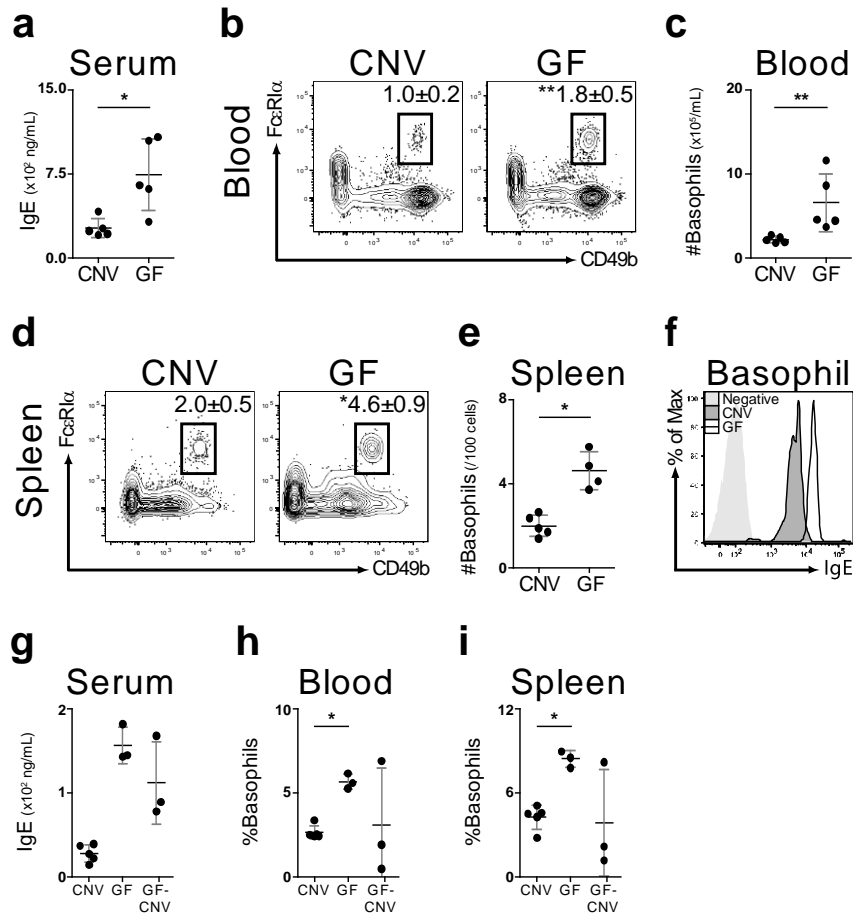


**Figure 13: Elevated steady-state serum IgE levels and circulating basophils in germ-free mice**

(**a**) Serum IgE from conventionally-reared (CNV) or germ-free (GF) mice as measured by ELISA. (**b**) Flow cytometric analysis of blood basophils from CNV or GF mice. Gated on CD3⁻, CD4⁻, CD8⁻, CD19⁻, CD117⁻ cells. (**c**) Number of basophils per mL of blood from CNV or GF mice. (**d**) Flow cytometric analysis of spleen basophils from CNV or GF mice. (**e**) Number of spleen basophils from CNV or GF mice. (**f**) Mean fluorescence intensity of surface-bound IgE on blood basophils from CNV or GF mice as determined by flow cytometry. (**g**) Serum IgE from CNV, GF or conventionalized germ-free (GF-CNV) mice as measured by ELISA. (**h**) Frequency of blood basophils from CNV, GF or GF-CNV mice. (**i**) Frequency of spleen basophils from CNV, GF or GF-CNV mice. Data representative of three or more independent experiments (mean ± S.D.; CNV, n=5-18; GF, n=3-10; CNV-GF, n=3; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Experiments performed in collaboration with Dmytro Kobuley.

### 3.4.2 Commensal bacteria limit basophil-mediated allergic skin inflammation

Given the enhanced frequencies and numbers of circulating steady-state basophils in ABX-treated mice, we next examined whether commensal-derived signals influence basophil effector function *in vivo*. Basophils contribute to the development of atopic dermatitis-like lesions in mice exposed to the vitamin D3 analogue MC903 (Li et al. 2006, Siracusa et al. In Press). To test whether commensal-derived signals influence MC903-elicited inflammation, CNV-reared or ABX-treated mice were exposed to MC903, and IgE levels, basophil responses and skin inflammation were examined. Compared to controls, MC903 exposure in ABX-treated mice resulted in significantly elevated serum IgE levels and blood basophil populations (**Figure 14a,b**), increased recruitment of basophils to inflamed skin (**Figure 14c**), and increased skin basophil surface-bound IgE levels (**Figure 14d**). Concurrently, compared to CNV-reared controls, ABX-treated mice displayed evidence of enhanced atopic dermatitis-like changes (**Figure 14e**) characterized by significantly more orthokeratosis compared to CNV-reared mice (**Figure 14f**), a pathological finding associated with atopic dermatitis in patients (Lobitz, Dobson 1956). Together, these results indicate that commensal bacterial-derived signals limit the development of basophil-mediated inflammation of the skin.
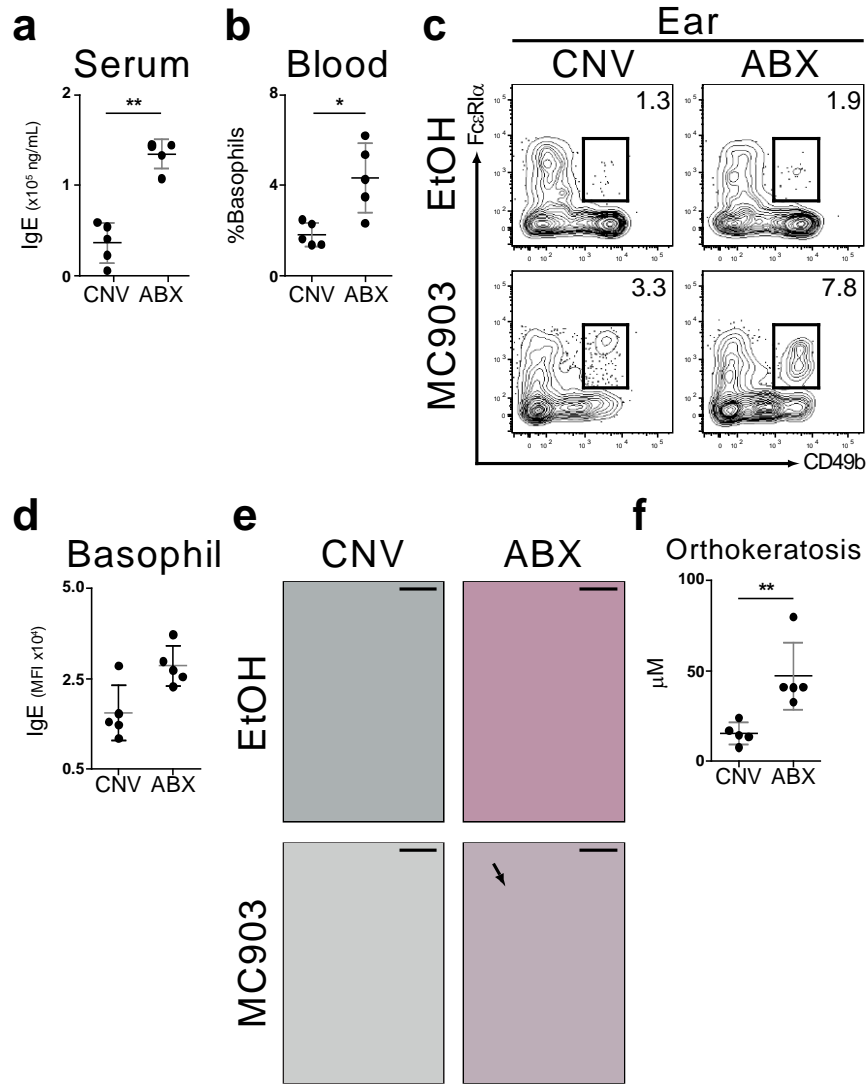
**Figure 14: Exaggerated MC903-induced skin inflammation in antibiotic-treated mice**

(**a**) Serum IgE from conventionally-reared (CNV) or antibiotic-treated (ABX) mice treated with MC903 for 7 days as measured by ELISA. (**b**) Frequency of blood basophils from CNV or ABX mice treated with MC903 for 7 days. (**c**) Flow cytometric analysis of skin basophils from control (EtOH) or MC903-treated, CNV or ABX mice. Gated on CD3⁻, CD4⁻, CD8⁻, CD19⁻, CD117⁻, CD45.1⁺ cells. (**d**) Mean fluorescence intensity of IgE on ear basophils from MC903-treated CNV or ABX mice. (**e**) Histological sections of skin from CNV or ABX mice treated for 7 days with ethanol (EtOH) or MC903 and stained with hematoxylin and eosin (H&E) (Bar = 100 μM). Arrow indicates orthokeratosis. (**f**) Orthokeratosis of skin from MC903-treated CNV or ABX mice. Data representative of two or more independent experiments (mean ± S.D.; CNV, n=2-5; ABX, n=1-5; *, $P \leq 0.05$; **, $P \leq 0.01$). Experiments performed in collaboration with Brian Kim.

### 3.4.3 Commensal bacteria limit basophil-mediated allergic airway inflammation

Basophils also contribute to allergic airway responses to inhaled house dust mite allergen (HDM) (Hammad et al. 2010). To test whether commensal-derived signals influenced the development of HDM-induced airway inflammation, CNV-reared or ABX-treated mice were exposed to HDM and $T_H2$ cell responses, systemic and local innate cell responses, and airway inflammation were examined. Compared to CNV-reared mice, ABX-treated mice displayed increased HDM-elicited $T_H2$ cell responses in the draining mediastinal lymph node (LN) as determined by the IL-4/eGFP reporter system (Mohrs et al. 2005) (**Figure 15a**), increased mediastinal LN $T_H2$ cell-derived IL-4 responses (**Figure 15b**), and increased blood basophil responses (**Figure 15c**). Concurrently, compared to CNV-reared controls, ABX-treated mice displayed increased HDM-elicited bronchoalveolar lavage (BAL) and lung eosinophil responses (**Figure 15d**) and exaggerated airway inflammation (**Figure 15e**) characterized by exaggerated cellular infiltrates and airway destruction. Together, these findings indicate that commensal-derived signals limit basophil-mediated allergic inflammation in the lung.
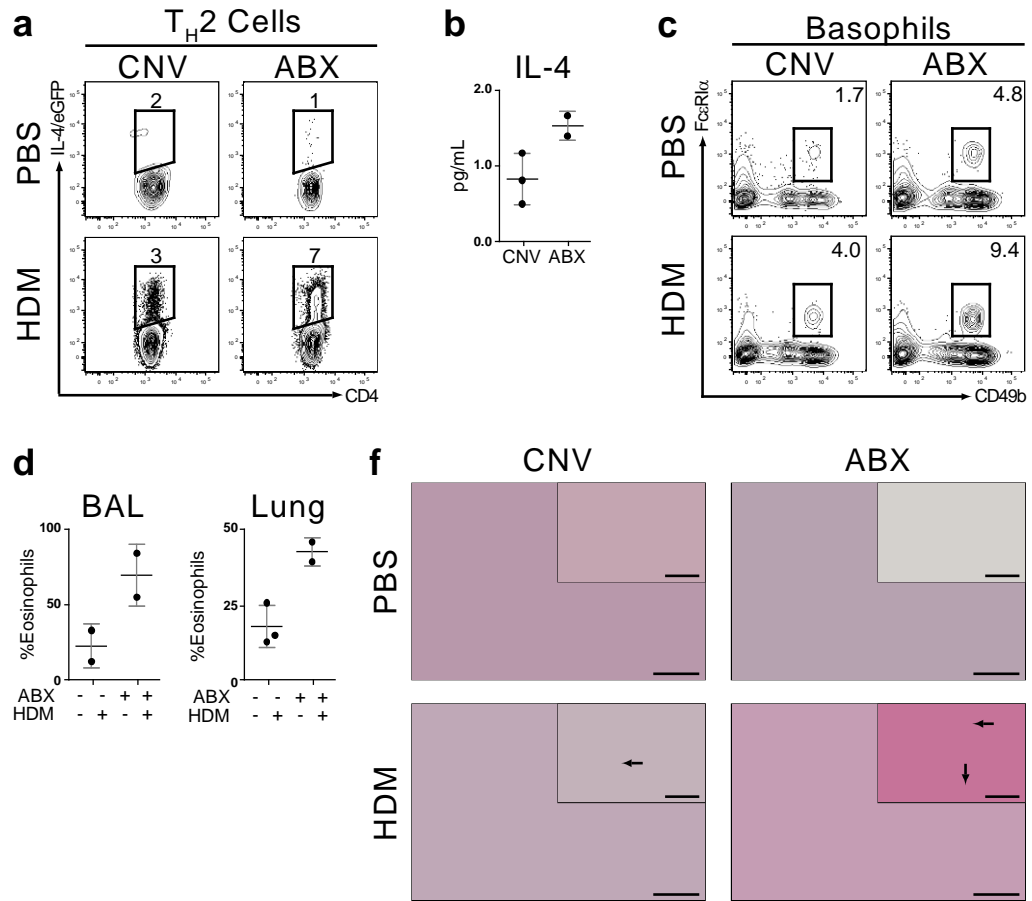
**Figure 15: Exaggerated HDM-induced airway inflammation in antibiotic-treated mice**

(**a**) Flow cytometric analysis of day 18 mediastinal lymph node (MLN) IL-4/eGFP$^+$CD4$^+$ T$_H$2 cells from conventionally-reared (CNV) or antibiotic-treated (ABX) mice treated with PBS or house dust mite (HDM) antigen. Gated on CD8$^-$, CD19$^-$, CD4$^+$ cells. (**b**) IL4 in in supernatants of MLN cell cultures from CNV or ABX mice treated with HDM as measured by ELISA. (**c**) Flow cytometric analysis of blood basophils from control CNV or ABX mice treated with PBS or HDM. Gated on CD3$^-$, CD4$^-$, CD8$^-$, CD19$^-$, CD117$^-$ cells. (**d**) Frequency of eosinophils in bronchoalveolar lavage or lung samples from HDM-treated CNV or ABX mice. (**e**) Histological sections of lungs from CNV or ABX mice treated with PBS or HDM and stained with H&E (Large Bar = 100 µM; Small Bar = 20 µM). Arrows indicate eosinophil infiltrates. Data representative of two or more independent experiments (mean ± S.D.; CNV, n=2-5; ABX, n=2-5).

### 3.4.4 Commensal bacteria limit basophil-mediated T$_H$2 cell responses

In addition to their roles as effector cells, basophils are recruited to draining LNs early in the response to infectious (Perrigoue et al. 2009) or allergic (Yoshimoto et al. 2009,

Sokol et al. 2008) stimuli where they cooperate with DCs to promote optimal $T_H2$ cell responses (Perrigoue et al. 2009, Sokol et al. 2009, Yoshimoto et al. 2009). To test whether ABX-treated mice exhibited altered allergen-induced $T_H2$ cell responses, CNV-reared or ABX-treated IL-4/eGFP reporter mice were exposed to papain, a cysteine protease associated with occupational allergy in humans (Novey et al. 1979) and $T_H2$ cell responses in mice (Sokol et al. 2008). Following PBS-treatment, similar frequencies of basophils (**Figure 16a**) and IL-4/eGFP$^+$CD4$^+$ $T_H2$ cells (**Figure 16b**) were observed in the LNs of CNV-reared or ABX-treated mice. However, following exposure to papain, ABX-treated mice exhibited increased frequencies of LN basophils (**Figure 16c**) and IL-4/eGFP$^+$CD4$^+$ $T_H2$ cells (**Figure 16d**) compared to controls. Depletion of basophils by administration of anti-FcεRIα antibody (Sokol et al. 2008) resulted in a reduction in the frequency of LN basophils (**Fig. 16e**), and reduced frequencies (**Fig. 16f**) and numbers (**Fig. 16g**) of papain-elicited IL-4/eGFP$^+$CD4$^+$ $T_H2$ cells, indicating that basophils contribute to the exaggerated $T_H2$ cell responses observed in ABX-treated mice.
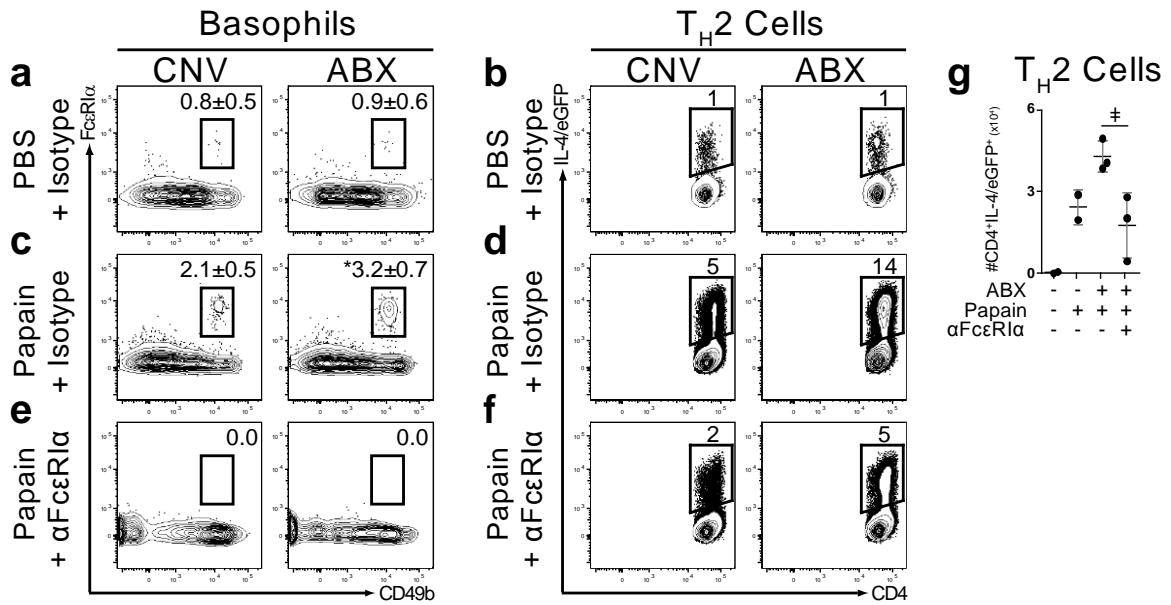
**Figure 16: Exaggerated Papain-induced $T_H2$ cell responses in antibiotic-treated mice**

(**a**) Flow cytometric analysis of day 3 popliteal LN (pLN) basophils from conventionally-reared (CNV) or antibiotic-treated (ABX) mice treated with PBS and Isotype control antibody. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on $CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD117^-$ cells. (**b**) Flow cytometric analysis of day 4 pLN IL-4/eGFP$^+$CD4$^+$ $T_H2$ cells from CNV or ABX mice treated with PBS and Isotype control antibody. Gated on $CD8^-$, $CD19^-$ cells. (**c**) Flow cytometric analysis of day 3 pLN basophils from CNV or ABX mice treated with Papain and Isotype control antibody. (**d**) Flow cytometric analysis of day 4 pLN IL-4/eGFP$^+$CD4$^+$ $T_H2$ cells from CNV or ABX mice treated with Papain and Isotype control antibody. (**e**) Flow cytometric analysis of day 3 pLN basophils from CNV or ABX mice treated with Papain and anti-FcεRIα (αFcεRIα) antibody. (**f**) Flow cytometric analysis of day 4 pLN IL-4/eGFP$^+$CD4$^+$ $T_H2$ cells from CNV or ABX mice treated with Papain and αFcεRIα antibody. (**g**) Number of day 4 pLN IL-4/eGFP$^+$CD4$^+$ $T_H2$ cells from CNV or ABX mice treated with PBS or Papain and Isotype control (-) or αFcεRIα (+) antibody (means of three experiments ± S.D.; CNV-PBS-ISO, n=4-5; CNV-PAP-ISO, n=4-5; ABX-PBS-ISO, n=5; ABX-PAP-ISO, n=5-8; CNV-PAP-αFcεRIα, n=2; ABX-PAP-αFcεRIα, n=2-6; significance determined by 2 way ANOVA). Data representative of three or more independent experiments ((mean ± S.D.; CNV, n=2-5; ABX, n=2-5; ‡, P ≤ 0.06; *, P ≤ 0.05). Experiments performed in collaboration with Mark Siracusa.

### 3.4.5 Basophils contribute to exaggerated $T_H2$ cell responses in antibiotic-treated mice

Inflammatory dendritic-cells (DCs) are important for the development of optimal $T_H2$ cell responses to some allergens (Hammad et al. 2010). To investigate the influence of commensal-derived signals on DC populations, DCs were examined in CNV-reared or ABX-treated mice. Steady-state DC frequencies were similar between CNV-reared or

ABX-treated mice (data not shown), as was DC expression of MHC-I, MHC-II, CD40, CD80, CD86, or FcεRIα (**Figure 17a,b**), indicating that commensal-derived signals do not significantly influence circulating DC populations in the steady-state.
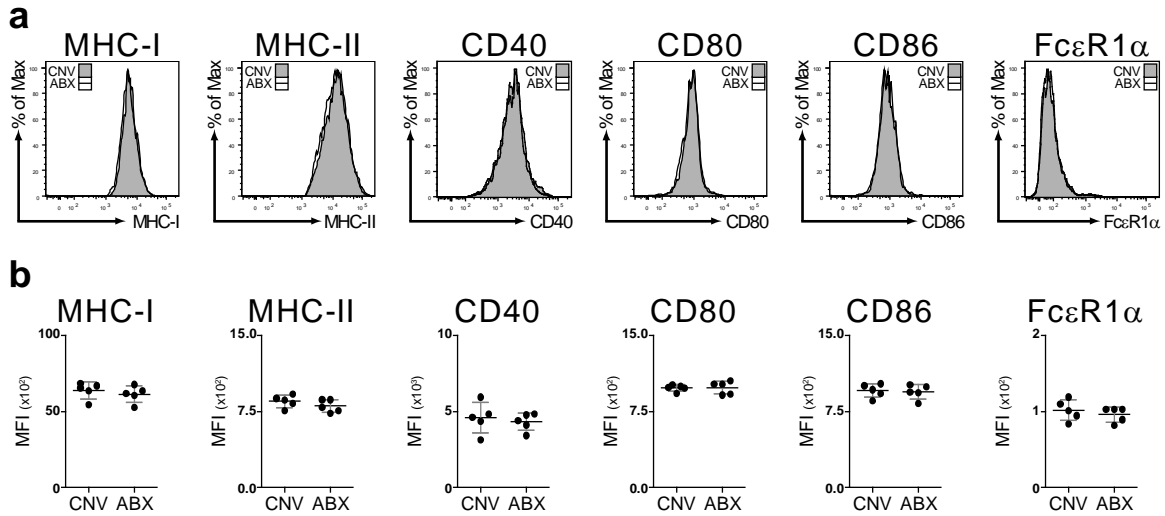


**Figure 17: Antibiotic treatment does not influence dendritic cell surface phenotype in mice**

(**a**) Mean fluorescence intensity of surface MHC-I, MHC-II, CD40, CD80, CD86 and FcεRIα on splenic DCs from conventionally-reared (CNV) or antibiotic-treated (ABX) mice as determined by flow cytometry. Gated on CD3⁻, CD4⁻, CD8⁻, CD19⁻, MHC-II⁺, CD11c⁺ cells. (**b**) Mean fluorescence intensity of MHC-I, MHC-II, CD40, CD80, CD86 and FcεRIα on splenic DCs from CNV or ABX mice. Data representative of three or more independent experiments (mean ± S.D.; CNV, n=5; ABX, n=5). Experiments performed in collaboration with Michael Abt.

As inflammatory DCs express FcεRIα (Hammad et al. 2010), we next examined whether commensal-derived signals influence allergen-induced non-basophil FcεRIα⁺ cell populations in the LN. Papain-elicited LN non-basophil FcεRIα⁺ cell populations were similar between CNV-reared or ABX-treated mice (**Figure 18a**) and ABX-treatment did not significantly influence non-basophil FcεRIα⁺ cell populations expressing MHC-II or CD40 (**Figure 18b,c**), indicating that non-basophil FcεRIα⁺ cell responses are not significantly influenced by commensal-derived signals in this setting. As inflammatory DCs express FcεRIα, and have been shown to be influenced by anti-FcεRIα antibody

treatment (Hammad et al. 2010), we examined the effect of anti-FcεRIα antibody treatment on LN non-basophil FcεRIα[+] cell populations. Compared to Isotype treated controls, we did not observe a significant difference in papain-elicited non-basophil FcεRIα[+] cell populations in the LNs of CNV-reared or ABX-treated mice upon anti-FcεRIα antibody treatment (**Figure 18a**), nor did we find a significant effect of anti-FcεRIα antibody treatment on non-basophil FcεRIα[+] cell populations expressing MHC-II or CD40 (**Figure 18c,b**). Together, these findings indicate that neither steady-state DC responses, nor papain-elicited non-basophil FcεRIα[+] LN cell responses, are significantly dysregulated in ABX-treated compared to CNV-reared mice.
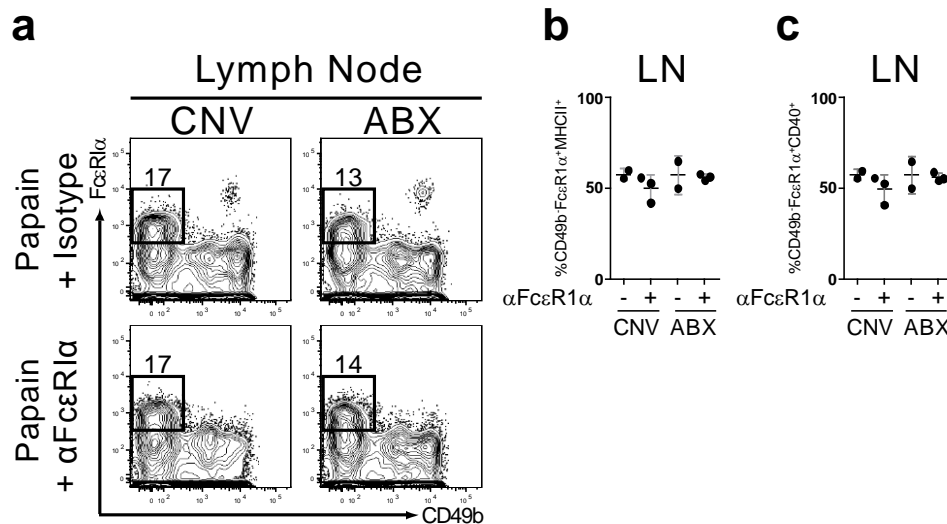


**Figure 18: Anti-FcεRIα treatment does not influence non-basophil FcεRIα^{+} lymph node antigen presenting cells in antibiotic-treated mice**

(**a**) Flow cytometric analysis of pLN CD49b^{-} FcεRIα^{+} cells from conventionally-reared (CNV) or antibiotic-treated (ABX) mice treated with Papain and isotype or αFcεRIα antibody. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on CD3^{-}, CD4^{-}, CD8^{-}, CD19^{-}, CD117^{-} cells. (**b**) Frequencies of pLN CD49b^{-}, FcεRIα^{+}, MHC-II^{+} cells from CNV or ABX mice treated with Papain and isotype or αFcεRIα antibody. (**c**) Frequencies of pLN CD49b^{-}, FcεRIα^{+}, CD40^{+} cells from CNV or ABX mice treated with Papain and isotype or αFcεRIα antibody. Data representative of two or more independent experiments (mean ± S.D.; CNV, n=2-3; ABX, n=2-3).

Due to concerns that αFcεRIα treatment might be influencing FcεRIα⁺ inflammatory DC populations, we adopted a loss-of-function approach complementary to anti-FcεRIα antibody treatment by utilizing a diphtheria toxin (DT)-dependent basophil depletion mouse system (BaS-TRECK) (Siracusa et al. In Press). ABX-treatment resulted in increased frequencies of circulating basophils (**Figure 19a**) and frequencies (**Figure 19b**) and numbers (**Figure 19c**) of papain-elicited $T_H2$ cells in the LN compared to CNV-reared mice. Importantly, DT-treatment resulted in efficient depletion of basophils (**Figure 19a**) and reductions in the frequency (**Figure 19b**) and number (**Figure 19c**) of papain-elicited $T_H2$ cells in BaS-TRECK but not control mice. Together, these data indicate that basophils contribute to the exaggerated allergen-induced $T_H2$ cell responses observed in ABX-treated mice.
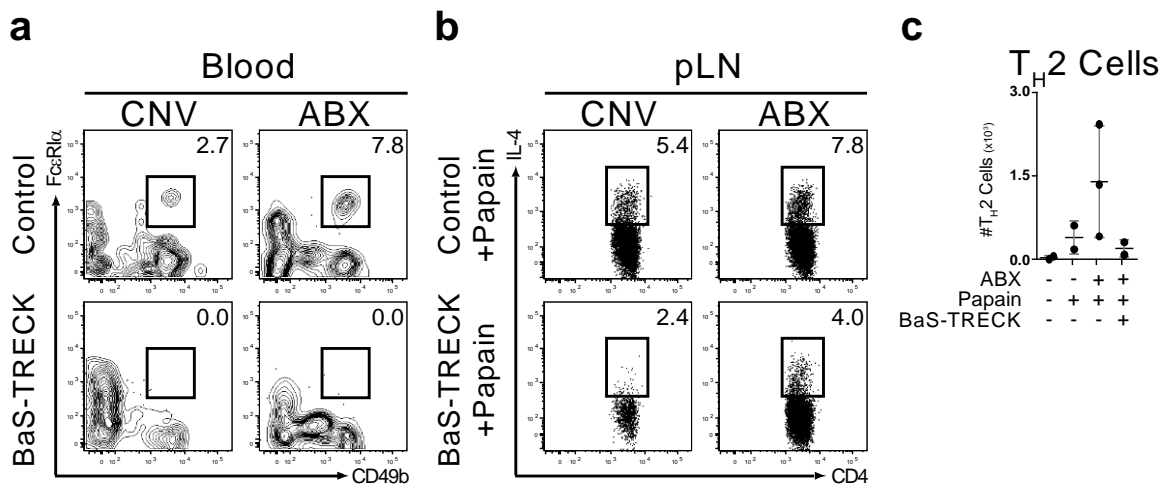


**Figure 19: Basophils contribute to exaggerated $T_H2$ cell responses in antibiotic-treated mice**

(**a**) Flow cytometric analysis of blood basophils from conventionally-reared (CNV) or antibiotic-treated (ABX), control or BaS-TRECK mice treated with diphtheria toxin (DT). Gated on CD3⁻, CD4⁻, CD8⁻, CD19⁻, CD117⁻ cells. (**b**) Flow cytometric analysis of pLN CD4+ $T_H2$ cells from CNV or ABX, control or BaS-TRECK mice treated with papain and DT. Gated on CD8⁻, CD19⁻, CD4⁺ cells. (**c**) Number of day 4 pLN CD4⁺, IL-4⁺ $T_H2$ cells from CNV (-) or ABX (+), control (-) or BaS-TRECK (+) mice treated with PBS (-) or Papain (+) and DT. Data representative of two independent experiments (Control, n=2-3; BaS-TRECK, n=1-3; $T_H2$ cells determined by expression of IL-4 or IL-5). Experiments performed in collaboration with Mario Noti.

## 3.5 Discussion

Although the prevalence and severity of allergic diseases have been increasing in the U.S. and U.K. for the past fifty years (Eder, Ege & von Mutius 2006), the genetic (Vercelli 2008) and environmental (Gilliland 2009, Zeiger 2003, Ege et al. 2011) factors that influence allergy susceptibility are not well understood. Recently, epidemiologic (Marra et al. 2009, Kummeling et al. 2007, Kalliomaki et al. 2001) and experimental (Bashir et al. 2004, Noverr et al. 2005, Noverr et al. 2004, Herbst et al. 2011) studies have identified associations between alterations in the composition of commensal bacterial communities and the development of allergic $T_H2$ cytokine-dependent inflammation. However, the influence of commensal bacterial-derived signals on innate immune cells known to influence the development of $T_H2$ cytokine-dependent inflammation remain poorly characterized (Hill, Artis 2010, Lambrecht, Hammad 2010, Paul, Zhu 2010).

Data in this chapter identify a role for commensal bacterial-derived signals in limiting steady-state serum IgE levels and circulating basophil populations. These findings are of particular interest given that basophils have recently been shown to express MHC class II (Charles et al. 2010, Skokos et al. 2003, Padigel et al. 2007), be potent sources of IL-4 and, depending on the stimulus, contribute to the development of optimal $T_H2$ cytokine-dependent allergic inflammation (Gessner, Mohrs & Mohrs 2005, Mohrs et al. 2005, Min et al. 2004). An alternative explination for the elevations in basophils observed in ABX-treated mice is the depletion of another cell population. Germ-free and antibiotic treated mice are considered to be lymphpenic, supporting this hypothesis (Hudson, Luckey 1964, Hooijkaas et al. 1984). However, there are lines of evidence that suggest that the elevations in circulating basophils we observe are not a consequence of depletion of another cell population. First, basophil numbers as well as

frequencies were significantly elevated in ABX-treated compared to CNV-reared mice. Second, a stringent gating strategy was utlilized to ensure that T and B cells were excluded from our basophil plots, minimizing the likelihood that lymphpenia would influence our results. Third, increases were selective to basophils as eosinophil and mast cell populations were not apparently influenced by ABX-treatment. Finally, ABX-treated mice displayed elevated basophil responses in three models of allergic inflammation. Together, these results suggest that commensal bacterial-derived signals act to actively limit circulating basophil populations in the steady-state.

Basophils from ABX-treated mice did not display alterations in surface phenotype, suggesting that commensal-derived signals influence quantitative rather than qualitative aspects of basophil biology. However, this does not exclude the possibility that functional heterogeneity exists between basophils from CNV-reared or ABX-treated mice. Additionally, antibiotic-treated mice displayed exaggerated basophil-mediated skin inflammation in an innate model of atopic-dermatitis and allergic airway inflammation in a model of asthma. While these findings are consistant with primarirly quantative alterations to basophil biology in ABX-treated mice, functional heterogeneity between basophils from CNV-reared or ABX-treated mice could also be contributing to these phenotypes. Together, these findings suggest that in addition to limiting circulating basophil populations in the steady-state, commensal bacterial-derived signals act to limit basophil-mediated allergic responses.

In addition to their roles as effector cells, basophils are recruited to draining LNs early in the response to infectious (Perrigoue et al. 2009) or allergic (Yoshimoto et al. 2009, Sokol et al. 2008) stimuli where they cooperate with DCs to promote optimal $T_H2$ cell responses (Perrigoue et al. 2009, Sokol et al. 2009, Yoshimoto et al. 2009). Data in

this chapter indicate that ABX-treated mice display elevated basophil LN recruitment and exaggerated LN $T_H2$ cell responses to allergens compared to CNV-reared mice. Non-basophils APC populations did not seem to be significantly influenced by commensal bacterial-derived signals in our experimental systems. Importantly, basophils were shown to contribute to the development of exaggerated allergen-induced $T_H2$ cell responses using two methods of basophil depletion. In both systems, depletion of basophils in ABX-treated mice reduced $T_H2$ cell numbers to levels similar to those seen in CNV-reared mice, however, $T_H2$ cell frequencies were consistently slightly higher upon basophil depletion in ABX-treated compared to CNV-reared mice. These findings suggest that cell types other than basophils may be contributing to the exaggerated $T_H2$ cell frequencies observed in ABX-treated mice. Together, these findings indicate that commensal bacterial-derived signals limit disease in models of basophil effector function, and basophil-mediated $T_H2$ cell responses. Together, they identify basophils as a potential therapeutic target to prevent the development or propagation of allergic diseases in patients.

# Chapter 4: Regulation of circulating basophil populations by immunoglobulin E

## 4.1 Abstract

Data presented in **Chapter 3** identified a role for commensal bacterial-derived signals in limiting serum immunoglobulin E (IgE) levels, steady-state circulating basophil populations, and basophil-mediated $T_H2$ cytokine-dependent allergic inflammation. However, the mechanisms through which commensal-derived signals limit circulating basophil populations is not understood. This chapter describes a previously unrecognized axis of basophil homeostasis that is mediated by IgE. Serum IgE levels correlated with circulating basophil populations in CNV-reared and ABX-treated mice, as well as human patients with hyperimmunoglobulinemia E syndrome. Steady-state serum IgE levels and circulating basophil populations were dysregulated by B cell-intrinsic deletion of MyD88, providing insights into one cell-intrinsic mechanism by which commensal-derived signals may influence circulating basophil populations.

## 4.2 Introduction

Data in **Chapter 3** identified a previously unappreciated role for commensal bacterial-derived signals in limiting serum IgE levels, circulating steady-state basophil populations, and basophil-mediated allergic inflammation. IgE sensitizes mast cells and basophils by binding to the high affinity IgE receptor (FcεRIα) and, upon re-exposure to the IgE-specific antigen, causes the release of cytokines, chemokines, and other chemical mediators that result in the pathological and clinical sequelae of immediate hypersensitivity reactions (Galli et al. 2005, Kalesnikoff, Galli 2008). Additionally, recent findings suggest that IgE may influence granulocyte homeostasis in the absence of an antigen. For example, IgE has been shown to upregulate the expression of FcεRIα on

mast cells and basophils (Lantz et al. 1997, Yamaguchi et al. 1997), and FcεRIα expression on basophils positively correlates with serum IgE levels in human blood (Saini et al. 2000). Antigen-independent immunoregulatory roles for IgE are supported by studies in humans and animal models that indicate that IgE is spontaneously produced at low levels in the absence of allergic or infectious stimuli (Gould, Sutton 2008). Induced by a mechanism independent of MHC class II cognate help, the function of so termed "natural" IgE is not well understood, although it has been hypothesized to provide a link between the innate immune system and the initiation and/or propagation of allergic responses (McCoy et al. 2006).

Data presented in this chapter identify a previously unappreciated role for IgE in influencing basophil homeostasis. Serum IgE levels were found to correlate with circulating basophil populations in CNV-reared and ABX-treated mice, as well as human patients with hyperimmunoglobulinemia E syndrome. Steady-state serum IgE levels and circulating basophil populations were elevated following B cell-intrinsic deletion of MyD88, providing insights into one cell-intrinsic mechanism by which commensal-derived signals may influence serum IgE levels and circulating basophil populations. Together, these results provide therapeutically relevant insights into the cellular and molecular mechanisms by which commensal bacterial-derived signals influence susceptibility to allergic disease.

## 4.3 Methods

### 4.3.1 Animals

BALB/c, C57BL/6 or Rag1-/- mice were purchased from Jackson or Charles River Laboratories. Germ-free mice were provided by the Penn Gnotobiotic Mouse Facility. Il4-/- mice were obtained from P. Scott, Igε-/- mice were obtained from H. Oettgen, NOD1-/- mice were obtained from J. Weiser, Myd88-/- mice were provided by D. LaRosa. All mice used were 8–24 weeks of age. Conventional animals were bred and housed in specific pathogen-free conditions at the University of Pennsylvania. All experiments were performed under Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with the guidelines of the IACUC of the University of Pennsylvania.

### 4.3.2 Reagents and Treatments

For antibiotic treatment, mice were fed autoclaved water or autoclaved water supplemented with ampicillin (0.5 mg/mL), gentamicin (0.5 mg/mL), metronidazole (0.5 mg/mL), neomycin (0.5 mg/mL), and vancomycin (0.25 mg/mL) continuously via water bottle for four weeks. In the occasional event of poor animal oral hydration, both control and antibiotic water was supplemented with artificial sweetener. For IgE transfer, $Rag1^{-/-}$ or $Ig\varepsilon^{-/-}$ mice were treated i.p. with 50 μg of control IgG or IgE (BD Bioscience) daily for seven days. For IgE depletion, mice were treated i.p. with 200 μg of control IgG or Omalizumab (Genentech) daily for seven days. For CpG treatment, mice were treated i.p. once weekly during the course of ABX treatment with 100 μl of PBS containing 100 μg of control GpC-Phos (5'-ZOOFZEFEOZZOOZEFEZOZT-3') or CpG-Phos (5'-ZOOFZEFOEZZOOZEFOEZZT-3').

### 4.3.3 Flow Cytometry and ELISA

Blood, spleen or bone marrow were collected at necropsy, homogenized by passing through a 70 μm nylon mesh filter, purified of red blood cells by histopaque (Sigma-Aldrich) or RBC lysis, and stained with anti-mouse fluorochrome-conjugated monoclonal antibodies against B220, CD3ε, CD4, CD8, CD19, CD49b, CD117, CD123, CD200R, FcεRIα, IgE, IgM (BD Bioscience, BioLegend, eBioscience). Human blood samples were obtained from *DOCK8*- or control patients with participant consent, assent, and/or parental consent as appropriate under protocols approved by the Children's Hospital of Philadelphia and the Ludwig Maximilians University Institutional Review Boards. PBMCs were isolated by Ficoll (GE) gradient and stained with anti-human fluorochrome-conjugated monoclonal antibodies against CD11c, CD19, CD117, CD123, FcεRIα, IgE or TCRβ (BD Bioscience, eBioscience) and fixed with 4% PFA. Cells were acquired on a FACSCanto II or LSR II with DiVa software (BD Bioscience) and analyzed with FlowJo software (version 8.7.1; Tree Star). Cells were sorted with a FACSAria (BD Bioscience). Purified B or T cells were transferred by intraperitoneal injection into *Rag1*$^{-/-}$ recipients and allowed to reconstitute for 6-8 weeks. Murine IgE was assayed by sandwich ELISA (BD Bioscience). Human IgE levels were determined by sandwich ELISA or chart review.

### 4.3.5 Statistics

Results shown as mean ± standard deviation for individual animals. Statistical significance was determined by two-tailed Mann-Whitney test or unweighted means analysis two-way ANOVA as indicated. Results were considered significant at $P \leq 0.05$ (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

## 4.4 Results

### 4.4.1 Serum IgE levels correlate with circulating basophil populations in antibiotic-treated mice

Previous reports demonstrated that IgE regulates mast cell homeostasis (Kalesnikoff et al. 2001, Kitaura et al. 2003) and IgE has been hypothesized to have similar effects on basophils (Xiang, Moller & Nilsson 2006). Based on the observation that serum IgE levels were increased in ABX-treated mice (**Figure 11a**), the relationship between elevated IgE levels and increased basophil populations observed in ABX-treated mice was examined. Serum IgE levels significantly correlated with circulating basophil numbers in CNV-reared or ABX-treated mice (**Figure 20a**), suggesting that IgE produced by B cells may influence basophil homeostasis. To test whether the increase in circulating basophils observed in ABX-treated mice was dependent on lymphocytes, recombination activating gene 1-deficient mice ($Rag1^{-/-}$) (Mombaerts et al. 1992) were employed. Consistent with a role for IgE produced by B cells in regulating basophil homeostasis, blood basophil frequencies and numbers were similar between CNV-reared and ABX-treated $Rag1^{-/-}$ (**Figure 20b,c**) or GF $Rag1^{-/-}$ (**Figure 20d,e**) mice. As IL-4 is necessary for IgE production by B cells (Kopf et al. 1993, Delphin, Stavnezer 1995), IL-4-deficient mice ($Il4^{-/-}$) were utilized to test the influence of IL-4 on IgE levels and basophil responses in ABX-treated mice. ABX-treatment of wild-type (WT) mice resulted in increased serum IgE levels (**Figure 20f**) and blood basophils (**Figure 20g**). In contrast, IgE was not detected in $Il4^{-/-}$ mice (**Figure 6f**) and ABX-treatment of $Il4^{-/-}$ mice had no effect on the frequency or number of blood basophils compared to CNV-reared controls (**Figure 20g,h**) suggesting that IL-4-induced IgE production by B cells contributes to the increased basophil responses observed in ABX-treated mice.
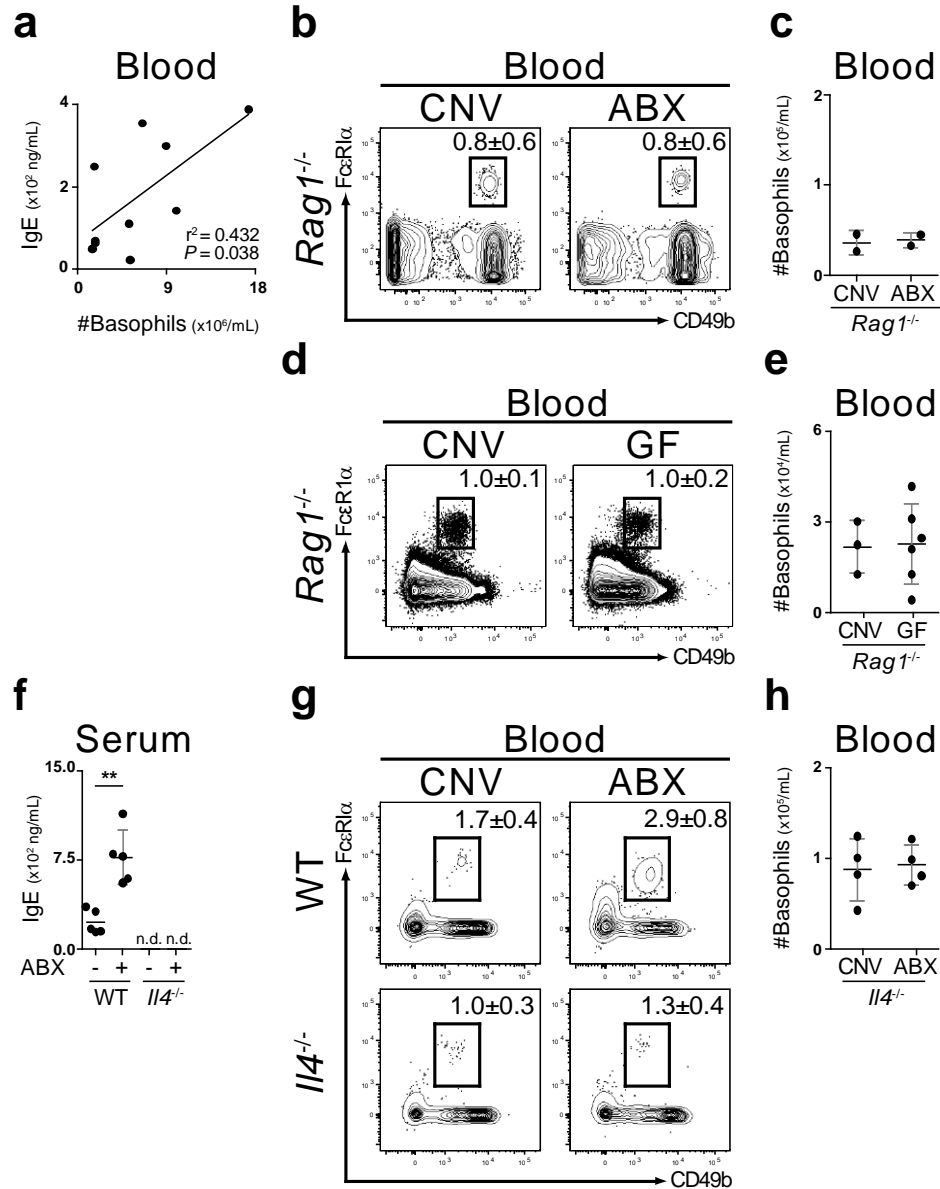
**Figure 20: Serum IgE levels correlate with circulating basophils in mice**

(**a**) Statistical correlation of blood basophil number and serum IgE concentration (linear regression; r2 = 0.432, P = 0.038). (**b**) Flow cytometric analysis of blood basophils from conventionally-reared (CNV) or antibiotic-treated (ABX) Rag1^{-/-} mice. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on CD3^{-}, CD4^{-}, CD8^{-}, CD19^{-}, CD117^{-} cells. (**c**) Number of basophils per mL of blood from CNV or ABX Rag1^{-/-} mice. (**d**) Flow cytometric analysis of blood basophils from CNV or germ-free (GF) Rag1^{-/-} mice. (**e**) Number of basophils per mL of blood from CNV or GF Rag1^{-/-} mice. (**e**) Serum IgE from CNV or ABX, wild-type (WT) or Il4^{-/-} mice as measured by ELISA (n.d., not detected). (**f**) Flow cytometric analysis of blood basophils from CNV or ABX, WT or Il4^{-/-} mice. (**g**) Number of basophils per mL of blood from CNV or ABX Il4^{-/-} mice. Data representative of three or more independent experiments (mean ± S.D.; CNV, n=2-5; ABX, n=2-5; GF, n=6; **, P ≤ 0.01).

110

### 4.4.2 Regulation of circulating basophil populations by commensal bacteria is mediated by IgE

To directly test the role of IgE in mediating commensal bacterial-dependent regulation of basophil responses, IgE-deficient ($Ig\varepsilon^{-/-}$) mice were utilized. While ABX-treatment of WT mice resulted in increased serum IgE levels (**Figure 21a**) and blood basophils (**Figure 21b,c**), IgE was not detected in $Ig\varepsilon^{-/-}$ mice (**Figure 21a**) and blood basophil populations were not significantly different between CNV-reared or ABX-treated $Ig\varepsilon^{-/-}$ animals (**Figure 21b,c**). Together, these results indicate that IgE is necessary to mediate the exaggerated steady-state basophil responses observed in ABX-treated mice. To test whether IgE was sufficient to promote the population expansion of circulating basophils, $Rag1^{-/-}$ mice were treated with monoclonal IgE and blood basophil populations were examined. Compared to controls, IgE transfer into $Rag1^{-/-}$ mice resulted in recovery of serum IgE levels (**Figure 21d**) and significantly elevated frequencies and numbers of blood basophils (**Figure 21e,f**). Collectively, these gain- and loss-of-function approaches identify IgE as an important regulator of peripheral basophil responses in mice.
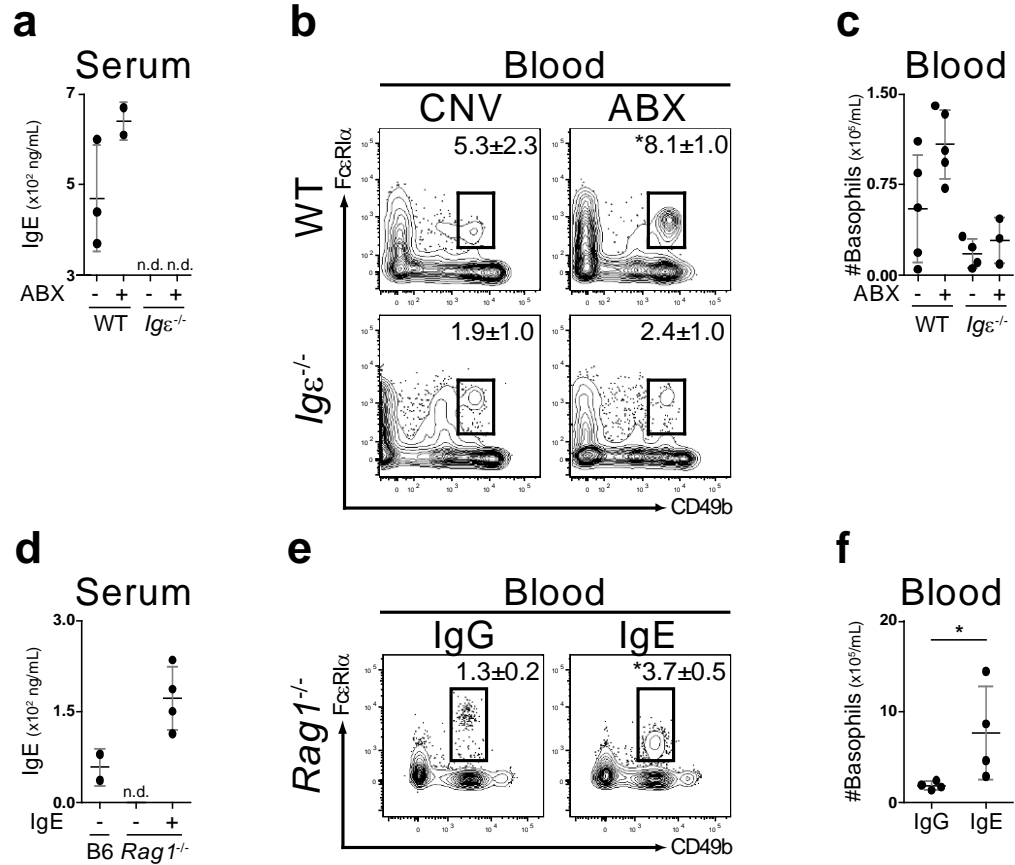
**Figure 21: IgE is necessary and sufficient to regulate circulating basophil populations in mice**

(**a**) Serum IgE from conventionally-reared (CNV) or antibiotic-treated (ABX), wild-type (WT) or Igε^{-/-} mice as measured by ELISA (n.d., not detected). (**b**) Flow cytometric analysis of blood basophils from CNV or ABX, WT or Igε^{-/-} mice. (**c**) Number of basophils per mL of blood from CNV or ABX Igε^{-/-} mice. (**d**) Serum IgE from IgG (-) or IgE (+) treated Rag1^{-/-} mice as measured by ELISA (n.d., not detected). (**e**) Flow cytometric analysis of blood basophils from IgG or IgE treated Rag1^{-/-} mice. (**f**) Number of basophils per mL of blood from IgG or IgE treated Rag1^{-/-} mice. Data representative of three or more independent experiments (mean ± S.D.; CNV, n=4-5; ABX, n=3-5; IgG, n=4; IgE, n=4; *, P ≤ 0.05.).

## 4.4.3 Serum IgE levels and circulating basophil populations correlate in patients with hyperimmunoglobulinemia E syndrome

Human patients with loss-of-function polymorphisms in the dedicator of cytokinesis 8 gene (*DOCK8-*) exhibit an autosomal recessive form of hyper-IgE syndrome (Engelhardt

et al. 2009, Zhang et al. 2009) characterized by recurrent infections, increased susceptibility to atopic eczema, and average serum IgE levels 10 times higher than found in control patients (Grimbacher, Holland & Puck 2005). Based on the identification of a role for IgE in regulating basophil responses in mice, we hypothesized that elevated serum IgE levels resulting from genetic alteration in *DOCK8-* patients would be associated with increased circulating basophil populations in these individuals. In collaboration with Dr. Jordan Orange (CHOP) and Dr. Ellen Renner (Ludwig Maximilians University), we examined serum IgE levels and circulating basophil populations in *DOCK8-* patients. *DOCK8-* patients displayed elevated serum IgE levels compared to controls (Grimbacher, Holland & Puck 2005) (**Figure 22a**). Human blood basophils (identified as TCRβ⁻, CD11c⁻, CD19⁻, CD117⁻, CD123⁺, FcεRIα⁺) comprised approximately 1% of NBNT compartment of controls (**Figure 22b,d**). In contrast, in four of five *DOCK8-* patients examined basophils comprised approximately 3% of the NBNT compartment (**Figure 22c,d**). Further, basophils isolated from *DOCK8-* patients exhibited increased surface-bound IgE compared to controls (**Figure 22e**). These data are consistent with IgE influencing basophil homeostasis in humans, and suggest that elevated basophil responses in *DOCK8-* patients may contribute to the increased susceptibility to atopic diseases observed in these patients.
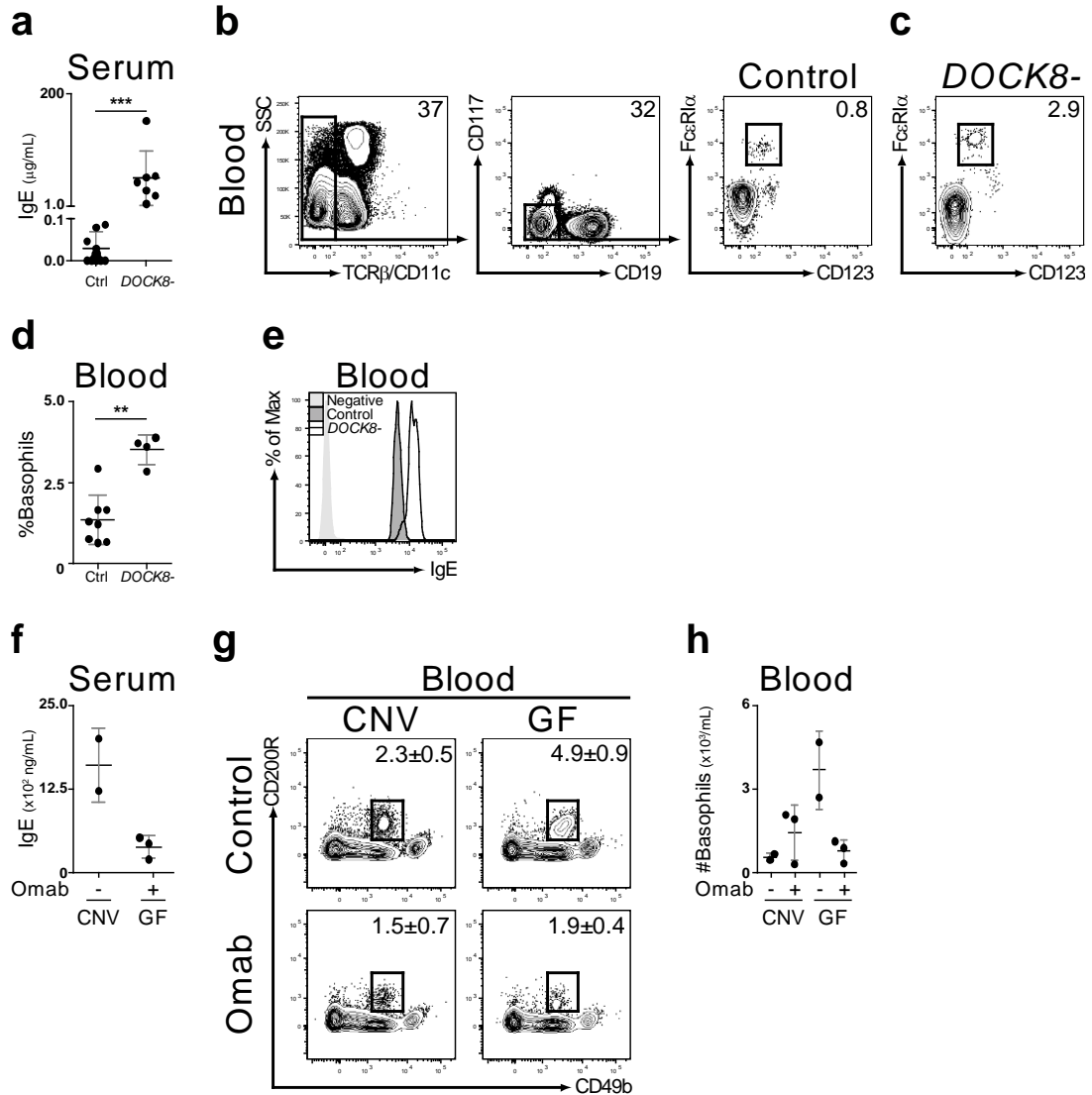
**Figure 22: Serum IgE and basophils correlate in human patients and anti-IgE treatment reduces serum IgE levels and circulating basophil populations in mice**

(**a**) Serum IgE levels from control (Ctrl) patients or patients with polymorphisms in *DOCK8* (DOCK8-) (mean ± S.D.; Ctrl, n=15; DOCK8-, n=7). (**b**) Identification of blood basophils from control patients by flow cytometric analysis as TCRβ-, CD11c-, CD19-, CD117-, FcεRIα+, CD123+ cells. Numbers adjacent to outlined areas indicate percent cells in each gate. (**c**) Analysis of blood basophils from DOCK8- patients. (**d**) Frequency of basophils in blood from control or DOCK8- patients (mean ± S.D.; Ctrl, n=8; DOCK8-, n=4). (**e**) Mean fluorescence intensity of surface-bound IgE on blood basophils from control or DOCK8- patients. (**f**) Serum IgE from conventionally-reared (CNV) or germ-free (GF) mice treated with control (-) or Omalizumab (Omab;+) antibody as measured by ELISA. (**g**) Flow cytometric analysis of blood basophils from CNV or GF mice treated with control or Omalizumab antibody. Gated on CD3-, CD4-, CD8-, CD19-, CD117- cells. (**h**) Number of basophils per mL of blood from CNV or GF mice treated with control or Omalizumab antibody. Data representative of two or more independent experiments (mean ± S.D.; CNV, n=2-3; GF, n=2-3; **, P ≤ 0.01; ***, P ≤ 0.001).

114

Omalizumab is a monoclonal, humanized mouse anti-human IgE-specific antibody and FDA approved anti-allergy drug (Pace et al. 2011, Holgate et al. 2009). To test whether Omalizumab treatment influenced circulating basophil populations, CNV-reared or GF mice were treated with Omalizumab or control antibody and serum IgE levels and blood basophils were examined. As Omalizumab decreases FcεRIα expression on basophils (Lin et al. 2004), basophils were identified as NBNT, CD117⁻, CD49b⁺, CD200R⁺ cells (Shiratori et al. 2005). Omalizumab treatment of GF mice resulted in reductions in serum IgE levels (**Figure 22f**), and reduced frequencies and numbers of blood basophils (**Figure 22g,h**). Together, these results identify Omalizumab as a potential therapeutic intervention to limit IgE-mediated increases in circulating basophils.

### 4.4.4 MyD88-dependent commensal-derived signals limit serum IgE levels and circulating basophil populations

It has been hypothesized that commensal bacterial-derived signals influence allergic responses by signaling through pattern recognition receptors (PRRs) (Bashir et al. 2004, Noverr et al. 2005). To interrogate the molecular mechanisms through which commensal-derived signals influence serum IgE levels and circulating basophil responses, mice deficient in myeloid differentiation primary response gene 88 (*Myd88*⁻/⁻), a critical adaptor molecule that regulates signaling through multiple PRRs (Schnare et al. 2001), were utilized. As previously reported, CNV-reared *Myd88*⁻/⁻ mice exhibited significantly increased serum IgE levels compared to littermate controls (Schnare et al.

2001) (**Figure 23a**). Associated with dysregulated IgE responses, compared to controls, *Myd88*^{-/-} mice displayed increased frequencies and numbers of peripheral basophils (**Figure 23b,c**) and increased basophil-surface-bound IgE levels (**Figure 23d**) indicating that MyD88 signaling pathways are important for limiting these responses. Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) is a MyD88-independent intracellular PRR that mediates innate and adaptive immunity by recognizing commensal bacterial-derived signals (Clarke et al. 2010). To test whether NOD1 influenced steady-state circulating basophil populations, serum IgE levels and circulating basophils were examined in control or NOD-1-deficient (*Nod1-/-*) mice. Neither serum IgE levels (**Figure 23e**) nor blood basophils were elevated in *Nod1-/-* compared to control mice (**Figure 23f,g**), indicating that NOD1 does not significantly influence steady-state serum IgE levels or circulating basophil responses. To examine whether a MyD88-dependent commensal bacterial-derived signal was sufficient to limit circulating basophils, ABX-treated mice were treated with CpG (Bashir et al. 2004, Hall et al. 2008), and serum IgE levels and circulating basophil populations were examined. CpG treatment of ABX-treated mice resulted in reductions in serum IgE levels (**Figure 23h**) and blood basophil frequencies and numbers (**Figure 23i,j**). Together, these findings implicate MyD88-dependent commensal bacterial-derived signals in limiting serum IgE levels and circulating basophil populations.
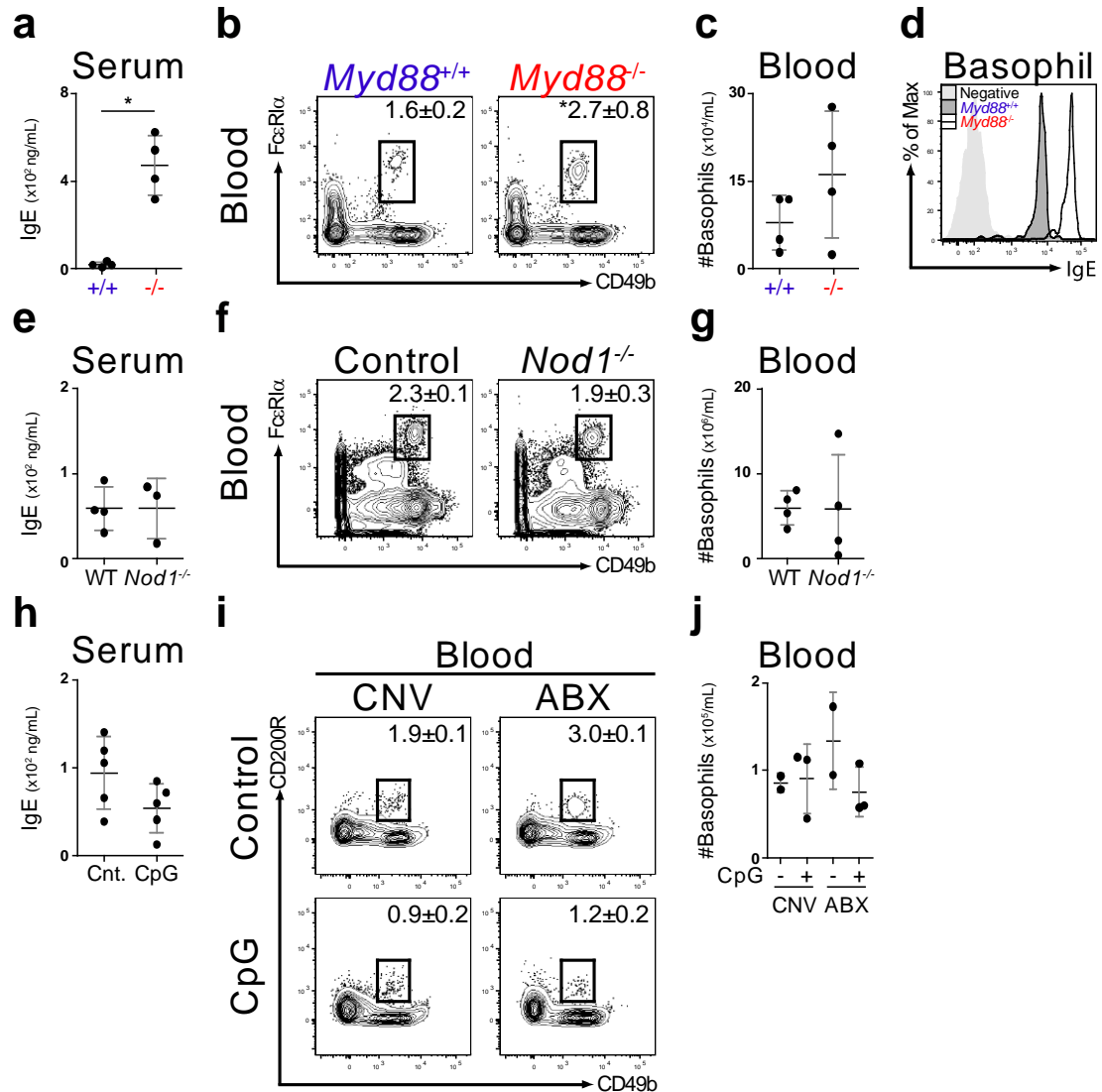
**Figure 23: MyD88^{-/-} mice exhibit elevated serum IgE levels and circulating basophil populations**

(**a**) Serum IgE from conventionally-reared (CNV) Myd88^{+/+} (+/+) or Myd88^{-/-} (-/-) mice as measured by ELISA. (**b**) Flow cytometric analysis of blood basophils from CNV Myd88^{+/+} or Myd88^{-/-} mice. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on CD3^{-}, CD4^{-}, CD8^{-}, CD19^{-}, CD117^{-} cells. (**c**) Number of basophils per mL of blood from CNV Myd88^{+/+} or Myd88^{-/-} mice. (**d**) Mean fluorescence intensity of surface-bound IgE on blood basophils from CNV Myd88^{+/+} or Myd88^{-/-} mice as determined by flow cytometry (mean ± S.D.; Myd88^{+/+}, n=4; Myd88^{-/-}, n=4). (**e**) Serum IgE levels from control (WT) or Nod1^{-/-} mice as measured by ELISA. (**f**) Flow cytometric analysis of blood basophils from control or Nod1^{-/-} mice. (**g**) Number of basophils per mL of blood from control (WT) or Nod1^{-/-} mice (mean ± S.D.; WT, n=2-4; Nod1^{-/-}, n=3-4). (**h**) Serum IgE from antibiotic-treated (ABX) mice treated with control or CpG as measured by ELISA. (**i**) Flow cytometric analysis of blood basophils from CNV or ABX mice treated with control or CpG. (**j**) Number of basophils per mL of blood from CNV or ABX mice treated with control or CpG. Data representative of two or more independent experiments (mean ± S.D.; CNV, n=2-5; ABX, n=3-5; *, P ≤ 0.05).

MyD88-dependent signaling in B cells can inhibit IgE class switching *in vitro* (Liu et al. 2003). To test whether B cell-intrinsic MyD88-dependent signaling influenced IgE production or basophil homeostasis *in vivo*, chimeras were generated by sorting and adoptively transferring B or T cells from $Myd88^{+/+}$ or $Myd88^{-/-}$ mice into $Rag1^{-/-}$ recipients (**Figure 24a**). Compared to controls, mice that received $Myd88^{-/-}$ B cells with $Myd88^{+/+}$ T cells displayed elevated serum IgE levels (**Figure 24b**), increased frequencies (**Figure 24c**) and numbers (**Figure 24d**) of blood basophils, and increased basophil-surface-bound IgE levels (**Figure 24e**). Together, these results indicate that B cell-intrinsic MyD88-dependent signaling limits steady-state serum IgE levels and circulating basophil responses *in vivo* and implicates B cell-intrinsic recognition of commensal bacterial-derived signals in mediating commensal-regulation of circulating basophils and basophil-mediated $T_H2$ cytokine-dependent allergic inflammation.
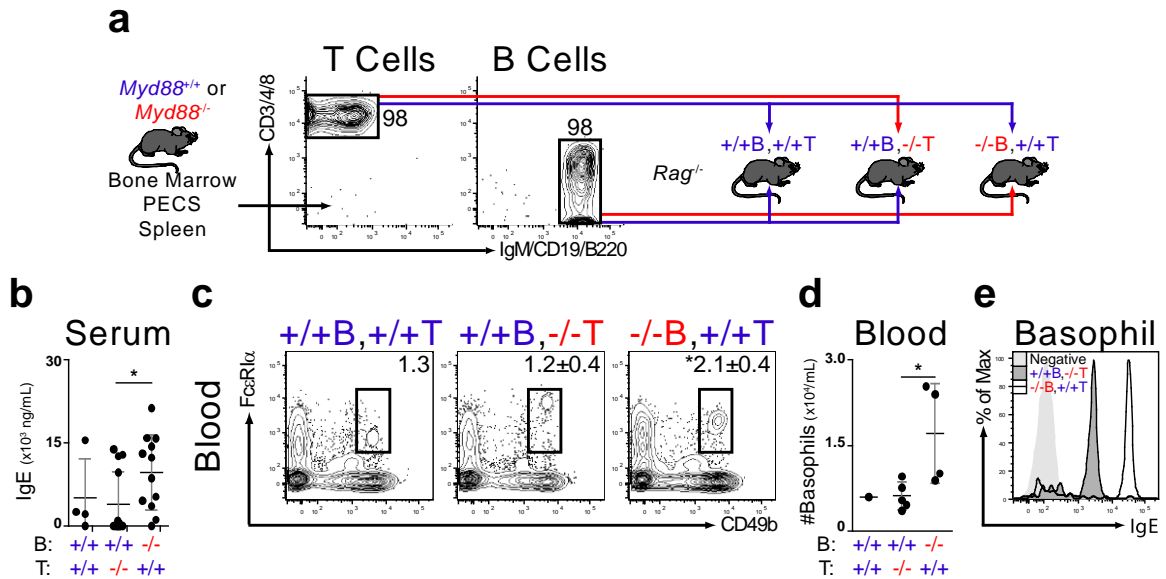
**Figure 24: B cell-intrinsic deletion of MyD88 results in elevated serum IgE levels and circulating basophil populations**

(**a**) Experimental diagram of sort and transfer of Myd88$^{+/+}$ or Myd88$^{-/-}$, B or T cells into Rag1$^{-/-}$ recipients. (**b**) Serum IgE from Rag1-/- mice reconstituted with Myd88$^{+/+}$ or Myd88$^{-/-}$, B or T cells as measured by ELISA. (**c**) Flow cytometric analysis of blood basophils from Rag1$^{-/-}$ mice reconstituted with Myd88$^{+/+}$ or Myd88$^{-/-}$, B or T cells. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on CD3$^-$, CD4$^-$, CD8$^-$, CD19$^-$, CD117$^-$ cells. (**d**) Number of basophils per mL of blood from Rag1$^{-/-}$ mice reconstituted with Myd88$^{+/+}$ or Myd88$^{-/-}$, B or T cells. (**e**) Mean fluorescence intensity of surface-bound IgE on blood basophils from Rag1$^{-/-}$ mice reconstituted with Myd88$^{+/+}$ or Myd88$^{-/-}$, B or T cells as determined by flow cytometry. Data representative of three or more independent experiments (mean ± S.D.; +/+B +/+T, n=1-4; +/+B -/-T, n=5-12; -/-B +/+T, n=4-12; *, P ≤ 0.05).

## 4.5 Discussion

Data in **Chapter 3** identified a previously unreconized role for commensal bacterial-derived signals in limiting steady-state serum IgE levels, circulating basophil populations, and basophil-mediated allergic inflammation. However, the mechanisms by which commensal bacterial-derived signals limit circulating basophil populations are not known. Data presented in this chapter indicate that elevations in circulating basophil populations observed upon antibiotic treatment of mice are critically dependent on IgE. This finding is consistent with findings from other groups that indicate that IgE influences granulocyte

119

homeostasis in the absence of an antigen (Lantz et al. 1997, Yamaguchi et al. 1997, Saini et al. 2000). Though presented in the context of allergy here, the observation that IgE alone is sufficient to increase the frequency and number of circulating basophils implies that regulation of basophils by IgE is likely relevant in immunity to helminth parasitic infections that are characterized by high serum IgE levels (Perona-Wright et al. 2008).

Data presented in this chapter also indicate that B cell-intrinsic MyD88 dependent signaling limits serum IgE levels and circulating basophil populations. It is interesting to note that while serum IgE levels and circulating basophil frequencies and numbers were significantly elevated in mice that received $Myd88^{-/-}$ B cells with $Myd88^{+/+}$ T cells compared to controls, there was variation in these peramaters within experimental groups. One potential explination for this variation is that IgE is not acting directly on basophils to mediate their population expansion, but rather is acting indirectly via one or more additional cell types or signaling molecules. Additionally, the TLR9-dependent commensal-derived signal CpG was found to be sufficient to limit serum IgE levels and circulating basophil populations in ABX-treated mice. CpG also reduced circulating basophil populations in CNV-reared mice suggesting that either the CpG-dependent pathways that limit these phenotypes are not saturated in CNV-reared mice, or that CpG is influencing circulating basophils through additional mechanisms other than suppression of serum IgE levels. Commensal-derived signals have been shown to influence B cell homeostasis in other contexts. For example, class switching of B cells to the IgA isotype and subsequent secretion of IgA into the intestinal lumen is dependent on commensal-derived signals (Macpherson, Slack 2007). Interestingly, this effect is mediated through both T cell-dependent and –independent mechanisms (Renshaw et al.

1994, Macpherson et al. 2000, Macpherson, Uhr 2004a, Bergqvist et al. 2006) indicating that innate recognition of commensal-derived signals is fundamental to B cell physiology. However, unlike commensal-regulation of IgA responses which are limited to mucosal tissues, a lack of commensal-regulation of IgE results in systemic elevations in serum IgE levels (Macpherson, Uhr 2004a).

Over the past decade, advances in therapeutics for asthma and other allergic diseases have been considerable, however, reliable interventions designed to prevent the development of allergic diseases or the progression from one to multiple allergies are yet to become a reality. Data presented in this chapter indicate that serum IgE levels and blood basophil populations correlate in human patients with hyper-IgE syndrome, and that treatment with Omalizumab reduces IgE-mediated increases in circulating basophil populations. Additionally, data presented here indicate that steady-state serum IgE levels and circulating basophil populations were limited by B cell-intrinsic MyD88 signaling pathways, providing insights into one cell-intrinsic mechanism by which commensal-derived signals may influence serum IgE levels. Together, these results provide therapeutically relevant insights into the cellular and molecular mechanisms by which commensal bacterial-derived signals regulate circulating basophil populations in the steady-state, and subsequently influence the development of $T_H2$ cytokine-mediated inflammation in response to an allergen.

# Chapter 5: Summary, discussion and future directions

## 5.1 The influence of commensal bacteria on allergic inflammation

It has been nearly fifty years since the first examinations of $T_H2$ cytokine-dependent immune responses in germ-free animals performed by Wescott and colleagues using the murine helminth parasite *Nippostrongylus brasiliensis* (Wescott, Todd 1964). Despite this, subsequent study of the influence of commensal-derived signals on the development and/or progression of $T_H2$ cytokine-mediated inflammation required technical advances in microbiology and immunology. It was not until the early 1980's, when Wannemuehler *et. al.* described an impaired ability for germ-free mice to induce systemic tolerance to oral antigens (Wannemuehler et al. 1982), that this field again began to garner attention. Wannemuehler went on to show that LPS, a commensal bacterial-derived signal from gram negative bacteria, was sufficient to induce oral tolerance to systemically administered sheep erythrocytes in germ-free animals (Wannemuehler et al. 1982). Sudo *et. al.* expanded on these initial observations using an OVA oral tolerance model by showing that germ-free mice develope robust total IgE, and OVA-specific IgE and IgG1 but not IgG2a responses compared to conventionally-reared mice (Sudo et al. 1997). Sudo went on to show that exaggerated Ig response in OVA-challenged mice correlated with increased production of IL-2, IL-4 and TGF-β by splenocytes when cultured with OVA antigen.

Subsequent mechanistic studies focused on identifying the specific microbial-derived signals and their receptors responsible for promoting oral tolerance. TLR4-defective mice were shown to have elevated allergic responses to peanut allergens co-administered orally with cholera toxin (Bashir et al. 2004). Antibiotic treatment of TLR4-sufficient mice recapitulated the elevated IgE levels seen in TLR-4-defective mice suggesting that commensal-derived LPS contributed to steady-state tolerance to food

antigens (Bashir et al. 2004). These results are consistent with Wannemuehler initial observation that LPS could induce oral tolerance in germ-free animals (Wannemuehler et al. 1982). The failure of oral tolerance induction in germ-free mice was also found to correlate with the absence of Peyer's patch T cells (Maeda et al. 2001), however, subsequent studies indicated that antigen-specific T cell tolerance to oral OVA was intact in germ-free mice (Walton et al. 2006a). Together, these findings indicated that commensal bacterial-derived signals influenced humoral oral tolerance more strongly than cellular oral tolerance, and prompted subsequent studies of how the innate immune system senses and responds to commensal-derived signals to influence allergic responses.

Beyond the field of oral tolerance, the majority of progress made in understanding the role that commensal-derived signals play in modulating allergic immune responses have come in the past 10 years from epidemiologic studies of human patients. Kalliomäki *et. al.* identified associations between alterations in commensal-bacterial populations and the subsequent development of allergic disease (Kalliomaki et al. 2001) and allergic patients were shown to have altered commensal bacterial community structure in the intestine compared to healthy controls (Penders et al. 2007). These findings implicate commensal bacterial-derived signals in influencing susceptibility to allergic disease in humans. More recently, antibiotic exposure in children was shown to significantly correlate with increased risk of developing asthma or eczema (Marra et al. 2009, Kummeling et al. 2007, Verhulst et al. 2008) suggesting that normal commensal-bacterial communities are important for protecting against the development allergies.

Despite these epidemiologic advances, mechanistic understandings of the mechanisms by which commensal-bacterial derived signals influence $T_H2$ cytokine-dependent allergic inflammation using animal models have been lacking. In one nice study Noverr and colleagues used an allergic airway model to show that antibiotic treatment of conventionally-reared mice resulted in exaggerated IL-13-mediated airway inflammation (Noverr et al. 2005). Similar results were obtained using the ovalbumin-aluminum allergic airway model (Herbst et al. 2011). However, these studies provide only limited understanding of the mechanisms by which the innate immune system recognizes commensal bacterial-derived signals and modulates the development of allergic $T_H2$ cytokine-dependent allergic inflammation.

## 5.2 The influence of antibiotics on intestinal bacteria

One limitation of previous studies that utilized antibiotic treatment in animal models is that they did not include a thorough characterization of the influence antibiotic treatment has on commensal bacterial communities. We therefore first carried out an in depth characterization of the effects of oral antibiotic treatment bacterial communities that colonize the murine intestine. Because a large percentage of intestinal bacteria are anaerobes that lack the enzymes necessary for the detoxification of oxygen, we opted for 454 deep sequencing molecular biologic methods in lieu of classical culture-based methods (Adlerberth, Wold 2009).

We initially adopted an antibiotic treatment protocol similar to previously published protocols (Sekirov et al. 2008, Bashir et al. 2004, Rakoff-Nahoum et al. 2004, Zaph et al. 2008, Hall et al. 2008, Kang et al. 2008). While this treatment protocol was

highly effective and reduced intestinal bacteria to the extent that DNA from food composed the majority of recovered 16S rDNA sequences, it was associated with animal dehydration that could complicate the interpretation of subsequent studies (Guseinov, Guseinova 2008). This finding highlights the need for minimizing or controlling for unintentional side-effects of antibiotic treatment that may confound results and interpretations. For example, in addition to potential for dehydration, commensal-derived signals have been shown to modulate nutrition and metabolism (Ley et al. 2005, Bollyky et al. 2009), fundamental aspects of mammalian physiology that likely influence the immune system. The utilization of both gain and loss of function approaches, such as conventional mouse strains deficient in innate pattern recognition receptors, can complement antibiotic-treated and germ-free mouse models to help control for some of these effects.

To circumnavigate potential complications of dehydration, acute administration of broad-spectrum antibiotics by gavage was employed, while allowing animal access to untreated drinking water; more closely mimicking antibiotic administration in humans. Antibiotic treatment by oral gavage reduced absolute bacterial numbers and mimicked anatomic, histologic and immunologic characteristics of reduced bacterial stimulation observed in germ-free mice. UniFrac analysis of bacterial communities indicated that antibiotic treatment promoted a novel community structure in the intestine that was more similar between antibiotic-treated samples as compared to samples from control-treated animals. Importantly, temporal analysis of stool pellet bacterial communities during treatment revealed a fast restructuring in community structure after only one day of antibiotic treatment. These findings indicate that even short exposure to oral antibiotics dramatically alters intestinal bacterial communities of the murine intestine and supposes

126

that similarly short courses of antibiotics in patients (days to weeks) might also significantly alter commensal community structure.

Early colonization with *Bacteroides fragilis* has been implicated as a risk factor for asthma development in humans (Vael et al. 2008). As such, the effects of antibiotic treatment on members of the Bacteroidies phylum were examined. Members of the Bacteroidies phylum, namely the Bacteroidales order, represented 60-70% of intestinal bacteria in control-treated animals. After one day, these bacteria represented a combined frequency of greater than 95% in samples from antibiotic-treated but not control-treated animals, and subsequently stabilized at a frequency of approximately 90% in all sampled compartments. It is intriguing to speculate that rapid increases in Bacteroidies frequency after antibiotic initiation may be responsible for associations between early antibiotic exposure in humans and increased susceptibility to allergic inflammation.

Naïve animals displayed significantly higher frequencies of *Lactobacillus* species in mucosal-associated as compared to luminal communities and *Lactobacillus* species were significantly reduced in frequency with antibiotic treatment. *Lactobacillus* species have been cited as "probiotic" bacteria with mixed effectiveness in treating human diseases (Betsi, Papadavid & Falagas 2008, Kozuch, Hanauer 2008). It may therefore be useful to examine whether reductions in this potentially beneficial mucosal-associated bacterial group are related to changes in immune cell homeostasis observed following antibiotic treatment. The mucosal-associated anatomical location of this bacterium also suggests that colonization of this anatomical location may be relevant for the beneficial host effects of this species. This is consistent with recent observations that segmented filamentous bacteria exist in an intimate relationship with the intestinal epithelium where

127

they regulate $T_H17$ and Treg cell populations (Gaboriau-Routhiau et al. 2009, Ivanov et al. 2009, Wu et al. 2010, Kriegel et al. 2011). Together, these findings indicate that antibiotic treatment results in temporal and spatial alterations to bacterial communities that colonize the mammalian intestine.

## 5.3 Basophils as a mediator of commensal-bacterial effects

Antibiotic-treated and germ-free mice were found to have elevated steady-state serum IgE levels and a selective population expansion of circulating basophils compared to conventionally-reared mice. Interestingly, elevations in basophils were limited to the blood and spleen, and were not observed in tissue or lymphoid tissues, indicating that steady-state effects of commensal-derived signals on basophils are compartmentalized to these anatomical sites. These findings are of particular interest given that basophils can be directly activated by allergens and contribute to the development of optimal $T_H2$ cytokine-dependent allergic inflammation (Gessner, Mohrs & Mohrs 2005, Mohrs et al. 2005, Min et al. 2004). Additionally, and perhaps more importantly, basophils have been shown to function as accessory cells by providing early sources of IL-4 and contributing to the initiation and/or propagation of CD4$^+$ $T_H2$ cell responses (Hida et al. 2005, Oh et al. 2007). Finally, basophils have been shown to endocytose soluble antigen, express MHC II and co-stimulatory molecules, and in select cases, act as antigen presenting cells that promote antigen-specific $T_H2$ cell differentiation (Perrigoue et al. 2009, Sokol et al. 2009, Yoshimoto et al. 2009). Regardless of whether basophils are sufficient to initiate and/or propagate $T_H2$ cell responses, their ability to augment $T_H2$ cell differentiation in response to an allergic stimulus identifies basophils as a key player determining whether an encounter with an allergen results in the development of a $T_H2$

128

cell response. As such, the observation that basophils are selectively expanded in antibiotic-treated or germ-free mice provides a mechanism to explain correlations between antibiotic treatment and increased susceptibility to allergic diseases in human patients.

We tested whether the elevations in steady-state basophils observed in antibiotic-treated mice contributed to the development of allergic inflammation using three basophil-mediated allergy models. Antibiotic-treated mice displayed exaggerated basophil responses and inflammation in a skin model of MC903-induced atopic dermatitis. Additionally, antibiotic-treated mice displayed exaggerated basophil responses and lung inflammation using a house dust mite-elicited allergic airway model. In both cases, basophils may be contributing to the development of $T_H2$ cytokine-mediated inflammation by either by augmenting the development of the $T_H2$ cell response or by being recruited to the site of tissue inflammation where they may be exerting more classical effector cell functions such as the release of histamine, leukotrienes, cytokines or chemokines. The differential contribution of basophils to the allergic inflammation observed in these models was not directly tested here, and could be explored in future studies.

To directly test whether the elevated steady-state basophil responses observed in antibiotic-treated mice were contributing to the development of $T_H2$ cell responses, we utilized the protease allergen papain. ABX-treated mice displayed elevated basophil LN recruitment and exaggerated LN $T_H2$ cell responses to papain compared to CNV-reared mice. Non-basophil APC populations were not significantly influenced by antibiotic-treatment in our experimental systems, however, they may contribute to exaggerated allergic responses in antibiotic-treated or germ-free mice in other settings. Basophils

were shown to be critical contributors to the exaggerated allergen-induced $T_H2$ cell responses observed in antibiotic-treated mice using two methods of basophil depletion. Together, these findings indicate that commensal-derived signals limit basophil effector function and basophil-mediated $T_H2$ cell responses, and identify basophils as a potential therapeutic target, the manipulation of which may prevent the development or propagation of allergic diseases in patients.

## 5.4 Regulation of circulating basophil populations by IgE

Natural IgE is produced in the absence of allergic stimuli and lacks antigen specificity, implicating IgE in functions other than antigen detection. Consistent with this hypothesis, IgE has been found to influence multiple aspects of granulocyte homeostasis in the absence of an antigen including regulation of surface protein expression, cell survival, and cell adhesion (Galli et al. 2005, Xiang, Moller & Nilsson 2006, Kitaura et al. 2005). We found that steady-state serum IgE levels correlate with circulating basophil populations in mice with reduced commensal stimulation and humans with hyper-IgE syndrome. IgE was found to be critical for elevations in circulating basophils observed with antibiotic treatment of mice, and IgE was sufficient to elevate murine circulating basophil populations. Although presented in the context of allergy here, the observation that IgE alone can increase the frequency and number of circulating basophils implies that regulation of basophils by IgE is likely relevant in immunity to helminth parasitic infections that are characterized elevations in serum IgE (Perona-Wright et al. 2008).

We found that B cell-intrinsic MyD88 dependent signaling limits serum IgE levels and circulating basophil populations *in vivo* and CpG was found to be sufficient to limit

130

serum IgE levels and circulating basophil populations. These findings implicate direct detection of commensal-derived signals by B cells in limiting IgE class switching and production, raising the question as to where B cells interact with commensal-derived signals. One possibility is that B cells circulate through intestinal-associated compartments such as the lamina propria where they are exposed to commensal bacterial-derived signals (**Figure 25**). Alternatively, B cells may encounter commensal-derived signals trafficked to the mesenteric lymph nodes by dendritic cells or other mechanisms. Finally, commensal-derived signals may be present systemically at high enough levels to impart effects on B cell populations.

## 5.5 Final thoughts

Over the past decade, advances in therapeutics for asthma and other allergic diseases have been considerable, however, reliable interventions designed to prevent the development of allergic diseases or the progression from one to multiple allergies are yet to become a reality. Data presented in this thesis indicate that commensal-derived signals limit serum IgE levels and circulating basophil populations in the steady-state. Antibiotic-treated mice displayed exaggerated $T_H2$ cytokine-dependent inflammation in two models of basophil-mediated allergic disease and basophils were shown to contribute directly to the development of exaggerated $T_H2$ cell responses in this setting. Together, these results provide therapeutically relevant insights into one mechanism by which commensal bacterial-derived signals influence susceptibility to the development of a $T_H2$ cell response, and to $T_H2$ cytokine-dependent allergic inflammation. It is hoped that identification of this commensal-basophil-$T_H2$ cell axis will stimulate studies by other investigators aimed at exploiting this knowledge to develop new preventative or therapeutic approaches for allergic diseases.
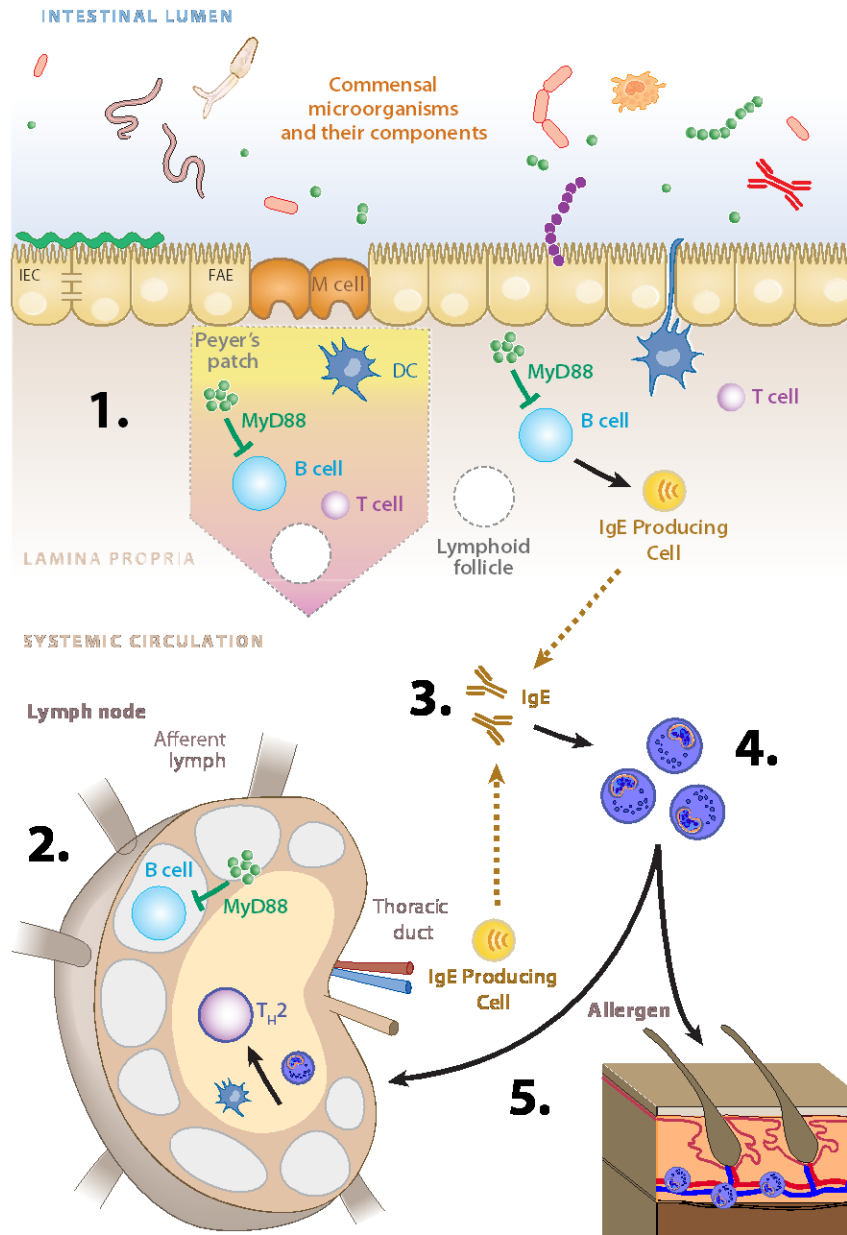
**Figure 25: Summary of thesis**

Commensal bacterial-derived signals act via B cell-intrinsic, MyD88-dependent signaling pathways to limit B cell production of IgE. B cells may detect commensal signals in intestinal compartments (**1**) or systemically (**2**). (**3**) In the absence of commensal signals, B cells produce increased amounts of IgE which enters the circulation. (**4**) IgE acts to expand the number of circulating basophils in the steady-state. (**5**) In the event of allergen exposure, basophils are recruited to draining lymph nodes or sites of tissue inflammation where they contribute to the generation of $T_H2$ cell responses and/or $T_H2$ cytokine-dependent allergic inflammation. Adapted from (Hill, Artis 2010).

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139

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152

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153

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160

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186

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