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Commensal Bacterial Communities Regulate Antiviral Immunity

Michael Christopher Abt
University of Pennsylvania, abtc@mail.med.upenn.edu

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Abstract
Alterations in the composition of commensal bacterial communities in the human intestine are associated with enhanced susceptibility to multiple inflammatory diseases. Further, studies in murine model systems have demonstrated that signals derived from commensal bacteria can influence immune cell development, function and homeostatic regulation within the intestinal environment, leading to altered host susceptibility to infectious or inflammatory diseases. However, whether commensal bacteria-derived signals regulate protective immunity to viral pathogens that infect sites outside of the gastrointestinal microenvironment remain poorly understood. Chapter 2 of this thesis examines this question and demonstrates that disruption or absence of commensal bacterial communities results in impaired protective immunity to respiratory influenza virus. Antibiotic (ABX)-mediated disruption of intestinal commensal bacteria significantly impaired the innate and adaptive immune response, abrogated viral clearance and increased host mortality following influenza virus infection indicating a crucial role for commensal bacterial communities in regulating antiviral immune defense. Chapter 3 interrogates the influence of commensal bacteria-derived signals on the antiviral immune response to systemic viral infection. Following infection with Lymphocytic Choriomeningitis virus (LCMV), ABX-treated mice exhibited functionally impaired LCMV-specific CD4 and CD8 T cell responses that correlated with significantly delayed viral clearance. Collectively, Chapters 2 and 3 indicate a global role for commensal bacteria-derived signals in regulating immunity to mucosal or systemic viral infection. Chapter 4 examines the mechanisms underlying commensal bacteria regulation of antiviral immunity and identifies a previously unrecognized role for commensal bacteria-derived signals in establishing the activation threshold of the innate immune system. Genome-wide transcriptional profiling of macrophages isolated from ABX-treated mice revealed decreased expression of genes associated with antiviral immunity. Moreover, macrophages isolated from ABX-treated mice prior to viral infection exhibited defective responses to type I/II IFNs and a reduced capacity to limit viral replication. Strikingly, adoptive transfer of macrophages from CNV mice into ABX-treated mice prior to LCMV infection resulted in significantly improved virus-specific adaptive immune responses and effectively restored the ability of ABX-treated mice to control viremia. Taken together, the results presented in this thesis indicate that commensal bacteria-derived signals calibrate the activation threshold of the innate immune system, providing tonic immune stimulation essential for optimal antiviral immunity.

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COMMENSAL BACTERIAL COMMUNITIES REGULATE ANTIVIRAL IMMUNITY

Michael Christopher Abt

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David Artis, PhD, Associate Professor of Microbiology and Pathobiology

Supervisor of Dissertation

E. John Wherry, PhD, Associate Professor of Microbiology

Graduate Group Chairperson

Dissertation Committee:

Yasmine Belkaid, PhD, Chief of Mucosal Immunology, NIAID

Michael P Cancro, PhD, Professor of Pathology and Laboratory Medicine

Christopher A. Hunter, PhD, Professor of Pathobiology

Hui Hu, PhD, Assistant Professor of Pathology and Laboratory Medicine

E. John Wherry, PhD, Associate Professor of Microbiology

COMMENSAL MICROBIAL COMMUNIITIES REGULATE ANTIVIRAL IMMUNITY

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ABSTRACT

COMMENSAL BACTERIAL COMMUNITIES REGULATE ANTIVIRAL IMMUNITY

Michael Christopher Abt

David Artis, PhD

Alterations in the composition of commensal bacterial communities in the human intestine are associated with enhanced susceptibility to multiple inflammatory diseases. Further, studies in murine model systems have demonstrated that signals derived from commensal bacteria can influence immune cell development, function and homeostatic regulation within the intestinal environment, leading to altered host susceptibility to infectious or inflammatory diseases. However, whether commensal bacteria-derived signals regulate protective immunity to viral pathogens that infect sites outside of the gastrointestinal microenvironment remain poorly understood. **Chapter 2** of this thesis examines this question and demonstrates that disruption or absence of commensal bacterial communities results in impaired protective immunity to respiratory influenza virus. Antibiotic (ABX)-mediated disruption of intestinal commensal bacteria significantly impaired the innate and adaptive immune response, abrogated viral clearance and increased host mortality following influenza virus infection indicating a crucial role for commensal bacterial communities in regulating antiviral immune defense. **Chapter 3** interrogates the influence of commensal bacteria-derived signals on the antiviral immune response to systemic viral infection. Following infection with Lymphocytic Choriomeningitis virus (LCMV), ABX-treated mice exhibited functionally impaired LCMV-specific CD4 and CD8 T cell responses that correlated with significantly delayed viral

clearance. Collectively, **Chapters 2** and **3** indicate a global role for commensal bacteria-derived signals in regulating immunity to mucosal or systemic viral infection. **Chapter 4** examines the mechanisms underlying commensal bacteria regulation of antiviral immunity and identifies a previously unrecognized role for commensal bacteria-derived signals in establishing the activation threshold of the innate immune system. Genome-wide transcriptional profiling of macrophages isolated from ABX-treated mice revealed decreased expression of genes associated with antiviral immunity. Moreover, macrophages isolated from ABX-treated mice prior to viral infection exhibited defective responses to type I/II IFNs and a reduced capacity to limit viral replication. Strikingly, adoptive transfer of macrophages from CNV mice into ABX-treated mice prior to LCMV infection resulted in significantly improved virus-specific adaptive immune responses and effectively restored the ability of ABX-treated mice to control viremia. Taken together, the results presented in this thesis indicate that commensal bacteria-derived signals calibrate the activation threshold of the innate immune system, providing tonic immune stimulation essential for optimal antiviral immunity.

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The contents of this thesis include portions of modified text and figures from the following published manuscripts:

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Chapter 1
Introduction

1.1 Commensal bacterial communities in health and disease

All mammals are born sterile, but colonization with beneficial microbial species begins soon after birth (Dethlefsen et al., 2007; Ley et al., 2006a). Commensal microbial communities colonize host organisms at multiple barrier surfaces including the skin, mouth, nasopharynx, vaginal, and gastrointestinal tract, and establish a symbiotic relationship with the host organism. (Clemente et al., 2012; Ley et al., 2006a; Round and Mazmanian, 2009). These microorganisms have an important role in host nutrition by breaking down food by-products and producing essential vitamins. Commensal microbes also have a passive role in host defense by competing with other potentially pathogenic organisms for space and resources in the gut microenvironment (Hooper and Gordon, 2001; Smith et al., 2007). Dysbiosis of commensal microbial communities, however, is associated with various disease states such as obesity (Turnbaugh et al., 2006), inflammatory bowl disease (Ott et al., 2004), and atopic disorders (Marra et al., 2009). These clinical observations highlight the need to better understand how commensal microbial communities shape human health and susceptibility to disease.

1.1.1 Composition of intestinal commensal bacterial communities

Intestinal commensal microbial communities are comprised of bacteria, fungi, protozoa, and viruses that inhabit the gastrointestinal tract (Breitbart et al., 2003; Ley et al., 2006a; Scupham et al., 2006). The bacterial component of these microbial communities is the most well defined and studied. A large degree of diversity of bacterial species is found throughout the intestine with the lowest total concentration of microbes found in the

duodenum and steadily increasing until the peak density of 10^{11} -10^{12} colony forming units (CFU) /mL of luminal content in the proximal large intestine (Marteau et al., 2001; Savage, 1977). Culture-based identification of the bacterial species residing in the human intestine have identified members of the Lactobacilli, Streptococci and Bacteroides genera, however, these techniques are limited because the majority of commensal bacterial species do not grow using current laboratory techniques (Adlerberth and Wold, 2009; Dubos et al., 1965). Use of high-throughput sequencing technology has expanded the ability to identify both culturable and non-culturable bacterial species residing in the human intestine and current estimates of total diversity range between 15,000 and 36,000 species (Frank et al., 2007). The majority of these bacteria belong to the Bacteroidetes (~68%) and Firmicutes (~31%) phyla, with smaller percentages from the Proteobacteria, Actinobacteria, Deferribacteres, and Tenericutes phyla (Figure 1) (Eckburg et al., 2005; Hayashi et al., 2002; Wang et al., 2003). Intestinal commensal bacteria exhibit spatial diversity along the intestinal tract with some classes of bacteria preferentially residing either in the lumen or the mucus that coats the apical surface of intestinal epithelial cells (Hill and Artis, 2010). Due to proximity, mucosal-associated bacteria are more likely to interact with host cells. Therefore, the location of colonization is likely to be equally, or more important in influencing intestinal homeostasis as total quantity of particular bacterial species.

1.1.2 Commensal bacterial communities and human disease

Alterations in the composition of intestinal commensal bacterial communities have been linked with several human diseases. The occurrence of obesity in both mice and humans has been associated with a decrease in the frequency of Bacteroidetes and an increase among Firmicutes (Turnbaugh et al., 2006). The shift in the Bacteroidetes/Firmicutes

ratio increases the ability of the commensal bacteria to break down indigestible fiber into short-chain fatty acids (SCFA), thus providing an additional energy source that is capable of being converted into fat. (Ley et al., 2006b; Schwiertz et al., 2008). In addition to obesity, clinical evidence has linked commensal bacteria to the unregulated immune responses observed in inflammatory bowel diseases (IBD) such as Crohn's disease (De Hertogh et al., 2006; Dicksved et al., 2008), ulcerative colitis (Rutgeerts et al., 2005), and coeliac disease (Tlaskalova-Hogenova et al., 2004), as well as colon cancer (Karin et al., 2006; Moore and Moore, 1995). Clinical investigations into the etiology of intestinal inflammatory disease has revealed decreased species diversity within the patients' intestinal commensal bacterial community (Ott et al., 2004) and aberrant immune responses such as IgG antibody production (Macpherson et al., 1996), T cell activation (Duchmann et al., 1995), and proinflammatory cytokine secretion (Medina et al., 2008) in response to bacteria found within the intestine. Furthermore, deliberate manipulation of commensal bacterial using antibiotics or diversion of the fecal stream away from the inflamed portion of the intestine has been moderately successful in treating Crohn's disease, thus strengthening the link between commensal bacterial communities and intestinal inflammation (Casellas et al., 1998; D'Haens et al., 1998; Rutgeerts et al., 1991; Sartor, 2004).

Perturbations of commensal bacterial communities have also been linked to other dysregulated inflammatory diseases at sites outside of the intestine, such as asthma and other atopic disorders (Penders et al., 2007a). In particular, there is a strong correlation between treating infants with multiple rounds of antibiotics and the development of asthma (Kozyrskyj et al., 2007; Marra et al., 2009). The presence of particular bacterial species in the intestine, such as *Bifidobacterium pseudocatenulatum*, *Clostridium*

difficile, and *Escherichia coli* are also associated with increased cases of eczema (Gore et al., 2008; Penders et al., 2007b). Because of the correlation between altered commensal bacterial communities and inflammatory diseases, administration of "probiotic" bacterial species has been employed to prevent or ameliorate certain atopic and intestinal inflammatory diseases (Gionchetti et al., 2000; Hart et al., 2003; Hedin et al., 2007; Kukkonen et al., 2007; Mimura et al., 2004). Despite encouraging results, the full potential of probiotic therapy may not be realized until the mechanisms by which commensal bacterial communities shape the immune responses that drive these inflammatory diseases are understood.

1.2 Commensal bacterial communities and the immune system

Associations between commensal bacterial communities and human inflammatory diseases have implicated commensal bacteria-derived signals in shaping the immune responses that drive pathogenesis. Studies using murine model systems demonstrate that manipulation of commensal bacteria can alter mammalian immune cell homeostasis and specific commensal bacteria-derived signals have been identified that can impact immune cell function both within the intestinal tract and in peripheral tissues (Littman and Pamer, 2011; Macpherson and Harris, 2004). These commensal bacteria-derived signals can either have an immunoregulatory effect, creating a state that is refractory to inflammation, or conversely, act as an adjuvant, aiding in the propagation of an immune response. Innate immune cells responding to commensal bacterial-derived signaling can modulate both the local and systemic immune response (Honda and Littman, 2012; Littman and Pamer, 2011; Round and Mazmanian, 2009). This section will discuss the

mechanisms through which commensal bacterial communities shape the host immune system.

1.2.1 Impact of commensal bacterial communities on immune system development and homeostasis

The commensal bacterial communities of the mammalian gastrointestinal tract are integral in shaping the development and function of the immune system. Mouse models enable the manipulation of the commensal bacterial communities and systematic investigation of how commensal bacteria influence immune responses of the host. Studies employing mice lacking bacterial communities (germ-free) demonstrate the need for commensal bacteria-derived signals in the development of gut-associated lymphoid tissues (GALT). Germ-free (GF) mice have fewer Peyer's patches along the small intestine and the mesenteric lymph nodes of these mice have decreased cellularity (Smith et al., 2007). Further, commensal bacteria-derived signals have been shown to stimulate intestinal epithelial cells (IECs) to stimulate production of cytokines that foster lymphoid tissue genesis (Bouskra et al., 2008). Defective development of lymphoid architecture is not limited to the GALT as the spleen of GF mice lack germinal centers and have poorly formed T and B cell zones (Macpherson and Harris, 2004).

Loss of commensal bacteria signaling alters the distribution, activation level, differentiation status, and inflammatory profile of dendritic cells, macrophages, NK cells, B cells, CD4 and CD8 T cells, both within the GALT and at distal sites within the host (Hill and Artis, 2010; Macpherson and Harris, 2004). GF mice exhibited fewer intestinal dendritic cells (DCs) (Williams et al., 2006), intraepithelial lymphocytes (Smith et al., 2007), lamina propria $CD4^+$ T cells (Macpherson and Harris, 2004), and IgA^+ B cells

(Macpherson et al., 2001). These inherent developmental defects in GF mice make it challenging to parse out the cause of altered immune cell function and have been the motivating factor to develop complementary methods to study the impact of commensal bacterial communities on immune cell function at steady-state.

Manipulation of commensal bacterial communities in mice reared in a conventional environment via antibiotic (ABX) treatment has demonstrated that commensal bacteria influence intestinal immune cell homeostasis even after the developmental stage. An example that illustrates this paradigm of interactions between commensal bacteria and immune cell populations is the role commensal bacteria have in influencing CD4 T cell differentiation in the intestinal lamina propria. GF mice exhibit fewer CD4 T helper 17 (T_H17) cells in the lamina propria of the small intestine compared to conventionally reared (CNV) mice (Atarashi et al., 2008; Niess et al., 2008). In addition to GF studies, selective depletion of commensal bacterial communities by oral administration with a specific antibiotic (vancomycin) also results in reduced percentage of T_H17 cells in the lamina propria of the small intestine (Ivanov et al., 2008). The commensal bacteria, Segmented Filamentous Bacteria (SFB), were identified to be the driving factor in promoting the development of CD4 T_H17 cells. The mechanisms through which SFB promotes of CD4 T_H17 cell differentiation is believed to involve conditioning of DCs by Serum amyloid A, which promote differentiation into a RORγt^+ T_H17 lineage in CD4 T cells (Ivanov et al., 2009). As will be discussed in further detail later in Section 1.2.5, the induction of CD4 T_H 17 cells by SFB has significant physiological consequences in immune homeostasis. While SFB can promote CD4 T_H17 cell differentiation, another class of commensal bacteria, *Clostridia*, promotes CD4 T cells in the intestine to a T regulatory cell fate (Atarashi et al., 2011). These observations emphasize that signals

from distinct bacterial species can have a differential role in influencing the immune cell development within the intestine.

Use of the GF mouse system has been critical in demonstrating a role for bacteria in the induction and propagation of intestinal inflammation. Production of inflammatory cytokines in the intestine is a hallmark characteristic of IBD and is believed to be driven by commensal bacteria stimulation (Duchmann et al., 1995; Fiocchi, 1998; Frank et al., 2007; Kaser et al., 2010). Several transgenic mouse strains that are defective in immunoregulatory mechanisms, such as IL-10 production (Kuhn et al., 1993) or regulatory T cell development (Sadlack et al., 1993) spontaneously produce inflammatory cytokines in the intestine and develop intestinal inflammation. When re-derived into a sterile, germ-free environment, however, these mouse strains no longer exhibit spontaneous disease, supporting the link between commensal bacteria and intestinal inflammation (Dianda et al., 1997; Schultz et al., 1999; Sellon et al., 1998). Furthermore, it has been demonstrated that transfer of colitis-inducing bacterial species from a genetically susceptible host into wild type recipients can initiate disease (Elinav et al., 2011; Garrett et al., 2010a; Garrett et al., 2007). Thus certain bacterial species are capable of inducing intestinal inflammation in otherwise healthy hosts. Conversely, probiotic bacterial species can ameliorate intestinal inflammation and disease (Dieleman et al., 2003; Round and Mazmanian, 2010). These divergent effects of commensal bacterial species on intestinal homeostasis highlight the need to understanding how commensal bacteria are detected by intestinal immune and non-immune cell populations.

1.2.2 Recognition of commensal bacteria-derived signals in the intestine

The immune system within the GALT must be able to initiate an appropriate immune response against invading pathogens yet remain hypo-responsive to bacterial signals from the commensal bacteria. The resulting balance between proinflammatory and immunoregulatory signals is thought to be dependent on the context in which the bacterial signals are recognized. DCs are a key cell type that modulates the immune response within the intestine (Coombes and Powrie, 2008; Jaensson et al., 2008; Sun et al., 2007). DCs within the intestine are exposed to bacterial products via M cell antigen sampling within Peyer's patches or directly by extending dendrites between tight junctions of IECs and into the intestinal lumen (Mach et al., 2005; Milling et al., 2005; Niess et al., 2005; Rescigno et al., 2001). Intestinal DCs are capable of phagocytizing live bacteria then migrating into the mesenteric lymph nodes to present commensal bacteria antigen to resident T and B cells (Macpherson and Uhr, 2004). This process is thought to be important in driving production of commensal bacteria specific IgA. DCs express a wide range of pattern recognition receptors (PRRs) that can be activated by microbial products. PRRs expressed by DCs include members of the transmembrane toll-like receptor (TLR) family and the intracellularly residing nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) (Iwasaki and Medzhitov, 2004; Medzhitov, 2007). Pathogen-associated molecular patterns (PAMPs) directly stimulate TLRs or NLRs leading to activation of the conserved Nuclear factor kappa B (NFκB), mitogen-activating pathway kinase (MAPK), and inflammasome signaling pathways resulting in the expression of a proinflammatory gene profile characterized by cytokines such as IL-12, IL-1β, and type I interferons (Akira and Takeda, 2004; Franchi et al., 2009; Girardin et al., 2003a; Girardin et al., 2003b; Strober et al., 2006). However, not all

DC/PAMP interactions in the intestine result in proinflammatory immune responses. Stimulation of intestinal DCs with the TLR4 ligand, lipopolysacchride (LPS), does not result in costimulatory molecule upregulation or IL-12p70 expression as is normally seen with DCs derived from other tissues of the body. Instead, LPS stimulation results in elevated IL-10 production by intestinal DCs (Cerovic et al., 2009; Monteleone et al., 2008; Takenaka et al., 2007). In addition, NOD2 signaling has been shown to negatively regulate TLR2 activation of the NFκB pathway and thereby attenuate expression of T_H1 associated cytokines IL-12, and IFN-γ (Watanabe et al., 2004; Yang et al., 2007).

The intestinal epithelium maintains a physical barrier between the commensal bacteria of the intestinal environment and the host. Located at this host/bacteria interface, IECs experience constant, direct exposure from commensal bacterial communities and are therefore in a position to sense and respond to commensal bacteria. IECs can express TLRs and NOD receptors that sense bacterial stimuli (Fritz et al., 2006; Hisamatsu et al., 2003; Medzhitov, 2007), though the anatomical location of these PRRs may be an important factor in determining responsiveness of IECs to bacterial stimulation. For example, TLR5, which recognizes bacterial flagellin, is only expressed on the basolateral surface of IECs, and therefore sequestered from bacterial contact on the apical surface of the cell (Gewirtz et al., 2001). Other cell membrane-associated PRRs, such as TLR2 and 4 are expressed at low or undetectable levels in IECs (Abreu et al., 2001; Melmed et al., 2003) while endosomally or cytosolically located PRRs, such as TLR3,7,8,9 and NOD protein are expressed at higher levels in IECs (Medzhitov, 2007). Commensal bacteria-derived signaling in IECs via these PRRs influence intestinal immune cell development and are critical in maintaining homeostasis (Bouskra et al., 2008; Hasegawa et al., 2006; Nenci et al., 2007; Rakoff-Nahoum et al., 2004; Zaph et al.,

2007). Commensal bacteria-derived signals acting upon IECs can induce an immunoregulatory environment characterized by cytokines such as IL-10, TGF-β, TSLP, and IL-25, which modulate DC, macrophage, T and B cell activation (Figure 2) (Dignass and Podolsky, 1993; He et al., 2007; Rimoldi et al., 2005a; Taylor et al., 2009; Zeuthen et al., 2008). Similar to DCs, PRR activation in IECs by invading pathogens can also elicit a proinflammatory response that is important in initiating the intestinal immune response to infection (Zilbauer et al., 2007). Thus IECs exhibits a PRR expression profile that limits continuous proinflammatory stimulation, but is poised to respond to invasive pathogens upon breakdown of the epithelial barrier. Therefore, while cells within the intestinal compartment express TLR and NOD receptors and the ligands for these PRRs are found within commensal bacterial communities, the outcome of PAMP/PRR interactions is dependent upon the context in which they occur. Recent work has elucidated several specific commensal bacteria-derived signals that can activate TLR or NOD signaling and influence immune cell homeostasis. These can be broadly categorized into signals that have an immunoregulatory effect or signals that promote proinflammatory immune responses.

1.2.3 Immunoregulatory signals derived from commensal bacterial communities

Commensal bacteria appear to be integral in inducing an immunoregulatory environment within the intestine. GF mice were reported to have reduced expression of IL-25 in the large intestine, an immunoregulatory cytokine involved in driving type 2 immune responses (Saenz et al., 2008; Zaph et al., 2008). Further, *Clostridium* species have been reported to induce accumulation of IL-10 producing $Foxp3^+$ CD4 regulatory T cells in the lamina propria of the colon (Atarashi et al., 2011). Medzhitov and colleagues

implicate the commensal bacteria-derived signals LPS or lipoteichoic acid (LTA) in regulating intestinal immune homeostasis via TLR4 and TLR2 signaling, respectively. In this study, mice depleted of commensal bacterial communities by antibiotics display exacerbated intestinal inflammation and increased mortality following administration of dextran sodium sulfate (DSS) in the drinking water, a chemically-induced colitis model (Rakoff-Nahoum et al., 2004). However, ABX-treated mice, subsequently treated with LPS, were able to limit intestinal inflammation and recover from chemically-induced colitis (Rakoff-Nahoum et al., 2004). Subsequent studies have identified commensal bacteria-mediated protection to be dependent on MyD88-mediated TLR signaling on hematopoietic cells, particularly intestinal B cells (Kirkland et al., 2012; Rakoff-Nahoum et al., 2006). These results indicate that commensal bacterial communities can modulate intestinal inflammation and that a specific bacterial product can orchestrate these immunoregulatory effects in the absence of living commensal bacteria.

Although TLR ligands have been predominantly shown to activate a proinflammatory response, TLR activation is capable of dampening an inflammatory response under certain conditions. A series of studies have demonstrated that signaling through TLR2 by polysaccharide A (PSA) could induce an environment refractive to inflammation. A unique form of PSA, specific to *Bacteroides fragilis*, induces an immunoregulatory phenotype in CD4 T cells that was highlighted by elevated production of the immunoregulatory cytokine IL-10 (Mazmanian et al., 2005; Mazmanian et al., 2008; Wang et al., 2006). Treatment with PSA isolated from *Bacteroides fragilis* prevents the induction of *Helicobacter hepaticus*-induced colitis, indicating that a single commensal bacteria-derived molecule can influence the immune response within the intestinal environment.

In addition to TLR ligands, commensal bacteria provide other signaling molecules that modulate the intestinal immune network and prevent pathologic inflammation. The NOD2 ligand muramyl dipeptide (MDP), a component of bacterial peptidoglycan, was found to negatively regulate the production of IL-12p40, IL-6 and TNF-α by DCs *in vitro* (Watanabe et al., 2008). Furthermore, *in vivo* administration of MDP resulted in a diminished proinflammatory cytokine profile of colonic lymphocytes and was able to protect against trinitrobenzene sulfonic acid (TNBS) induced colitis (Watanabe et al., 2008). Van Kooyk and colleagues described a specific surface layer A protein (SlpA) present on the intestinal bacterial specie *Lactobacillus acidophilus* NCFM that enables bacterial binding to dendritic cells resulting in the production of IL-10 and inhibition IL-12p70 (Konstantinov et al., 2008). Also, butyrate, a SCFA byproduct of intestinal bacteria fermentation of dietary fibers (Roediger, 1980), has been shown to be a major energy source of colonic epithelial cells and can reduce the expression of TNF-α, TNF-β, IL-6 and IL-1β in lamina propria lymphocytes via NFκB inhibition in Crohn's and ulcerative colitis patients (Luhrs et al., 2002; Segain et al., 2000). SCFA, via binding and signaling through the G-protein receptor 43 (GPR43) has been shown to limit inflammatory response (Le Poul et al., 2003; Maslowski et al., 2009). Mice deficient in GPR43 exhibited exacerbated intestinal inflammation following treatment with DSS. Similarly, GF mice, which lack the bacteria to break down fiber into SCFA also exhibited increased weight loss and rectal bleeding following DSS administration (Maslowski et al., 2009). Treatment of these GF mice with acetate in the water to supplement SCFA deficiency markedly diminished disease severity and proinflammatory cytokine levels in the intestine, demonstrating that a commensal bacteria-derived metabolite could limit inflammatory responses and disease progression (Maslowski et al., 2009). Each of these

commensal bacteria-derived products described in this section is a potential conduit through which the intestinal commensal bacteria can regulate the immune system.

1.2.4 Proinflammatory signals derived from commensal bacterial communities

Although signals from commensal bacterial communities can help establish an immunoregulatory environment, there is also evidence from human and mouse studies linking commensal bacteria to promotion of intestinal inflammatory diseases. As described in Section 1.2.1, GF and ABX-treated mouse studies have demonstrated the role for commensal bacteria in dictating the balance between proinflammatory T_H17 and regulatory T cells populating the GALT (Atarashi et al., 2008; Ivanov et al., 2008; Niess et al., 2008). Takeda and colleagues attributed this capability to bacterial-derived ATP preferentially inducing T_H17 $CD4^+$ T cells. ATP was shown to act on a population of $CD70^{high}$ $CD11c^{low}$ accessory cells that express P_2X receptors, a family of ATP-binding receptors. ATP stimulation induced these cells to express IL-6 and IL-23p19, cytokines that can promote T_H17 cell development and survival(Atarashi et al., 2008). In addition to ATP, several commensal bacterial species have been demonstrated to have proinflammatory properties. *Klebsiella pneumoniae* and *Proteus mirabilis* were identified by high-throughput sequence screening of intestinal bacterial communities from the *T-bet*$^{-/-}$ x *Rag2*$^{-/-}$ ulcerative colitis (TRUC) mouse model. These commensal bacterial species could elicit intestinal inflammation when transferred to wild type or *Rag2*$^{-/-}$ recipients (Garrett et al., 2010a). In another study, commensal bacterial species *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron* isolated from the intestine bacterial communities of a *Il10r2, Tgrbr2* double knockout mice, an IBD-susceptible mouse strain, induced spontaneous intestinal inflammation where as *Enterobacteriaceae* species identified from the same IBD-susceptible mice did not elicit onset of disease

(Bloom et al., 2011). While these studies have identified particular bacterial species that can elicit proinflammatory effects on the host, the specific bacterial products and mechanism of action by which these commensal bacterial species have proinflammatory effects remains poorly defined. This gap in knowledge highlights the need to identify specific bacterial signals and the immune pathways that are activated by commensal bacteria.

1.2.5 Commensal bacterial communities influence the systemic inflammatory immune response

The effect of commensal bacterial communities on the immune system go beyond the intestine and have been shown to influence immune responses and cell populations at peripheral sites. For example, GF mice exhibit diminished proinflammatory cytokine responses to subcutaneous LPS injection or ischemic perfusion (Amaral et al., 2008; Souza et al., 2004). In addition, mice depleted of commensal bacterial communities via ABX treatment exhibit an exacerbated, T_H2 cytokine mediated, allergic response to intranasal challenge OVA or house dust mite antigen (Hill et al., 2012; Noverr et al., 2005). Furthermore, it was demonstrated that commensal bacterial communities were responsible for ameliorating the severity of spontaneous diabetes in MyD88$^{-/-}$ NOD mice (Wen et al., 2008). In addition, peptidoglycans derived from commensal bacteria were demonstrated to stimulate recruitment of bone marrow-derived neutrophils to the peritoneal cavity through the NOD1 receptor and enhance phagocytosis of bacteria (Clarke et al., 2010). Many of the bacterial species and products identified in regulating intestinal immune homeostasis also influence systemic immune responses to autoimmune inflammatory diseases. SFB, which can promote T_H17 cell differentiation in the intestinal mucosa, can exacerbate T_H17 dependent peripheral autoimmune

inflammatory disease such as arthritis, and experimental autoimmune encephalomyelitis (a murine model for multiple sclerosis) (Kriegel et al., 2011; Wu et al., 2010b). In addition to regulating intestinal inflammation, signaling through commensal bacteria-derived SCFA, can limit inflammatory immune responses in murine models of arthritis and asthma (Maslowski et al., 2009). This body of literature has been crucial in demonstrating that the commensal bacterial communities can influence the immune response in multiple tissues.

The mechanism by which signals originating from the intestine have a systemic effect on the immune response remains unclear. One possible explanation is that bacterial products translocate across the intestinal epithelium and circulate throughout the host to stimulate the systemic immune system. In known incidences of bacterial translocation of commensal bacteria, mice exhibit elevated proinflammatory cytokines in the serum and spleen and increased expression of immune activation markers (Brenchley et al., 2006; Sonnenberg et al., 2012). Restifo and colleagues found that bacterial translocation of intestinal commensal bacteria had an adjuvant effect on the cytotoxic capability of tumor-specific CD8 T cells (Paulos et al., 2007). Combined, these studies suggest that signals derived from bacteria within the intestine can aid in immune activation throughout the host. Whether commensal bacteria-derived signals are influencing the systemic immune system at steady-state, either via basal bacterial translocation or an alternative mechanism has yet to be examined and will be the focus of **Chapter 4** of this thesis.

1.2.6 Commensal bacterial communities influence the immune response to infection

Commensal bacterial communities can also augment the immune response and aid in protective immunity against pathogenic infection. For example, SFB, described in

previous sections to exacerbate immune response in autoimmune inflammatory diseases, has a protective role following infection with a murine enteropathic bacterium, *Citrobacter rodentium* (Ivanov et al., 2009). Clearance of *C. rodentium*, which induces a robust T_H17 immune response (Mangan et al., 2006), was enhanced in mice colonized with SFB, a commensal bacteria that drives CD4 T_H17 cell differentiation (Ivanov et al., 2009). In addition, following selective depletion of commensal bacterial communities with metronidazole, mice are more susceptible to *Citrobacter* infection, further indicating a role for commensal bacteria in host immunity (Wlodarska et al., 2011). Belkaid and colleagues reported that mice depleted of commensal bacterial communities with antibiotics had diminished IFN-γ and IL-17 production following infection with the intestinal parasite *Encephalitozoon cunniculi*. This diminished immune response correlated with a higher parasite burden compared to wild type mice. In this report, oral administration of the specific TLR ligand CpG, but not LPS, was sufficient to recapitulate the immune response in the ABX-treated mice and resulted in parasite clearance in a TLR9 dependent manner (Hall et al., 2008). ABX-mediated depletion of commensal bacteria also revealed a critical role for commensal bacteria in protective immunity following intestinal infection with *Toxoplasma gondii,* via commensal bacteria driven stimulation of intestinal DCs (Benson et al., 2009). Studies conducted using another intestinal pathogen, *Salmonella enterica*, found that ABX-treatment increased host susceptibility to *Salmonella* infection, characterized by increased bacterial translocation and intestinal inflammation (Croswell et al., 2009; Garner et al., 2009; Sekirov et al., 2008). Susceptibility to infection-induced colitis persisted in mice for up to two weeks after termination of ABX treatment suggesting that enduring alterations in the composition of commensal bacterial communities may be responsible for the inability to

limit inflammation (Croswell et al., 2009). GF mice, which lack production of the commensal bacteria induced antimicrobial peptide RegγIII, also exhibit increased bacterial dissemination when orally challenged with *Salmonella typhimurium* (Vaishnava et al., 2008). In contrast to these studies demonstrating that commensal bacteria augment immunity to infection, commensal bacteria-derived signals can also suppress immune responses to the benefit of the pathogen. Two studies have demonstrated that enteric viral infections can use immunoregulatory signals from commensal bacteria to improve viral infectivity (Kane et al., 2011; Kuss et al., 2011). Combined, these reports establish an important role for commensal bacterial communities in influencing the immune response following intestinal bacterial, parasitic, or viral infection.

Few studies, however, have investigated the influence of commensal bacteria-derived signals on host immune responses to pathogens that infect tissues outside of the gastrointestinal tract. Early studies reported that GF mice were more susceptible to influenza A and coxsackie B viral infection (Dolowy and Muldoon, 1964; Schaffer et al., 1963). However, these studies were limited in the immunological and pathological parameters examined and failed to address the underlying mechanisms for increased susceptibility. More recently, Oliveira and colleagues found that GF Swiss/NIH mice infected with *Leishmania major* failed to heal skin lesions and had increased parasite burden at 13 weeks post-infection (Oliveira et al., 2005). Impaired control of *Leishmania* in GF mice was attributed to defective macrophage activation and killing of the parasite (Oliveira et al., 2005). GF mice were also found to have increased susceptibility to *Listeria monocytogenes* infection due to impaired T cell homing to sites of infection (Inagaki et al., 1996). Another study by Tanaka and colleagues reported that GF mice infected with murine cytomegalovirus had a reduced virus-specific CD8 T cell response

in the lung compared to conventionally-reared (CNV) mice despite comparable viral levels (Tanaka et al., 2007). As described in section 1.2.1, GF mice have impaired development of the immune system. Therefore, the underlying cause of defective immune responses in GF mice to infection may be an underdeveloped immune system. To account for this possibility, antibiotics have been employed prior to infection to allow for normal immune development before disruption of commensal bacteria-derived signaling. Iwasaki and colleagues reported that antibiotic-mediated manipulation of commensal bacteria resulted in impaired adaptive immune responses to viral infection in the lung (Ichinohe et al., 2011). This effect was associated with defective activation of the inflammasome, a pathway activated by bacterial products that is also important in conferring protective immunity against a subset of viruses, including influenza virus and poxviruses (Lamkanfi and Dixit, 2011). This study indicates that commensal bacterial communities aid in the induction of the immune response to a non-intestinal, mucosal-associated viral infection. Whether depletion of commensal bacteria selectively regulates inflammasome-dependent pathways or this observation represents broader immunological crosstalk mechanisms between commensal bacteria and other antiviral will be addressed in **Chapters 2** and **3**.

1.3 Immune responses to viral infection

Viruses consist of DNA or RNA encoding genes encapsulated by a protein and/or lipid coat to protect the genetic material (Alberts et al., 2002). A virus does not possess the capacity to replicate independently and therefore must gain entry into a cell to access the molecular machinery necessary to synthesize more copies of itself (Alberts et al., 2002). The biological goal of a virus upon infection is to replicate without detection by the

host organism. Viral replication can cause stress and potentially death to the host cell as resources are diverted to replication of the virus causing accumulation of viral copies and rupture of the host cell membrane (Alberts et al., 2002). Higher order organisms have developed an immune system to rapidly detect and clear the virus from the host or, as is the case in persistent viral infections, control replication and limit virus-induced tissue damage. The immune system accomplishes this goal through the coordinated response of multiple specialized cells of the innate and adaptive immune system. This section will discuss the mechanisms of viral detection and the cells involved in the antiviral immune response.

1.3.1 Recognition of viral pathogens by the immune system

Detection of a viral infection by the immune system is primarily accomplished by distinct PRRs that are expressed by immune and non-immune cells and recognize PAMPs associated with viruses. Viral PRRs initiate a cascade of signaling events that lead to transcription of a gene program specified for antiviral defense (Seth et al., 2006). A virus must gain entry into a cell to establish infection and correspondingly most of the PRRs that detect viruses are located in the cytosol or endosomal compartments. TLRs consist of a extracellular domain of leucine rich repeats and a cytoplasmic Toll/Interleukin-1 (TIR) signaling domain (Akira and Takeda, 2004). The leucine rich repeat region of the TLR contains the ligand binding domain of the receptor and provides specificity for PAMP recognition (Akira and Takeda, 2004). A subset of the TLRs (TLR 3,7,8, and 9) exhibit binding affinity for various viral derivatives and are expressed in the endosomal compartment of cells (Akira and Takeda, 2004). TLR3 recognizes double-stranded RNA and is expressed in most innate immune cells except of plasmacytoid DCs (pDCs) and neutrophils (Schroder and Bowie, 2005). Deficiency in TLR3 expression can lead to

decreased production of proinflammatory cytokines and has been shown to correlate with an impaired ability to suppress herpes simplex virus (Tabeta et al., 2004; Zhang et al., 2007). TLR7 and TLR8 share the same activating ligand, long single-stranded RNA, and both drive interferon alpha (IFN-α) production in pDCs (Heil et al., 2004; Lee et al., 2007). Unmethylated CpG DNA motifs, a common feature found in both bacterial and viral genomes are the ligands for TLR9, and, therefore, TLR9 has a critical role in detecting infection by DNA viruses (Hemmi et al., 2000). For example, infection of *Tlr9*$^{-/-}$ mice with a DNA virus such as murine cytomegalovirus results in diminished induction of type I IFNs and increased mortality compared to wild-type controls (Tabeta et al., 2004). These endosomal TLRs represent one group of the PRRs that detect viral infection.

Host cells have also evolved to detect viruses replicating in the cytoplasm with a family of cytosolic PRRs that sense intracellular nucleic acid. The two most well characterized cytoplasmic nucleic acid sensors of this recently described family are retinoic acid-inducible gene (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Thompson et al., 2011). Both of these proteins sense RNA in the cytoplasm, with RIG-I detecting 5'-triphosphorylated(Hornung et al., 2006; Pichlmair et al., 2006), uncapped single-strand RNA and MDA5 responding to long double-stranded RNA (Kato et al., 2006). Ligand binding to these two PRRs leads to recruitment of mitochondrial antiviral signaling protein (MAVS) followed by activation of NFκB and interferon response factor (IRF) 3 and 7, which bind to the *Ifn*β promoter and initiate transcription (Honda et al., 2005; Kawai et al., 2005). Mice with genetic deletion of either RIG-I or MDA5 exhibit increased susceptibility to several viral infections including paramyxoviruses, influenza virus, Japanese encephalitis virus, and picornavirus, demonstrating the *in vivo* relevance of these PRRs in mounting protective antiviral immunity (Kato et al., 2006). In addition to

RIG-I and MDA5, a growing list of RIG-I-like receptors have been identified and the importance of these receptors in antiviral defense are just starting to be understood (Thompson et al., 2011). Collectively, the main function of viral PRRs appears to be to detect virus inside a host cell and to rapidly initiate a gene program to limit viral replication and to trigger the immune system into action

1.3.2 Type I IFNs are critical initiators of antiviral immunity

The initial host cell response to viral infection leads to production of several proinflammatory factors including the type I IFN cytokine family. Type I IFNs, which were originally identified as a secreted factor that "interfered" with viral replication in culture (Isaacs and Lindenmann, 1957), are critical in defense against most viruses (Hwang et al., 1995; Muller et al., 1994). Type I IFNs coordinate the antiviral immune response in three distinct steps; i.) induction of a gene program to limit viral replication, ii.) activation innate immune cells, iii) drive CD4 and CD8 T cell differentiation and B cell antibody production (Figure 3).

The Type I IFNs family consists of IFN-β (a single gene) and IFN-α (gene with multiple isoforms) and are direct downstream targets of PRRs signaling, such as RIG-I, MDA5 and TLR3, as discussed in the previous section (Thompson et al., 2011). The cellular source of type I IFNs during a viral infection, in addition to the virus-infected cell itself, are innate immune cells such as pDCs and macrophages, which are stimulated via IFN signaling in a positive feedback manner to produce more type I IFNs (Jung et al., 2008; Kumagai et al., 2007). Production of type I IFNs from either of these cell types have been demonstrated to be critical in supporting the expansion of the antiviral immune response and control of viral infection (Jung et al., 2008; Kumagai et al., 2007). The type

I IFN receptor is composed of two subunits (IFNAR1 and IFNAR2) that are expressed ubiquitously in hematopoietic and non-hematopoietic cells and signal through Janus kinases prophorylation of the transcription factors STAT1 and STAT2 (Li et al., 1997). Phosphorylation of STAT1 and STAT2 results in formation of a heterodimer that associates with IRF9, forming a transcription factor complex (ISGF3), that translocates to the nucleus and initiates transcription of interferon response genes (IRG) (Trinchieri, 2010). Ablation of type I IFN signaling has lead to well characterized impairments in host antiviral immunity to multiple viruses, highlighting the ubiquitous need for these cytokines in defense against viral infection (Moskophidis et al., 1994; Muller et al., 1994).

Expression of IRGs following IFN signaling leads to induction of numerous defense mechanisms that make the cell refractory to viral replication. For example, IFN signaling leads to expression of a family of enzymes named 2'-5' oligoadenylate synthases (OAS) that activate RNase L, an enzyme that degrades host and viral RNA and therefore limits viral replication as well as produces dsRNA for RIG-I/MDA5-mediated recognition and subsequent production of type I IFNs (Malathi et al., 2007; Stark et al., 1998). Other IFN-inducible genes such as the Mx and PKR proteins have similar effects of inhibiting the replication process of the virus (Haller et al., 2007). Further, IFN signaling can cause infected cells to undergo apoptosis and therefore deny the virus access to the cellular machinery needed for self-replication (Stetson and Medzhitov, 2006). Programmed cell death via apoptosis also prevents viral-induced cell lysis and release of newly synthesized virus. Combined, these type I IFN driven mechanisms of prevention and containment represent the first line of defense against viruses.

1.3.3 Induction of the innate immune response to viral infection

In response to viral infection the innate immune system is activated by detection of cytokines produced by virally infected host cells or cellular debris caused by virus-induced cell death. Innate immune cell types consist of macrophage, DCs, pDCs, neutrophils, basophils, eosinophils, mast cells, NK cells, and innate lymphoid cells. These cells exhibit diverse characteristics and functions in response to distinct classes of pathogens. The broad function of innate immune cells is to be early responders to an invasive pathogen and coordinate induction of a protective adaptive immune response. Macrophages and DCs are two innate cell types that are critical antiviral defense and induction of the adaptive immune system.

Macrophages, crucial sentinel cells of the innate immune system, rapidly respond to type I IFNs secreted by virally-infected cells by increasing phagocytic activity and releasing nitric oxides into the local environment which are capable of limiting viral replication (Fujimoto et al., 2000; Guidotti et al., 2000; Saura et al., 1999). In addition, macrophages produce an array of proinflammatory chemokines and cytokines that lead to the recruitment of other effector immune cells and create a proinflammatory cytokine milieu to shape the immune response (Guidotti and Chisari, 2001; Peschke et al., 1993). Consistently, studies using macrophage-deficient mice or macrophage depletion techniques have reported impaired viral clearance and diminished induction of the adaptive immune response demonstrating the importance of this innate immune cell following viral infection (Kim et al., 2008; Kumagai et al., 2007; Seiler et al., 1997).

In addition to macrophages, DCs are another innate immune cell population critical in the propagation of the antiviral immune response. The main purpose of DCs is to function as antigen presenting cells (APCs) that prime the adaptive immune response.

DCs specialize in the uptake and processing of antigens into short peptide segments that are packaged on major histocompatability complex (MHC) class I and II molecules and presented to T cells (Bevan, 2006; Yewdell and Haeryfar, 2005). DC exposure to proinflammatory cytokines and/or PRR stimuli creates a feedforward loop, inducing DCs to produce more proinflammatory chemokines and cytokines, increase antigen uptake, and upregulate costimulatory molecules such as CD80, CD86 and CD40 (Kato et al., 2006; Masson et al., 2008; West et al., 2004). DCs migrating from the site of infection to the draining lymph nodes act in concert with lymphoid resident DCs to present antigen on MHC class I and II to naïve CD8 and CD 4 T cells (Allenspach et al., 2008; Yewdell and Haeryfar, 2005). Costimulatory molecule interactions and proinflammatory cytokine stimulation provide a critical second and third stimulatory signals, along with the initial T cell receptor-MHC-peptide engagement, to prime naïve T cells and drive T cell differentiation (Masson et al., 2008). DCs either remain at the site of infection to stimulate and direct newly recruited innate and adaptive immune cells or migrate to draining lymph nodes to present viral antigen to naïve CD4 and CD8 T cells leading to initiation of the adaptive immune response (McGill et al., 2009).

1.3.4 Induction of the adaptive immune response to viral infection

When the initial antiviral mechanisms of the innate immune system are insufficient to control viral infection the adaptive arm of the immune provides crucial cell-mediated (T cells) and humoral (antibody producing B cells) immune responses to control viral infection. The importance of the adaptive immune response has been demonstrated in several models of viral infection where mice lacking an adaptive immune system can not control infection and succumb to the viral infection (Franco and Greenberg, 1995;

Palladino et al., 1995; Xu et al., 2004). Three cell types comprise the adaptive immune response; Cytotoxic CD8 T cells, CD4 T helper cells and B cells. All of these cell types express antigen specific surface receptors, the T cell receptor (TCR) or B cell receptor (BCR), that enable the adaptive immune response to target effector responses against specific pathogens. Upon stimulation of the TCR or BCR with their cognate antigen CD4, CD8 T cells and B cells undergo rapid clonal expansion, creating a pool of effector cells (Hugues et al., 2004). Effector functions include direct cell lysis of virus-infected cells, production of antiviral effector cytokines, and production of antibodies. Expansion of the adaptive immune response corresponds with viral clearance and is followed by a contraction of the effector cells and establishment of a pool of memory cells (Figure 4). Memory CD4, CD8 T cells and B cells establish long-lasting immunity and prevent recurrent infections with the same virus (Harrington et al., 2008; Harty and Badovinac, 2008; Slifka et al., 1998).

During a viral infection, in addition to MHC/TCR binding and costimulatory molecule interaction, CD4 T helper cells receive a third signal from proinflammatory cytokines, such as type I IFNs and IL-12, that activates a gene program that directs the differentiation of the CD4 T cell into a T_H1 phenotype (Whitmire, 2011). T_H1 cells act to coordinate the adaptive and innate immune response against the virus by producing cytokines such as IFN-γ and IL-2 that reinforce the antiviral response and aid in T cell proliferation (Williams and Bevan, 2007). T_H1 cells also support B cell antibody production and isotype class switching through CD40-CD40L interactions (Bishop and Hostager, 2001). These functions are critical for the antiviral response as loss of the CD4 T cell responses severely impairs the size and quality of the adaptive immune

response and can lead to persisting viral infection and host mortality (Leist et al., 1989; Louten et al., 2006; Xu et al., 2004).

Cytotoxic CD8 T cells limit viral infection by directly targeting and eliminating virally-infected cells or producing antiviral cytokines. CD8 T cell-mediated cell lysis can occur via ligand mediated cell death (Fas/FasL interaction) (Topham et al., 1997) (Trapani and Smyth, 2002) or production and release of cytotoxic lytic granules, such as perforin and granzyme, at the interface of the CD8 T cell and target cell (Hou and Doherty, 1995; Peters et al., 1991). CD8 T cells also produce of effector cytokines IFN-γ and TNF-α that can directly induce cell programmed death or stimulate surrounding innate cells, such as macrophages and NK cells, to increase cytolytic activity (Kaech et al., 2002). Studies in mice deficient of CD8 T cells have reported impaired viral clearance and decreased host survival demonstrating the essential role of the CD8 T cell response has in protective antiviral immunity (Fung-Leung et al., 1991; Qi et al., 2006).

Through the BCR, B cells can detect virus as soluble antigens in the lymphatics or in the virus' native form on the surface of macrophages or DCs (Depoil et al., 2008; Junt et al., 2007; Qi et al., 2006). Endocytosis of antigen through BCR binding, leads to antigen processing and presentation on MHC-II molecules allowing B cells function as an addition APC driving T cell proliferation (Crawford et al., 2006). In addition, B cells secrete virus-specific antibodies that can either neutralize free virus or bind to a virally-infected cell and target it for antibody dependent cell-mediated cytotoxicity. The importance of the B cell response during primary viral infection varies for different viruses, but in general, the primary B cell response aids in the latter stages of viral clearance (Franco and Greenberg, 1995; Graham and Braciale, 1997; Palladino et al., 1995). Following primary infection, germinal center reactions and B cell affinity

maturation leads to the differentiation of long lived plasma cells that secrete high affinity antibodies into circulation and are highly effective at preventing secondary infection (Franco and Greenberg, 1995; Tarlinton, 2006). Combined the cells of the adaptive immune system direct a targeted response to control viral infection in the host.

1.4 Two models of viral infections: mucosal-associated influenza virus and systemic Lymphocytic Choriomeningitis virus

1.4.1 Influenza virus and pathogenicity

Seasonal influenza virus infection results in a significant burden on the health system causing approximately 200,000 hospitalizations and 36,000 deaths in the United States annually (Thompson et al., 2003). The most susceptible populations are infants, the elderly, or individuals with pre-existing health concerns (Thompson et al., 2003). However, influenza pandemics, caused by introduction of a new subtype of influenza virus into the population can cause death in otherwise healthy adults, as was the case in 1918 with the Spanish flu epidemic or the more recent H5N1 highly pathogenic avian influenza virus, (Taubenberger and Morens, 2008; Webster and Govorkova, 2006). Inflammation and tissue damage resulting from influenza virus infection is predominantly localized to the lung and is characterized by pulmonary hemorrhaging and edema, inflammatory infiltrates, and necrotizing bronchitis (Taubenberger and Morens, 2008). Mouse-adapted influenza viral strains mimic disease pathogenesis in humans and provide an excellent model to study the immune response to the virus and formation of protective immunity (Kuiken et al., 2010). The murine viral infection model will be used in **Chapter 2** to study virus-induced pathology and immune responses to a local, non-

intestinal, mucosal-associated viral infection following disruption of commensal bacterial communities.

Influenza A virus is an enveloped, single-stranded RNA virus member of the *Orthomyxoviridae* family (Cheung and Poon, 2007). The virus is composed of eight RNA gene segments that encode ten proteins, two of these proteins, hemagglutinin (HA) and neuraminidase (NA), are expressed on the viral capsid and are highly immunogenic epitopes that serve as direct targets for neutralizing antibodies (Cheung and Poon, 2007). Influenza virus primarily infects respiratory epithelial cells but can also infect alveolar macrophages and other immune cells that are recruited to the site of infection causing cell death by cytolysis or apoptosis within forty hours of infection (Julkunen et al., 2001). Severity of the influenza virus infection depends on a combination of host-derived and viral-specific factors. Multiple influenza virus strains have been adopted for the murine model and cause different degrees of pathology. In this thesis the H1N1 influenza A/Puerto Rico/8/34 strain (PR8) and the A/X-31 H3N2 strain (X31) will be employed. In immunocompetent mice, infection with a low dose of PR8 virus (~300 $TCID_{50}$) causes significant morbidity and lung tissue damage lasting 1-2 weeks. X31 viral infection, even when administered at a much higher dose (~$1x10^5$ $TCID_{50}$), is cleared from the mouse at a quicker rate and causes substantially less morbidity (Bouvier and Lowen, 2010; Decman et al., 2010)(personal observations). Morbidity can be monitored by weight loss and/or blood oxygen saturation levels, enabling non-invasive measures of disease progression (Verhoeven et al., 2009). These two influenza virus strains will be used in **Chapter 2** to assess the protective capacity of the antiviral immune response.

1.4.2 The immune response to influenza virus infection

Successful clearance of influenza virus infection requires a coordinated innate and adaptive immune response. Defects in PRR expression (Ichinohe et al., 2011; Kato et al., 2006), the IFN signaling pathway (Mordstein et al., 2010; Price et al., 2000), DC (McGill et al., 2008), macrophage (El Bakkouri et al., 2011; Kumagai et al., 2007), CD8 T cells (Epstein et al., 1993) and B cells (Graham and Braciale, 1997) all lead to increased host mortality following challenge with influenza virus demonstrating the importance of each arm of the immune system in controlling influenza virus infection. Infected respiratory epithelial cells initiate the immune response by producing type I IFNs and chemokines to recruit and activate innate immune cells into the lung (Julkunen et al., 2001; Sanders et al., 2011). Resident alveolar macrophages and DCs are activated by IFN stimulation and produce proinflammatory factors to increase the magnitude of the response (McGill et al., 2009). Neutrophils, NK cells and inflammatory monocytes are recruited to the lung and in combination with alveolar macrophages clear virally-infected cells and cellular debris (Hermesh et al., 2010; Tate et al., 2009; Tumpey et al., 2005). Meanwhile, DCs migrate to the draining mediastinal lymph nodes to present antigen to naïve CD4 and CD8 T cells to initiate the adaptive immune response (Belz et al., 2004; Kim and Braciale, 2009). Following clonal expansion, virus-specific T cells home to the lung starting around 6-7 days post-infection and produce effector cytokines and induce apoptosis via secretion of lytic granules and Fas/FasL interactions (Topham et al., 1997). Induction and recruitment of the adaptive immune response rapidly leads to viral clearance by approximately day 10-12 post-infection (Kohlmeier and Woodland, 2009). However, if the immune response is delayed or defective, influenza virus infection can cause severe damage to the lung tissue, disrupting gas exchange that can potentially be

fatal. This fine balance highlights the critical nature for a rapid and robust immune response to viral infection.

1.4.3 Lymphocytic Choriomeningitis virus and pathogenicity

Lymphocytic Choriomeningitis Virus (LCMV) is an enveloped, single-stranded RNA virus belonging to the *Arenaviridae* family (Oldstone, 2002a). Unlike influenza virus, LCMV is a natural murine pathogen that results in a systemic infection with virus circulating through the blood to infect multiple tissues of the host (Ahmed et al., 1987; Sydora et al., 1996). As a non-cytolytic virus, most of the inflammation and tissue damage associated with LCMV infection is induced by the cytotoxic effects of the inflammatory immune response (Khanolkar et al., 2002). LCMV gains access to a cell through binding with the α-dystroglycan receptor, a cell surface receptor for proteins of the extracellular matrix that is ubiquitously expressed in multiple tissues (Cao et al., 1998; Rojek et al., 2007). The cell tropism and replicative capacity of LCMV varies from between strains. For example, the Armstrong (Arm) strain of LCMV establishes an acute infection that is rapidly controlled and cleared by the host within a week (Ahmed et al., 1984a; Ahmed et al., 1984b). On the other end of the spectrum the clone 13 strain (cl-13), which carries two point mutations increasing α-dystroglycan receptor affinity and viral polymerase activity (Cao et al., 1998; Matloubian et al., 1993), exhibits increased macrophage-tropism and results in prolonged viremia and persisting virus in immunoprivileged organs such as the kidney and central nervous system (Ahmed et al., 1984b; King et al., 1990). The LCMV strain used in this thesis, T1b, exhibits a lymphocyte-tropism and results viremia lasting approximately 2 weeks (Blackburn et al., 2009; King et al., 1990). The intermediate phenotype of this LCMV strain can be exploited to identify defects in

antiviral immunity that might otherwise be unobserved with an acute or chronic LCMV strain. Characterization of the immune response to LCMV infection has led to the development of molecular tools, such as identification of virus-specific T cells by MHC-tetramer staining, which enable a comprehensive analysis of the antiviral immune response (Altman et al., 1996) (Oldstone, 2002b). These studies have greatly improved our understanding of the innate and adaptive antiviral immune response and the tools developed in the LCMV infection system will be employed in **Chapter 3** to address the influence of commensal bacterial communities on the immune response to a systemic viral infection.

1.4.4 The immune response to Lymphocytic Choriomeningitis virus

The immune response to LCMV has been extensively characterized and has served as a prototypic model to study the immune response to viral infection. Because LCMV infects multiple organs of the host, there is not one anatomical location that recruits activated immune cells as is the case in influenza virus infection of the lung. Instead activated effector cells of the innate and adaptive immune system can be found in multiple organs.

Similar to other viral infections, LCMV is capable of stimulating PRRs RIG-I, MDA5, TLR3, 7, and 9 to induce an early burst of type I IFN production that initiate the immune response (Lee et al., 2009; Zhou et al., 2010). However, signaling via TLR7 and 9, particularly in plasmacytoid DCs, is the predominant source of type I IFN production immediately following LCMV infection. (Jung et al., 2008; Montoya et al., 2005). Disruption of TLR signaling via genetic knockout of the TLR signaling protein MyD88, results in significantly decreased type I IFN levels in the serum and diminished innate

and adaptive immune response leading to persisting viral titers in the host (Chen et al., 2005; Jung et al., 2008; Kolumam et al., 2005; Zhou et al., 2005). DCs and macrophage have separate but critical roles in initiating a protective immune response. As early as 48 hours post-infection, splenic DCs upregulate costimulatory markers CD80, CD86, and CD40 and are producing proinflammatory cytokines to direct T cell expansion and differentiation (Belz et al., 2005; Montoya et al., 2005). Deletion of DCs results in a diminished LCMV-specific CD8 T cell population demonstrating that DCs are the predominant APC in LCMV infection (Probst and van den Broek, 2005). Macrophages, stimulated by IFN signaling, increase phagocytic activity and are important in limiting spread of viral infection (Lang et al., 2010). Macrophage activation at this early stage of infection before the adaptive immune response has been primed is a critical determinant in whether the virus is ultimately cleared (Lang et al., 2010; Oehen et al., 2002; Seiler et al., 1997).

LCMV infection induces both a cell-mediated and humoral arms of the adaptive immune system, but the T cell response, particularly the CD8 T cell response has been the more extensively studied and characterized (Hangartner et al., 2006; Khanolkar et al., 2002; McDermott and Varga, 2011). The virus-specific CD8 T cell response robustly expands and migrates to infected tissue, aiding in viral clearance through cell-mediated cytotoxicity and production of effector cytokines such as IFN-γ and TNF-α (Ahmed et al., 1984a; Khanolkar et al., 2002). Control of acute LCMV infection is dependent on the CD8 T cell response as loss of these cells or ablation of their effector mechanisms results in persisting viral infection (Fung-Leung et al., 1991; Intlekofer et al., 2008; Kagi et al., 1995). Infection with chronic strains of LCMV lead to defective CD8 T cell responses, termed CD8 T cell exhaustion, that is characterized by upregulation of

inhibitory receptors and progressive loss of effector function (Blackburn et al., 2009; Wherry, 2011; Wherry et al., 2003; Zajac et al., 1998). Because of the tight link between the LCMV-specific CD8 T cell response and viral progression, characterizing the quality of the CD8 T cell response is an excellent indicator of the overall ability of the host to respond to and control viral infection (Fuller et al., 2004; Wherry et al., 2005). As such, it will be used in **Chapter 3** of thesis to assess the quality of the antiviral immune response to a systemic viral infection following depletion of commensal bacterial communities.

1.5 Outline of Thesis

It is increasingly apparent that the commensal bacterial communities can shape immune cell development and function. Commensal bacterial communities have been demonstrated to have either an adjuvant or immunoregulatory effect in the context of intestinal infection. The focus of this thesis will be to explore the influence of commensal bacterial communities on antiviral immunity to non-intestinal viral infections. Through use antibiotic-mediated disruption of commensal bacterial communities or GF mice, **Chapter 2** will address whether commensal bacterial communities aid in induction of a host protective immune response following infection with influenza virus. The impact of commensal bacteria-derived signals on immunity to a systemic viral infection will be investigated in **Chapter 3** through probing the innate and adaptive immune response to LCMV following depletion of commensal bacterial communities. Lastly, **Chapter 4** will examine the fundamental role for commensal bacteria-derived signals in calibrating the activation threshold of the innate immune system required for immunity to viral infection.

Figure 1. Composition of intestinal bacterial communities.

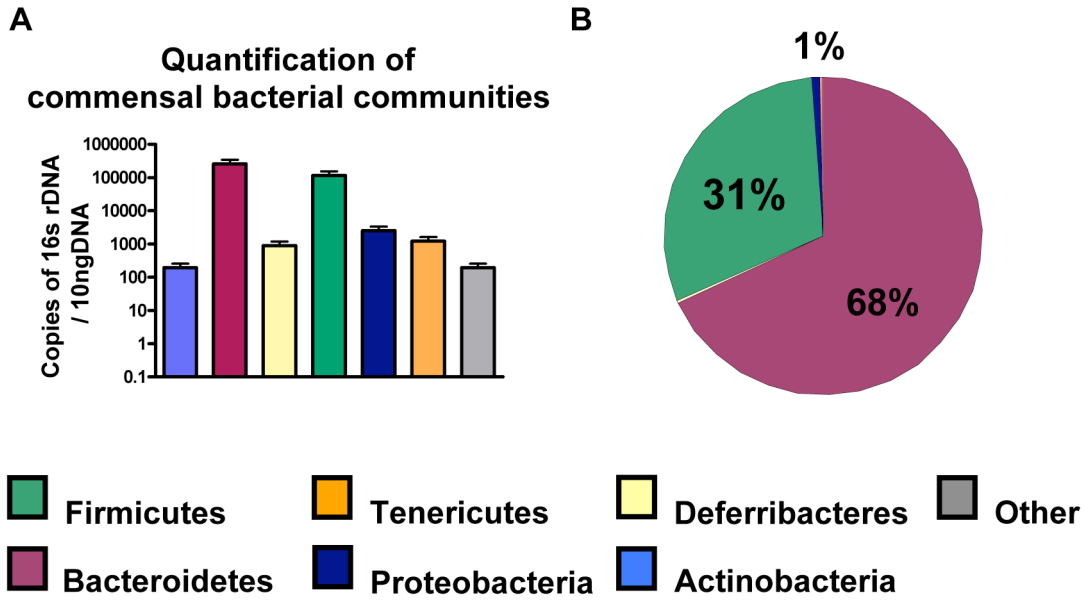


Figure 1. (A) Quantification of bacterial phyla based on total bacterial 16S rDNA in stool samples isolated from naïve, conventionally-reared, C57BL/6 mice. **(B)** Phylum level classification of intestinal bacterial communities of naïve C57BL/6 mice based on 16S rDNA frequencies in stool sample. (Adapted from (Hill et al., 2010)).

Figure 2. Commensal bacteria-derived signals regulate intestinal immune homeostasis.

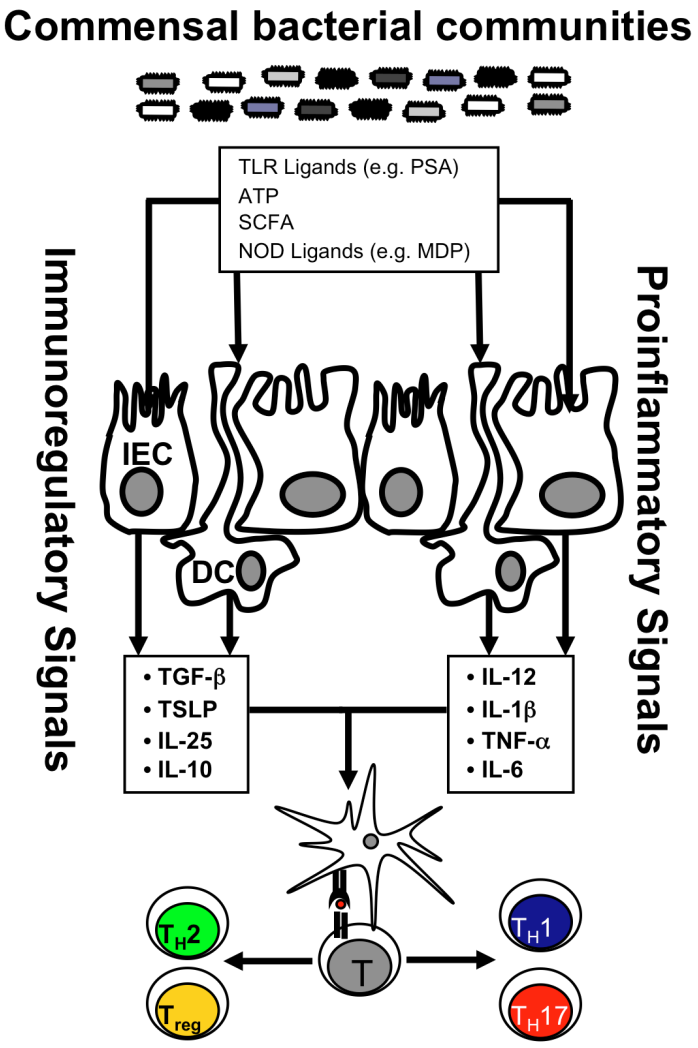


Figure 2. Specific signals, derived from intestinal bacterial communities, stimulate different cell types within the intestine (e.g. DCs and IECs). Commensal bacteria-derived signals can influence DCs and IECs to induce either an immunoregulatory or proinflammatory cytokine environment. This cytokine mileu of the intestinal microenvironment can condition antigen presenting cells and influence $CD4^+$ T cell differentiation within the intestinal mucosa.

Figure 3. Type I Interferons initiate multiple phases of host antiviral defense.

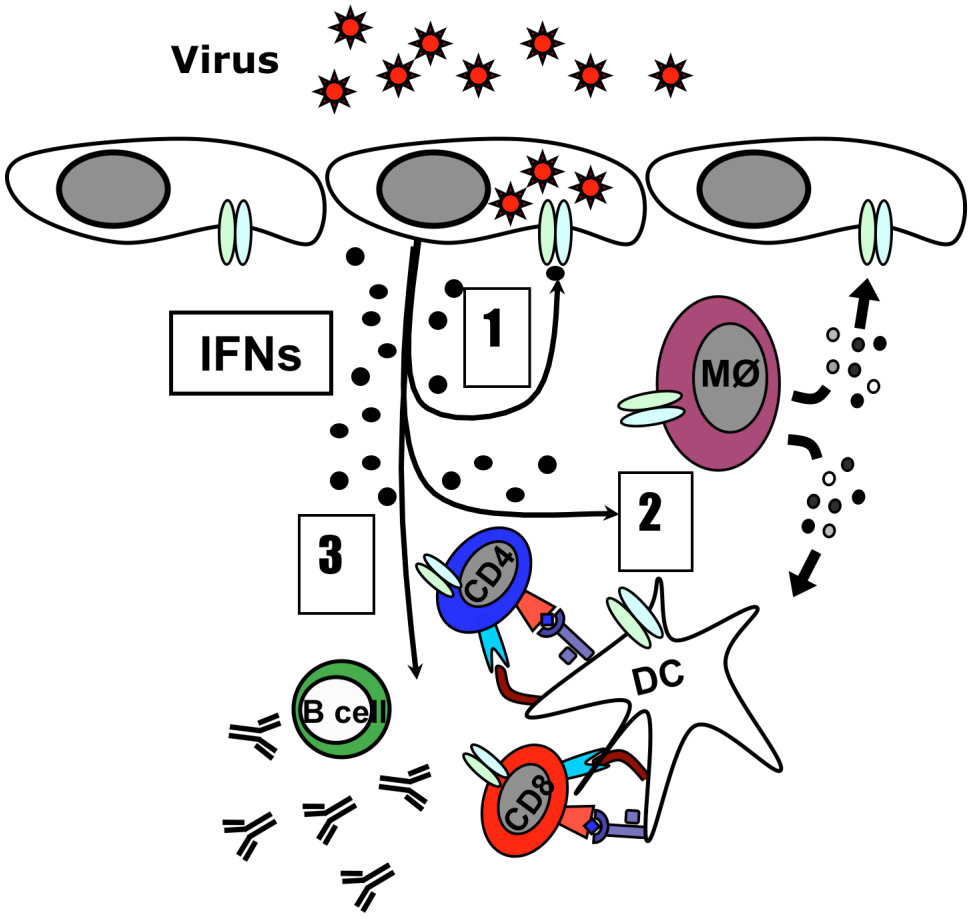


Figure 3. Following viral infection of a cell, PRR signaling initiates Type I IFN production (IFN-α/β), which shapes the host antiviral response in three phases. **(1)** Type I IFNs act in an autocrine or paracrine manner to induce transcription of interferon response genes (IRGs) that limit viral replication. **(2)** Sentinel innate immune cells, such as macrophages and DCs, are stimulated by Type I IFN to produce proinflammatory cytokines, increase antigen processing and presentation, and upregulate costimulatory molecules. **(3)** Type I IFN stimulation contributes to CD4 and CD8 T cell expansion and B cell antibody prodution.

Figure 4. Kinetics of the adaptive immune response to viral infection.

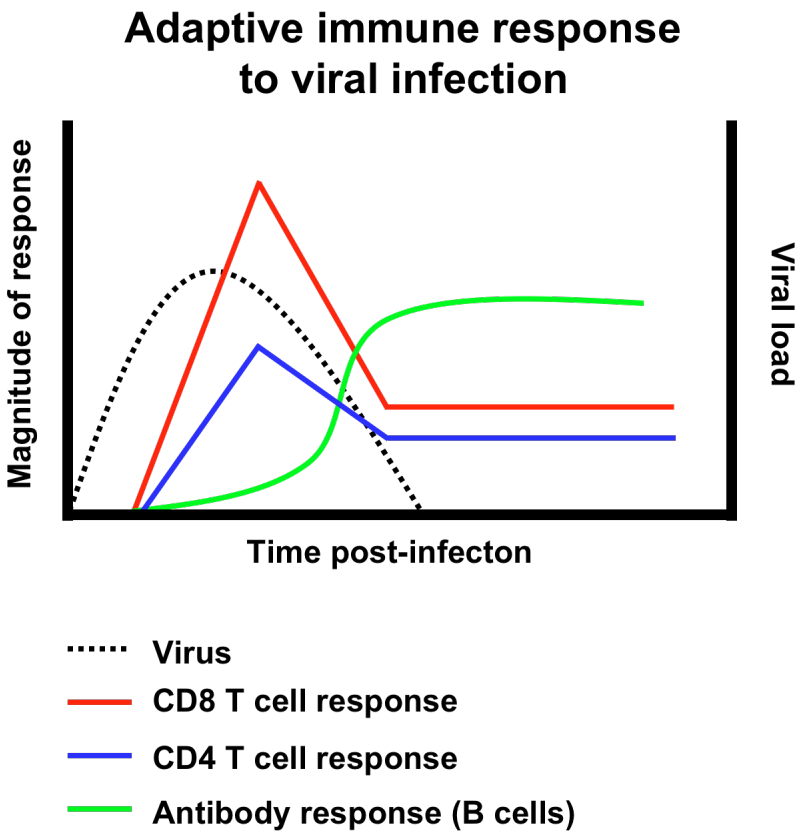


Figure 4. The cell-mediated (CD4 and CD8 T cells) and humoral (B cells) arms of the adaptive immune response expand in response to viral infection. Following control of viral infection, memory populations of all three adaptive immune cell types are established and prevent recurrent infection.

Chapter 2
Loss of commensal bacterial communities results in impaired immunity to influenza virus infection in the lung

2.1 Abstract

Commensal bacterial communities regulate intestinal immune cell development and differentiation. Furthermore, commensal bacteria-derived signals can act as an adjuvant in augmenting the immune response against intestinal infections. However, whether alterations in commensal bacterial communities can influence host protective immunity to viral infection in tissues outside of the intestinal tract remains poorly defined. In this chapter, I identify a role for commensal bacteria-derived signals in promoting protective antiviral immunity following influenza virus infection. Antibiotic (ABX)-treated mice challenged with two different strains of influenza A virus (PR8 or X31) exhibited impaired lung function and severe bronchiole epithelial degeneration following infection, resulting in increased host mortality. ABX-treated mice exhibited a diminished adaptive immune response that correlated with elevated viral titers in the lung. Further, ABX-treated mice exhibited a defective innate immune response characterized by decreased production of proinflammatory cytokines and chemokines in the lung and reduced expression of IFN-induced antiviral defense genes in alveolar macrophages at day 3 post-infection. Restoration of IFN responsiveness in ABX-treated mice re-established protective antiviral immunity after influenza virus infection. Taken together, these data indicate that signals derived from commensal bacterial communities regulate the innate and adaptive immune response that is critical for host survival following respiratory viral infection.

2.2 Introduction

Commensal microbial communities that colonize the barrier surfaces of the skin, vaginal, upper respiratory, and gastrointestinal tract of mammals consist of parasites, bacteria, fungi and viruses (Breitbart et al., 2003; Ley et al., 2006a; Scupham et al., 2006). The largest and most diverse microbial communities reside within the intestine where non-culture based methods have identified an estimated 15,000-36,000 individual bacterial species (Eckburg et al., 2005; Frank et al., 2007; Wang et al., 2003). These commensal bacteria have multiple beneficial properties ranging from aiding in metabolism to competing with invasive pathogens to occupy an environmental niche (Hooper and Gordon, 2001; Macpherson and Harris, 2004; Sonnenburg et al., 2006). Recent studies in patients have identified that alterations in the composition of bacterial communities are associated with susceptibility to diabetes, obesity, cancer, inflammatory bowl disease (IBD), allergy and other atopic disorders, highlighting the potential impact of host-commensal interactions in influencing multiple metabolic and chronic inflammatory diseases (Ley et al., 2006b; Manichanh et al., 2006; Moore and Moore, 1995; Penders et al., 2007a; Penders et al., 2007b).

Studies utilizing gnotobiotic (germ-free), antibiotic-treated or selectively colonized mice have demonstrated that the absence or alterations in composition of commensal bacterial communities results in impaired lymphoid tissue development, dysregulated immune cell homeostasis and altered susceptibility to multiple infectious or inflammatory diseases in the gastrointestinal tract (Amaral et al., 2008; Herbst et al.; Smith et al., 2007; Souza et al., 2004). For example, Mazmanian and colleagues identified a zwitterionic polysaccharide (PSA) isolated from *Bacteroides fragilis* that ameliorated intestinal inflammation in two murine models of IBD (Mazmanian et al., 2008). In contrast

colonization of the intestine with Segmented Filamentous Bacteria (SFB), is associated with increased frequencies of $CD4^+$ T_H17 cells in the lamina propria of mice, a proinflammatory subset of CD4 T cells. (Ivanov et al., 2009; Ivanov et al., 2008). Consistent with a proinflammatory role, signals derived from commensal bacteria can act as an adjuvant, augmenting immune responses following parasitic or bacterial infections of the intestine (Benson et al., 2009; Hall et al., 2008; Ivanov et al., 2009). Thus, depending on the context, commensal bacteria-derived signals are capable of dampening or exacerbating inflammatory immune responses in the intestinal microenvironment. However, the role of commensal bacteria-derived signals in augmenting the immune response to infection in tissues distal to the gastrointestinal tract remains largely unexplored.

The immune response to respiratory viral infections must efficiently clear virus from the lung tissue while limiting tissue damage to this important barrier surface. Proper regulation of this delicate balance is vital to host survival and requires rapid induction of the antiviral immune response followed by prompt cessation of effector mechanisms upon control of the pathogen. It is unknown whether signals derived from commensal bacterial communities contribute to modulating the immune response to respiratory viral infection in a manner similar to what has been observed during intestinal infections. To address this question, the commensal bacterial communities of mice were deliberately manipulated by antibiotic treatment and challenged with influenza virus.

Influenza virus is a cytolytic virus that infects the epithelial cells of the respiratory tract leading to severe lung pathology (Julkunen et al., 2001). Host protection is dependent on a coordinated innate and adaptive immune response to control viral replication (Kohlmeier and Woodland, 2009; McGill et al., 2009). Following alteration or absence of

commensal bacterial communities, however, influenza virus-infected mice were unable to clear the virus, exhibited severe degeneration of the bronchiole epithelial layer and succumbed to infection. These antibiotic-treated (ABX) mice exhibited an impaired innate and adaptive immune response indicating that commensal bacteria-derived signals are important in augmenting the immune response to viral infection of the respiratory tract.

2.3 Methods

2.3.1 Mice and viruses

C57BL/6 mice (4-6 weeks old) were purchased from the National Cancer Institute (Frederick, MD). Mice were maintained in specific pathogen–free facilities at the University of Pennsylvania. GF C57BL/6 mice were maintained in plastic isolator units and fed autoclaved chow and water. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved all protocols, and all experiments were performed according to the guidelines of the University of Pennsylvania IACUC.

Mice were inoculated intranasally (i.n.) with recombinant influenza viruses expressing the LCMV GP33 (PR8-GP33: 368 $TCID_{50}$, X31-GP33: $1x10^5$ $TCID_{50}$). Recombinant influenza virus strains containing the LCMV GP33–41 epitope inserted in the neuraminidase stalk region were obtained from Dr. Richard J. Webby (St. Jude Children's Research Hospital, Memphis, TN), propagated in eggs, and stored at -80°C. The replication and pathogenicity of these recombinant strains of X31 and PR8 were not substantially different from their non-recombinant counterparts (data not shown). Prior to i.n. infections, mice were anesthetized by intraperitoneal (i.p.) injection of 0.2 mL of

Avertin (2-2-2 Tribromoethanol dissolved in tert-amyl alcohol (Sigma-Aldrich); working stock 20 mg/mL diluted in PBS).

2.3.2 Oral antibiotic treatment

Mice were provided autoclaved drinking water supplemented with ampicillin (0.5 mg/mL, Sigma), gentamicin (0.5 mg/mL, Gemini Bio-Products), metronidazole (0.5 mg/mL Sigma), neomycin (0.5 mg/mL, Med-Pharmex), vancomycin (0.25 mg/mL, Novaplus), and sucralose (4mg/mL, Splenda, McNeil Nutritionals, LLC). Splenda was added to make the antibiotic cocktail more palatable. Antibiotic treatment was started 2-4 weeks prior to infection and continued for the duration of the experiment.

2.3.3 16s rDNA isolation and tracheal bacterial cultures

Stool pellets were collected 2 weeks following antibiotic treatment and total DNA was extracted using QIAamp DNA stool Mini Kit (Qiagen). Quantification of 16S rDNA was performed by qRT-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).

Trachea tissue segment was isolated under sterile conditions with particular attention to ensuring the trachea sample was separated from the esophagus. An approximate 2-3 cm portion of the trachea from the bottom of the larynx to where the trachea splits into the bronchioli was removed and mechanically homogenized in PBS. The resultant homogenate was plated by serial dilution on LB agar plates and grown under aerobic or anaerobic conditions for 48 hours.

2.3.4 Pulse oximetry

Mice were anesthetized with Avertin then neck and throat hair was removed with a chemical depilatory agent (Nair, Church & Dwight Co.) one day prior to infection. MouseOx Tm Pulse-Oximeter neck sensors (Starr Life Sciences, Oakmount, PA) were placed on exposed skin and blood oxygen saturation was recorded with Starr MouseOx software v. 5.1. Pulse oximetry readings were allowed to stabilize and fifteen seconds of measurements were averaged.

2.3.5 Isolation of cells from the lung parenchyma and bronchiole alveolar lavage fluid

Bronchoalveolar lavage cells (BAL) were obtained by inflating the lung with PBS via the tracheal cannula and recovering the PBS wash. This was repeated 2x to obtain increased cellular yield. The lung was perfused by cutting the hepatic vein and injecting PBS into the right ventricle. The excised lung was cut into small pieces and digested with 1 mg/mL collagenase D (Roche) and 20 µg/mL DNase Type IV (Sigma-Aldrich) in complete media (DMEM supplemented w/ 10% FBS, 10µg/mL penicillin/streptomycin, 10mM HEPES, 0.5mM β-mercaptoethanol, 10µg/mL L-glutamine, 5mM sodium pyruvate) for 30 min at 37°C under constant agitation. Single cell suspensions were obtained by mechanical disruption through 70µm cell strainers followed by red blood cell (RBC) lysis.

2.3.6 Flow cytometry, cell sorting, tetramer, and intracellular cytokine staining

Single cell suspensions were surface stained in FACS Buffer (PBS, 2% BSA, 0.2mg Sodium Azide, 2mM EDTA) using standard flow cytometric staining protocol with

fluorescently conjugated antibodies specific to CD3ε, CD4, CD8α, CD11c, CD19, CD25, CD43, CD45, CD69, CD160, F4/80, Ly6c, MHC-I (H-2K^b), MHC-II (I-A/I-E) (eBioscience), CD5, CD44, CD80, CD86, Ly6g (clone 1A8), ICOS, LAG-3, PD-1 (clone RMP1-30) (Biolegend), CD40 (BD Biosciences), CXCR3 (R&D Systems), 2B4 (BD Pharmingen), CD11b, and granzyme B (Invitrogen). Major histocompatibility complex (MHC) class I peptide tetramers were made and used to identify virus-specific CD8 T cells (Altman et al., 1996). Alveolar macrophages were identified as Non-T, Non-B, Non-NK cells (NTNBNNK), CD11c$^+$, F4/80$^+$. Inflammatory monocytes were identified as NTNBNNK, CD11c$^-$, F4/80$^-$, Ly6g$^-$, Ly6c$^+$, CD11b$^+$. Neutrophils were identified as NTNBNNK, CD11c$^-$, F4/80$^-$, Ly6g$^+$, CD11b$^+$. Dendritic cells were identified as NTNBNNK, F4/80$^-$, CD11c$^+$, MHC-II^{hi}. Plasmacytoid dendritic cells were identified as NTNBNNK, non-alveolar macrophage, non-inflammatory monocyte, non-neutrophil, CD11c^{int}, PDCA-1$^+$. Alveolar macrophages from naïve or d3 PR8-gp33 virus infected CNV or ABX-treated mice were sorted directly into TRIzol LS (Invitrogen) using a BD Aria (Beckson Dickson). Additional macrophages from the sample were separately sorted to ensure sort purity (≥95%). For intracellular cytokine analysis, 10^6 splenocytes or 10^5 cells isolated from the lung were cultured in a 96-well plate in complete media with viral peptides (0.2 μg/ml) and brefeldin A (Golgiplug, eBioscience) for 5 hrs at 37°C. After staining for surface antigens as described above, cells were stained for intracellular cytokines using the Cytofix/Cytoperm kit (BD/Pharmingen) and fluorescently conjugated antibodies specific for IL-2 (eBioscience), IFN-γ (BD Pharmingen), TNF-α (Biolegend), MIP-1α (R&D Systems). The CD107a assay was performed as previously described (Blackburn et al., 2009). Cell viability was assessed with Live/Dead AQUA stain (Invitrogen). Samples were collected by using a LSR II flow cytometer (Becton

Dickinson). All flow cytometry data was analyzed by FlowJo v 8.8 (Treestar). Pie charts were created using the Pestle and SPICE programs (Mario Roederer; Vaccine Research Center, NIAID, NIH).

2.3.7 Virus-specific antibody and cytokine ELISA

PR8 IgM and IgG serum antibody concentrations were determined by ELISA as previously described (Ahmed et al., 1984b; Mozdzanowska et al., 2000). In brief, 1,000 HAU/mL of purified PR8 was adsorbed to plates and bound antibody was detected using anti-IgM or -IgG conjugated to alkaline phosphatase (Sigma) and developed with *p*-nitrophenol phosphate (Sigma). PR8 antibody concentrations were determined using anti-HA antibody standards of IgM (clone H35-C7-2R1) or IgG (clone H36-4-5.2). Optical densities ($OD_{405-750}$) were measured using an ELISA reader and concentrations calculated using SoftMax Pro software (Molecular Devices). BAL wash supernatant were analyzed for cytokines and chemokines by luminex multiplex bead array (Millipore).

2.3.8 RNA isolation, cDNA preparation, and RT-PCR

RNA was isolated from cells using an RNeasy mini-kit (Qiagen) and from lung tissue using mechanical homogenization and TRIzol isolation (Invitrogen) according to the manufacturer's instructions. cDNA was generated using SuperScript reverse transcriptase (Invitrogen). RT-PCR was performed on cDNA using SYBR green chemistry (Applied Biosystems) and commercially available primer sets (Qiagen). PA influenza-specific primers and probes (Sense: CGGTCCAAATTCCTGCTGAT; Anti-Sense: CATTGGGTTCCTTCCATCCA; Probe: CCAAGTCATGAAGGAGAGGGAATACCGCT) were used in combination with Taqman PCR Master Mix (ABI) to determine influenza virus genome copies then converted to

$TCID_{50}$/ gram of lung tissue. Reactions were run on an RT-PCR system (ABI7500; Applied Biosystems). Genes of interest were normalized to *β-actin* and displayed as fold difference relative to uninfected CNV control mice.

2.3.9 Histological sections and pathology

Lungs were inflated with 4% paraformaldehyde, embedded in paraffin and 5-μm sections were cut and stained with hematoxylin and eosin. Blind scoring of H&E-stained lung tissue sections by a board-certified veterinary pathologist reflect degree of luminal exudates (0-5) and degree of bronchiole epithelial degeneration and necrosis (0-5), for an overall maximum score of 10.

2.3.10 Poly I:C administration in influenza virus infected mice

ABX-treated mice were administered 30μg of polyinosinic-polycytidylic acid (poly I:C - Sigma) i.n. at d -1 and 100μg i.p. at d3 post influenza virus infection (PR8-GP33). Control CNV and ABX-treated mice received 30μl of PBS i.n. at d -1 and 100μl i.p. at d3.

2.3.11 Statistical analysis

Results represent means ± SEM. Statistical significance was determined by the unpaired, two tailed, Student's *t*-test for individual timepoints, two-way ANOVA test for timecourse experiments, logrank test for survival curve, or two-part *t*-test for comparison of groups that contained samples that were below the limit of assay detection. Statistical analyses were performed using Prism GraphPad software v4.0. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2.4 Results

2.4.1 Antibiotic-mediated alterations of commensal bacterial communities result in enhanced susceptibility to influenza A virus infection.

Signals derived from commensal bacterial communities regulate intestinal immune cell homeostasis and susceptibility to multiple inflammatory diseases in the gastrointestinal tract (Garrett et al., 2010b; Honda and Littman, 2012; Rakoff-Nahoum et al., 2004; Round and Mazmanian, 2009). Furthermore, depletion of commensal bacteria leads to impaired anti-pathogen immune responses to intestinal parasitic and bacterial infections, indicating an adjuvant-like role for commensal bacteria-derived signals in augmenting the immune response to intestinal infection (Benson et al., 2009; Hall et al., 2008; Ivanov et al., 2009). However, it is not clear whether commensal bacteria-derived signals regulate immunity to viral pathogens that infect sites distal to the gastrointestinal tract. To address this question, naïve C57BL/6 mice were orally administrated broad-spectrum antibiotics for two weeks, subsequently infected with influenza virus (H1N1 influenza A/Puerto Rico/8/34 strain with LCMV GP33 epitope) and immunologic, virologic and pathologic parameters were analyzed.

As reported previously, antibiotic treatment resulted in a significant reduction of commensal bacteria along the intestinal tract as well as a dramatic shift in the composition of intestinal bacterial communities (Figure 5A) (Hill et al., 2010; Hill et al., 2012). In addition, trachea homogenate samples from conventionally-reared (CNV) or antibiotic (ABX) treated mice were cultured under aerobic and anaerobic conditions to examined the impact of antibiotic treatment on the composition of commensal bacterial communities of the upper respiratory tract. Similar to the gastrointestinal tract, there was

a loss of culturable aerobic and anaerobic commensal bacteria in the upper respiratory tract of ABX-treated mice compared to CNV mice (Figure 5B). Despite the loss of culturable commensal bacteria in the upper respiratory tract, the lung architecture (Figure 5C) and composition of lung resident immune cells (Figure 5D) remained unchanged in the steady-state in ABX-treated mice compared to CNV mice.

Following exposure to influenza virus infection, CNV mice lost approximately 20% of their original body weight (Figure 6A) and had reduced lung function as measured by blood oxygen saturation at day 10 (d10) post-infection (Figure 6B). Approximately 80% of the mice were able to recover and clear virus from the lungs by d12 post-infection (Figure 6C,D). In contrast, ABX-treated mice lost significantly more weight and had a drastic drop in blood oxygen saturation following influenza virus infection (Figure 6A,B). Further, ABX-treated mice had significantly higher viral titers in the lung (Figure 6C) and increased mortality (Figure 6D), demonstrating a deleterious physiological consequence of altering commensal bacterial communities in the context of respiratory influenza virus infection.

2.4.2 ABX-treated mice exhibit severe lung pathology following influenza virus infection

Histopathological examination of lung sections from CNV mice at d12 post-infection compared to uninfected control mice (Figure 5C) revealed peribronchiolar inflammation and epithelial hyperplasia, suggestive of wound healing and tissue repair (Figure 7A,B). Consistent with impaired lung function and increased mortality, lung sections from ABX-treated mice at d12 post-infection revealed more pronounced epithelial cell necrosis (Figure 7C,D), increased exudate and dead cells in the bronchiolar lumen (Figure 7E,

arrows), and in the most severe cases, complete loss of the bronchiole epithelial layer (Figure 7F, arrows). Scoring of histological sections of the lung confirmed increased prevalence of epithelial cells with morphologic features of degeneration and necrosis in ABX-treated mice relative to CNV mice (Figure 7G). In agreement with histologic analysis, increased cell death was observed in the bronchiole alveolar lavage (BAL) fluid of ABX-treated mice at d12 post-infection compared to CNV mice following staining with a cellular viability dye (Figure 7H,I).

To further explore the relationship between commensal bacterial communities and antiviral immunity, gnotobiotic or “germ-free” (GF) mice were infected with influenza virus and compared to CNV and ABX-treated mice. Similar to a previously published report (Dolowy and Muldoon, 1964), GF, as well as ABX-treated mice, exhibited increased weight loss (Figure 8A), impaired viral clearance (Figure 8B) and reduced virus-specific antibody titers (Figure 8C) compared to CNV mice. Histological examination of the lung at d12 post-infection revealed more severe bronchiole epithelial degeneration in GF and ABX-treated mice compared to CNV mice (Figure 8D-F). These data support a critical role for commensal bacteria-derived signals in promoting optimal antiviral immunity.

2.4.3 ABX-treated mice exhibit a diminished influenza virus-specific adaptive immune response

Increased lung pathology and impaired viral clearance in ABX-treated mice suggests an impaired immune response to influenza virus infection. The cell-mediated and humoral adaptive immune response is critical for viral clearance and host survival (Palladino et al., 1995; Wu et al., 2010a). To interrogate the adaptive immune response CNV and ABX-treated influenza virus-infected mice were sacrificed at d7 post-infection.

Histological examination of the lung revealed significant recruitment of lymphocytes into the alveolar spaces of CNV mice while ABX-treated mice had relatively diminished lymphocyte infiltration (Figure 9A). Quantification by flow cytometric analysis confirmed significantly fewer CD8 T cells in the lung parenchyma and bronchiole alveolar lavage fluid (BAL) of ABX-treated mice compared to CNV mice (Figure 9B,C).

Major histocompatability complex (MHC) class I tetramer specific for the gp33-41 epitope (Altman et al., 1996), expressed by recombinant PR8-GP33 virus, or tetramer specific for the endogenous np366-374 amino acid sequence of nucleoprotein of influenza virus (the two dominant CD8 T cell epitopes for influenza virus) were employed to more thoroughly investigate the influenza virus-specific CD8 T cell response. CNV mice infected with PR8-GP33 virus generated large populations of influenza virus-specific (H-2Db/GP33^{+} and H-2Db/NP366^{+}) CD8 T cells present in the BAL fluid (Figure 9D) and lung parenchyma (Figure 9E,F) by d7 post-infection. In contrast, the total number of influenza virus-specific CD8 T cells present in these tissues was significantly reduced in ABX-treated mice (Figure 9D-F). In addition to an impaired CD8 T cell response, ABX-treated mice exhibited lower titers of PR8-specific IgM and IgG in the serum at d12 post-infection (Figure 9G). Collectively, these data demonstrate diminished humoral and cell-mediated adaptive immune responses to influenza virus infection in ABX-treated mice that correlates with impaired viral clearance from the lung and increased host susceptibility.

Expression of activation markers such as CD69, CD25, and the cytotoxic granulated protein Granzyme-B is rapidly upregulated early following acute viral infection and then downregulated as the infection is controlled (Harty et al., 2000; Lawrence and Braciale, 2004; Wherry et al., 2007). The expression of these molecules on DbGP33 tetramer^{+}

CD8 T cells (Figure 10A) at day 7, 10, and 12 p.i was assessed for an in depth phenotypic profile of the CD8 T cell activation over the course of influenza virus infection (Figure 10B). While DbGP33 tetramer$^+$ CD8 T cells from CNV mice downregulated early activation markers between d7 to 12 post-infection, expression of CD69 and CD25 remained elevated in DbGP33 tetramer$^+$ CD8 T cells isolated from ABX-treated mice (Figure 10, Table 1). Elevated expression of these molecules, along with Granzyme-B and CD43, persisted on influenza virus-specific CD8 T cells isolated from the lung of ABX-treated mice at d12 post-infection. These data are consistent with a delay in the adaptive immune response to influenza virus and sustained activation of virus-specific CD8 T cells due to prolonged viral persistence.

2.4.4 ABX-treated mice exhibit an impaired CD8 T cell response and rapidly succumb to the weakly pathogenic X31-GP33 influenza A virus.

Infection with the PR8 strain of influenza virus results in substantially increased morbidity and impaired viral clearance in ABX-treated mice compared to CNV mice, making comparisons of the functional quality of the CD8 T cell response at a particular timepoint challenging. To better study the influenza virus-specific CD8 T cell response in ABX-treated mice, CNV or ABX-treated mice were infected with X31-GP33 virus (H3N2 strain), a less pathogenic strain of influenza virus that causes minimal morbidity and mortality in CNV mice (Bouvier and Lowen, 2010; Decman et al., 2010). Despite infection with a less virulent influenza virus strain, ABX-treated mice still exhibited increased weight loss (Figure 11A), elevated viral titers (Figure 11B), increased epithelial degeneration (Figure 11D) and increased mortality (Figure 11C) following exposure to X31-GP33 virus compared to CNV mice. X31-gp33 virus-infected CNV mice exhibited

robust adaptive immune responses by d7 post-infection characterized by large population of influenza virus-specific CD8 T cells in the BAL fluid (Figure 12A), lung parenchyma (Figure 12B), mediastinal lymph nodes (Figure 12C) and spleen (Figure 12D) Further, following *ex vivo* stimulation with GP33 peptide infiltrating virus-specific CD8 T cells in the lung were capable of simultaneously producing multiple effector molecules (IFN-γ, TNF-α, IL-2, Mip-1α, CD107a) (Figure 12E). In contrast, ABX-treated mice exhibited a significant reduction in the number of influenza virus-specific CD8 T cells isolated from multiple compartments (Figure 12A-D) and these virus-specific T cells were less capable of simultaneously producing multiple effector molecules indicating a functionally impaired virus-specific CD8 T cell response in ABX-treated mice. (Figure 12E). Thus, following infection with two separate strains of influenza virus, ABX-treated mice exhibited a diminished influenza virus-specific CD8 T cell response that correlated with impaired viral clearance and increased host mortality.

2.4.5 Impaired innate antiviral immune responses in ABX-treated mice

The impaired antiviral CD8 T cell response in ABX-treated mice following either PR8-GP33 or X31-GP33 virus infection suggested a potential defect in the early innate immune response. To test this hypothesis, the recruitment and activation of early responding innate immune cells was assessed at d3 following PR8-GP33 virus infection. There was a comparable influx of macrophages, inflammatory monocytes, neutrophils, plasmacytoid dendritic cells (Figure 13A), and conventional dendritic cells (Figure 13B) into the lung of CNV versus ABX-treated mice. Further, conventional dendritic cells exhibited a similar activation profile in CNV versus ABX-treated mice at d3 post-infection

(Figure 13C). These data suggested that recruitment of innate immune cells and upregulation of costimulatory molecules was intact in the ABX-treated mice.

The cytokine environment in the lung early following viral infection is a critical factor that shapes the quality of the adaptive immune response (McGill et al., 2009; Sanders et al., 2011), therefore the cytokine milieu in CNV or ABX-treated mice prior to and early following infection was assessed. IL-10 is an immunoregulatory cytokine that has been demonstrated to influence the antiviral immune response (Brooks et al., 2006; McKinstry et al., 2009; Sun et al., 2009; Zajac et al., 1998) and expression of this cytokine can be modulated by the microbiota (Amaral et al., 2008; Mazmanian et al., 2008; Ueda et al., 2010). However, assessment of IL-10/eGFP reporter Vertex mice following administration of antibiotics revealed no significant differences in the frequency of IL-10/eGFP^{+} macrophages, dendritic cells, CD4 T cells, CD8 T cells, or B cells isolated from the lung parenchyma (Figure 14A) or spleen (Figure 14B) of CNV versus ABX-treated mice. Further, no difference in mRNA expression of *Il10* was observed in the spleen (Figure 14C) or lung (Figure 14D) of C57BL/6 CNV versus ABX-treated mice. In addition, following influenza virus infection, a similar increase in IL-10 was detected in the BAL fluid of CNV versus ABX-treated mice (Figure 14E). Combined, these analyses indicate that impaired antiviral immune responses in ABX-treated mice were not associated with dysregulated IL-10 production prior to or following influenza virus infection. In contrast, reduced levels of proinflammatory cytokines and chemokines were detected in the BAL fluid of ABX-treated mice early following influenza virus infection (Figure 15A). Induction of these proinflammatory chemokines and cytokines are critical to the propagation of the innate immune response and suggest that the functional quality

of the innate immune response to respiratory viral infection is impaired following ABX-mediated alterations in commensal bacteria.

2.4.6 Diminished induction of IFN responsive genes and protection against influenza virus challenge following restoration of IFN responsiveness in ABX-treated mice

While there was no difference in the total numbers of innate immune cells that accumulated in the lung, less proinflammatory cytokines and chemokines were present following influenza virus infection in ABX-treated mice compared to CNV mice (Figure 13, 15). This observation lead to the hypothesis that the quality of the innate antiviral response was impaired in ABX-treated mice. To test the magnitude of the innate immune response, mRNA expression of key antiviral defense genes in the lung was assessed early following influenza virus infection. Despite a similar number of innate immune cells present in the lung between CNV and ABX-treated mice, there was significantly reduced expression of interferon-related antiviral defense genes (*Ifnb*, *Irf7, Mx1*, *Oas1a*, *Il28b [Ifnλ]*, *Il6*, *Tnfa*, *Ccl3 [Mip1α]* and *Il1b*) in ABX-treated mice compared to CNV mice at d3 post-infection (Figure 15B). Combined, the decreased expression of proinflammatory cytokines and antiviral defense genes demonstrate a diminished innate immune response in ABX-treated mice following influenza virus infection.

Diminished expression of these macrophage-associated interferon response genes suggested an impaired macrophage response in ABX-treated mice following viral infection. Alveolar macrophages are resident immune cells in the lung and are among the first immune cells to encounter influenza virus in the respiratory tract (McGill et al.,

2009). The response of these cells is crucial for the rapid induction of immune defense mechanisms (Peschke et al., 1993; Tumpey et al., 2005). To assess the quality of the macrophage response following influenza virus infection, alveolar macrophages were sorted from d3 post-infection CNV or ABX-treated mice and assessed for expression of key antiviral defense genes (Figure 16A). At d3 post-infection alveolar macrophages from ABX-treated mice had a reduced induction of interferon response genes such as *Mx1*, *Irf7*, *Stat1*, *Ifit3* and *Ifnb* (Figure 16B). These results identify macrophages as an innate cell type that exhibits a diminished antiviral immune response to influenza virus infection following antibiotic-mediated perturbation of commensal bacterial communities. Combined, these findings demonstrate that ABX-mediated alterations in commensal bacteria result in impaired induction of innate antiviral defense mechanisms following respiratory viral infection.

To test whether if restoring the IFN response in ABX-treated mice could provide protective immunity to influenza virus infection, the synthetic TLR ligand poly I:C, known to stimulate endogenous type I IFN production (Proietti et al., 2002), was administered to ABX-treated mice 1 day prior to and 3 days following influenza virus infection. ABX-treated mice receiving PBS had drastic weight loss (Figure 17A), loss of lung function (Figure 17B) and high mortality following influenza virus infection (Figure 17C). In contrast, administration of poly I:C to influenza virus infected ABX-treated mice resulted in less weight loss (Figure 17A) as well as marked improvements in lung function and survival (Figure 17B,C), suggesting that re-calibrating the immune response to IFN stimulation could overcome the need for commensal bacteria-derived signaling in mounting an efficient immune response to influenza virus infection.

2.5 Discussion

This chapter demonstrates that antibiotic-mediated disruption of commensal bacterial communities impairs host protective antiviral immunity. Following mucosal-associated influenza virus infection ABX-treated mice exhibited an impaired innate and adaptive immune response. Impaired antiviral immunity was characterized by diminished induction of antiviral defense genes by alveolar macrophages, fewer virus-specific CD8 T cells, reduced circulating viral-specific antibodies and elevated viral titers. Consistent with a role for commensal bacteria-derived signals in promoting protective antiviral immunity, commensal bacteria-depleted hosts exhibited severe bronchiole epithelial degeneration, impaired oxygen exchange and increased mortality following influenza virus infection. Protective immunity was partially restored in ABX-treated mice by administering the TLR ligand poly I:C to boost the diminished type I IFN response observed in ABX-treated mice. These data provide evidence of a role for commensal bacteria in modulating the immune response to an ongoing viral infection in a tissue distal to the intestinal tract. It is unclear, however, whether commensal bacteria-derived signals can only influence the immune response to mucosal-associated viruses that infect tissues in close proximity to resident bacterial communities or if these signals influence the systemic immune response against viruses that infect multiple peripheral tissues. This question will be addressed in **Chapter 3**.

In a recent report Iwasaki and colleagues also observed that deliberate manipulation of commensal bacteria resulted in impaired antiviral immunity in the lung (Ichinohe et al., 2011). This defect was associated with decreased expression of genes involved in the inflammasome pathway in the lung of antibiotic-treated mice compared to CNV mice following influenza virus infection (Ichinohe et al., 2011). While our studies also observed

decreased *Il-1b* mRNA expression in whole lung tissue following infection, alveolar macrophages isolated from CNV or ABX-treated mice did not exhibit significant differences in expression of inflammasome related genes such as *Il-1b, Il-18,* and *Nlrp3* (data not shown). Instead, alveolar macrophages isolated from ABX-treated mice exhibited significantly reduced expression of antiviral interferon response genes such as *Mx1* and *Oas1a*, suggesting a novel interplay between commensal bacteria and interferon signaling pathways that are critical in response to most viruses. Further, two recent reports have identified a fundamental interaction between the intestinal microbiota and enteric viruses where the virus utilizes the presence of commensal bacteria to enhance infectivity of the gastrointestinal tract (Kane et al., 2011; Kuss et al., 2011). Antibiotic treatment did not alter influenza virus infectivity of the respiratory mucosa in our studies suggesting that enteric and respiratory viruses have evolved different mechanism to promote replication. Collectively, these reports highlight the dynamic interrelationship between viral pathogens, commensal bacteria and the immune system and support a model where commensal bacteria-derived signals can coordinately influence multiple innate immune cell types to impact the immune response in the lung in an infection setting. Further these data provoke the possibility that commensal bacteria may provide tonic stimulation on innate immune cells prior to infection, thereby predisposing the immune system to rapidly respond to pathogens upon initial encounter. This hypothesis will be the focus of **Chapter 4.**

Figure 5. Oral antibiotic treatment alters commensal bacterial communities in the intestine and upper respiratory tract, but not immune cell populations in the lung.

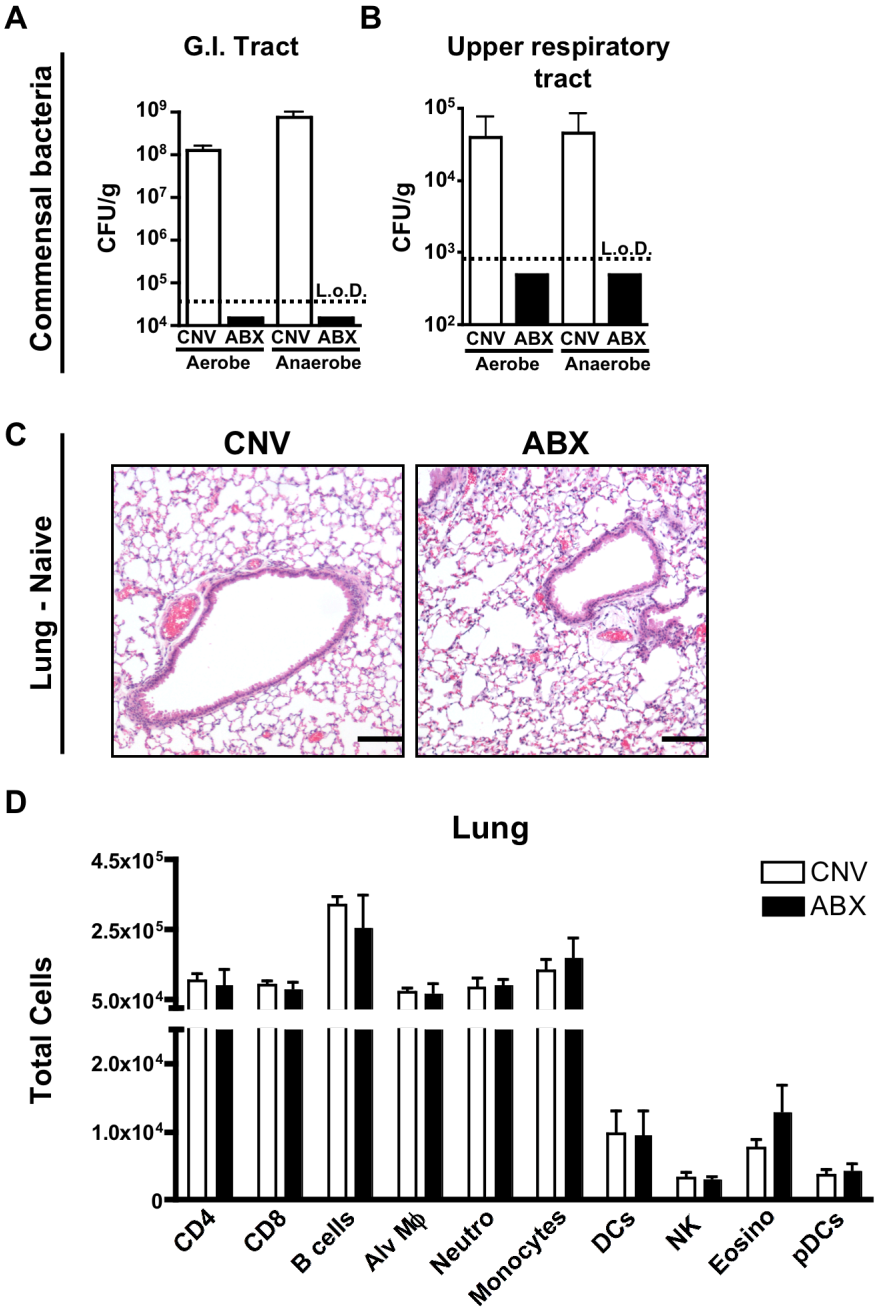


Figure 5. Culturable bacteria from **(A)** stool samples or **(B)** trachea homogenates of naïve CNV or ABX-treated mice following two weeks of antibiotic treatment. Samples were plated on **(A)** Schaedler's or **(B)** LB agar and incubated for 48hrs in aerobic or anaerobic conditions (L.o.D., limit of detection). **(C)** H&E stained lung sections from naïve CNV or ABX-treated mice (scale bar: 200μm). **(D)** Total number of hematopoietic cells in the lung parenchyma of CNV or ABX-treated mice at steady-state. Cells quantified by flow cytometric analysis. CD4 T cells gated on $CD45^+$, $CD3\varepsilon^+$, $CD4^+$. CD8 T cells gated on $CD45^+$, $CD3\varepsilon^+$, $CD8\alpha^+$. B cells gated on $CD45^+$, $CD19^+$, MHC-II^{hi}. Alveolar macrophages gated on $CD45^+$, NTNB, $CD11c^+$, $F4/80^+$. Neutrophils gated on $CD45^+$, NTNB, $CD11b^+$, $Ly6g^+$. Monocytes gated on $CD45^+$, NTNB, $CD11b^+$, $Ly6c^+$. Dendritic cells gated on $CD45^+$, NTNB, $F4/80^-$, $Ly6g^-$, $CD11c^+$, MHC-II^{hi}. NK cells gated on $CD45^+$, NTNB, $NK1.1^+$. Eosinophils gated $CD45^+$, NTNB, $F4/80^-$, SSC^{hi}, Siglec-F^+. Plasmacytoid dendritic cells gated on $CD45^+$, NTNB, non-alveolar macrophage, non-neutrophil, non-monocyte, $CD11c^{int}$, PDCA-1^+. Data representative of three independent experiments n = 3-5 mice per group. Data shown are the mean ± SEM. **p <0.01.

Figure 6. ABX-treated mice have a higher morbidity and mortality rate following influenza virus infection.

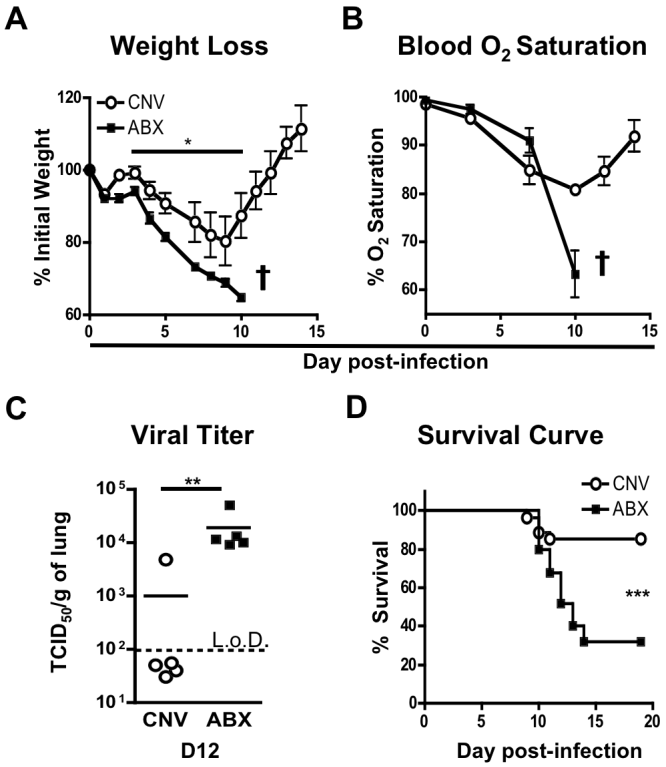


Figure 6. CNV or ABX-treated C57BL/6 mice were infected intranasally (i.n.) with 368 $TCID_{50}$ recombinant influenza virus PR8-GP33. Timecourse of **(A)** weight loss and **(B)** blood oxygen saturation following infection (representative exp. n=5: † signifies mice below 70% initial weight were sacrificed). **(C)** Influenza virus genome copies in the lung at d12 post-infection assessed by qPCR and displayed as $TCID_{50}$/gram of lung tissue based on a standard curve of genome copies versus $TCID_{50}$ (L.o.D., limit of detection). **(D)** Survival curve following PR8-GP33 infection. Survival curve is a combination of five independent experiments; CNV n=27, ABX n=25. Data representative of five independent experiments with n=5-6 mice per group. Survival statistics determined by logrank test. Viral titer statistics determined by two-part *t*-test. *p<0.05, **p <0.01, ***p<0.001.

Figure 7. ABX-treated mice exhibited increased lung pathology following influenza virus infection.

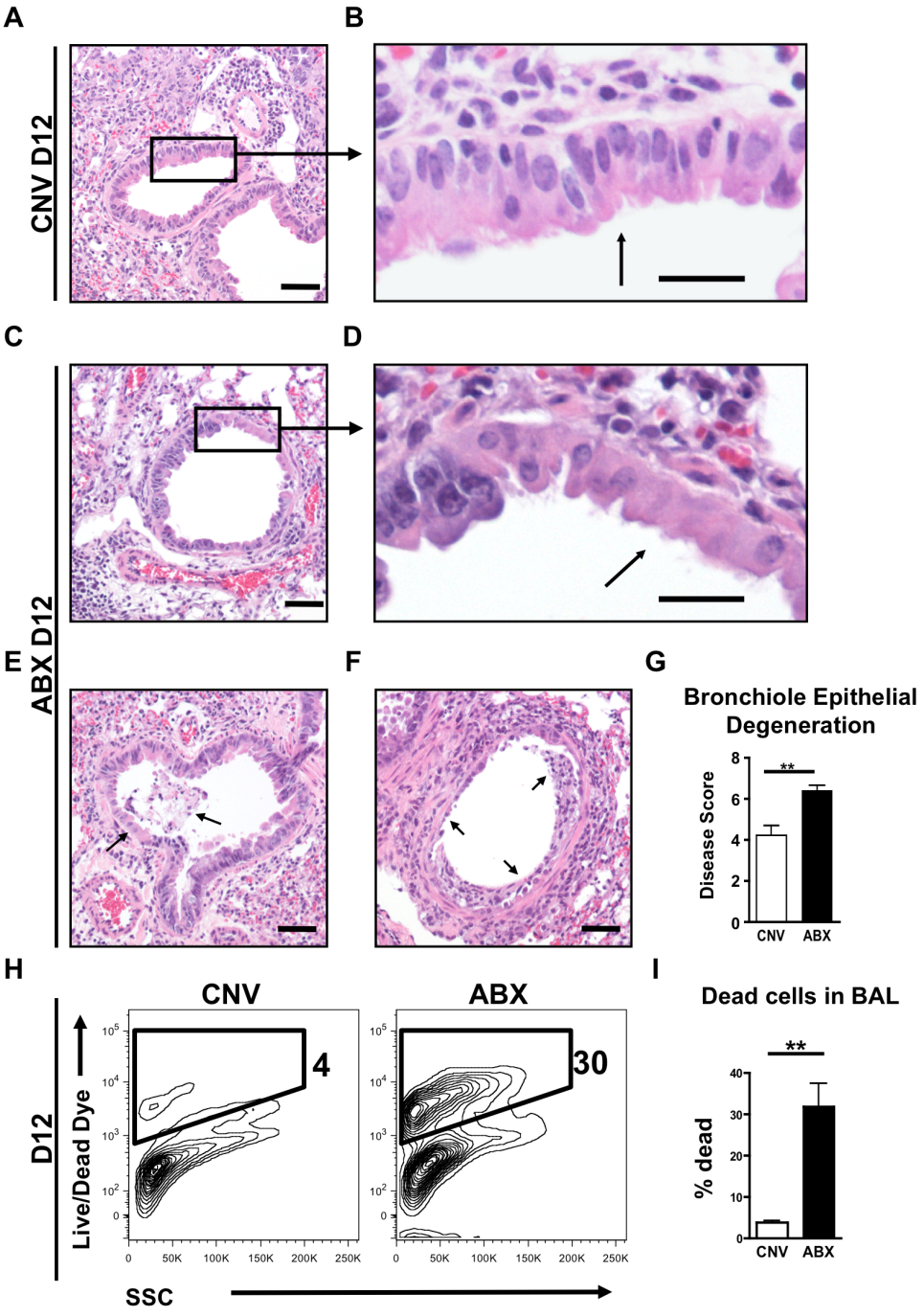


Figure 7. H&E stained lung section of **(A,B)** CNV or **(C-F)** ABX-treated mice at d12 post-infection. Black box/arrows highlight **(A,B)** epithelial hyperplasia, **(C,D)** epithelial cell necrosis, **(E)** cellular debris and exudate in lumen and **(F)** loss of bronchiole epithelium (scale bar: 50μm A,C,E,F; 20μm B,D). **(G)** Disease score of bronchiole epithelial degeneration at d12 post-infection. **(H)** Flow cytometric analysis and **(I)** frequency of dead cells in the BAL fluid of CNV or ABX-treated at d12 post-infection as assessed by an amine reactive viability dye. Data representative of three independent experiments with n=5 mice per group. Data shown are the mean ± SEM. **p <0.01.

Figure 8. GF mice have increased morbidity, impaired viral clearance and exacerbated lung pathology following influenza virus infection.

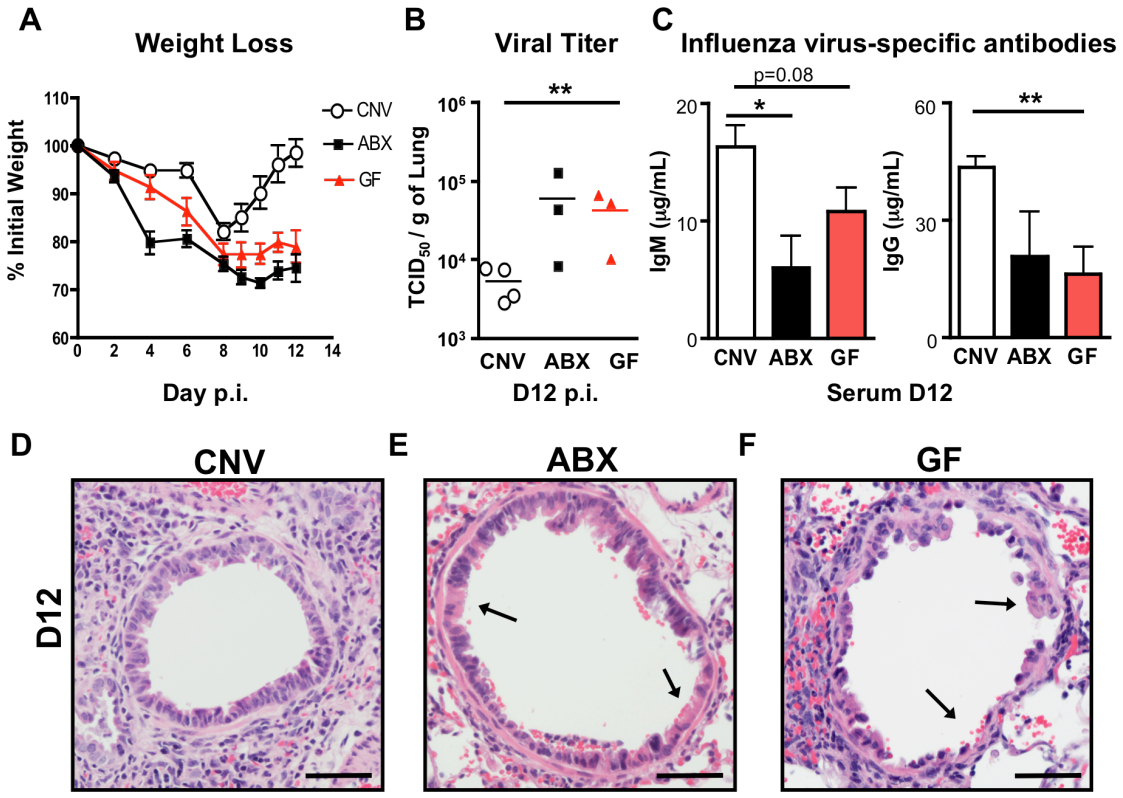


Figure 8. CNV, ABX-treated or GF C57Bl/6 mice were infected i.n. with 368 $TCID_{50}$ recombinant influenza virus (PR8-GP33 strain). **(A)** Timecourse of weight loss following infection. **(B)** Influenza virus genome copies in the lung at d12 p.i. assessed by qPCR and displayed as $TCID_{50}$/gram of lung based on a standard curve of genome copies versus $TCID_{50}$ tissue. **(C)** PR8-specific IgM and IgG titers in serum at d12 post-infection. H&E stained lung sections of **(D)** CNV, **(E)** ABX, or **(F)** GF mice at d12 post infection. Black arrows highlight bronchiole epithelial necrosis/loss (scale bar: 50μm). Data representative of two independent experiments with n=5 mice per group. Viral titer statistics determined by two-part t test. Data shown are the mean ± SEM. *p<0.05, ** p <0.01.

Figure 9. ABX-treated mice exhibit a diminished adaptive immune response following influenza virus infection.

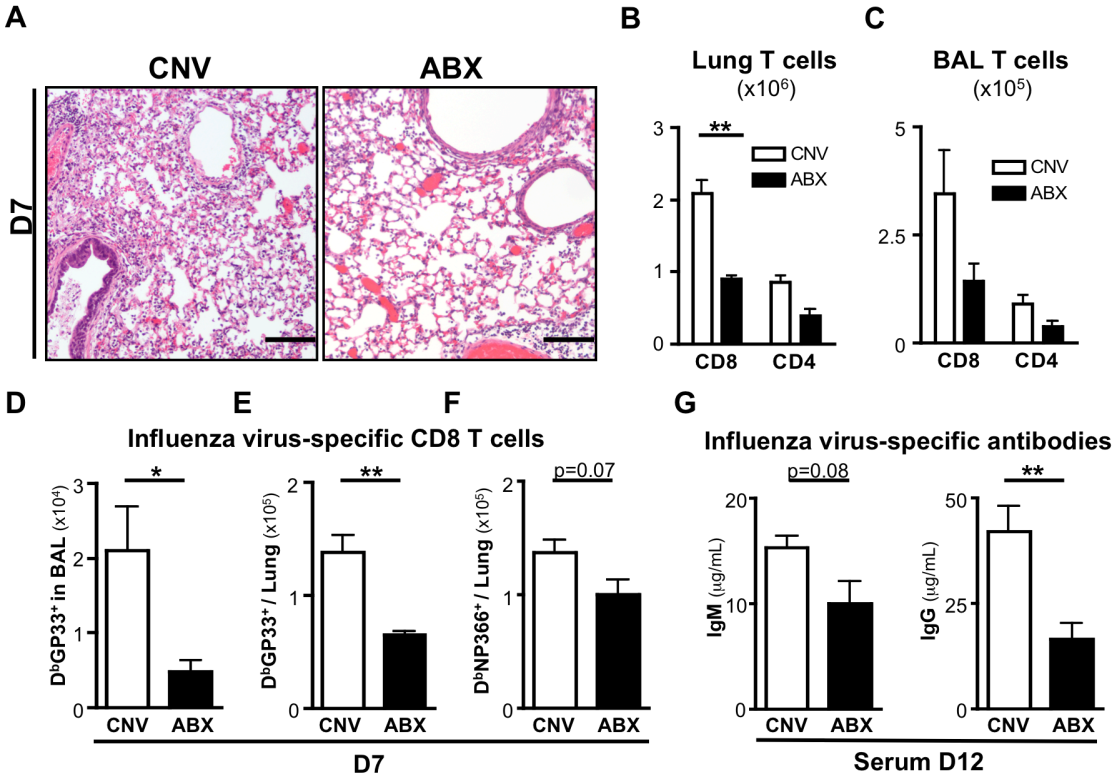


Figure 9. (A) H&E stained lung sections demonstrate lymphocyte infiltration into alveolar spaces at day 7 p.i. (scale = 200μm) **(B)** Total number of T cells in the lungs and **(C)** BAL fluid at day 7 p.i. **(D-F)** Total number of influenza virus-specific DbGP33 tetramer$^+$ CD8 T cells isolated from the **(D)** BAL fluid or **(E)** lung parenchyma at d7 post-infection. **(F)** DbNP366 tetramer$^+$ CD8 T cells isolated from the lung parenchyma at d7 post-infection. **(G)** PR8-specific IgM and IgG titers in the serum at d12 post-infection. Data representative of three independent experiments with n=4-5 mice per group. Data shown are the mean ± SEM. *p<0.05, **p< 0.01.

Figure 10. Influenza virus-specific CD8 T cells have an altered phenotype in ABX-treated mice that correlates with impaired activation.

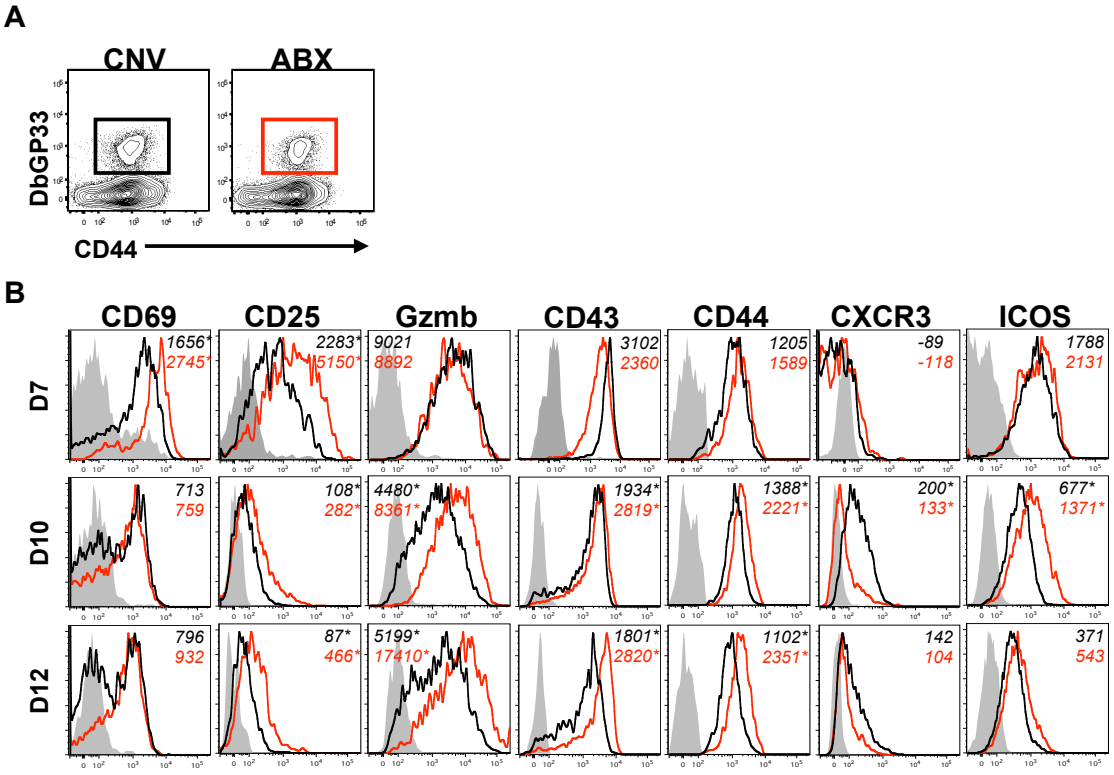


Figure 10. Phenotypic profile of DbGP33 tetramer^{+} CD8 T cells isolated from the lung of CNV (black line) or ABX-treated (red line) mice at d7,10,12 post-infection. Gray shaded histograms are CD44^{lo} CD8 T cells isolated from the lung. Numbers in italics represent MFI. Data representative of three independent experiments with n=4-5 mice per group. *p<0.05, **p< 0.01.

Table 1. Expression of activation markers on influenza virus-specific CD8 T cells.

Day		CD69$^+$	CD25$^+$	Gzmb$^+$	CD43$^+$	CXCR3$^+$
7	**CNV**	67.9 ± 3.9*	67.4 ± 3.4**	93.8 ± 3.9	94.6 ± 4.6	1.7 ± 0.4
	ABX	79.1 ± 6.7*	80.7 ± 2.3**	94.2 ± 3.4	94.6 ± 3.1	2.4 ± 2.8
10	**CNV**	58.5 ± 2.3	18.3 ± 6.4**	76.2 ± 5.4***	87.5 ± 3.8	34.2 ± 6.5**
	ABX	63.2 ± 4.3	35.5 ± 6.5**	96.5 ± 0.1***	90.0 ± 3.4	17.9 ± 2.5**
12	**CNV**	32.3 ± 4.5	11.4 ± 2.4	55.7 ± 4.8***	89.0 ± 4.2	16.8 ± 5.2
	ABX	30.7 ± 5.7	34.6 ± 11.7	84.5 ± 1.4***	90.6 ± 4.1	10.4 ± 0.3

Table 1. Frequency of DbGP33 tetramer$^+$ CD8 T cells that express CD69, CD25, Granzyme-b, CD43 or CXCR3 at d7, 10, or 12 post influenza virus infection (PR8-GP33 strain). Positive gate determined by expression of markers on naïve CD44^{lo} CD62L^{hi}, CD8 T cells. Data shown are the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Figure 11. ABX-treated mice are susceptible to weakly pathogenic X31-gp33 influenza virus strain.

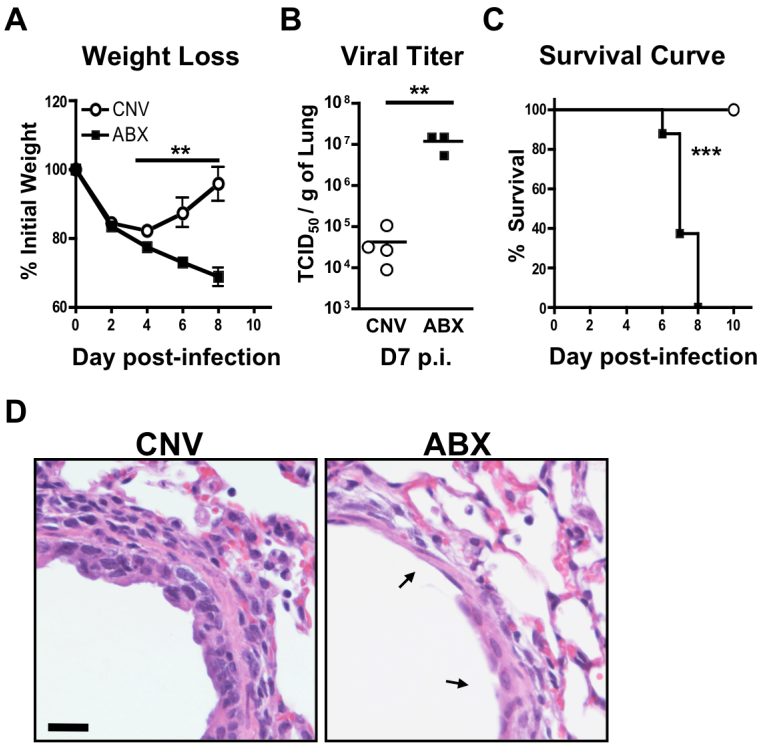


Figure 11. CNV or ABX-treated C57Bl/6 mice were infected i.n. with $1x10^5$ $TCID_{50}$ recombinant influenza virus X31-GP33. **(A)** Timecourse of weight loss following infection. **(B)** Influenza virus genome copies in the lung at d7 post-infection assessed by qPCR and displayed as $TCID_{50}$/gram of lung tissue based on a standard curve of genome copies versus $TCID_{50}$. **(C)** Survival curve following infection. **(D)** H&E stained lung section of CNV or ABX lungs at d7 post-infection (black arrow highlights epithelial necrosis/loss; scale 20μm). Data representative of three independent experiments with n=4-5 mice per group. Survival curve statistics determined by logrank test. Viral titer statistics determined by two-part *t*-test. *p<0.05, **p <0.01, ***p<0.001.

Figure 12. Diminished and functionally impaired influenza virus-specific CD8 T cell response in ABX-treated mice following X31 virus infection.

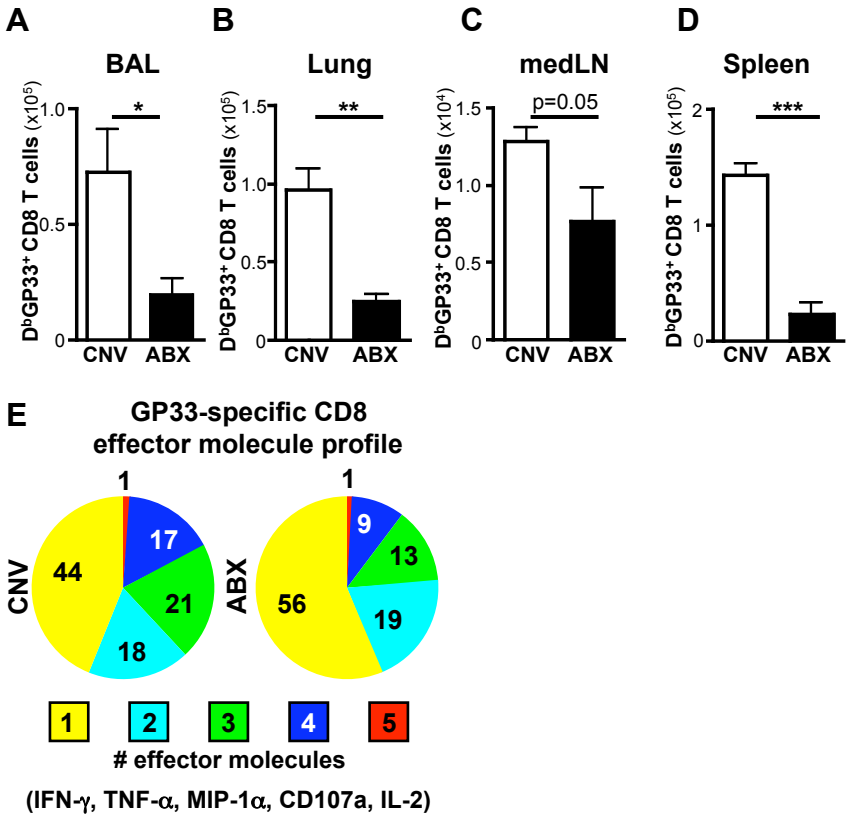


Figure 12. Total number of DbGP33 tetramer^{+} CD8 T cells in the **(A)** BAL, **(B)** lung, **(C)** mediastinal lymph nodes, and **(D)** spleen at d7 post-infection. **(E)** Lung cells were stimulated with GP33 peptide for 5hrs in the presence of BFA. Proportion of GP33 peptide responsive CD8 T cells in the lung producing multiple effector molecules (IFN-γ, TNF-α, IL-2, MIP-1α, CD107a). Data representative of three independent experiments with n=4-5 mice per group. Data shown are the mean ± SEM. *p<0.05, **p <0.01, ***p<0.001.

Figure 13. Recruitment and activation of innate immune cells in the lung is equivalent in CNV and ABX-treated mice following influenza virus infection.

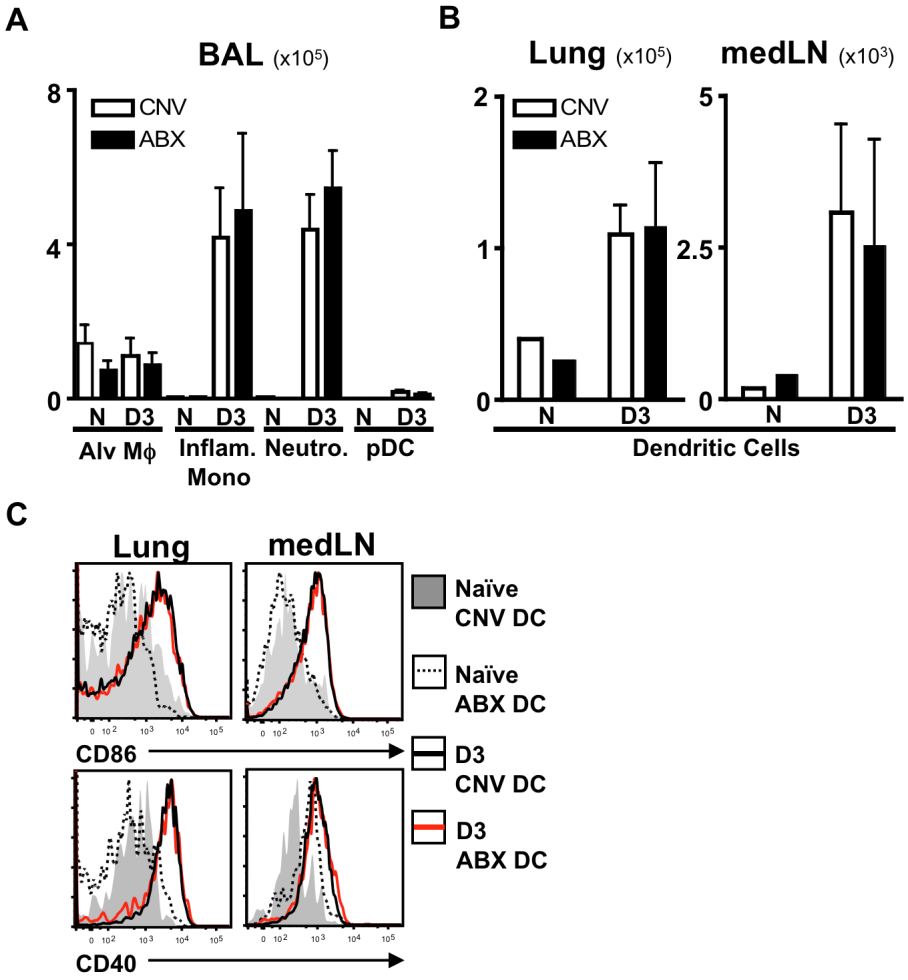


Figure 13. (A) Total numbers of alveolar macrophages, inflammatory monocytes, neutrophils and plasmacytoid dendritic cells (pDC) isolated from BAL fluid at d3 post-infection. Alveolar macrophages gated on $CD3\varepsilon^-$, $CD5^-$, $CD19^-$, $NK1.1^-$ (Non T, Non B, Non NK cells - NTNBNNK), $CD11c^+$, $F4/80^+$. Inflammatory monocytes gated on NTNBNNK, $CD11c^-$, $F4/80^-$, $Ly6g^-$, $Ly6c^+$, $CD11b^+$. Neutrophils gated on NTNBNNK, $CD11c^-$, $F4/80^-$, $Ly6g^+$, $CD11b^+$. pDC gated on NTNBNNK, non-alveolar macrophage, non-neutrophil, non-inflammatory monocyte, $CD11c^{int}$, $PDCA\text{-}1^+$. **(B)** Total numbers of dendritic cells (DCs) isolated from the lung parenchyma and mediastinal lymph node (medLN). DCs are gated on NTNBNNK, $F4/80^-$, $CD11c^+$, $MHC\text{-}II^{hi}$ cells. **(C)** Expression of CD86 and CD40 on naïve or d3 post-infection DCs isolated from the lung and medLN of CNV or ABX-treated mice. Data representative of four independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM.

Figure 14. Equivalent IL-10 expression in CNV and ABX-treated mice at steady-state and following influenza virus infection.

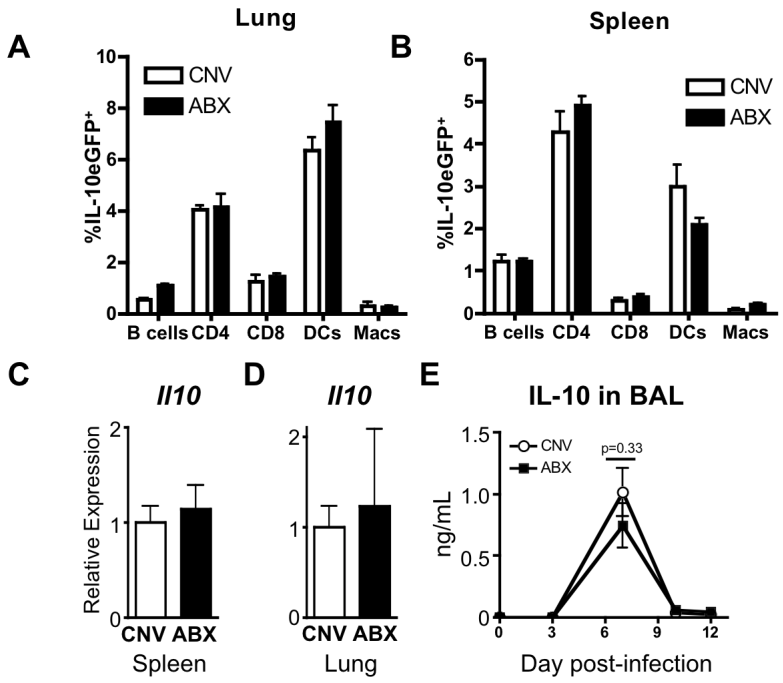


Figure 14. Frequency of the IL-10 competent immune cells in the **(A)** lung, or **(B)** spleen of naïve, CNV or ABX-treated IL-10/eGFP reporter Vertex mice. B cells were gated on live, $CD45^+$, $CD19^{+,}$ $MHC\text{-}II^{hi}$. CD4 T cells were gated on live, $CD45^+$, $CD3\varepsilon^+$, $CD4^+$. CD8 T cells were gated on live, $CD45^+$, $CD3\varepsilon^+$, $CD8^+$. Dendritic cells were gated on live, $CD45^+$, $CD3\varepsilon^-$, $CD5^-$, $CD19^-$ (Non-T, Non-B - NTNB) $CD11c^+$, $MHC\text{-}II^{hi}$. Splenic macrophages from the spleen were gated on live, $CD45^+$, NTNB, $CD11b^+$, $F4/80^+$. Alveolar macrophages were gated on live, $CD45^+$, NTNB, $CD11c^+$, $F4/80^+$. **(C,D)** Relative expression of *Il-10* mRNA in the **(C)** spleen or **(D)** lung of naïve CNV or ABX-treated mice. **(E)** IL-10 protein present in the BAL fluid of influenza virus infected CNV or ABX-treated mice (PR8-GP33). IL-10 levels determined by luminex multiplex bead array. Statistical analysis of individual timepoints was done by Student's *t*-test. Data representative of two independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM. *$p<0.05$, **$p<0.01$.

Figure 15. Diminished induction of antiviral defense genes and proinflammatory cytokines in the lung of ABX-treated mice at D3 following influenza virus infection.

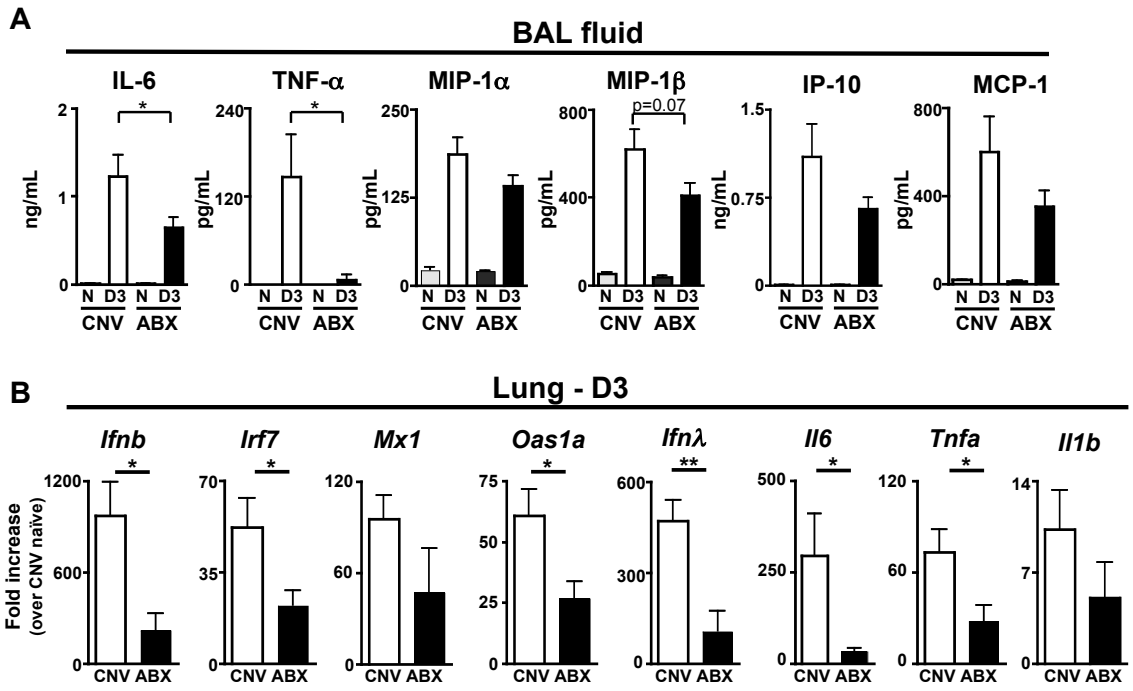


Figure 15. (A) Proinflammatory cytokines and chemokines present in BAL fluid d3 post influenza virus infection. **(B)** Fold induction of antiviral defense gene expression in the lung at d3 post-influenza virus infection compared to lung of naive CNV mice as assessed by qPCR. Data representative of two or more independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM. *p< 0.05, **p<0.01.

Figure 16. Alveolar macrophages from influenza virus infected, ABX-treated mice exhibit diminished expression of antiviral defense genes.

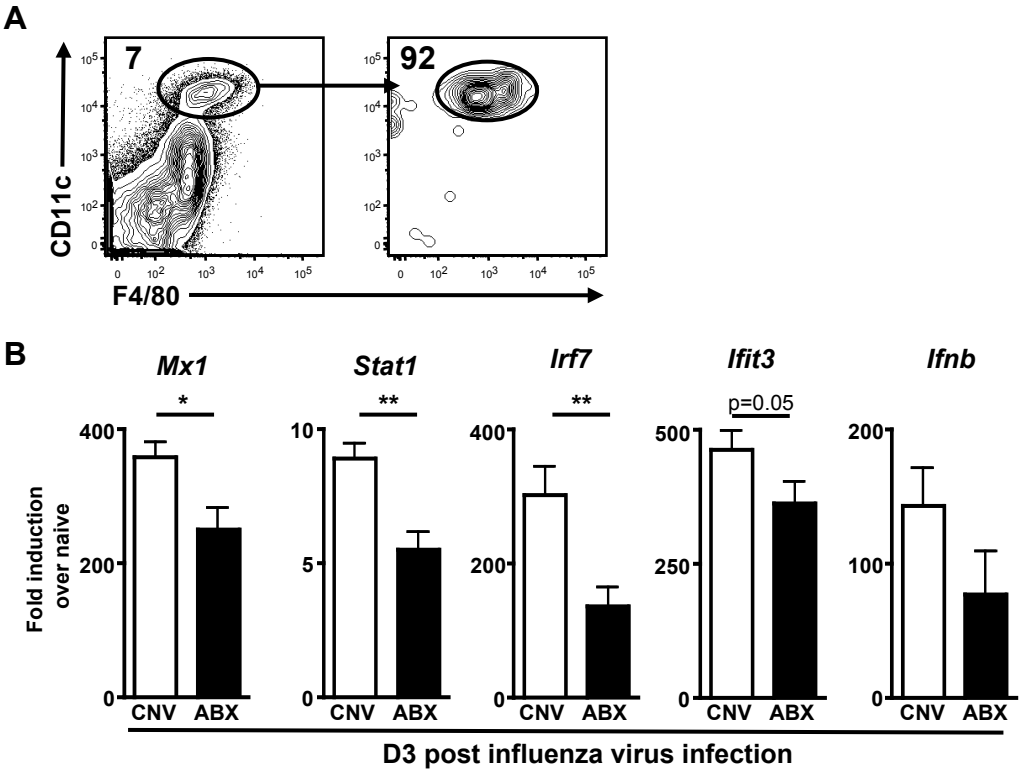


Figure 16. CNV or ABX-treated mice were infected with influenza virus (PR8-GP33). At d3 post-infection, alveolar macrophages (NTNBNNK, F4/80^{+}, CD11c^{+}) were sorted from the BAL and RNA was purified to assess *in vivo* induction of antiviral defense genes by qPCR. **(A)** Representative FACS plot demonstrating pre & post-sort purity of alveolar macrophages. FACS plots gated on CD45^{+} cells. **(B)** Gene expression displayed as fold induction over naïve alveolar macrophages from CNV mice. Data representative of two independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM. *p <0.05, **p<0.01.

Figure 17. Restoration of IFN responsiveness partially protects ABX-treated mice against influenza virus challenge.

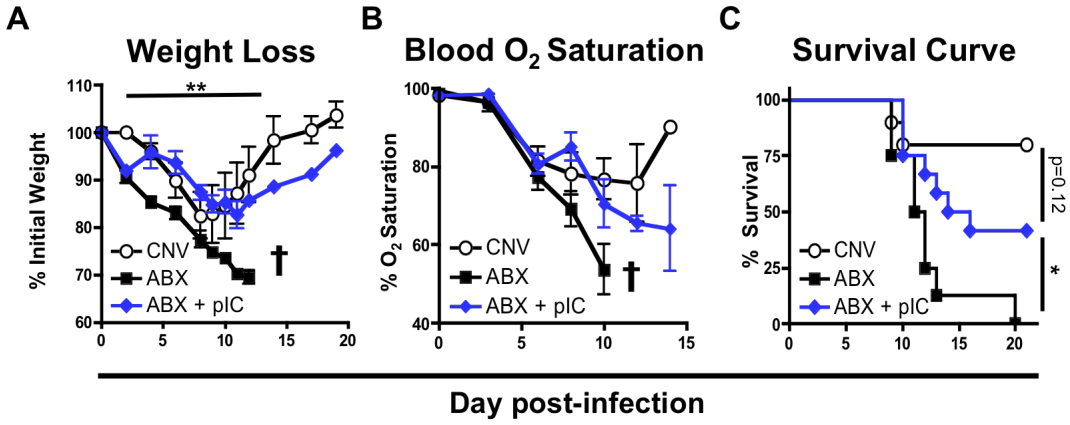


Figure 17. CNV or ABX-treated mice were infected with influenza virus (PR8-GP33). Mice received 30μg of poly I:C (ABX+pIC group) or PBS (CNV & ABX group) i.n. at d -1 and 100μg of poly I:C or PBS i.p. at d3. **(A)** Weight loss and **(B)** blood oxygen saturation following infection (representative exp. n=4-6: † signifies mice below 70% initial weight were sacrificed). Weight loss statistics determined by two-way ANOVA comparing ABX+pIC to ABX ($p<0.01$) or CNV to ABX ($p<0.01$). **(C)** Survival curve following influenza virus infection. Survival curve is a combination of 2 independent experiments. CNV n=10, ABX n=8, ABX+pIC n=12. Survival statistics determined by logrank test comparing ABX+pIC to ABX ($p<0.05$) or ABX+pIC to CNV ($p=0.12$). *p <0.05, **p<0.01.

Chapter 3
Commensal bacteria-derived signals augment innate and adaptive immune responses following systemic viral infection

3.1 Abstract

Commensal bacterial communities can modulate intestinal immune homeostasis and response to infection in the gastrointestinal tract. Data presented in **Chapter 2** demonstrated a role for commensal bacteria-derived signals in promoting the antiviral immune response to a localized respiratory viral infection (influenza virus). Whether commensal bacteria-derived signals influence immunity to systemic viral infection, however, remains poorly defined. To test this, CNV or ABX- treated mice were infected with Lymphocytic Choriomeningitis Virus (LCMV) and the induction and quality of the innate and adaptive immune responses were analyzed. Macrophages isolated from ABX-treated mice exhibited lower expression of co-stimulatory molecules and impaired activation the type I interferon (IFN) signaling pathway. The diminished innate immune response was associated with a defective adaptive immune response. ABX-treated mice exhibited a functionally impaired LCMV-specific CD8 T cell response and delayed control of viral replication compared to CNV control mice. Collectively, these studies indicate that signals derived from commensal bacteria aid in the induction of a rapid antiviral immune response required for immunity to systemic viral infection.

3.2 Introduction

Intestinal commensal bacteria are important in the development and homeostasis of the GALT (Artis, 2008; Macpherson and Harris, 2004). Immunologic analysis of mice that were reared in the absence of live commensal bacteria (GF mice), highlight the significance of the relationship between commensal bacterial communities and the intestinal immune system. For example, the Peyer's patches and mesenteric lymph nodes of GF mice are reduced in cellularity and fewer CD4 and CD8 T cells reside in the intraepithelial compartment compared to CNV control mice (Glaister, 1973) (Macpherson and Harris, 2004). In addition to developmental defects, loss of commensal bacteria-derived signaling impacts intestinal immune cell homeostasis and inflammation, altering immune cell differentiation, migration, and antibody production (Ivanov et al., 2008; Macpherson and Uhr, 2004; Niess and Adler, 2010; Obata et al., 2010; Round and Mazmanian, 2010). Several murine models of spontaneous intestinal inflammation, in part characterized by overproduction of proinflammatory cytokines in the intestinal microenvironment, exhibit amelioration of disease when re-derived in a sterile, germ-free environment (Dianda et al., 1997; Schultz et al., 1999; Sellon et al., 1998). Collectively, these studies demonstrate a fundamental relationship between commensal bacteria and the intestinal immune system.

However, whether commensal bacteria-derived signals shape the systemic immune system, which is not in direct contact with commensal bacteria that reside at barrier surfaces of the body, is less well characterized. GF mice have fewer germinal centers and plasma cells in the spleen supporting a role for commensal bacteria in immune development beyond the GALT (Macpherson and Harris, 2004). It has also been reported that the commensal specie Segmented Filamentous Bacteria can impact

disease progression in murine models of autoimmune inflammatory disorders such as arthritis, multiple sclerosis and diabetes (Ivanov et al., 2009; Kriegel et al., 2011; Lee et al., 2011; Wu et al., 2010b), suggesting that commensal bacterial communities can have an impact on systemic inflammatory responses. It is unknown, however, whether commensal bacteria-derived signals help shape the systemic immune response to a pathogen that infect multiple tissues throughout the host. This chapter addresses the impact of commensal bacteria-derived signals on the host immune response to a systemic pathogen, Lymphocytic Choriomeningitis Virus (LCMV).

Unlike influenza virus, which was employed in studies described in **Chapter 2**, LCMV is a non-cytolytic virus that can infect multiple organs, such as the spleen, liver, kidney, lung as well as the gastrointestinal tract (Buchmeier et al., 1980; Sydora et al., 1996). Infection can result in a chronic viremia if not efficiently controlled by the antiviral immune response with viral reservoirs persisting at immuno-privileged sites such as the CNS and kidney (Ahmed et al., 1987; Ahmed et al., 1984b; Zajac et al., 1998). LCMV infection elicits a potent CD8 cytotoxic T lymphocyte (CTL) response that is crucial for viral control through the production of proinflammatory cytokines (e.g. TNF-α and IFN-γ) and cell-mediated cytotoxicity (Ahmed et al., 1984a; Kaech et al., 2002; Welsh et al., 1990). Dendritic cells and macrophages of the innate immune system are critically important in the priming and expansion of the LCMV-specific CD8 T cell response via antigen presentation, co-stimulatory molecule interaction, and production of proinflammatory cytokines such as type I IFNs that provide a 3rd signal to initiate T cell activation (Belz et al., 2005; Cousens et al., 1999; Montoya et al., 2005; Oehen et al., 2002; Seiler et al., 1997). In this chapter, we demonstrate that following LCMV infection mice depleted of commensal bacteria exhibit a functionally defective LCMV-specific CD8

T cell response that correlates with delayed control of viral replication. Compared to CNV mice, ABX-treated mice exhibited diminished induction of innate proinflammatory cytokines and antiviral defense genes early following infection. Specifically, macrophages from ABX-treated mice exhibited an impaired response to type I IFN signaling and a diminished activation profile. Thus, commensal bacteria-derived signals are critical in the intiation of the early innate immune response to systemic viral infection. Lack of sufficient commensal bacteria-derived signaling lead to downstream defects in the adaptive immune response and ultimately an inability to control viral infection.

3.3 Methods

3.3.1 Mice and viruses

C57BL/6 mice (4-6 weeks old) were purchased from the National Cancer Institute (Frederick, MD). Mice were maintained in specific pathogen–free facilities at the University of Pennsylvania. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved all protocols, and all experiments were performed according to the guidelines of the University of Pennsylvania IACUC. Mice were inoculated intravenously (i.v.) with LCMV T1b ($4x10^6$ PFU). LCMV viral titers were determined by plaque assay on Vero cell monolayers (Ahmed et al., 1984b).

3.3.2 Oral antibiotic treatment

Mice were provided autoclaved drinking water supplemented with ampicillin (0.5 mg/mL, Sigma), gentamicin (0.5 mg/mL, Gemini Bio-Products), metronidazole (0.5 mg/mL Sigma), neomycin (0.5 mg/mL, Med-Pharmex), vancomycin (0.25 mg/mL, Novaplus), and sucralose (4mg/mL, Splenda, McNeil Nutritionals, LLC). Splenda was added to

make the antibiotic cocktail more palatable. Antibiotic treatment was started 2-4 weeks prior to infection and continued for the duration of the experiment.

3.3.3 Isolation of cells and flow cytometric staining

Lymphocytes were isolated from the spleen or lymph node by mechanical disruption through 70μm cell strainers followed by red blood cell (RBC) lysis. Peripheral blood mononuclear cells (PBMCs) were isolated using a Histopaque gradient (Sigma). Peritoneal exudates cells (PECs) were obtained by injecting and recovering PBS from the peritoneal cavity. Single cell suspensions were surface stained in FACS Buffer (PBS, 2% BSA, 0.2mg Sodium Azide, 2mM EDTA) using standard flow cytometric staining protocols with fluorescently conjugated antibodies specific to CD3ε, CD4, CD8α, CD11c, CD19, CD25, CD43, CD45, CD69, CD160, F4/80, Ly6c, MHC-I (H-2K^b), MHC-II (I-A/I-E) (eBioscience), CD5, CD44, CD80, CD86, Ly6g (clone 1A8), ICOS, LAG-3, PD-1 (clone RMP1-30) (Biolegend), CD40 (BD Biosciences), CXCR3 (R&D Systems), 2B4 (BD Pharmingen), CD11b, and granzyme B (Invitrogen). Major histocompatibility complex (MHC) class I peptide tetramers were made and used to identify virus-specific CD8 T cells (Altman et al., 1996). For intracellular cytokine analysis, 10^6 splenocytes were cultured in a 96-well plate in complete media with viral peptides (0.2 μg/ml) and brefeldin A (Golgiplug, eBioscience) for 5 hrs at 37°C. After staining for surface antigens as described above, cells were stained for intracellular cytokines using the Cytofix/Cytoperm kit (BD Pharmingen) and fluorescently conjugated antibodies specific for IL-2 (eBioscience), IFN-γ (BD Pharmingen), TNF-α (Biolegend), MIP-1α (R&D Systems). The CD107a assay was performed as previously described (Blackburn et al., 2009). Cell viability was assessed with Live/Dead AQUA stain (Invitrogen). Samples

were collected by using a LSR II flow cytometer (Becton Dickinson). All flow cytometry data was analyzed by FlowJo v 8.8 (Treestar). Pie charts were created using the Pestle and SPICE programs (Mario Roederer; Vaccine Research Center, NIAID, NIH).

3.3.4 Virus-specific antibody and cytokine ELISA

LCMV-specific IgM and IgG serum antibody concentrations were determined by ELISA as previously described (Ahmed et al., 1984b; Mozdzanowska et al., 2000). In brief, LCMV was adsorbed to plates and bound antibody was detected using anti-IgM or -IgG conjugated to alkaline phosphatase (Sigma) and developed with *p*-nitrophenol phosphate (Sigma). Optical densities ($OD_{405-750}$) were measured using an ELISA reader and concentrations calculated using SoftMax Pro software (Molecular Devices). Serum samples were analyzed for cytokines and chemokines by luminex multiplex bead array (Millipore). Tissue from the spleen was mechanically homogenized in DMEM, centrifuged, and the resultant supernatant was measured for cytokine secretion by ELISA (eBioscience). IFN-β protein levels in the serum or spleen homogenates were measured by ELISA (PBL Interferonsource).

3.3.5 RNA isolation, cDNA preparation, and RT-PCR

RNA was isolated from cells using an RNeasy mini-kit (Qiagen) and from spleen tissue using mechanical homogenization and TRIzol isolation (Invitrogen) according to the manufacturer's instructions. cDNA was generated using SuperScript reverse transcriptase (Invitrogen). RT-PCR was performed on cDNA using SYBR green chemistry (Applied Biosystems) and commercially available primer sets (Qiagen). Reactions were run on an RT-PCR system (ABI7500; Applied Biosystems). Genes of

interest were normalized to hypoxanthine guanine phosphoribosyl transferase (*Hprt)* and displayed as fold difference relative to uninfected CNV control mice.

3.3.6 *In vivo* Phosflow STAT1 staining following LCMV infection

LCMV-infected CNV or ABX-treated mice were sacrificed at 6 or 12 hrs post-infection. Cells were immediately fixed to preserve phosphorylation status as described in (Krutzik et al J.I. 2005). Briefly, cells were directly resuspended in DMEM w/ 1.6% PFA. Following a 10 minute incubation at room temperature, cells were centrifuged and resuspended in 90% methanol for 30 minutes at 4 ^{o}C then washed 3x with FACS buffer followed by staining with surface markers and pSTAT1 with PE-conjugated anti-STAT1 (pY701) antibody (BD biosciences).

3.3.7 Statistical analysis

Results represent means ± SEM. Statistical significance was determined by the unpaired, two tailed, Student's *t*-test for individual timepoints, two-way ANOVA test for timecourse experiments, logrank test for survival curve, or two-part *t*-test for comparison of groups that contained samples that were below the limit of assay detection. Statistical analyses were performed using Prism GraphPad software v4.0. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3.4 Results

3.4.1 Diminished adaptive immune response and delayed clearance of LCMV following ABX-mediated depletion of commensal bacteria

Signals from commensal bacteria regulate immune cell homeostasis and susceptibility to multiple infectious and inflammatory diseases in the gastrointestinal tract (Garrett et al., 2010b; Honda and Littman, 2012; Round and Mazmanian, 2009). However, it is not clear whether commensal bacteria-derived signals regulate immunity to pathogens that infect sites distal to the gastrointestinal tract. To address this question, naïve C57BL/6 mice were orally administrated broad-spectrum antibiotics for two weeks and subsequently infected with $2x10^6$ PFU LCMV T1b, a strain of virus that results in viremia for 1-2 weeks and requires a robust innate and adaptive immune response for viral clearance (Blackburn et al., 2009; King et al., 1990). Consistent with our earlier studies, exposure to antibiotics resulted in loss of culturable bacteria from enteric contents, a reduction in total bacterial 16S rDNA in the intestine and a dramatic reorganization of the commensal community structure (Figure 18A) (Hill et al., 2010). CNV mice and ABX-treated mice were longitudinally bled following LCMV infection to assess viral clearance and induction of virus-specific cellular and humoral adaptive immune cell response. CNV mice exhibited maximal viremia at day 7 (d7) post-infection and successfully controlled viremia by d23 (Figure 18B). Control of infection was associated with the population expansion of LCMV-specific H-2D^b gp33$^+$ and H2-D^b gp276$^+$ tetramer$^+$ (H-2Db/GP33$^+$ and H-2Db/GP276$^+$) CD8 T cells (Figure 18C,D), the two dominant CD8 T cell epitopes, as well as the presence of LCMV-specific IgG in the serum (Figure 18E). In contrast, ABX-treated mice exhibited a significant delay in clearance of LCMV (Figure 18B) that was associated with a delayed expansion of LCMV-specific CD8 T cell response and

reduced IgG antibody titers in the serum (Figure 18C-E). Thus, longitudinal bleeds to monitor a systemic viral infection in mice depleted of commensal microbial communities revealed a significant defect in the ability to control viremia and mount an adaptive immune response to LCMV infection in ABX-treated mice.

CNV or ABX-treated mice were sacrificed one month following LCMV infection and virus-specific immune responses and persisting viral reservoirs in peripheral tissues were analyzed. At d31 post-infection, ABX-treated mice exhibited increased viral titers in the kidneys (Figure 19A) that correlated with fewer total numbers of LCMV-specific tetramer$^+$ (H-2Db/GP33$^+$, H-2Db/GP276$^+$ and H-2Db/NP396$^+$) CD8 T cells in the spleen (Figure 19C-D). Elevated viral titers and decreased numbers of LCMV-specific CD8 T cells in ABX-treated mice indicate a significant impaired ability to control systemic viral infection in ABX-treated mice.

3.4.2 ABX-treated mice exhibit a functionally impaired LCMV-specific CD8 T cell response

Persisting viral reservoirs and fewer virus-specific CD8 T cells has been closely linked to functionally impaired CD8 T cell responses (Fuller et al., 2004; Wherry et al., 2005; Zajac et al., 1998), which can be characterized by a hierarchical increase in expression of multiple inhibitory receptors and decreased expression of effector molecules, attributes considered to be hallmarks of CD8 T cell exhaustion (Blackburn et al., 2009; Wherry et al., 2003). Therefore, LCMV-specific CD8 T cells isolated from the spleen of CNV or ABX-treated mice were assessed for expression of inhibitory receptors and effector molecules to measure CD8 T cell function. At d31 post-infection, LCMV-specific H2-Db/GP33 tetramer$^+$ CD8 T cells isolated from ABX-treated mice expressed increased

levels of multiple inhibitory receptors PD-1, 2B4, CD160 and LAG-3 compared to H2-Db/GP33 tetramer$^+$ CD8 T cells from CNV mice (Figure 20A,B).

Following *ex vivo* stimulation with viral peptides, cytotoxic CD8 T cells (Figure 21A,B from CNV mice readily produced effector cytokines such as IFN-γ and TNF-α, critical effector molecules with potent antiviral properties (Leist et al., 1989; Ramshaw et al., 1997). In contrast, GP33 responsive CD8 T cells from ABX-treated mice were less efficient producers of multiple effector molecules (IFN-γ, TNF-α, IL-2, MIP-1α, CD107a) (Figure 21A,B). To evaluate whether the impaired DbGP33 tetramer$^+$ CD8 T cell response in ABX-treated mice is indicative of the global LCMV-specific CD8 T cell response, splenocytes were stimulated with a pool of 20 LCMV-specific peptides (Kotturi et al., 2007). Following stimulation with pooled LCMV-specific peptide, fewer IFN-γ^+/TNF-α^+ dual producing CD8 T cells were observed in ABX-treated mice compared to CNV mice (Figure 21C,D) confirming that the functionally impaired response to the GP33-41 epitope was representative of the total LCMV-specific CD8 T cell response in ABX-treated mice. Together, these data are consistent with more severe T cell exhaustion in the ABX-treated group, a characteristic sign of impaired immunity to LCMV infection (Wherry, 2011; Yi et al., 2010).

In addition, while there was no significant difference in the total number of LCMV-specific I-Ab/GP61 tetramer$^+$ CD4 T cells in the spleen at d31 post-infection (Figure 22A), a significant increase in PD-1 expression on GP61 tetramer$^+$ CD4 T cells was observed in the spleen ABX-treated mice compared to CNV mice (Figure 22B), indicating impairment in the quality of the virus-specific CD4 T cell response. Stimulation of splenocytes with GP61 peptide revealed that fewer GP61 tetramer$^+$ CD4 T cells isolated from ABX-treated mice produced cytokines (IFN-γ and IL-2) compared to CD4 T

cells from CNV mice (Figure 22C). Combined, these data indicate that deliberate manipulation of commensal bacteria-derived signals results in defective virus-specific adaptive CD4 and CD8 T cell responses and impaired control of viral replication following systemic infection.

3.4.3 Impaired innate immune response to LCMV infection in ABX-treated mice

Studies discussed in **Chapter 2** demonstrated that depletion of commensal bacteria resulted in reduced adaptive immune responses and impaired viral clearance that correlated with diminished induction of innate proinflammatory cytokines and antiviral defense genes early following infection. To determine whether a diminished innate antiviral response was a generalized phenomenon in ABX-treated mice, expression of macrophage-associated antiviral response genes and production of proinflammatory cytokines were examined during LCMV infection. As early as 12 hours post-LCMV infection significantly reduced expression of mRNA encoding *Ifnb*, *Irf7*, *Mx1*, *Oas1a* and *Stat1* in the spleen (Figure 23A) and decreased IFN-β in the serum (Figure 23B) was observed in ABX-treated mice compared to CNV mice. Furthermore, reduced levels of proinflammatory cytokines were detected in the spleen homogenates of ABX-treated mice following LCMV infection (Figure 23C), indicating a diminished innate antiviral responses following systemic viral infection. In addition, expression of costimulatory molecules on macrophages and dendritic cells from CNV or ABX-treated mice was assessed as a measure of innate immune activation following infection. Macrophages isolated from ABX-treated LCMV-infected mice exhibited reduced expression of MHC-I and CD86 (Figure 23D) and DCs expressed reduced MHC-II and CD86 at d1 post-infection (Figure 23E). Collectively, these findings indicate that ABX-mediated alterations

in commensal bacteria result in selective dysregulation of innate immune responses following systemic viral infection.

3.4.4 Impaired *in vivo* antiviral macrophage response following LCMV in ABX-treated mice

Induction of type I IFNs in response to viral infection is a critical step in initiating early innate antiviral defense mechanisms (Hwang et al., 1995; Muller et al., 1994). IFN signaling leads to transcription of antiviral defense genes, the recruitment and activation of innate immune cells, and aid in the induction of T cell expansion and differentiation (Ramshaw et al., 1997; Sen, 2001). Diminished activation of innate immune cells and induction of proinflammatory cytokines in ABX-treated mice early following LCMV infection suggests defects in the IFN response. STAT1 is a transcription factor downstream of the IFN receptor signaling pathway (Li et al., 1997). Following IFN stimulation, STAT1 is phosphorylated, translocates into the nucleus, and initiates transcription of multiple antiviral defense genes including a positive feedback loop of more type I IFN production (Schindler et al., 1992; Shuai et al., 1992). Therefore, STAT1 phosphorylation can be used as a bio-marker for IFN signaling. To determine whether defects in macrophage interferon signaling exist *in vivo* following viral infection, CNV or ABX-treated mice were infected with LCMV and the STAT1 phosphorylation status of innate immune cells in the spleen and peritoneal cavity was assessed by fixing cells directly *ex vivo*. This procedure preserves the phosphorylation status of the STAT protein in the cell as it existed *in vivo* (Krutzik et al., 2005). As early as 6 hr post-infection, macrophages isolated from the spleen of ABX-treated mice displayed reduced pSTAT1 compared to splenic macrophages in CNV mice (Figure 24A,B). By 12hr post-infection, peritoneal macrophages from ABX-treated mice had yet to display a significant

phosphorylation of STAT1 while pSTAT1 induction was detectable in peritoneal macrophages from CNV mice (Figure 24C,D). These results indicate that antiviral responses in macrophages from ABX-treated mice following LCMV infection are impaired *in vivo*. These data, in combination with the data presented in **Chapter 2** identifying diminished expression of antiviral defense genes in alveolar macrophages isolated from ABX-treated mice following influenza virus infection (Figure 16), suggest a global defect in macrophage antiviral response in hosts lacking exposure to commensal bacteria-derived signals.

3.5 Discussion

Commensal bacterial communities can modulate immune cell homeostasis and disease by providing either immunoregulatory or proinflammatory signals (Round and Mazmanian, 2009). For example, the microbial product polysaccharide A, isolated from *Bacteroides fragilis,* can reduce the severity of intestinal inflammation in two murine models of IBD (Mazmanian et al., 2008). In addition, Segmented Filamentous Bacteria, an enteric commensal bacteria induces a $CD4^+$ T_H17 cell population in the small intestine leading to increased susceptibility to autoimmune arthritis and experimental autoimmune encephalomyelitis (Lee et al., 2011; Wu et al., 2010b), demonstrating that intestinal bacteria can shape the inflammatory response at sites outside of the local gastrointestinal environment. These studies provoke the hypothesis that commensal bacteria-derived signals might influence the systemic immune response to infection.

The results presented in this chapter demonstrate a role for commensal bacterial communities in influencing the immune response to a systemic viral infection. Antibiotic-mediated disruption of commensal bacterial communities compromised innate and

adaptive antiviral immunity following LCMV infection. Severe defects in the adaptive immune response to LCMV as well as poor control of viral replication pointed toward early defects in the initiation of antiviral responses. Indeed, significant differences in the macrophages between CNV and ABX-treated mice were apparent as early as 6 hours following infection. For rapidly replicating viruses, such a delay in initiating antiviral pathways and activating downstream events, such as humoral and cell-mediated adaptive immune responses, can lead to failure to control infection, as well as more dramatic consequences such as host mortality, as was observed with influenza virus in **Chapter 2**. Together, these data suggest a model in which signals from commensal bacteria are priming the innate immune system to ensure a rapid and robust immune response upon encounter with viral pathogens. The impact of commensal bacteria-derived signals on shaping innate immune cells prior to infection will be addressed in **Chapter 4**.

This chapter established that commensal bacteria influence activation of IFN signaling, a ubiquitous innate antiviral response pathway. Induction of a type I IFN response is fundamental and critical for defense against the majority of viruses and is a hard-wired response usually induced within minutes to hours of viral infection (Ramshaw et al., 1997; Sen, 2001). Macrophages isolated from ABX-treated mice, however, displayed defects in phosphorylation of STAT1, an immediate downstream molecule of type I IFN/ IFN receptor interactions, early following viral infection compared to macrophages from CNV mice. This reduction in STAT1 phosphorylation was associated with a significant decrease in proinflammatory cytokine production and reduced induction of antiviral defense genes.

It was notable that macrophages isolated from ABX-treated mice that had not been infected with LCMV also displayed less detectable *in vivo* STAT1 phosphorylation compared to macrophages from naïve CNV counterparts (Figure 23). This trend was consistent over several experiments, however did not reach statistical significance. Potentially, commensal bacteria-derived signals may induce tonic, low-level STAT1 activation at steady-state impacting basal transcription of antiviral defense genes prior to infection. This possibility will be further explored in **Chapter 4**. Notwithstanding this, the mechanisms through which commensal bacteria-derived signals stimulate immune cells in the periphery are poorly understood. One possibility is that lymphoid tissue-resident immune cells are directly exposed to commensal bacteria or their products. Small numbers of live commensal bacteria can be found in the Peyer's patches and mesenteric lymph nodes of mice at steady-state, and there is some evidence that absorbed commensal products circulate throughout the host (Clarke et al., 2010; Macpherson and Uhr, 2004; Obata et al., 2010). Thus, direct interaction between peripheral immune cells and microbial products is plausible; however, the mechanism of microbial translocation from sites of bacterial colonization remains poorly defined. Another possibility is that commensal bacteria act indirectly on peripheral immune cells via responses evoked from epithelial or other mucosal-associated cells. Commensal-driven secretion of epithelial-derived cytokines can regulate intestinal immune cell homeostasis (Bouskra et al., 2008; He et al., 2007; Rimoldi et al., 2005b; Zaph et al., 2007), raising the possibility that epithelial cells exposed to commensal bacteria in the steady-state could secrete immunomodulatory factors into the circulation that influence other hematopoietic cells. Defining the potential pathways involved in microbial sensing

by the peripheral immune system will be crucial to understanding how microbial crosstalk influences immune cell homeostasis and host protective immunity.

In summary, these studies have uncovered a previously unrecognized role for commensal bacteria-derived signals in influencing the immune response to a systemic virus. In addition, these studies provide novel insights into how distinct microbial sensing and response pathways are integrated and provide evidence for critical cross-regulation of antiviral immunity by bacterial communities. These findings could lead to new opportunities to improve antiviral immunity and to understand the role of commensal bacteria in influencing patients' predisposition to distinct immunologic and pathologic outcomes from viral infections.

Figure 18. Systemic LCMV T1b infection results in delayed viral clearance and reduced expansion of the LCMV-specific CD8 T cell response in ABX-treated mice.

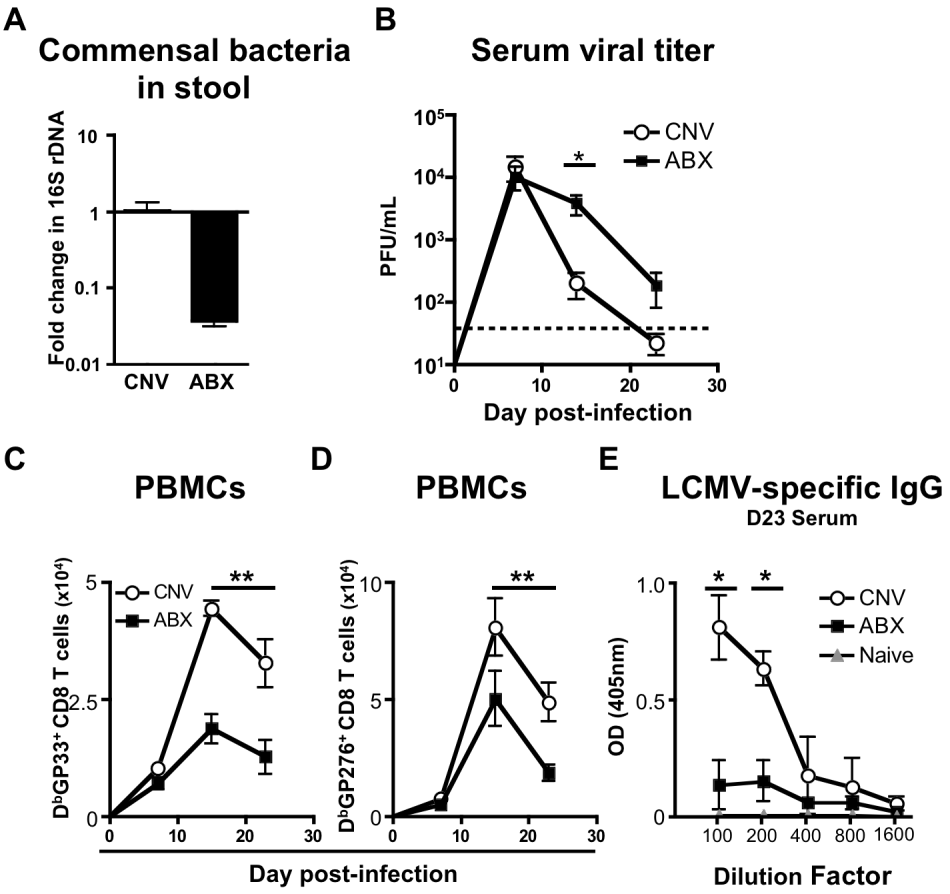


Figure 18. (A) Total bacterial 16S rDNA in stool samples from naïve CNV or ABX-treated mice following two weeks of antibiotic treatment. **(B-E)** CNV or ABX-treated C57BL/6 mice were infected i.v. with $2x10^6$ PFU of LCMV T1b. **(B)** Viral titer in the serum following infection (L.o.D., limit of detection). LCMV-specific **(C)** DbGP33 and **(D)** DbGP276 tetramer$^+$ CD8 T cells per 10^6 peripheral blood mononuclear cells (PBMCs) at d7, 14, and 23 post-infection. **(E)** Serial dilution of LCMV-specific IgG antibody titers in serum of CNV or ABX-treated mice at d23 post-infection. Naïve serum from CNV mice used for baseline. Data representative of three independent experiments with n=5 mice per group. Data shown are the mean ± SEM. Serum viral titer statistics determined by two-part *t*-test for each timepoint. *p<0.05,

Figure 19. ABX-treated mice exhibit persisting viral reservoirs and fewer LCMV-specific CD8 T cells at D31 post-infection.

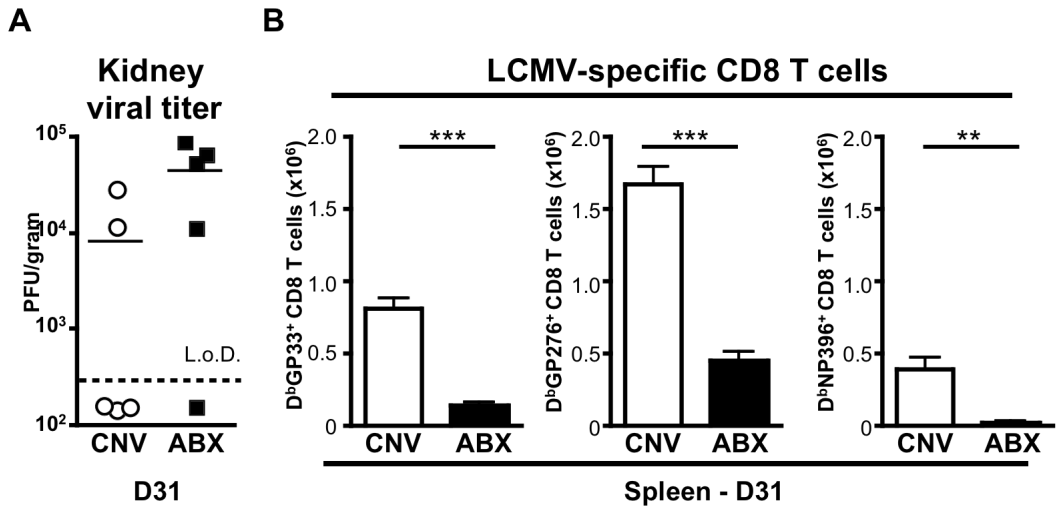


Figure 19. (A) Viral load in the kidney of CNV or ABX-treated mice at d31 post-LCMV infection. **(B)** Total number of DbGP33, DbGP276, and DbNP396 tetramer^{+} CD8 T cells in the spleen. Data representative of two-three independent experiments with n=5 mice per group. Data shown are the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Figure 20. Increased expression of inhibitory receptors on LCMV-specific CD8 T cells in ABX-treated mice.

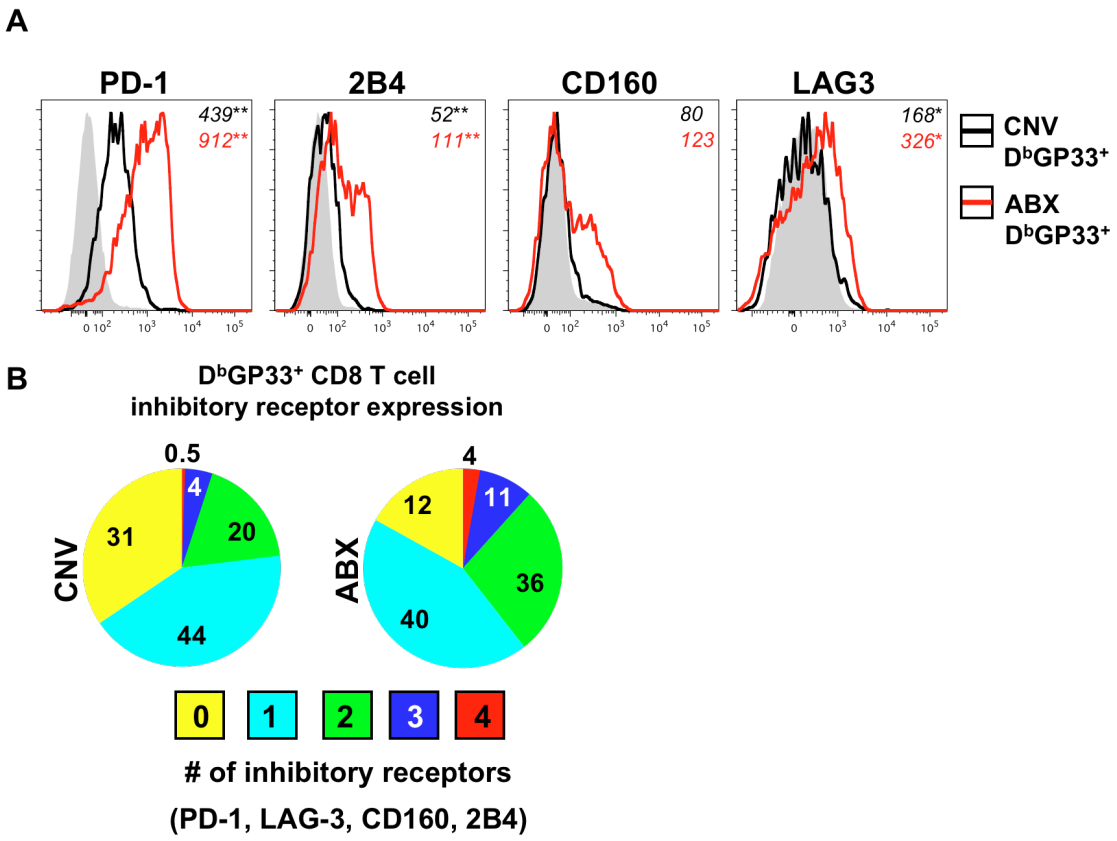


Figure 20. (A) Expression of inhibitory receptors PD-1, 2B4, CD160, LAG-3 on DbGP33 tetramer^{+} CD8 T cells isolated from the spleen of CNV (black line) or ABX-treated (red line) mice at d31 post-LCMV infection. Shaded histograms represent CD44^{lo} CD8 T cells. Numbers in italics represent mean fluorescence intensity (MFI). **(B)** Proportion of DbGP33 tetramer^{+} CD8 T cells expressing multiple inhibitory receptors. Data representative of two-three independent experiments with n=5 mice per group. Data shown are the mean. *p<0.05, **p<0.01.

Figure 21. LCMV-specific CD8 T cells isolated from ABX-treated mice produce less effector cytokines.

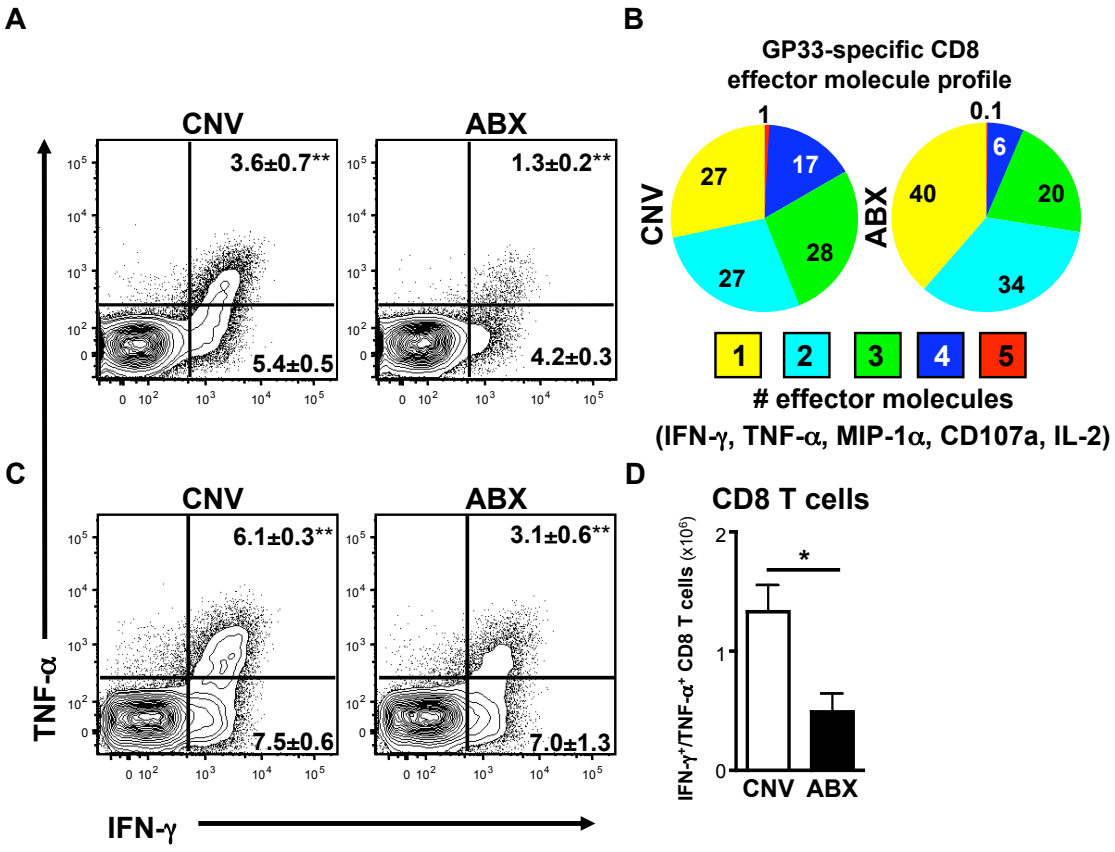


Figure 21. Splenocytes from d31 LCMV-infected mice were incubated with GP33 peptide **(A,B)** or a pool of 20 LCMV-specific peptides **(C,D)** (Kotturi et al., 2007) for 5 hrs in the presence of BFA and assessed for production of effector molecules (IFN-γ, TNF-α, MIP-1α, CD107a, IL-2). **(A)** Frequency of IFN-γ/TNF-α dual producing CD8 T cells in the spleen following GP33 peptide stimulation. **(B)** Proportion of GP33 peptide responsive CD8 T cells producing multiple effector molecules. **(C)** Frequency and **(D)** total number of IFN-γ/TNF-α dual producing CD8 T cells in the spleen following stimulation with pooled LCMV peptides. FACS plots gated on live, CD8α$^+$ cells. Data representative of two-three independent experiments with n=5 mice per group. Data shown are the mean ± SEM. *p<0.05, **p<0.01.

Figure 22. ABX-treated mice exhibited a functionally impaired CD4 T cell response following LCMV infection.

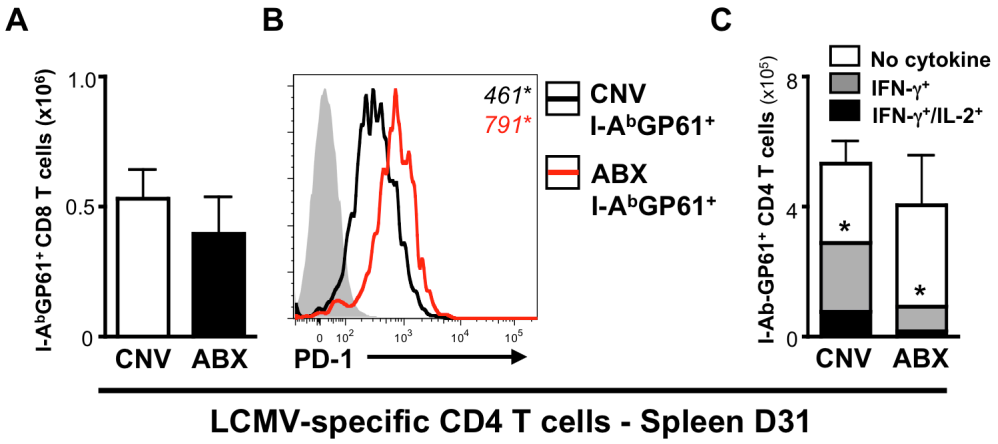


Figure 22. (A) Total number of I-AbGP61 tetramer$^+$ CD4 T cells in the spleen of CNV or ABX-treated mice at d31 post-LCMV infection. **(B)** Expression of PD-1 on I-AbGP61 tetramer$^+$ CD4 T cells from the spleen of CNV (black line) or ABX-treated (red line) mice at d31 post-infection. Shaded histogram represents CD44^{lo} CD62L^{hi} naïve CD4 T cells. Numbers in italics represent MFI. **(C)** Splenocytes from d31 infected mice were either stained with I-AbGP61 tetramer or incubated with LCMV peptide (GP61-80) for 5 hrs in the presence of BFA and assessed for production of IFN-γ and IL-2. Data shown are the mean ± SEM. *p<0.05.

Figure 23. Innate antiviral immune response is diminished in ABX-treated mice following LCMV infection.

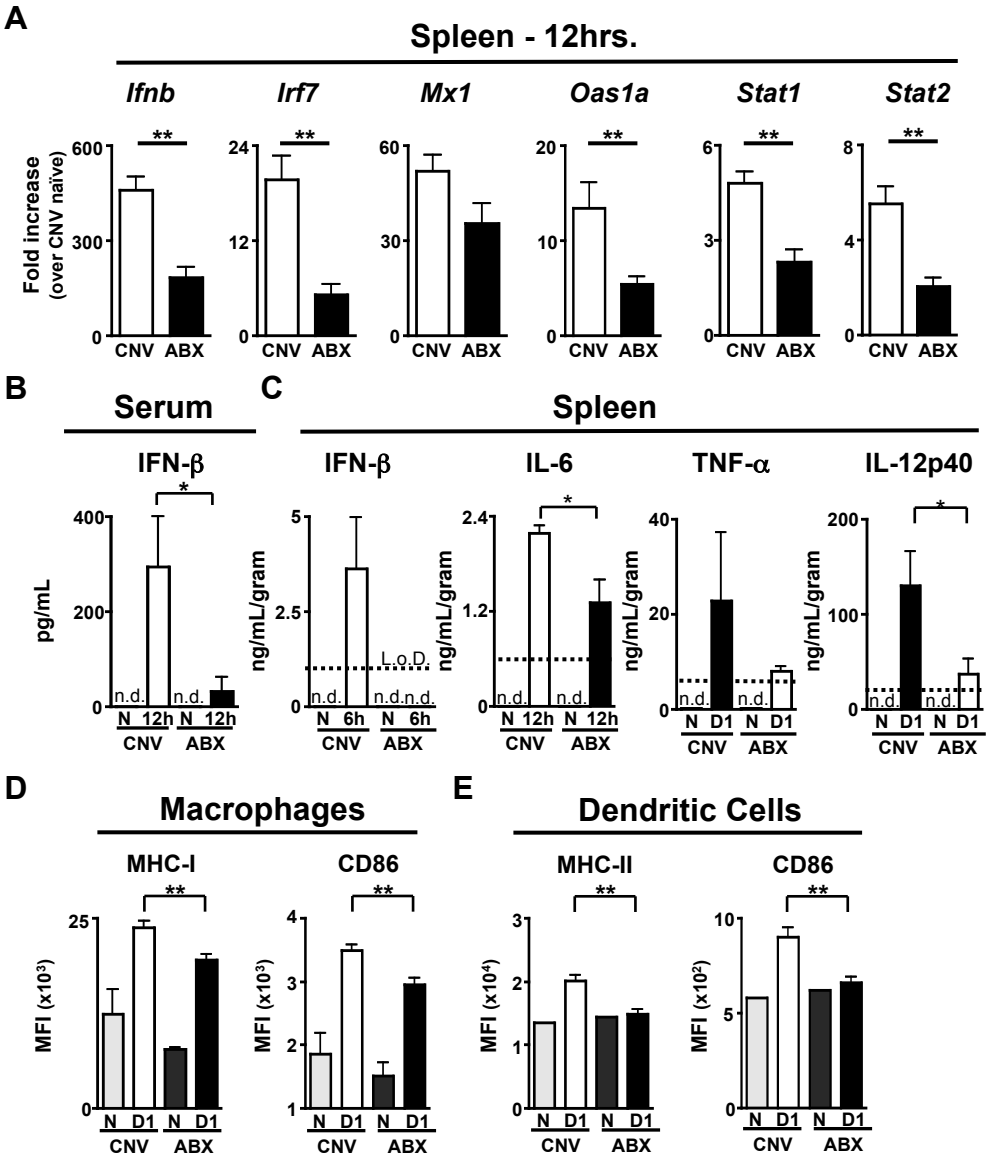


Figure 23. (A) Fold induction of antiviral defense genes in the spleen 12 hrs following LCMV infection compared to expression in the spleen of naïve CNV mice. **(B)** IFN-β levels in the serum at 12 hrs post-LCMV infection as detected by ELISA. **(C)** Proinflammatory cytokine levels in spleen homogenates at 6-24hr post LCMV infection (n.d., not detectable). **(D)** Expression of MHC-I and CD86 on peritoneal macrophages and **(E)** MHC-II and CD86 on peritoneal dendritic cells from CNV or ABX-treated mice 24 hrs post LCMV infection. Data representative of two or more independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM. *$p<0.05$, **$p<0.01$.

Figure 24 Antiviral macrophage response is impaired in ABX-treated mice following LCMV infection.

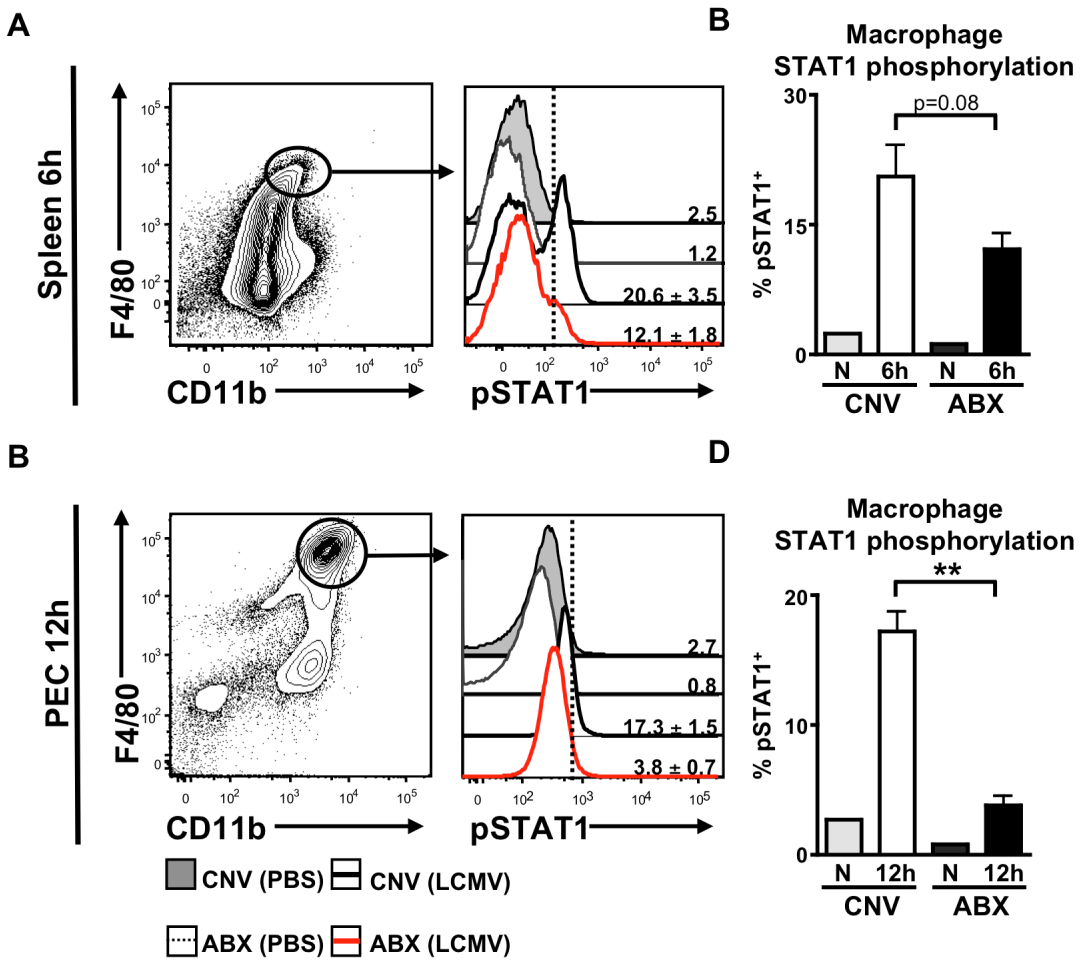


Figure 24. CNV or ABX-treated mice were inoculated with LCMV or PBS i.v. and **(A,B)** splenocytes at 6 hrs post-infection or **(C,D)** peritoneal exudate cells at 12 hrs post-infection were isolated and immediately fixed to preserve the *in vivo* STAT1 phosphorylation status of macrophages. **(A,B)** Frequency of $pSTAT1^+$ splenic macrophages. Splenic macrophages gated on Non T, Non-B, Non NK cells (NTNBNNK), $CD11c^-$, $Ly6g^-$, $Ly6c^-$, $F4/80^+$, $CD11b^{lo}$. **(C,D)** Frequency of $pSTAT1^+$ peritoneal macrophages. Peritoneal macrophages gated on NTNBNNK, $F4/80^+$, $CD11b^+$. Data representative of two independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM. *p<0.05, **p<0.01.

Chapter 4
Commensal bacteria-derived signals calibrate the activation threshold of the innate immune system

4.1 Abstract

Commensal bacterial communities shape intestinal immune cell homeostasis and can influence the predisposition to intestinal and systemic inflammatory and autoimmune diseases. Further, commensal bacteria can act as an adjuvant and boost the immune response to an ongoing infection. However, it is unclear whether commensal bacteria-derived signals can shape immune cell activation prior to infection rendering the immune system more or less susceptible to a potential pathogenic challenge. Defective macrophage responses to viral infection in mice depleted of commensal bacterial communities, as reported in **Chapters 2** and **3,** suggested an inherent impairment in the ability of macrophages from ABX-treated mice to respond to viruses. Genome-wide transcriptional profiling of macrophages isolated from naïve ABX-treated mice revealed decreased expression of genes associated with antiviral immunity. Moreover, macrophages isolated from ABX-treated mice exhibited defective responses to type I/II IFNs and impaired capacity to limit viral replication. Transfer of macrophages from CNV mice into ABX-treated mice immediately prior to LCMV infection improved the virus-specific adaptive immune response and restored the ability to control viremia. Collectively, these data indicate that commensal bacteria-derived signals provide tonic immune stimulation that calibrates the activation threshold of the innate immune system required for optimal antiviral immunity.

4.2 Introduction

Host defense against invasive pathogens requires a coordinated response by the innate and adaptive immune system. The role of the innate immune system is detection of pathogens and initiation of the proper immune response needed to eliminate infection. In the absence of antigen-specificity, which evolved with the adaptive immune system to enable a targeted immune response against a specific pathogen, the innate immune system relies on germ-line encoded PRRs that recognize distinct PAMPs to distinguish between types of pathogens. PAMP recognition by PRRs expressed by innate immune cells activates defense pathways that initiate immune responses tailored to effectively combat the particular class of pathogen from which the PAMP originates (Medzhitov, 2007). For example, the innate immune response to parasitic infection leads to activation of genes programmed to prevent colonization and expel the parasite (Reese et al., 2007; Stetson et al., 2004) while viral infection activates genes that limit cytosolic RNA synthesis and thus viral replication (Seth et al., 2006). These innate defense mechanisms can be constitutively expressed, thereby limiting the ability of pathogens to establish a productive infection, but must also be carefully balanced, as overexpression of host defense genes can be detrimental to the host. For example, constitutive expression of type I IFN, a potent antiviral cytokine, can lead to autoimmune diseases when unchecked by negative regulators (Arakura et al., 2007; Hida et al., 2000). In addition, overexpression of proinflammatory cytokines in the intestine, which have important roles in maintaining the intestinal barrier, are associated with an increased incidence of IBD and colonic cancer (Karin et al., 2006; Papadakis and Targan, 2000). Thus, the innate immune system must be dynamically calibrated to enable rapid

response upon encounter with pathogens while limiting detrimental side effects to the host.

The mammalian innate immune system has evolved diverse strategies to detect and respond to a vast array of microbial signals, allowing induction of distinct defense mechanisms tailored to different types of pathogens (anti-bacterial vs. antiviral vs. anti-protozoan response) (Medzhitov, 2007). Despite this apparent specialization in pattern recognition and induction of innate immune responses, crosstalk between pathways has been reported where stimulation from one class of pathogens influences the immune response against another class (Actor et al., 1993; Barton et al., 2007; Spencer et al., 1977). However, it is unclear if commensal bacteria influence innate immune pathways in the steady-state and, if so, whether these interactions modulate the systemic immune response to viral pathogens. Iwasaki and colleagues reported impaired antiviral immunity in the lung following deliberate manipulation of commensal bacteria (Ichinohe et al., 2011). This effect was associated with defective activation of the inflammasome, a pathway activated by bacterial products that is also important in conferring protective immunity against a subset of viruses, including influenza virus and poxviruses (Lamkanfi and Dixit, 2011). Whether depletion of commensal bacteria selectively regulates inflammasome-dependent pathways or represents broader immunological crosstalk between commensal bacteria and other antiviral pathways remains to be determined.

In this chapter, I examine this fundamental question and demonstrate a critical role for commensal bacteria in setting the immune activation threshold of macrophages required for antiviral immunity. Manipulating commensal bacterial communities dramatically impaired host protective immunity following either mucosal (influenza virus, **Chapter 2**) or systemic (LCMV, **Chapter 3**) viral infection, leading to dysregulated adaptive immune

responses and underlying defects in innate antiviral defense pathways. Genome-wide transcriptional profiling of macrophages isolated from naive ABX-treated mice revealed reduced expression of genes associated with IFN activation and antiviral immunity indicating an inherent impairment in responsivness to viruses in ABX-treated mice prior to infection. Moreover, transfer of macrophages isolated from CNV mice into ABX-treated mice re-established antiviral immunity to LCMV infection in ABX-treated mice. Taken together, these data indicate that commensal bacteria provide tonic signals that calibrate the activation threshold and sensitivity of the innate antiviral immune system.

4.3 Methods

4.3.1 Mice

C57BL/6 mice (4-6 weeks old) were purchased from the National Cancer Institute (Frederick, MD). Mice were maintained in specific pathogen–free facilities at the University of Pennsylvania. GF Swiss/Webster mice were maintained in plastic isolator units and fed autoclaved chow and water. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved all protocols, and all experiments were performed according to the guidelines of the University of Pennsylvania IACUC.

4.3.2 Oral antibiotic treatment

Mice were provided autoclaved drinking water supplemented with ampicillin (0.5 mg/mL, Sigma), gentamicin (0.5 mg/mL, Gemini Bio-Products), metronidazole (0.5 mg/mL Sigma), neomycin (0.5 mg/mL, Med-Pharmex), vancomycin (0.25 mg/mL, Novaplus), and sucralose (4mg/mL, Splenda, McNeil Nutritionals, LLC). Splenda was added to make the antibiotic cocktail more palatable. Antibiotic treatment was started 2-4 weeks prior to infection and continued for the duration of the experiment.

4.3.3 Isolation of cells and flow cytometric staining

Single cell suspensions were isolated from the spleen by mechanical disruption through 70μm cell strainers followed by red blood cell (RBC) lysis. Peritoneal exudates cells (PECs) were obtained by injecting and recovering PBS from the peritoneal cavity. Cells were surface stained in FACS Buffer (PBS, 2% BSA, 0.2mg Sodium Azide, 2mM EDTA) using standard flow cytometric staining protocol with fluorescently conjugated antibodies specific to CD3ε, CD4, CD8α, CD11c, CD19, CD25, CD43, CD45, CD69, CD119, CD160, F4/80, Ly6c, MHC-I (H-2K^{b}), MHC-II (I-A/I-E) (eBioscience), CD5, CD44, CD80, CD86, Ly6g (clone 1A8), ICOS, LAG-3, PD-1 (clone RMP1-30) (Biolegend), CD40, IFNαRI (BD Biosciences), CD11b (Invitrogen).

4.3.4 Cell sorting and microarray-data analysis

Peritoneal macrophages (CD3ε^{-}, CD19^{-}, CD11b^{+}, F4/80^{+}) from naïve CNV or ABX-treated mice were sorted directly into TRIzol LS (Invitrogen) on a BD Aria (Beckson Dickson). Additional macrophages from the sample were separately sorted to ensure sort purity (≥95%). For microarray analysis, RNA was extracted from sorted peritoneal macrophages from three biological replicates of naïve CNV or ABX-treated mice. cDNA was amplified using NuGen WT Ovation Pico kit and hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST microarray at the University of Pennsylvania's Microarray facility. Expression levels were summarized using the Robust Multichip Averaging (RMA) algorithm (Irizarry et al., 2003), as implemented by the Affymetrix Power Tool (apt-probeset-summarize). The ClassNeighbors module of GenePattern (Broad Institute, Cambridge MA) was used to identify differentially expressed genes. Gene transcripts with greater than 1.6-fold difference in expression were analyzed using Ingenuity

pathway analysis software (Ingenuity® Systems, www.ingenuity.com). A right-tailed Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the pathway or biological function could be explained by chance. GSEA was performed as described (Subramanian et al., 2005).

4.3.5 IFN stimulation and Phosflow STAT1 staining of macrophages

PECs from naïve CNV or ABX-treated mice were plated on a 6 well plate for 3 hrs to allow macrophage adherence. Non-adherent cells were washed away with PBS and remaining cells were counted and seeded at $1x10^5$ cells per well in complete media in a 96 well plate and incubated at 37°C for 3 hrs to allow cell adherence. Media was then removed and cells washed 2x with PBS. Macrophages were stimulated with recombinant IFN-γ (R&D Systems) or IFN-β (PBL Interferonsource) in complete media. Following stimulation, media was removed and replaced with 0.05% trypsin and incubated at 37°C for 2 minutes. Cells were then fixed with 1.6% PFA in complete media for ten minutes at room temperature, transferred to a 96-well round bottom plate, centrifuged, and permeabilized with ice cold 90% methanol for 10 minutes. Cells were washed 3x with FACS buffer and stained for surface markers and with PE-conjugated anti-STAT1 (pY701) antibody (BD biosciences) as described in (Krutzik and Nolan, 2003).

4.3.6 *In vitro* viral infection of peritoneal macrophages

Sort-purified macrophages isolated from naïve CNV or ABX-treated mice were seeded at $1x10^5$ cells per well in a 96-well plate and incubated at 37°C for 3 hrs to allow cell adherence. Macrophages were then infected with either LCMV clone 13 strain (MOI of 0.2) or influenza virus X31-GP33 strain (MOI of 5) for 1 hr then washed 2x to remove

virus. At indicated timepoints, supernatants were collected to assess viral titers by plaque assay or macrophages were directly lysed using RLT lysis buffer (Qiagen) and RNA was isolated using an RNeasy mini-kit (Qiagen). cDNA was generated using SuperScript reverse transcriptase (Invitrogen). RT-PCR was performed on cDNA using SYBR green chemistry (Applied Biosystems) and commercially available primer sets (Qiagen).

4.3.7 Macrophage purification and transfer

PECs from CNV or ABX-treated mice were isolated on day -1, 0, 1 of LCMV (T1b strain) infection. PECs were incubated with biotin CD3ε, CD5, CD11c, CD19, B220, and Ter-119 for 15 min at 4 °C followed by incubation with strepavidin conjugated magnetic beads (Dynabeads) for 20 min at 4 °C. Samples were separated by magnet and the negatively selected cells were collected. An aliquot was set aside to determine macrophage ($CD11b^+$, $F4/80^+$) purity (~60-80%). $3x10^5$ macrophages were transferred retro-orbitally into day of infection matched ABX-treated recipient mice.

4.3.8 Statistical analysis

Results represent means ± SEM. Statistical significance was determined by the unpaired, two tailed, Student's *t*-test for individual timepoints, two-way ANOVA test for timecourse experiments, logrank test for survival curve, or two-part *t*-test for comparison of groups that contained samples that were below the limit of assay detection. Statistical analyses were performed using Prism GraphPad software v4.0. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

4.4 Results

4.4.1 Macrophages, but not dendritic cells, exhibit an altered activation profile prior to viral infection in ABX-treated mice

Chapters 2 and **3** demonstrated an integral role for commensal bacterial communities in shaping the innate and adaptive immune response to mucosal or systemic viral infection. This chapter will investigate the mechanisms through which commensal bacteria-derived signals modulate antiviral immunity. Early defects in the innate antiviral immune response following influenza virus or LCMV infection in ABX-treated mice provoke the hypothesis that commensal bacteria-derived signals regulate the basal activation status of innate immune cells prior to viral infection. Therefore, to interrogate the potential mechanisms through which signals derived from commensal bacteria regulate innate immune responses and antiviral immunity, a phenotypic characterization of macrophages and dendritic cells isolated from the peritoneal cavity of naïve CNV or ABX-treated mice was performed. Macrophages (Figure 25A), but not dendritic cells (Figure 25B), isolated from ABX-treated mice selectively displayed decreased expression of a class of molecules (MHC-I, IFNγRI, CD40 and CD86) that are critical for the early propagation of the immune response to viral infection. These observations provide evidence that in the steady-state commensal bacteria-derived signals can shape immune cell activation status outside of the intestinal tract and contribute to maintaining macrophages in a poised state capable of rapidly respond to an invading pathogen.

4.4.2 Defective expression of antiviral defense genes in macrophages isolated from ABX-treated mice prior to viral infection

Differential expression of activation molecules on macrophages isolated from naive ABX-treated mice suggested an inherent impairment in the ability of macrophages to respond to infection. To interrogate the potential mechanisms through which signals derived from commensal bacteria regulate macrophage responses and innate antiviral immunity, we employed genome-wide transcriptional profiling of macrophages isolated from ABX-treated or CNV mice prior to viral infection. Fundamental differences in transcriptional profiles were readily apparent between macrophages isolated from CNV versus ABX-treated mice (Figure 26A). To further define these transcriptional differences, two separate analyses were conducted. First, Ingenuity Pathways Analysis (IPA) was used to identify enriched biological processes and networks. In total, there were 367 gene transcripts upregulated by at least 1.6 fold in macrophages isolated from CNV mice relative to macrophages isolated from ABX-treated mice (Figure 26B). Analysis of the 367 gene transcripts using IPA identified the IFN signaling pathway as the most significantly enriched pathway in macrophages isolated from CNV mice versus ABX-treated mice (Figure 26C). IPA can also identify complex biological networks by cross-referencing gene lists with a database consisting of empirically defined biological interactions between different genes. This analysis enables visualization of how different molecules interact to form complex biological networks. IPA identified the STAT1/STAT2 transcription factor complex as one of the central molecules in the network of inflammatory immune response genes elevated in macrophages isolated from CNV mice compare to ABX-treated mice (Figure 27A) Further analysis of this complex immune network revealed that macrophages isolated from ABX-treated mice had defects in

immune response genes critical in detecting viral infection and limiting viral replication (Figure 27B). Thus IPA suggested an inherent defect in the antiviral immune response following antibiotic-mediated disruption of commensal bacterial communities.

In addition to IPA, a second approach using gene set enrichment analysis (GSEA) was undertaken to compare the gene transcription profile of macrophages isolated from CNV mice to macrophages isolated from ABX-treated mice. GSEA enables an unbiased analysis of the whole genome microarray data to identify coordinated patterns of gene expression based on empirically defined gene sets (Haining and Wherry, 2010; Subramanian et al., 2005). GSEA revealed that six of the top eight most enriched gene sets in macrophages from CNV mice were related to IFN responses (Figure 28A,B). In addition, macrophages isolated from CNV mice were relatively enriched in a set of genes, independently reported by Agarwal et al. (Agarwal et al., 2009), responsive to IFN-α stimulation (Figure 28C). Specific gene expression analysis of macrophages isolated from ABX-treated mice revealed a relative downregulation of several genes that regulate detection of virus (*Ifih1* (MDA5), *Ddx58* (RIG-I)), the response to interferon signaling (*Irf7*, *Ifngr1*, *Stat1*, *Stat2*) or inhibition of viral replication (*Mx1*, *Oas1a*) (Figure 29A), but not genes associated with the inflammasome (Figure 29B) or TLR signaling pathways (Figure 29C), two alternative innate pathogen recognition mechanisms. Differential expression of IFN-pathway related antiviral defense genes was independently confirmed by qRT-PCR analysis on sorted macrophages from naïve CNV or ABX-treated mice (Figure 29D). Taken together, genome-wide transcriptional profiling and computational analyses suggest that signals derived from commensal bacteria calibrate the activation threshold of antiviral immune response pathways in macrophages.

4.4.3 Alteration or absence of commensal bacteria renders macrophages less responsive to IFN stimulation or viral infection

Genome-wide transcriptional profiling suggested that commensal bacteria-derived signals modulate the responsiveness of macrophages to viral infection or IFN stimulation. To functionally test this hypothesis, the response of macrophages isolated from naive CNV or ABX-treated mice to IFN stimulation or viral infection was assessed *ex vivo*. IFN receptor signaling by type I or II IFNs results in phosphorylation of STAT1 followed by translocation into the nucleus, where pSTAT1 initiates transcription of an array of IRGs crucial to the early antiviral response (Schindler et al., 1992; Shuai et al., 1992). To test IFN responsiveness, macrophages isolated from naïve, CNV or ABX-treated mice were stimulated with IFN-γ (0.4-200ng/mL) or IFN-β ($1x10^3$U/mL) for 5-15 minutes *in vitro*, and phosphorylation of STAT1 was measured by flow cytometry. Stimulated macrophages isolated from ABX-treated mice exhibited significantly reduced pSTAT1 levels compared to CNV macrophages following IFN-γ (Figure 30A,B) or IFN-β (Figure 30C,D) stimulation. Similar deficiencies in STAT1 phosphorylation were observed following IFN-γ (Figure 31A,B) or IFN-β (Figure 31C,D) stimulation of macrophages isolated from GF mice. These biological observations are consistent with diminished expression of antiviral defense genes identified by transcriptional profiling (Figure 26-29), and, together, suggest that signals from commensal bacteria are crucial to maintain optimal responsiveness to IFN stimulation in macrophages.

To examine whether these deficiencies impaired macrophage responsiveness to viral infection, macrophages isolated from CNV or ABX-treated mice were infected *ex vivo* with influenza virus (X31-gp33) and induction of antiviral defense genes was examined. This method of direct *ex vivo* viral infection of isolated macrophages assesses whether

macrophages are intrinsically impaired in responding to viruses. Expression of multiple antiviral defense genes (*Mx1, Oas1a, Ifnb, Stat2*), identified from our microarray analysis (Figure 29A,D), was reduced in macrophages from ABX-treated mice compared to CNV mice at 6-24 hrs after infection, indicating an impaired ability to respond to viral infection (Figure 32). Further, macrophages isolated from ABX-treated (Figure 33A) or GF (Figure 33B) mice supported significantly more viral replication upon *ex vivo* infection with LCMV demonstrated by higher titers of virus present in the supernatant at 48-72 hrs post-infection compared to CNV macrophages. Combined, these data demonstrate that manipulation of commensal bacterial communities by ABX treatment fundamentally alters expression of antiviral defense genes, rendering cells of the innate immune system less able to respond to viral infection.

4.4.4 Adoptive transfer of macrophages isolated from CNV mice improves control of viral replication and quality of antiviral immune response in ABX-treated mice

Tonic signaling from commensal bacteria primes macrophages to rapidly respond to virus and virus-induced interferon stimulation (Figure 29-32). Further, in **Chapter 2** and **3** we reported that ABX-mediated disruption of commensal bacterial communities renders mice susceptible to viral infection. To assess if commensal bacteria-derived tonic signaling on macrophages is sufficient restore antiviral immune responses and control viral replication in ABX-treated mice, macrophages from CNV mice were adoptively transferred into ABX-treated mice at d -1,0, and 1 following LCMV infection (Figure 34A). As an additional control, macrophages from ABX-treated mice were adoptively transferred to another group of ABX-treated mice. Thus, upon LCMV infection ABX-treated recipient mice received either commensal bacteria “exposed” or “unexposed” donor macrophages. Following LCMV infection, the immune response and control of

viral replication in macrophage recipient ABX-treated mice was compared to CNV mice and ABX-treated mice that did not receive macrophage transfers.

Following macrophage transfer and LCMV infection, the four groups (CNV, ABX, ABX + CNV Macs, ABX + ABX Macs) were longitudinally assessed for serum viral titers and LCMV-specific CD8 T cell responses. Consistent with experiments reported in **Chapter 3** (Figure 18), CNV mice cleared virus from the serum within 2 weeks following infection while ABX-treated mice exhibited persisting viremia for a month (Figure 34B). In contrast, ABX-treated mice receiving "commensal exposed" macrophages exhibited significantly improved control of virus in the serum compared to ABX-treated mice (Figure 34B). ABX-treated mice, receiving "commensal unexposed" macrophages, however, exhibited persisting viremia for a month following infection (Figure 34B). Thus, only donor macrophages harvested from an environment where commensal bacteria-derived signals were present were able to improve viral control in ABX-treated mice.

Fewer LCMV-specific H-2D^{b} gp33 (Figure 34C) and H2-D^{b} gp276 CD8 tetramer^{+} (Figure 34D) T cells were detected within the peripheral mononuclear cells (PBMC) isolated from the blood of ABX-treated mice at d23 post-infection. Phenotypic characterization of LCMV-specific CD8 T cells isolated from ABX-treated mice revealed increased expression of the inhibitory receptor PD-1 (Figure 34E,F), which has been shown to be associated with the quality of the antiviral immune response (Barber et al., 2006; Blackburn et al., 2009), indicating ABX-treated mice had an impaired antiviral immune response that correlated with delayed viral clearance, consistent with the results reported in **Chapter 3**. However, ABX-treated mice receiving "commensal exposed" macrophages, but not ABX-treated mice receiving "commensal unexposed" macrophages, exhibited significantly increased populations of LCMV-specific CD8 T

cells in the blood at d23 post-infection (Figure 34C,D) compared to ABX-treated mice not receiving a macrophage transfer. The LCMV-specific CD8 T cells from ABX + CNV Macs mice also expressed less PD-1 (Figure 34E,F) suggesting the macrophage transfer also improved the quality of the antiviral immune response.

At d31 post-infection the four groups of mice were sacrificed and splenocytes were stimulated with a pool of 20 LCMV-specific peptides (Kotturi et al., 2007) and assessed for effector cytokine production to interrogate the functional quality of the LCMV-specific CD8 T cell response. Consistent with previous observations (**Chapter 3**, Figure 21), ABX-treated mice exhibited significantly decreased frequency (Figure 35A) and total numbers (Figures 35B) of IFN-γ^+/TNF-α^+ dual producing splenic CD8 T cells compared to CNV mice. In contrast, ABX-treated mice receiving "commensal exposed" macrophages, but not ABX-treated mice receiving "commensal unexposed" macrophages, exhibited a significant increase in the frequency and total number of IFN-γ^+/TNF-α^+ dual producing CD8 T cells compared to ABX-treated mice (Figure 35A,B), indicating a significant improvement in the quality of the adaptive antiviral immune response. Thus, macrophages previously exposed to commensal bacteria-derived signals were sufficient to partially restore control of viral replication and improve the antiviral immune response in an environment devoid of commensal bacteria-derived signals. Collectively, these data demonstrate the *in vivo* relevance of tonic signaling by commensal bacteria in setting the immune activation threshold of macrophages required for antiviral immunity.

4.5 Discussion

The results described in this chapter reveal a novel interplay between commensal bacteria and antiviral IFN signaling pathways that is critical in response to viral infection. Genome-wide transcriptional profiling uncovered a global defect in expression of antiviral defense genes in macrophages isolated from ABX-treated mice compared to macrophages from CNV mice prior to viral infection. This reduction in steady-state transcription of antiviral defense genes was associated with a significant deficiency in responsiveness to virus or the virus-induced type I/II IFNs. Furthermore, adoptive transfer of macrophages isolated from CNV mice, but not ABX-treated mice, was sufficient to restore antiviral immunity in ABX-treated mice. Collectively, these data indicate a previously unrecognized aspect of innate immune cross-talk where low-level tonic signaling by commensal bacteria calibrate the activation threshold of innate antiviral immune responses.

Tonic signaling has been proposed to be a mechanism to maintain optimal responsiveness of signaling pathways in other settings (Macia et al., 2009). For example, naïve T cells use low affinity interactions with self MHC to generate tonic signals thought to be important for naïve T cell homeostasis and for optimal dynamic responsiveness upon engagement of cognate antigen (Takeda et al., 1996; Tanchot et al., 1997). Similarly, pre-TCR and pre-BCR are used during lymphocyte development to generate low-level signals that help guide these cells to maturity (Rajewsky, 1996; Scott et al., 1989). The tonic signaling involved in maintaining responsiveness of antiviral pathways described in this chapter was not dependent on signals that normally evoke antiviral IFN responses but rather on commensal bacteria-derived signals. Such crosstalk between anti-bacterial and antiviral immunity can occur in other settings. For

example, latent viral infections can render mice less susceptible to bacterial challenge, an effect attributed to basal macrophage activation driven by antiviral IFN-g production (Barton et al., 2007). Conversely, the symbiotic bacteria, *Wolbachia,* confers protection against viral infections in *Drosphilia* (Teixeira et al., 2008). Anti-bacterial responses driven by LPS triggered TLR4 signaling can also upregulate transcription of antiviral genes (Amit et al., 2009; Doyle et al., 2002). In the case of LPS/TLR4 signaling, antiviral gene expression is initially induced, but rapidly limited by the polycomb repressor Cbx4 (Amit et al., 2009).

This latter observation suggests a potential explanation for the commensal-antiviral immune fitness axis at the transcriptional level. Induction of transcription followed by repression might maintain key antiviral genes in a state of poised transcriptional regulation, rather than a repressed or inactive state. Transcriptional poising, or the presence of both activating and repressive chromatin, can allow for faster or more efficient transcriptional induction upon exposure to a true inducer of the gene of interest (Cuddapah et al., 2010; Zediak et al., 2011). This state of dynamic transcriptional regulation provided by tonic commensal bacteria stimulation may enable rapid induction of antiviral defense genes upon infection. Examples of this type of regulation exist in other settings. In yeast, the Hog1/MAPK pathway uses basal signaling and a negative feedback loop to maintain rapid and sensitive responses to environmental stimuli, suggesting that a pathway with active low-grade signaling might be biologically more responsive than one that lies dormant until a de novo signal is received (Macia et al., 2009). Our results suggest commensal bacteria provide such a signal that maintains antiviral innate immune pathways in a state of optimal readiness allowing dynamic and robust responses upon challenge by systemic or mucosal viral infections.

Figure 25. Macrophages isolated from ABX-treated mice exhibit reduced expression of activation molecules in the steady-state.

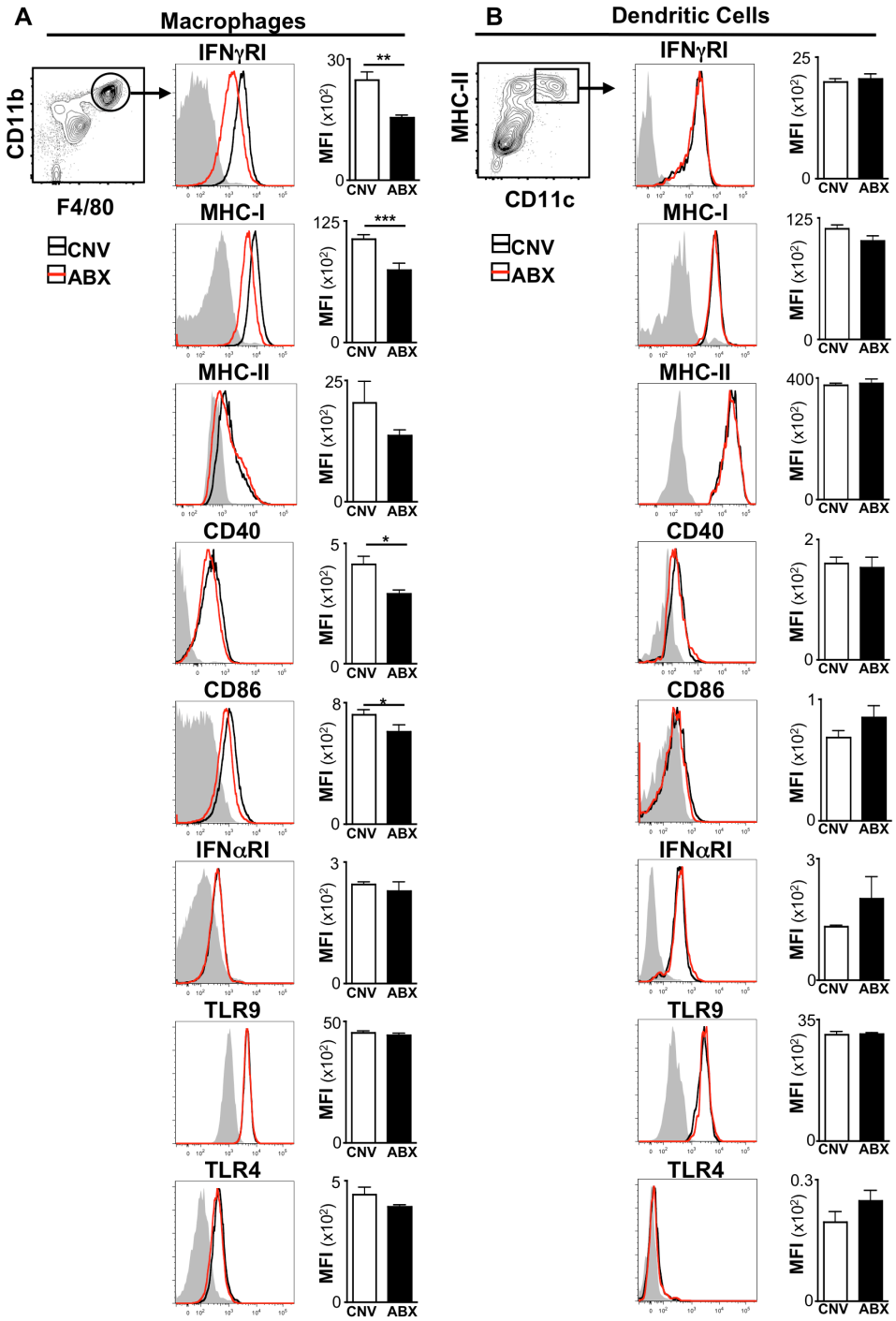


Figure 25. Phenotypic characterization of **(A)** macrophages or **(B)** dendritic cells isolated from the peritoneal cavity of naïve CNV (black line) or ABX-treated (red line) mice. Shaded histogram represents isotype control (or FMO for MHC-I and TLR-9). FACS plots gated on live, $CD45^{+}$, non-T, non- B, non-NK cells. Data representative of three independent experiments. n=4-5 mice per group. Data shown are the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Figure 26. Macrophages isolated from ABX-treated mice have an altered gene expression profile.

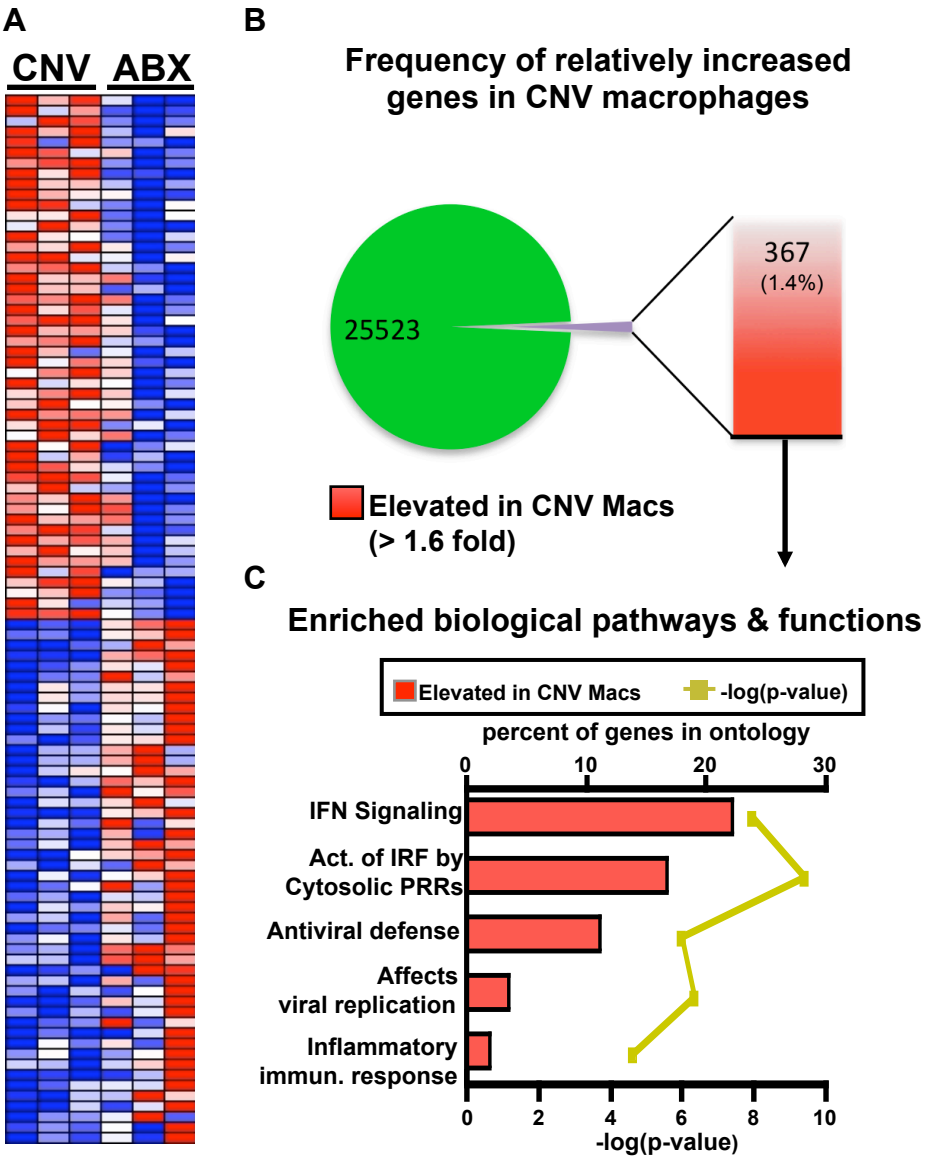


Figure 26. RNA was extracted from sort-purified peritoneal macrophages (sorted on $CD3\varepsilon^-$, $CD19^-$, $F4/80^+$, $CD11b^+$) isolated from naïve CNV or ABX-treated mice. Extracted RNA was hybridized to an Affymetrix GeneChip microarray to assess gene expression. **(A)** Heat map of differentially expressed genes in macrophages isolated from CNV or ABX-treated mice. Red = high expression, blue = low expression. **(B)** Frequency and total number of elevated gene transcripts in CNV macrophages compared to macrophages isolated from ABX-treated mice. (C) Highly enriched biological pathways and functions found within the subset of elevated gene transcripts from CNV macrophages as assessed by Ingenunity Pathways Analysis. Red bars indicate the percent of genes in a pathway upregulated in macrophages isolated from CNV mice. Yellow line indicates p-value calculated by Fisher's Exact test.

Figure 27. Key molecules in the inflammatory immune response network are relatively elevated in macrophages isolated from CNV mice.

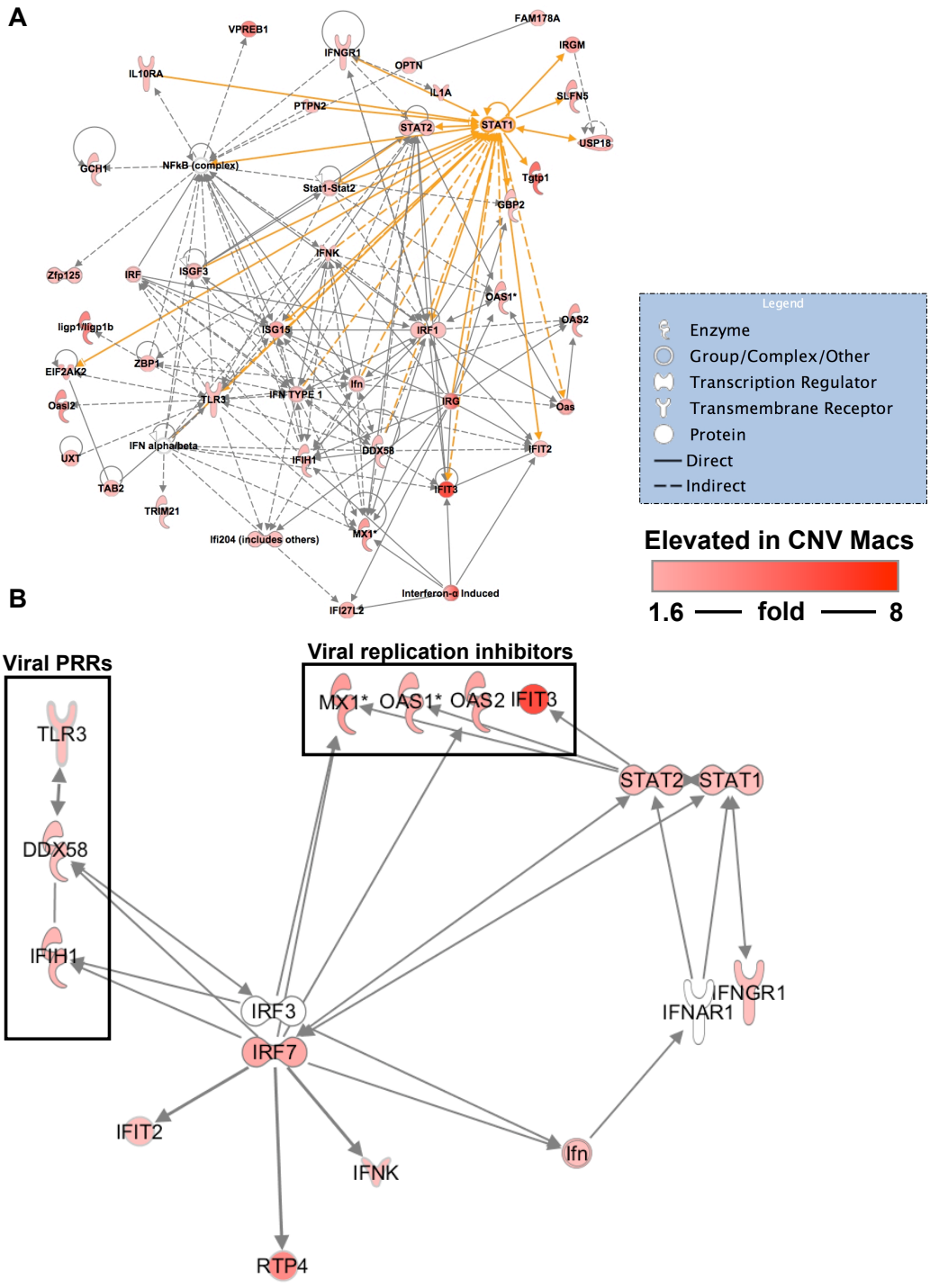
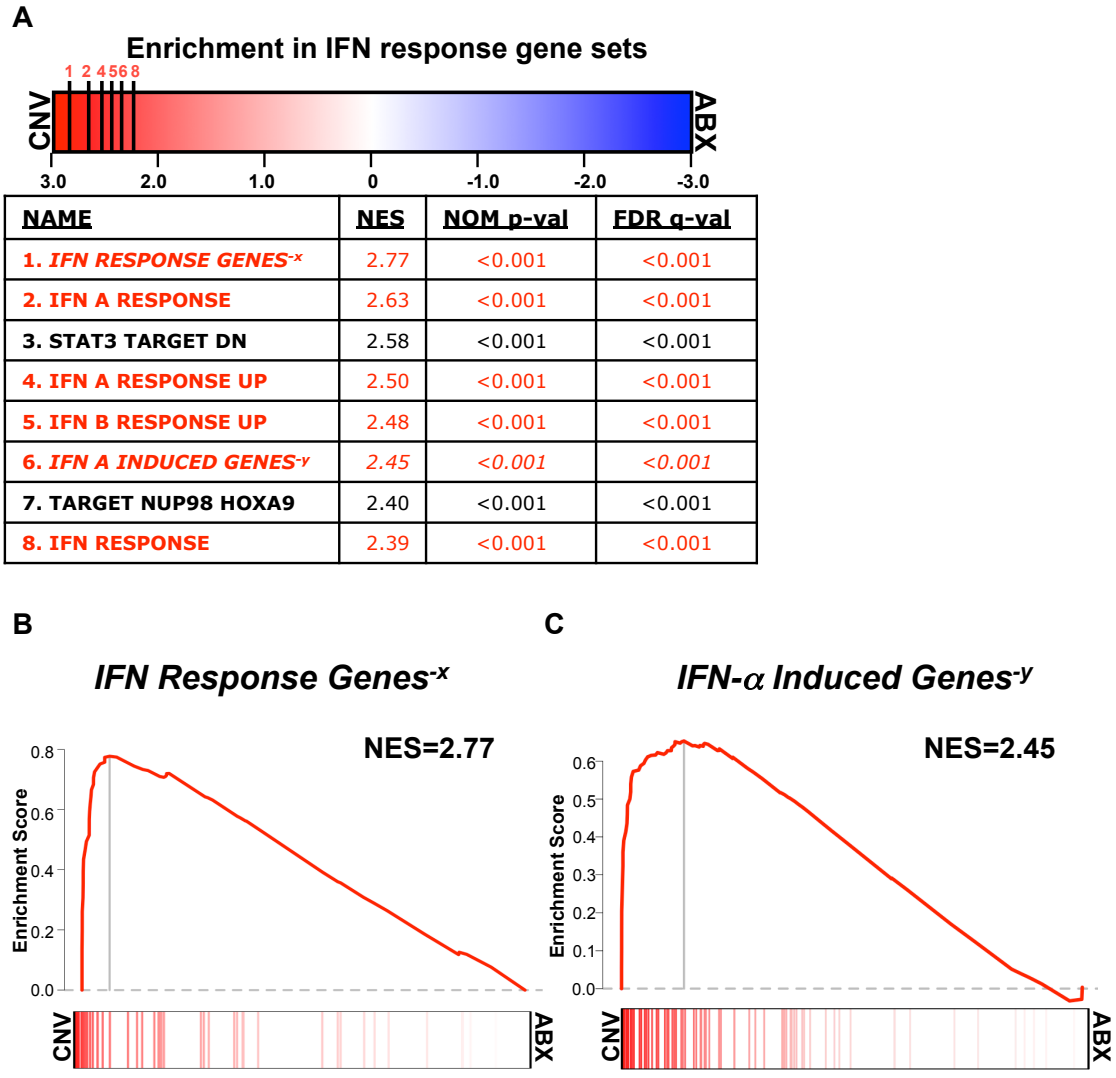


Figure 27. (A) Ingenuity Pathway analysis identified direct and indirect biological interactions amongst the subset of 367 genes relatively elevated in CNV macrophages creating an inflammatory immune response network. Connection lines in yellow highlight the interrelationship of STAT1 with other genes in the network. Molecules shaded in red represent gene expressed at relatively higher levels in CNV macrophages compared to macrophages isolated from ABX-treated mice. **(B)** Simplified network identifying molecules relatively elevated in CNV macrophages that are crucial in the detection and inhibition of viral replication. Network maps were created using Ingenuity pathway analysis software (Ingenuity® Systems, www.ingenuity.com)

Figure 28. Macrophages isolated from CNV mice exhibit a relative enrichment in IFN responsive genes.



NAME	NES	NOM p-val	FDR q-val
1. *IFN RESPONSE GENES-x*	2.77	<0.001	<0.001
2. IFN A RESPONSE	2.63	<0.001	<0.001
3. STAT3 TARGET DN	2.58	<0.001	<0.001
4. IFN A RESPONSE UP	2.50	<0.001	<0.001
5. IFN B RESPONSE UP	2.48	<0.001	<0.001
6. *IFN A INDUCED GENES-y*	*2.45*	*<0.001*	*<0.001*
7. TARGET NUP98 HOXA9	2.40	<0.001	<0.001
8. IFN RESPONSE	2.39	<0.001	<0.001

Figure 28. (A) Gene set enrichment analysis (GSEA) of CNV macrophages compared to macrophages isolated from ABX-treated mice. The most highly enriched gene sets determined by the normalized enrichment score (NES). **(B)** Enrichment curve of the top ranked gene set as defined by GSEA. **(C)** GSEA enrichment curve of genes upregulated following IFN-α stimulation, empirically defined from (Agarwal et al., 2009).

Figure 29. Decreased expression of antiviral defense genes but not inflammasome or TLR signaling components in macrophages isolated from ABX-treated mice.

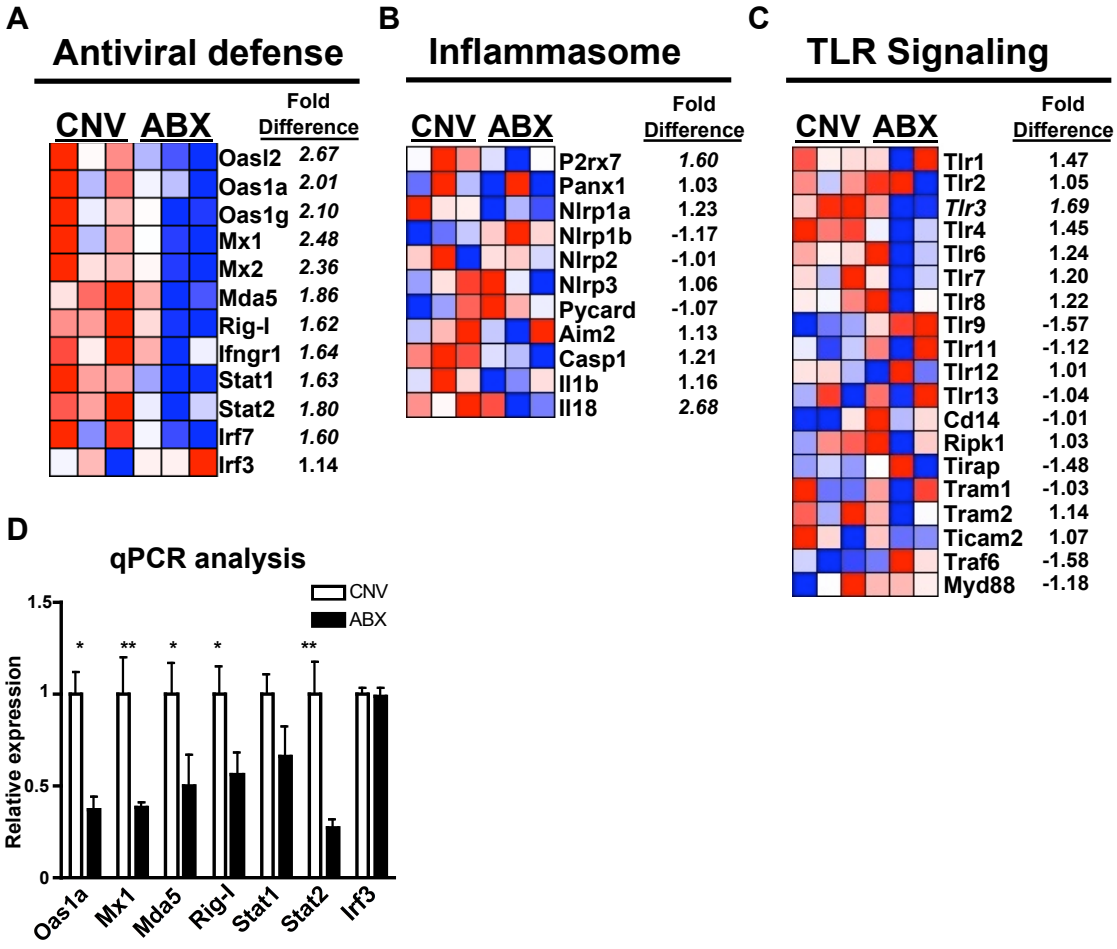


Figure 29. (A-C) Heat map of genes encoding **(A)** antiviral defense mechanisms, **(B)** inflammasome components or **(C)** TLR signaling components in macrophages isolated from naïve CNV or ABX-treated mice. **(D)** Expression of antiviral defense genes by qPCR analysis of RNA isolated from sort-purified macrophages from two independent experiments with n=3-5 mice per group. *p<0.05, **p<0.01.

Figure 30. Macrophages isolated from ABX-treated mice have an impaired response to type I/II IFN stimulation.

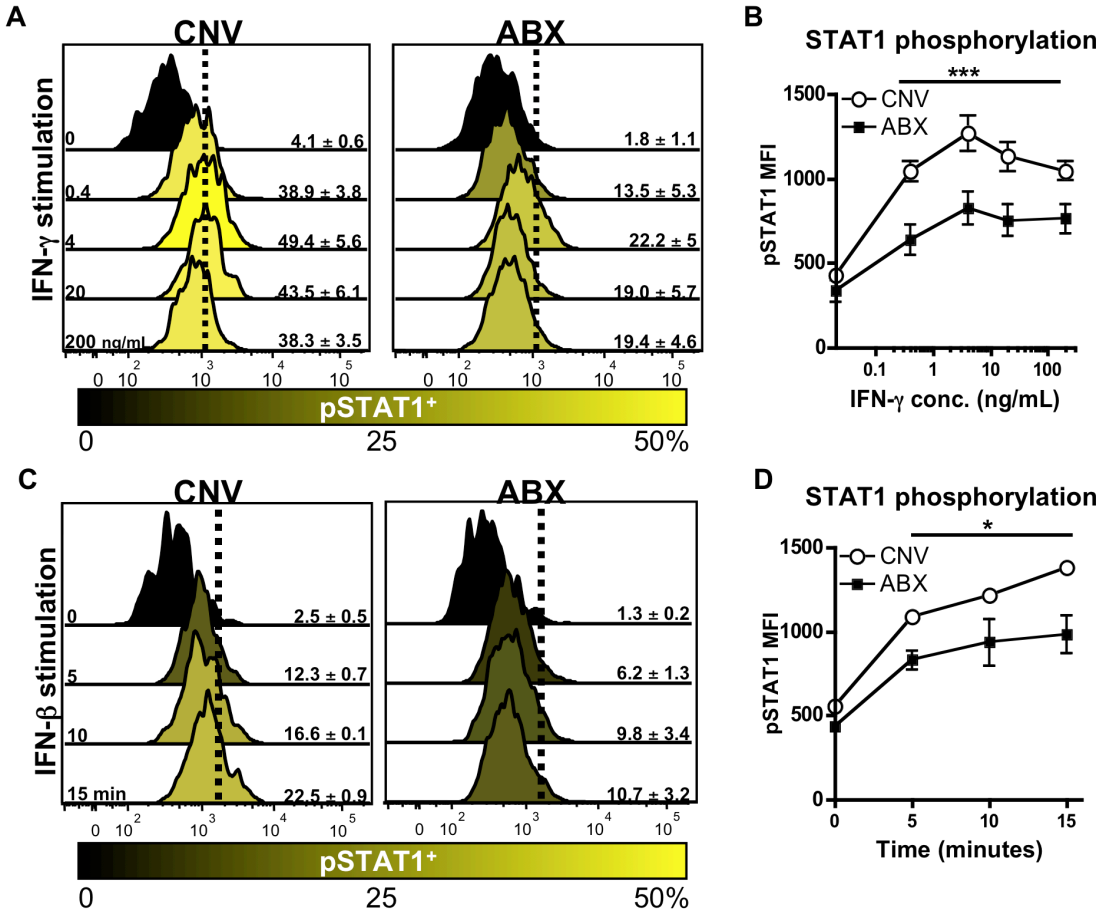


Figure 30. (A-D) Peritoneal macrophages isolated from CNV or ABX-treated mice were stimulated with IFN-γ or IFN-β *in vitro*. Histograms of STAT1 phosphorylation in macrophages following **(A)** IFN-γ stimulation (0.4, 4, 20, 200ng/mL) or **(C)** IFN-β stimulation (10^3 Units/mL for 5, 10, and 15 minutes). Mean fluorescence intensity (MFI) of pSTAT1 in macrophages following **(B)** IFN-γ or **(D)** IFN-β stimulation. Data representative of two or more independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM. *p <0.05, ***p <0.001.

Figure 31. Macrophages isolated from GF mice also display an impaired response to type I/II IFN stimulation.

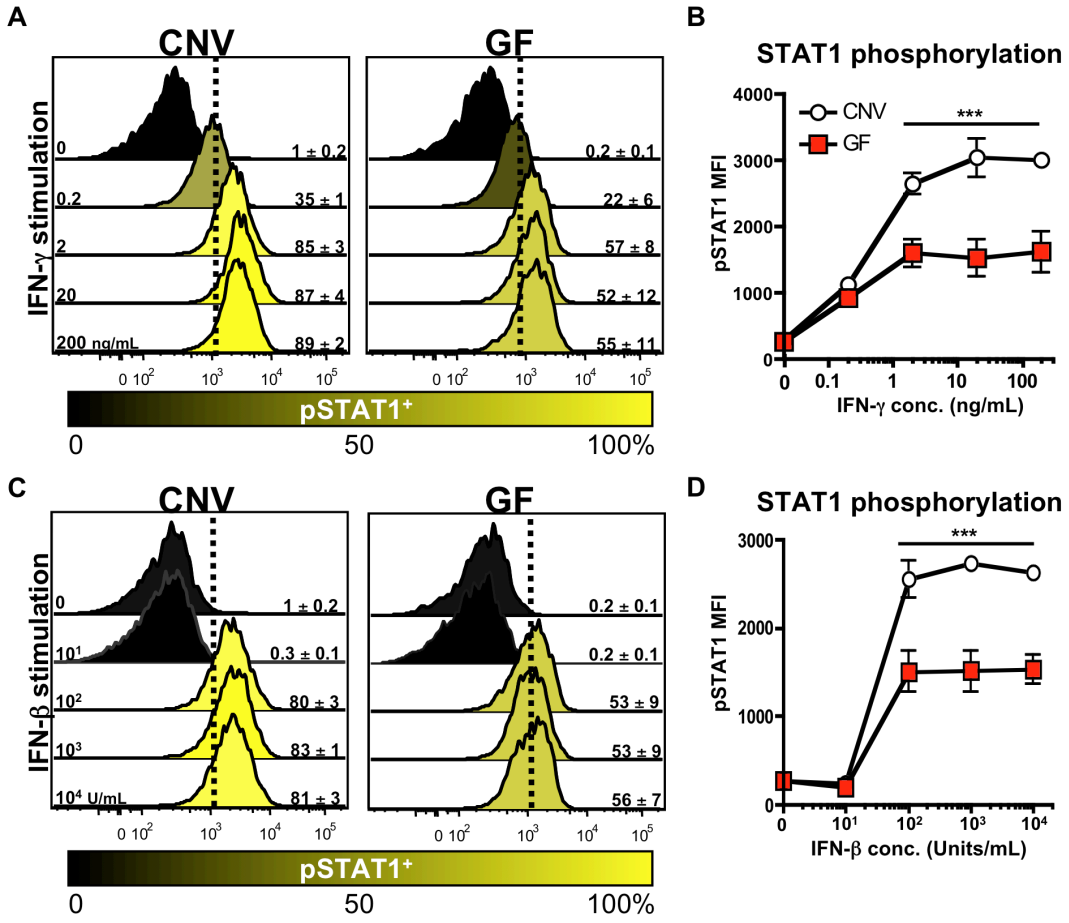


Figure 31. (A-D) Peritoneal macrophages isolated from naïve CNV or GF Swiss/Webster mice were stimulated with IFN-γ or IFN-β *in vitro*. Histograms of STAT1 phosphorylation in macrophages following **(A)** IFN-γ (0.2, 2, 20, 200ng/mL) or **(C)** IFN-β (10^1, 10^2, 10^3, 10^4 Units/mL) stimulation for 15 minutes. Mean florescence intensity (MFI) of pSTAT1 in macrophages following **(B)** IFN-γ or **(D)** IFN-β stimulation. Data representative of two independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM. ***p <0.001.

Figure 32. Macrophages isolated from ABX-treated mice exhibit delayed induction of antiviral defense genes.

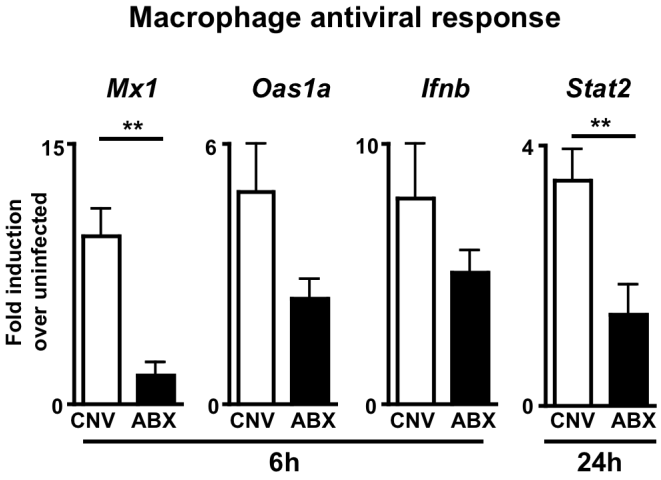


Figure 32. Peritoneal macrophages sorted from naive CNV or ABX-treated mice were infected with influenza virus *in vitro* (X31-GP33, MOI of 5). RNA was purified at 6 and 24 hrs post-infection and analyzed by qPCR for upregulation of antiviral genes. Data representative of three independent experiments with n=3-5 mice per group. Data shown are the mean ± SEM. **p<0.01.

Figure 33. Macrophages isolated from naive ABX-treated or GF mice have a diminished ability to respond to viral infection *in vitro.*

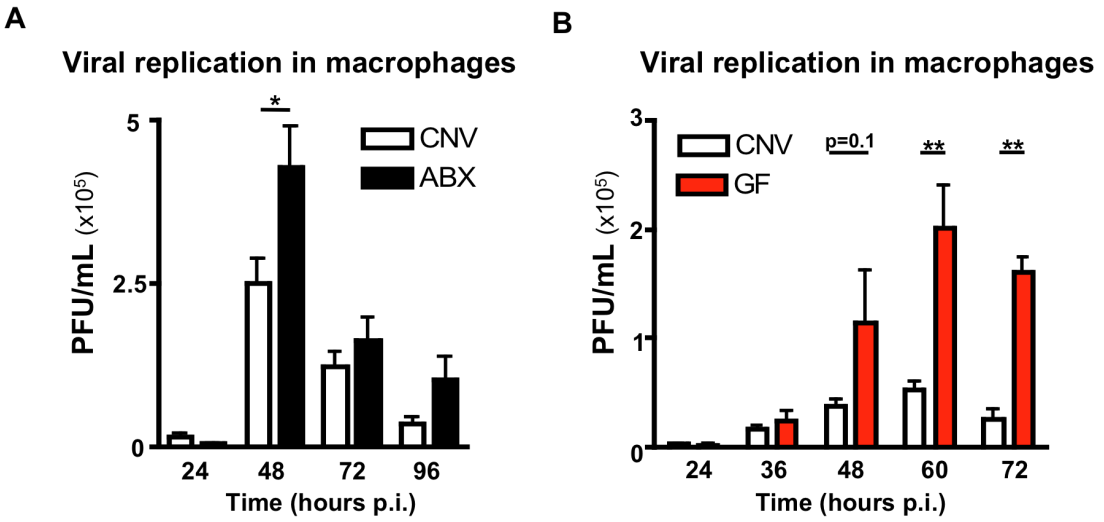


Figure 33. Peritoneal macrophages isolated from **(A)** naïve C57BL/6 CNV or ABX-treated mice or **(B)** naïve CNV or GF Swiss/Webster mice were infected with LCMV cl-13 strain (MOI of 0.2) *in vitro*. LCMV viral titers in supernatant of *in vitro* infected macrophages at 24-96 hrs post-infection were assessed by viral plaque assays. Data representative of three independent experiments; n=3-5 mice per group. Data shown are the mean ± SEM. *p<0.05, **p<0.01.

Figure 34. Macrophages transferred from CNV mice into ABX-treated mice improve viral control following LCMV infection.

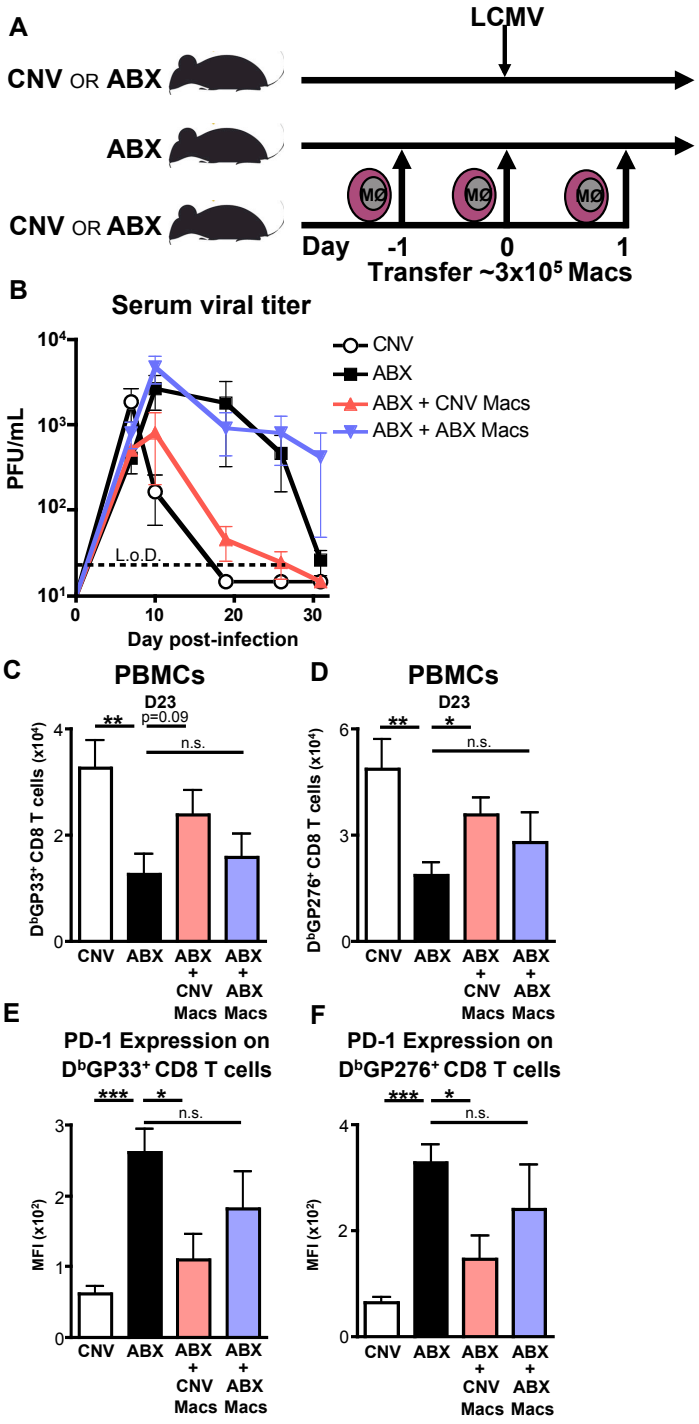


Figure 34. **(A)** Diagram schematic of macrophage transfer from CNV or ABX-treated donor mice into ABX-treated recipients. CNV, ABX-treated, ABX-treated + CNV Macs, ABX-treated + ABX Macs mice were infected i.v. with $2x10^6$ PFU of LCMV T1b. **(B)** Viral titer in the serum following infection (L.o.D., limit of detection). LCMV-specific **(C)** DbGP33 and **(D)** DbGP276 tetramer$^+$ CD8 T cells per 10^6 peripheral blood mononuclear cells (PBMCs) at d23 post-infection. **(E-F)** Expression of inhibitory receptor PD-1 on **(E)** DbGP33 or **(F)** DbGP276 tetramer$^+$ CD8 T cells isolated from the blood at d23 post-infection. Viral titer statistics determined by two-way ANOVA comparing CNV to ABX ($p<0.01$), ABX + CNV Macs to ABX ($p<0.01$), or ABX + ABX Macs (n.s.). Data shown are the mean ± SEM. *$p<0.05$, **$p<0.01$.

Figure 35. Adoptive transfer of CNV macrophages improves quality of LCMV-specific CD8 response in ABX-treated mice.

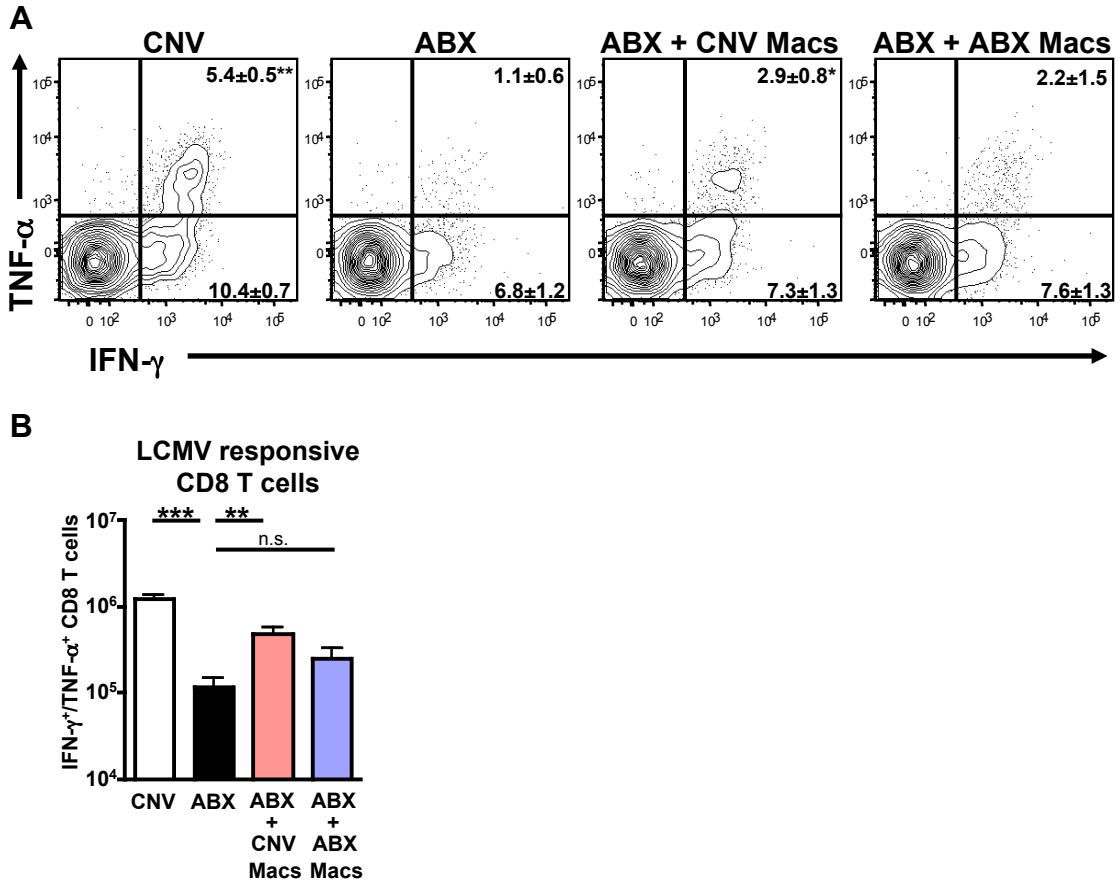


Figure 35. (A) Splenocytes from d31 infected mice were incubated with a pool of 20 LCMV-specific peptides (Kotturi et al., 2007) for 5 hrs in the presence of BFA and CD8 T cells were assessed for production of IFN-γ and TNF-α. FACS plots gated on live, CD8α$^+$ cells. **(B)** Total number of IFN-γ/TNF-α dual producing CD8 T cells in the spleen at d31 post-infection. Data shown are the mean ± SEM. *p<0.05, **p<0.01.

Chapter 5
Summary, Discussion and Future Directions

5.1 Commensal bacterial communities modulate immune responses to infection

The innate immune system has evolved to detect distinct molecular patterns conserved within specific groups of pathogens. This enables the innate immune system to be able to respond to a multitude of pathogens with a relatively limited number of pathogen recognition mechanisms. For example, TLR4 detects lipopolysaccarides (LPS) that are prominently displayed on the outer membrane of all gram-negative bacteria (Miller et al., 2005), while cytosolic proteins such as RIG-I and MDA5 can detect cytoplasmic dsRNA, a common intermediate in the replication cycle of viruses (Medzhitov, 2007). Detection of PAMPs initiates a cascade of signaling pathways that results in induction of innate immune response genes and activation of the adaptive immune system.

Intestinal commensal bacteria express the same molecular motifs as pathogenic bacteria, yet the immune system has evolved to interact with commensal bacterial communities without causing overt, persistent inflammation. This is accomplished, in part, by physical separation of intestinal commensal bacteria from the intestinal epithelial lining. Specialized IECs, called goblet cells, secrete mucin glycoproteins into the intestinal lumen creating a mucus layer coating the intestinal epithelia and limiting bacterial attachment to epithelial cells (Deplancke and Gaskins, 2001). Antimicrobial peptides and commensal bacteria-specific IgA are secreted into the mucus layer further preventing bacterial penetrance. (Macpherson et al., 2001; Vaishnava et al., 2011). Physical separation of commensal bacteria from host cells is not absolute, however, and

intestinal immune cells regularly come in direct contact with live commensal bacteria (Ivanov et al., 2009; Macpherson and Uhr, 2004; Obata et al., 2010). These interactions occur continuously throughout the life of the host and result in a dynamic relationship between commensal bacteria and the host immune system. Intestinal immune cell populations shaped by these interactions, can be driven to a proinflammatory or immunoregulatory differentiated state. This homeostatic regulation of the immune system by commensal bacterial communities can shape host fitness rendering the host more or less susceptible to inflammation, infection, allergy, or autoimmunity (Honda and Littman, 2012).

The data presented in this thesis describe a fundamental role for commensal bacterial communities in setting the activation threshold the innate immune system requires to respond to mucosal-associated or systemic viral infections. ABX-mediated disruption of commensal bacterial communities resulted in diminished expression of antiviral defense genes in macrophages isolated from ABX-treated mice compared to macrophages isolated from CNV mice prior to viral infection. This observation suggests that tonic stimulation by commensal bacteria-derived signals induces basal transcription of antiviral defense genes in innate immune cells in the steady-state that enables rapid immune responses upon exposure to viruses. Induction of innate immune response genes trigger robust expansion of adaptive immune responses, and, ultimately, successful control of infection. Even a slight delay in innate immune activation can swing the host versus virus equilibrium in favor of the pathogen and can have severe physiological consequences for the host. This was demonstrated in **Chapter 2**, in which influenza virus infection of ABX-treated mice resulted in impaired induction of innate proinflammatory cytokines and chemokines leading to diminished induction of adaptive

immune responses. Impaired immune responses to influenza virus in ABX-treated mice compared to CNV mice resulted in ineffective viral clearance, severe damage to the bronchiole epithelium, defective gas exchange, and ultimately host mortality.

The results presented in **Chapter 3** demonstrate defective antiviral innate and adaptive immune responses to a systemic viral infection (LCMV) following ABX-mediated disruption of commensal bacterial communities. Antiviral immunity could be rescued in mice depleted of commensal bacteria by either restoring IFN responsiveness as described in **Chapter 2** or supplementing ABX-treated mice with macrophages harvested from CNV mice and therefore exposed to commensal bacteria-derived signals, as reported in **Chapter 4**. Collectively, these data indicate that in the steady-state, commensal bacteria provide tonic signals that regulate the activation threshold of the innate immune system required for optimal antiviral immune responses (Figure 36).

5.2 Dynamic interrelationship between the immune system, commensal bacteria and viruses

The immune system has evolved distinct anti-bacterial or antiviral immune mechanisms that enable efficient and targeted responses tailored to each particular class of pathogen (Cousens et al., 1999; Curtis and Way, 2009; Kumar et al., 2009). Therefore, it was surprising that ABX-mediated disruption of commensal bacterial communities resulted in an impaired antiviral immune response. The results presented in this thesis suggest a conserved element of the innate immune system that utilizes tonic signaling from commensal bacteria to calibrate the activation threshold required to respond to viral infections. It has been previously demonstrated that components classically considered part of the anti-bacterial response, such as the LPS/TLR4 signaling axis, can also trigger

transcription of an antiviral gene program (Amit et al., 2009; Doyle et al., 2002). Further, the bacterial colonization with *Wolbachia* in *Drosphilia* flies confers protection against viral infections (Teixeira et al., 2008). Conversely, latent viral infections can basally activate innate immunity rendering latently-infected hosts less susceptible to bacterial challenge (Barton et al., 2007). These studies provide novel insights into how distinct microbial sensing and response pathways are interwoven and provide evidence for critical cross-regulation of antiviral immunity by bacterial communities.

Commensal bacteria-driven gene transcription of immune response genes in the steady-state may be a universal mechanism to modulate the pathogen response profile of immune and non-immune cell types. Hooper and colleagues first reported marked differences in immune response gene expression in cells derived from the intestinal epithelium (Hooper et al., 2001). The findings reported in **Chapter 4** of thesis demonstrate a role for commensal bacteria-driven gene transcription in cells outside the gastrointestinal tract. Disruption of microbial signaling by commensal bacteria reduced expression of antiviral defense genes in macrophages (**Chapter 4**; Figure 28). Further, while analysis of macrophages in this thesis did not reveal changes in expression of inflammasome related genes, Ichinohe and colleagues reported decreased expression of inflammasome components in the lung of ABX-treated mice both in the steady-state and following influenza virus infection (Ichinohe et al., 2011). These data suggest that gene expression of the inflammasome in a non-macrophage resident lung cell population may be mediated by commensal bacteria-derived signals. Collectively, these studies support a model where commensal bacteria-derived signals can coordinately influence the gene expression profile of multiple cell types thereby modulating the immune system at homeostasis and in an infection setting.

5.3 Mediators of constitutive type I IFN expression

Genome-wide transcriptional profiling of macrophages isolated from naïve CNV or ABX-treated mice revealed an altered gene expression profile following disruption of commensal bacterial communities by ABX treatment. Examination of the top 50 relatively upregulated genes in macrophages isolated from CNV mice compared to ABX-treated mice revealed increased basal expression of several interferon response genes (IRGs) that regulate or are regulated by the IFN signaling pathway (Table 2). Analysis of the gene expression microarray data with Ingenuity Pathways software and GSEA independently identified the interferon response as one of the main pathways disrupted in macrophages isolated from ABX-treated mice. Functional assays confirmed macrophages isolated from ABX-treated exhibited a defect in responding to IFN stimulation and, in turn, an impaired ability to control viral replication. Basal transcription of IRGs is thought to be maintained by constitutive type I IFN expression (Gough et al., 2012). It has long been appreciated that even in the absence of viral infection, low-level expression of type I IFNs can be detected in multiple tissues of mice and humans (Bocci, 1980; Tovey et al., 1987; Yaar et al., 1986). Constitutive IFN expression is important in maintaining a state of antiviral readiness as ablation of constitutive IFN signaling by using anti-interferon-α/β neutralizing antibodies or genetic deletion of the IFN-α/β receptor results in decreased expression of IRGs such as STAT1, STAT2 and IRF7 (Gough et al., 2010; Hata et al., 2001). Further, macrophages isolated from mice treated with anti-IFN neutralizing antibodies exhibited delayed induction of antiviral defense mechanisms and impaired control of viral replication, demonstrating the physiological relevance of constitutive IFN in priming immune cells to be ready to respond to viruses (Belardelli et al., 1984). Currently, the origins of the stimuli for constitutive IFN

expression are not known, although the observation that macrophages isolated from C3H/HeJ mice, which have defective TLR4 signaling, do not express constitutive IFN and have impaired phagocytic and antiviral function has lead to speculation that commensal bacteria-derived signals may be important regulators of constitutive IFN expression (Gough et al., 2012; Musso et al., 2011; Vogel and Fertsch, 1984).

While type I IFN production following viral infection is critically dependent on IRF3/IRF7, these two transcription factors do not have a role in mediating constitutive IFN expression (Hata et al., 2001; Sato et al., 2000). Rather, relA and c-Jun, subunits of the NFκB and AP-1 transcription factor complex respectively, bind to distinct positive regulatory domains of the *Ifnb* promoter and are essential for constitutive IFN expression (Balachandran and Beg, 2011; Gough et al., 2012; Maniatis et al., 1998). Loss of constitutive IFN expression in c-Jun-deficient fibroblasts result in decreased STAT1 protein levels and impaired responsiveness to IFN-γ stimulation (Gough et al., 2012). Meanwhile, reduced expression of *Ifnb* mRNA in *rela*$^{-/-}$ MEFs correlated with decreased expression of genes involved in viral detection (MDA5 and RIG-I) and propagation of the IFN signaling pathway (STAT1, STAT2 and IRF7) (Basagoudanavar et al., 2011). Notably, these same genes were also decreased in macrophages isolated from ABX-treated mice (**Chapter 4**, Figure 28). Further, similar to macrophages isolated from ABX-treated mice, following viral infection *rela*$^{-/-}$ cells exhibited a delayed induction of antiviral defense genes and impaired control of viral replication (**Chapter 4**, Figures 31-32) (Basagoudanavar et al., 2011). In a study by Basagoudanavar et al., constitutive IFN expression was induced by continuous degradation of NFκB by IKK-β, releasing relA to translocate to the nucleus and bind to the *Ifnb* promoter region (Basagoudanavar et al., 2011). At present, the source of IKK-β activation leading to NFκB degradation is unclear;

however, it has been reported that commensal bacteria-mediated activation of NFκB in intestinal epithelial cells is important in maintaining intestinal homeostasis and initiating a protective immune response following intestinal parasitic infection (Nenci et al., 2007; Zaph et al., 2007). Thus, it is tempting to speculate that commensal bacteria, perhaps through the LPS/TLR4 signaling axis, regulate constitutive IFN expression via NFκB degradation and relA-induced transcription. Consistent with this, LPS signaling can lead induction to of *Ifnb* as well as transcription of IRGs, such as *Mx1, Irf7,* and *Ifi1* in a NFκB dependent manner (Doyle et al., 2002). In this scenario, two potential models arise: i.) commensal bacteria-derived signals circulate through the host and directly induce constitutive IFN expression in innate immune cells or ii.) commensal bacteria-derived signaling is localized to the intestinal environment and stimulates constitutive IFN production by intestinal epithelial and/or intestinal immune cells. Constitutive IFN is then released into the circulation and can stimulate basal transcription of IFN response genes in peripheral immune and non-immune cells. Cell lineage-specific deletion of type I IFNs or the molecular machinery leading to constitutive IFN expression in mice will give valuable insight how commensal bacterial communities regulate IFN responses (Figure 37).

5.4 Regulation of constitutive type I IFN expression

Type I IFN expression can initiate a potent antiviral immune response. However, uncontrolled expression of IFNs can also be detrimental to the host and has been linked to autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and scleroderma (Banchereau and Pascual, 2006; Hooks et al., 1979). Therefore, the host must tightly regulate constitutive IFN expression to promote rapid responses to viral

pathogens while limiting destructive host tissue damage. Constitutive IFN expression is regulated in part by the transcription factor IRF2, an IFN-inducible gene that is constitutively expressed in multiple tissues (Senger et al., 2000; Taniguchi et al., 2001). IRF2 is believed to repress *Ifnb* transcription by competitively binding to the positive regulatory domain of the *Ifnb* promoter and via its transcriptional repression domain in the carboxyl terminal region (Taniguchi et al., 2001; Yamamoto et al., 1994). Therefore, basal IFN production may induce transcription of its own negative regulator, IRF2, creating a negative feedback loop to limit constitutive IFN expression. Genetic deletion of IRF2 in mice leads to increased baseline expression of IRGs and spontaneous induction of a psoriasis-like skin disease (Arakura et al., 2007; Hida et al., 2000) indicating unchecked regulation of constitutive IFN expression in these mice. Onset of the autoimmune skin disease could be ameliorated by backcrossing the $IRF2^{-/-}$ mice onto a IFN signaling deficient background ($IFNAR^{-/-}$ or $IRF9^{-/-}$) demonstrating that the disease pathogenesis was type I IFN-dependent (Hida et al., 2000). Thus transcription of constitutive IFN expression is continuously regulated by repressors (IRF2) and activators (relA) binding to the promoter region, possibly preventing histone deacetylation of the chromatin and maintaining the gene locus in an open formation (Figure 38). Dynamic transcriptional regulation, as discussed in **Chapter 4**, could keep the gene locus in an active state that can be quickly accessed upon detection of viral infection by PRRs and phosphorylation of IRF3/7, both primary inducers of type I IFN expression. The removal of commensal bacteria-derived signals could reduce activator and repressor signals for constitutive IFN expression and leave the *Ifn* gene in a closed dormant state resulting in decreased expression of IRGs in cells in the steady-state as observed in **Chapter 4** with macrophages from ABX-treated mice.

In addition to IFN signaling, IRF2 expression can also be modulated by other commensal bacteria-derived signals such as LPS (Marson et al., 2004), butyric acid (a SCFA produced by degradation of dietary fibers by commensal bacteria) (Joseph et al., 2004), and oxidized low density lipoprotein (oxLDL) (Marson et al., 2004), which has been reported to be elevated in the serum of GF mice (Velagapudi et al., 2010). Potential regulation of IRF2 expression by commensal bacteria via IFN-dependent or independent mechanisms may result in shaping additional arms of the immune system. For example, NK cells, whose cytotoxic activity can be augment by administration of probiotics (Matsuzaki and Chin, 2000), exhibit impaired target cell killing following stimulation with poly I:C or in a tumor rejection model in IRF2$^{-/-}$ mice (Lohoff et al., 2000). Further, *Irf2*$^{-/-}$ mice exhibit a skewed type-2 inflammatory phenotype with elevated serum IgE, increased basophils in the spleen and blood, and an expanded, basophil-dependent CD4 T_H2 cell population (Hida et al., 2005), attributes which are phenocopied in both ABX-treated and GF mice (Hill et al., 2012). Notably, in agreement with these findings, genetic polymorphisms in IRF2 and alteration in commensal bacterial communities have each been associated with atopic dermatitis, a type 2 cytokine-mediated inflammatory disease in human patients (Gao et al., 2011; Penders et al., 2007a). Cell-lineage specific deletion of IRF2 and manipulation of commensal bacterial communities in these murine models will be needed to better understand the possible connection between commensal bacteria, IRF2 expression, and immune cell homeostasis. Combined these findings suggest that commensal bacteria-derived signals may differentially modulate distinct immune cell populations and highlight the importance of understanding how commensal bacteria-derived signals mediate immune regulation and via what cell type these signals are eliciting their immunomodulatory effect.

5.5 Therapeutic potential of commensal bacterial communities

Modulating commensal bacterial communities has great therapeutic potential. Clinical probiotic treatment can ameliorate intestinal inflammatory diseases (Mimura et al., 2004; Sartor, 2004), and success of bacteriotherapy in cases of viral gastroenteritis demonstrates the potential use of probiotics as a treatment strategy to combat viral infection (Fang et al., 2009; Isolauri et al., 1994; Majamaa et al., 1995; Szajewska and Mrukowicz, 2005). Further, prophylactic probiotic administration can limit the duration and severity of respiratory viral infections and correlates with increased cell-mediated immune responses in human subjects, suggesting that the beneficial effects of probiotics on antiviral immunity are not limited to the gastrointestinal tract (de Vrese et al., 2006; Pregliasco et al., 2008). Conversely, the data presented in this thesis suggest that removing or limiting exposure to commensal bacterial communities, such as in populations receiving long-term antibiotic treatment, may increase an individual's predisposition to viral infection. This interpretation should be tempered, however, by findings demonstrating how enteric viruses use commensal bacteria to their benefit to improve viral infectivity and limit the host's immune response (Kane et al., 2011; Kuss et al., 2011). These two studies highlight the need to further characterize the specific bacterial products that can mediate susceptibility to infection.

Despite many recent advances in defining the diverse microbiome in humans and animal models (Peterson et al., 2009), only a few commensal species or microbial products that elicit an immunomodulatory effect have been defined (Reading and Kasper, 2011). Thus, it is currently unclear which commensal species or specific microbial products are associated with the beneficial clinical observations described above or the impact on antiviral immunity described in this thesis. The experiments

presented in this thesis, as well as numerous other published reports, have used a broad spectrum antibiotic cocktail designed to target all classes of bacteria (Gram positive, Gram negative; Aerobes, Anaerobes) to address how commensal bacteria modulate the immune system. This strategy indiscriminately, albeit successfully, eliminates signals derived from multiple commensal bacteria and therefore makes identification of specific species or commensal bacteria-derived products that elicit observed immunomodulatory effects difficult. It will be important to determine which bacterial products are immunomodulatory and to investigate the distinct signaling pathways these products stimulate. Such studies should allow for the development of novel strategies to therapeutically administer specific commensal bacteria or commensal bacteria-derived products and selectively manipulate host immunity.

5.6 Alternative mechanisms of immune regulation by manipulating commensal bacterial communities

The most direct interpretation of the data presented in this thesis is that in the steady-state, commensal bacteria provide tonic signals that regulate the activation threshold of the innate immune system required for optimal antiviral immune responses. However, disruption of commensal bacterial communities may be indirectly impairing antiviral immunity via alteration of the host viral microbiome. Disruptions of intestinal commensal bacterial communities may also alter the bacteriophage population, endogenous viruses residing in intestinal bacteria that substantially contribute to the pool of genes that comprise the intestinal metagenome (Letarov and Kulikov, 2009). It is possible that depletion of commensal bacteria could be indirectly leading to immune dysregulation via loss of a bacterial population that harbors viruses capable of interacting with host

immune cells and modulating immune responses. Currently, characterization of the human microvirome is still in its infancy and the technologic tools needed to manipulate the microvirome to probe the impact of these endogenous viruses on health and disease have yet to be developed (Reyes et al., 2010). Thus the role of the intestinal microvirome in modulating immune response remains largely unexplored.

Alternatively, loss of commensal bacteria may influence host immune responses by disrupting the breakdown of metabolites that have immunoregulatory properties. Intestinal commensal bacteria have a crucial role in the metabolic breakdown of food products and loss of commensal bacterial species alters nutrients available to be absorbed by the host (Velagapudi et al., 2010). Some food products are already known to modulate immune cell homeostasis. For example, vitamin A via its metabolite, retinoic acid, can shape immune cell differentiation and mediate tolerance and immunity in the gastrointestinal tract (Hall et al., 2011). While vitamin A conversion is not a process governed by commensal bacteria, it is possible to envision a scenario where loss of commensal bacterial species crucial in the breakdown and release of a metabolite with immunomodulatory function would result in shifts in immune homeostasis. Indeed, short chain fatty acids (SCFA), metabolic byproducts of fermentation of dietary fibers by commensal bacteria, can bind to the G-protein-coupled receptor 43 that is expressed on immune cells (Brown et al., 2003; Le Poul et al., 2003) and can limit inflammatory responses (Maslowski et al., 2009). GF mice, which cannot produce SCFA due to loss of commensal microbes (Hoverstad and Midtvedt, 1986), do not have this immunoregulatory mechanism and are more susceptible to chemically-induced intestinal inflammation (Maslowski et al., 2009). Further studies are needed to understand how disruption commensal bacterial communities are interwoven with changes in metabolism

in order to tease apart the precise mechanisms of action used by commensal bacterial communities to influence the immune system.

5.7 Concluding remarks

This thesis demonstrates the importance of commensal bacteria-derived signals in regulating antiviral immunity. In the absence of commensal bacteria-derived signals, the immune machinery for antiviral defense, while functionally intact, may lay dormant, waiting for activating signals to initiate it into action. This passive state can prove to be dangerous to the host if the invading pathogen can establish a replicative advantage before the host organism's immune system is activated to respond. Evolutionarily speaking, it would be beneficial for an organism to have its immune system in a state of constitutive readiness, producing anti-pathogen factors to prophylactically prevent infection and ensure the organism's fitness for survival and reproduction. Two obstacles lie in the way of this strategy. First, continuous expression of immune response genes would exact a substantial metabolic burden on the organism and require diversion of a large amount of energy resources to sustain. Second, a prolonged fully activated immune response could only be tolerated for a certain period of time before eventually resulting in pathologic damage to the host's tissues and organs. The immune system may have evolved to simultaneously overcome both of these obstacles by engaging with surrounding commensal bacterial communities and utilizing commensal bacteria-derived signals as an immuno-rheostat to calibrate itself for efficient and rapid responses to potential dangers while limiting energy consumption and exposure to potentially harmful immune responses. Using this kind calibration mechanism would give an organism a distinct evolutionary survival advantage by establishing basal protection against a threat

that has yet to be encountered and thus cannot be predicted, in terms of type, timing and location. Therefore, the immune system may have evolved to incorporate signals from commensal bacterial communities as part of its repertoire of host defense mechanisms, as important to host defense as any other arm of the immune system.

Figure 36. Commensal bacteria-derived signals calibrate the activation threshold of macrophages required for a rapid and robust antiviral immune response.

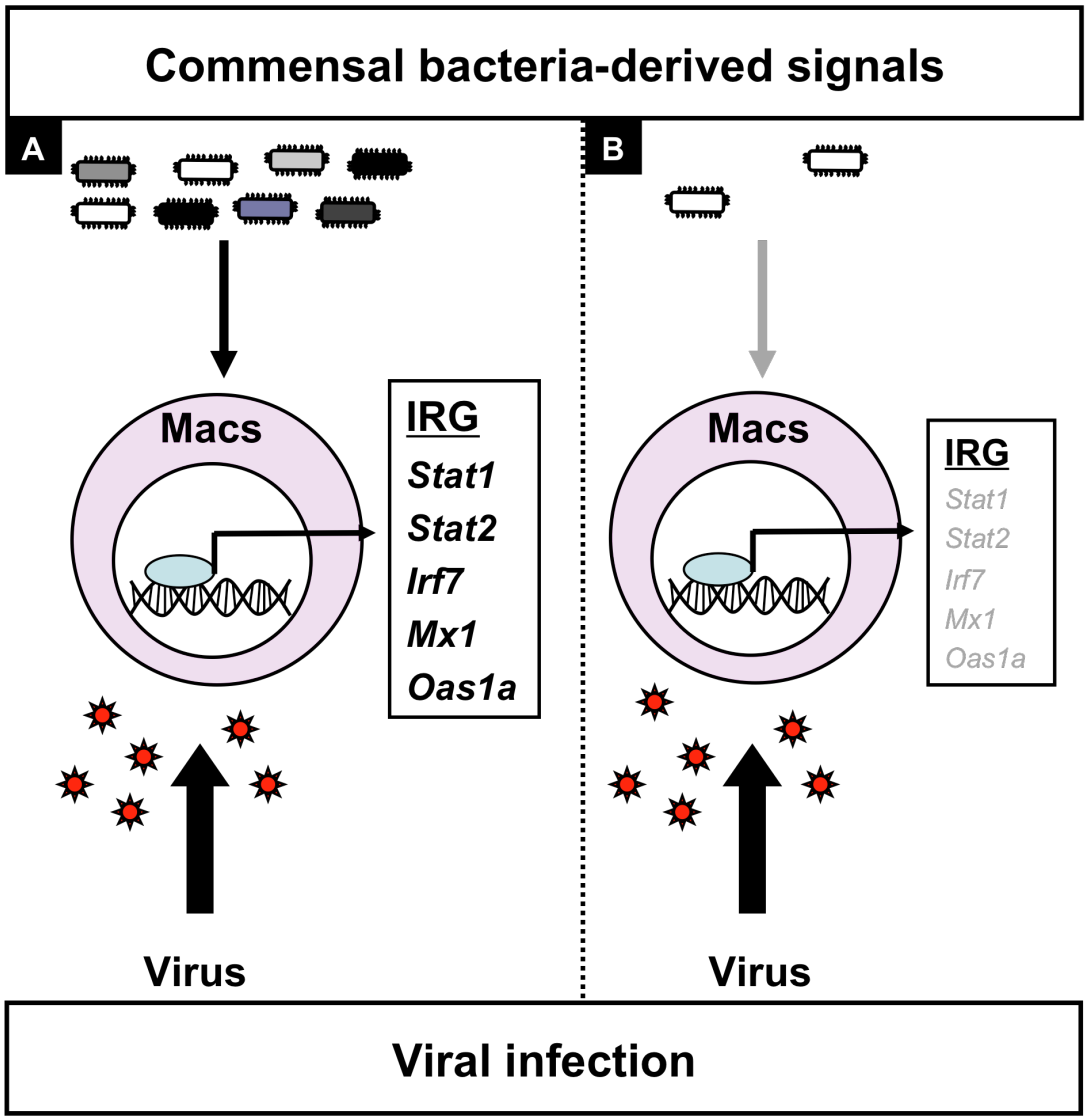


Figure 36. (A) Commensal bacteria-derived signals tonically stimulate macrophages and initiate basal transcription of IFN response genes (IRG). **(B)** Decreased commensal bacteria-derived signaling results in diminished expression of IRGs. (A,B) Upon viral infection, macrophages conditioned by commensal bacteria-derived signals induce a rapid and robust response, while macrophages that have been deprived of tonic stimulation from commensal bacteria exhibit a delayed and impaired antiviral response.

Table 2. Top 50 relatively increased genes in CNV macrophages compared to ABX macrophages.

Gene Symbol	Fold Difference	Gene Name
Ifit3	**7.88**	**IFN-induced protein w/ tetratricopeptide repeats 3**
Olfr99	4.19	Olfactory receptor 99
Rik	3.96	Riken cDNA gene
Tgtp	**3.52**	**T-cell specific GTPase 1**
Spa17	3.45	Sperm autoantigenic protein 17
Rbm45	3.44	RNA binding motif protein 45
Iigp1	**3.05**	**Interferon inducible GTPase 1**
Vpreb1	3.00	Pre-B lymphocyte gene 1
Rtp4	**2.92**	**Receptor transporter protein 4**
Fam126b	2.82	Family with sequence similarity 126
Aoah	2.76	Acyloxyacyl hydrolase
Snora44	2.73	Small nucleolar RNA, H/ACA box 44
Xaf1	2.69	BIRC4-binding protein
Myo9a	2.69	Myosin IXa
Il18	2.68	Interleukin 18
Oasl2	**2.67**	**2'-5' oligoadenylate synthetase-like 2**
S100a9	2.64	S100 calcium binding protein A9
Clec2i	2.60	C-type lectin domain family 2, member i
Ccr5	**2.50**	**Chemokine receptor 5**
Mx1	**2.48**	**Myxovirus resistance 1**
Ddx60	**2.44**	**DEAD box polypeptide 60**
BC057170	**2.44**	**Guanylate-binding protein 9**
Ifi47	**2.44**	**Interferon gamma inducible protein 47**
Stk39	2.40	Serine threonine kinase 39
D14Ertd668e	2.40	DNA segment, Chr 14, ERATO Doi 668
Mx2	**2.36**	**Myxovirus resistance 2**
Irgm1	**2.36**	**Immunity-related GTPase family M member 1**
Prkch	2.35	Protein kinase C, eta
Rnf160	2.34	Isterin E3 ubiquitin protein ligase 1
Oas2	**2.34**	**2'-5' oligoadenylate synthetase 2**
Gmnn	2.34	Geminin
Tmem195	2.33	Alkylglycerol monooxygenase
Rnf213	2.28	Ring finger protein 213
Irgm2	**2.28**	**IFN gamma inducible immunity related GTPase 2**
Tbc1d19	2.27	TBC1 domain family, member 19
Chd7	2.27	Chromodomain helicase DNA binding protein 7
Gm71	2.26	Methyltransferase like 21D
Mblac2	2.21	Metallo-beta-lactamase domain containing 2
Dnajc18	2.19	DnaJ (Hsp40) homolog, subfamily C, member 18
Fabp3	2.19	Fatty acid binding protein 3
Isg15	**2.19**	**IFN-stimulated gene 15 ubiquitin-like modifier**
Slfn5	2.18	Schlafen 5
Aif1	2.17	Allograft inflammatory factor 1
Stox2	2.16	Storkhead box 2
Adap2	2.16	ArfGAP with dual PH domains 2
Usp18	**2.13**	**Ubiquitin specific peptidase 18**
Syne2-1	2.12	Synaptic nuclear envelope 2
Eif2ak2	**2.11**	**Eukaryotic translation initiation factor 2- αK2**
F7	2.11	Coagulation factor VII
Oas1g	**2.11**	**2'-5' oligoadenylate synthetase 1G**
Oas1a	**2.10**	**2'-5' oligoadenylate synthetase 1A**

Table 2. Top 50 genes that are expressed at relatively elevated levels in macrophages isolated from naïve CNV mice compared to macrophages isolated from naïve ABX-treated mice. Bold highlighted genes denote genes that are related to the IFN signaling pathway.

Figure 37. Potential models for commensal bacteria-driven constitutive type I IFN expression.

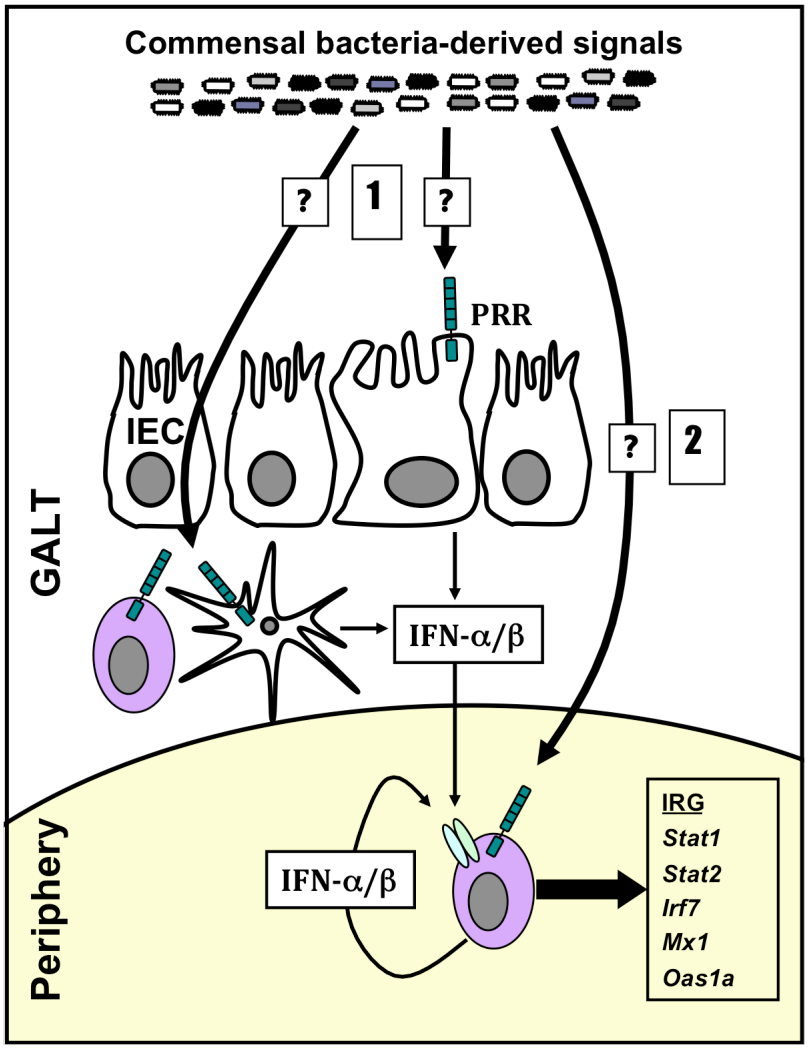


Figure 37. (1) Commensal bacteria may signaling via IECs or resident intestinal immune cells to induce constitutive type I IFN expression that can be secreted into circulation and stimulate peripheral immune cells. **(2)** Alternatively, Commensal bacteria-derived products could translocate from the intestine and directly stimulate innate immune cells to produce low levels of constitutive type I IFN that induce basal transcription of IFN response genes (IRG).

Figure 38. Dynamic versus dormant transcriptional regulation of constitutive type I IFN expression.

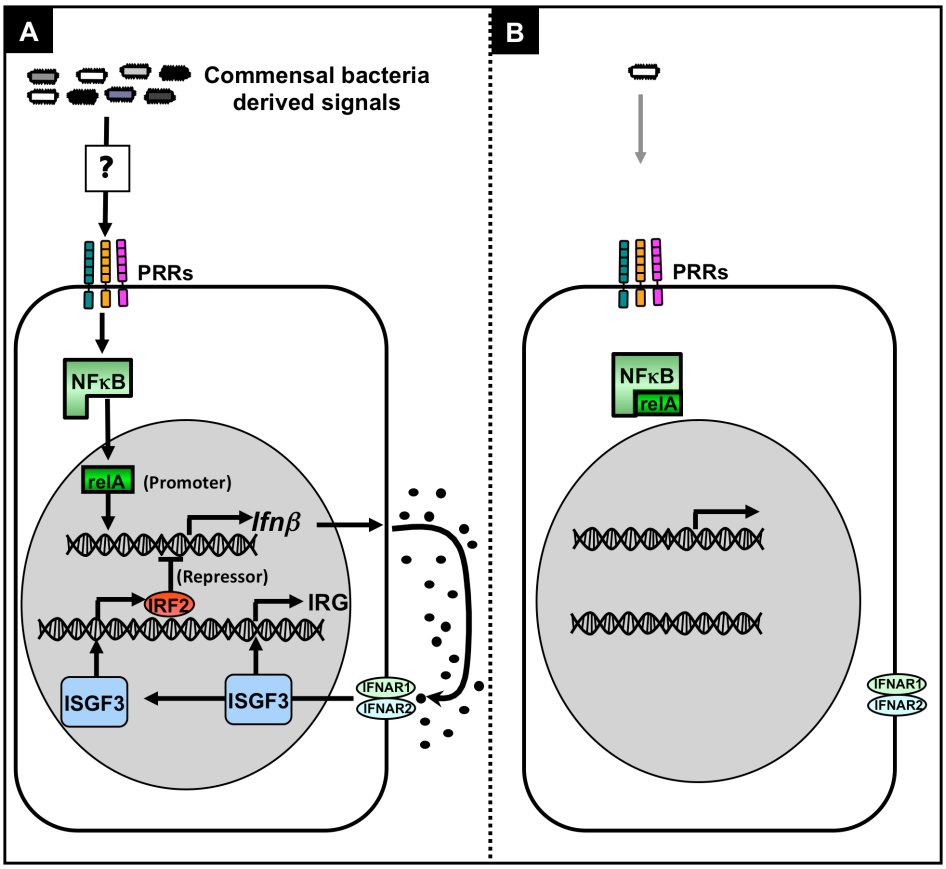


Figure 38. Expression of constitutive type I IFN is regulated by transcription factors relA (promoter) and IRF2 (repressor). Constitutive degradation of NFκB releases the relA subunit , which translocates into the nucleus. relA binds to the *Ifnβ* promoter region and induces expression of type I IFN. Type I IFN signaling leads to the formation of the IFN-stimulated gene factor 3 transcription factor complex (ISGF3), which initiates transcription of IRGs including IRF2, a transcriptional repressor of type I IFN. **(A)** Presence of both promoting and repressing factors can maintain genes in a state of dynamic transcriptional regulation and may allow for faster or more efficient transcriptional induction upon activation by a primary inducer (Viral PAMPs) compared to a **(B)** dormant or inactive state. Commensal bacterial-derived signals may influence constitutive type I IFN expression by regulating either promoter or repressor activation.

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