Alterations in preconception, antenatal, and postnatal maternal gut microbiota influence offspring intestinal microbiota and immunity


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Plagiarism Declaration

This thesis has been submitted to the Turnitin module and I confirm that my supervisor has seen my report and any concerns revealed by such have been resolved with my supervisor.

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Abbreviations

CD	Cluster of differentiation
IL-4Rα	Interleukin-4 receptor alpha
OTU	Operational taxonomic unit
ELISA	Enzyme Linked Immunosorbent Assay
Nb	*Nippostrongylus brasiliensis*
PI	Previously infected
C	Controls
G	Gestation
N	Nursing
GN	Gestation plus Nursing
WT	WildType
HMO	Human Milk Oligosaccharides
APC	Antigen Presenting Cells
NETs	Neutrophil Extracellular Traps
PRRs	Pattern Recognition Receptors
PCoA	Principal coordinates analysis

Abstract

Maternal microbiota during pregnancy, as well as maternal disease state, may impact offspring gut bacterial colonisation. Here, we explore the impact of maternal antibiotics during gestation and/or nursing on offspring gut microbiota. Further, we investigate the effect of preconception helminth infections on maternal and infant gut microbiota. For maternal antibiotic experiments, dams were fed vancomycin, polymyxin B, or both, in drinking water during gestation, nursing or gestation plus nursing, and their offspring microbiota analysed at 14 days of life, alongside immunity in the spleens. Offspring born to vancomycin treated mothers had significantly higher relative abundance of Proteobacteria and Tenericutes while maternal oral polymyxin B led to significantly lower abundance of Proteobacteria and Deferribacteres in infants. Maternal oral vancomycin led to significant reduction in proportions of infant central memory CD4+ T cells (CD4+CD44hiCD62Lhi) regardless of antibiotic timing. Effector memory CD4+ T cells were significantly lower in pups born to dams treated with polymyxin B while nursing while proportions of central memory CD4 T cells were significantly increased in gestation only or gestation plus nursing pups. In addition, oral vancomycin in dams during nursing resulted in significantly reduced proportions of both total and follicular B cells in offspring born to antibiotic treated dams. Pups born to Vancomycin treated mothers had a significant delay in growth when infected with Respiratory Syncytial Virus (RSV). On the other hand, pups born to mothers treated with Polymyxin B during gestation or gestation plus nursing were susceptible to *Nippostrongylus brasiliensis (Nb)* infections. In the second study, we infected female BALB/c mice with 500*Nb* L3 three weeks prior to mating and examined the effect of preconception helminth infection on offspring microbiota and immunity. Preconception *Nb* infections led to alterations of maternal gut microbiota during pregnancy. In addition, we observed dramatic differences in offspring microbiota in pups born to previously helminth infected dams. Coriobacteriaceae were predominant in pups born to previously *Nb* infected dams when compared to uninfected dams. Overall, manipulation of maternal microbiota during gestation or lactation profoundly impacts offspring growth, intestinal microbiota and immunity to RSV and helminths.

CHAPTER 1.

1.0 Introduction

1.1 Mammalian gut microbiota

Virtually all multicellular organism live in close proximity with microbes and the human host is no exception. The human body is home to a range of microorganism including viruses, bacteria, fungi, archae and unicellular eukaryotes. These collection of microbes that coexist peacefully with their host has been referred to as the microbiota, normal flora, or microflora (Neish, 2009). While the gut is in continuous contact with these microbes, cells such as the endothelium must remain sterile for local function and for the health of an individual. These commensal bacteria have been demonstrated to affect our health in profound ways, bringing into the forefront the symbiotic relationship that exists between the microbes and the human host. The microbiota colonises virtually every part of our skin and body that is exposed to the external environment with the highest colonisation being in the gastrointestinal tract; particularly the colon which contains about 70% of all microbes in the human body (Chiller et al., 2001; Ley et al., 2006b). Additionally, the gut is rich in various nutrients which are needed by commensals, making it a preferred site of colonisation. In terms of function, the gut microbiome plays key roles in regulating metabolic, immunological, physiological and gene expression patterns in the body (O'Hara & Shanahan, 2006; Qin et al., 2010). In the current study, we were particularly interested in understanding how maternal helminth infections or antibiotic treatment impact neonatal gut colonisation and the ramifications of the inherited neonatal microbiome on neonatal immunity. Recently, it has been demonstrated that gut colonisation in neonates within a critical window period has lasting metabolic consequences (Cox et al., 2014; Nobel et al., 2015). This section will review some relevant literature on factors that influence gut colonisation in infants, host microbiome

interactions and the impact of early microbial gut composition on offspring susceptibility to disease.

1.2 Factors that influence gut colonization

Until a recent study by Aagaard *et al*, the placenta had been thought to be sterile, and the prevailing belief was that gut colonisation begins after birth when the infant comes into contact with the extra uterine environment. However, the placenta has been shown to harbour a low abundance but metabolically rich microbiome composed of non-pathogenic commensal bacteria from Firmicutes, Tenericutes, Fusobacteria, Bacteriodetes and Proteobacteria phyla. This profile was most akin to oral microbiome of nonpregnant humans (Aagaard et al., 2014; Carmen Collado et al., 2016). These data has bought into question the idea that the fetus is sterile, and indeed, recently, a number of studies have found bacteria present in meconium, the first infant stools (Ardissone et al., 2014; Hu et al., 2013; Jiménez et al., 2008). Regardless of when gut colonisation begins, the microbiome develops rapidly in the first years following birth, changing in both diversity and complexity before becoming stable at adulthood (Nylund et al., 2014). A series of factors influence gut colonisation in infants including gestational age, mode of delivery, host genetics, antibacterial drugs, lifestyle and diet (Alderete et al., 2015; Dominguez-Bello et al., 2010).

1.2.1 Age

The development of the microbiome is a rapidly changing process where individuals' express different microbial profiles at different life stages (Fig. 1) (Power et al., 2014). Enterobacteriaceae, Streptococci and Staphylococci are predominant early in life (Marques et al., 2010). However, the recent improvement of hygienic conditions during delivery alongside shorter hospital stays have led to an alteration of the initial colonisation pattern with skin derived staphylococci being among

the first colonisers and not the fecal Enterobacteriaceae (Marques et al., 2010). Increase in the Caesarean (C-section) deliveries has fundamentally contributed to these colonisation pattern.

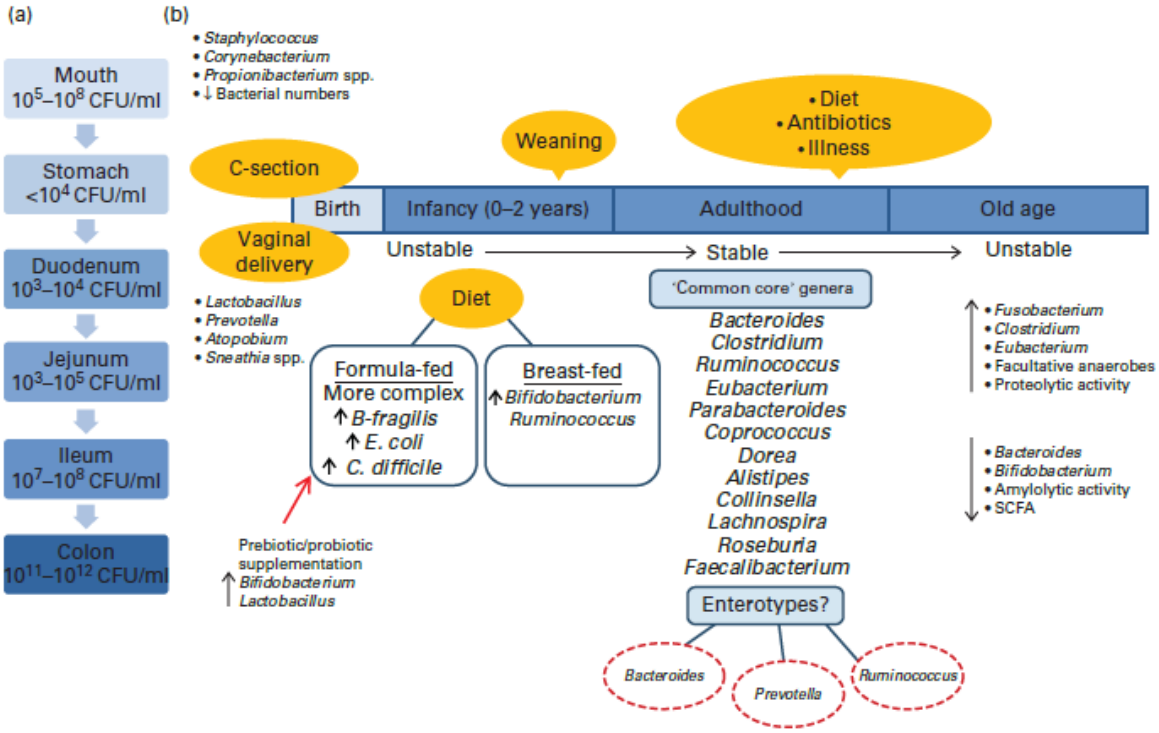


Figure 1.1: (a) Variation in microbial numbers across the length of the gastrointestinal tract. (b). Factors affecting the establishment and maintenance of the microbiota. (Power et al., 2013).

1.2.2 Mode of delivery

The mode of delivery also impacts the microbiome. Vaginally born infants encounter microbes in the birth canal while infants born by caesarean section derive their first major microbe source from the environment, mainly the skin. Therefore, infants born via C section have lower intestinal bacteria counts and reduced diversity in the first weeks of life (Morelli, 2008). Neonates have been found to harbor communities that are undifferentiated across multiple body habitats regardless of delivery mode (Dominguez-Bello et al., 2010). Vaginally born infants acquire bacteria communities resembling their mothers vaginal microbiome dominated by *Lactobacillus*,

Prevotella or *Sneathia* species while C section infants harbored bacteria similar to those found on the skin surface dominated by *Staphylococcus, Corynebacterium* and *Propionibacterium* species (Dominguez-Bello et al., 2010). A recent systematic analysis by Rutayisire et al. observed that cesarean delivery was associated with both lower abundance and diversity of Actinobacteria and Bacteroidetes, and higher abundance and diversity of Firmicutes from birth to 3 months of life (Rutayisire et al., 2016).

1.2.3 Mode of feeding and diet

Breast-fed infants have a microbiota dominated by Bifidobacteria and Ruminococcus (Favier et al., 2002). In contrast, their formula-fed counterparts have a complex more diverse microbiota comprising Streptococcus, *Bacteroides*, *Clostridium* and *Atopobium* (Bezirtzoglou et al., 2011). Human milk oligosaccharides (HMO) are thought to function as growth factors for beneficial bacteria, inhibitory receptors for binding of various pathogens and may promote development of the early immune system (Kunz et al., 2000). Recently, more physiological formulas has been developed which contain prebiotics such as galactooligosaccharide and fructooligosaccharide which have been proven to increase the abundance of Bifidobacteria and Lactobacilli in the gut (Chen et al., 2014).

Postpartum, diet is the greatest environmental factor that impacts the microbiome. Diet not only provides nutrients for the host but also bacteria in the gastrointestinal tract. Most enzymes required for the breakdown of the plant polysaccharides are not encoded by the mammalian genomes. Alterations in the bacteria composition in the gut occurs because different bacteria utilize different substrates as sources of energy (Bellei et al., 2012). Dietary history is associated with lifestyle, geography, cultural beliefs, and socioeconomic status. Significant differences in gut microbiota have been reported in people living in different environments and social status. In a study

comparing the intestinal microbiome of rural children in Burkina Faso who consumed plant derived diet with Italian children who consumed a low fiber diet, authors reported profound differences in the gut composition between the two groups (De Filippo et al., 2010). African children exhibited higher levels of Firmicutes than Bacteroidetes while the European children had an abundance of Enterobacteriaceae (*Shigella* and *Escherichia*). Others have compared the gut microbiota composition of Korean residents with those from other countries including USA and Japan (Nam et al., 2011). UniFrac analysis revealed slight differences at the phyla level for each cohort with Americans exhibiting higher Firmicutes, Japanese having higher Actinobacteria while the Koreans had enrichment of Bacteroides (Nam et al., 2011). Therefore, it is plausible that the gut microbial composition is tied to long-term dietary patterns.

1.2.3.1 Effect of breast milk on microbiota composition

The human milk provides the infant gut with a rich bacteria consortium promoting the colonization of beneficial microbes that impact metabolism and help in programming immunity (Jeurink et al., 2013). Human milk oligosaccharides (HMO) are vital in promoting development of normal infant physiology and immune development. As has been discussed above, the gut microbiota of breast-fed infants has been characterized to be less diverse and as having a high abundance of Bifidobacteria and Lactobacillus when compared to their nonbreast-fed counterparts. Recently, Bifidobacteriaceae (61%), Enterobacteriaceae (8%) and Coriobacteriaceae (6%) were identified as the most abundant taxa in the stool of breast-fed babies (Tannock et al., 2013). Regardless of mode of feeding, the composition of the gut bacteria begins to change with the introduction of solid foods at weaning (Palmer et al., 2007). Although the source of bacteria in human milk is not clear, it is now established that the human milk plays a central role in shaping the neonatal gut early in life.

1.2.4 Antibiotics

Antibiotics were designed to help in the treatment of infections by targeting pathogenic organisms. Depending on their spectrum of activity, they can potentially disrupt the native gut community. Previous studies have described this as the etiology of some antibiotic-associated diarrhea and why probiotics are often administered during broad-spectrum antibiotic therapy (Hickson, 2011; McFarland, 2006). However, antibiotic administration can interfere with the coevolved interactions of the commensals with their host. Various studies have demonstrated the role of antibiotics in driving dramatic alterations of the microbiome both in adults and neonates (Cox et al., 2014; Nobel et al., 2015; Tormo-Badia et al., 2014). Generally, antibiotic treatment leads to reduction in diversity and richness in the gut communities (Jernberg et al., 2007). Nonetheless, the microbiome has potential to recover after weeks of withdrawal of treatment to the pre-treatment state (Claesson et al., 2011; Dethlefsen & Relman, 2011). However, in some cases, microbial changes persist long after withdrawal from treatment and some bacteria are permanently lost from the native community (Dethlefsen & Relman, 2011; Jakobsson et al., 2014; Jernberg et al., 2007).

1.2.5 Probiotics

The World Health Organization's (WHO) 2001 definition of probiotics is, "live micro-organisms which when administered in adequate amounts can confer health benefits to the host (WHO, 2001). Studies have shown their impact in favorable influence of the stability of the microflora, inhibition of pathogen colonization and stimulation of specific and nonspecific components of the immune system (Kalliomaki et al., 2003). Furthermore, probiotics have been suggested as potential therapeutic agents in lowering necrotizing enterocolitis among preterm infants and preventing infections in immunocompromised patients (Guillemard et al., 2010; Martin & Walker, 2008). In addition, probiotics have been shown to have a role in ameliorating various disorders. Lee et al. have shown the anti-obesity effects of *Lactobacillus rhamnosus* PL60 in diet induced obesity study

conducted in mouse models (Lee et al., 2006). Johnson-Henry et al. demonstrated that probiotics (*Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) attenuate the effects of *Citrobacter rodentium* in mouse models (Johnson-Henry et al., 2005). Moreover, a proliferative Bifidobacterium strain in the gut has been reported to ameliorate progression of metabolic disorders via microbiota modulation and acetate elevation (Aoki et al., 2017).

1.2.6 Prebiotics

Prebiotics are nondigestible food components that stimulate the selective growth of particular microbes in the gut that confer beneficial outcomes to the host (Roberfroid, 2007). Some prebiotic rich-foods have been reported to regulate the gut microbiota by elevating the levels of Bifidobacteria and Lactobacilli (Kleessen et al., 2007).

1.3 Microbiome and health

The human gut microbiota confers health benefits to the host in multiple ways, including by protecting against colonization by pathogens. The microbiota serves as a physical barrier mainly by formation of microbial biofilms (Macfarlane & Dillon, 2007), and protect against pathogen colonization by competitive exclusion and by secretion of antimicrobial compounds (Belkaid & Hand, 2014). For example, members of the genera Lactobacillus release lactic acid which have an inhibitory activity towards the growth of bacteria and also potentiates the antimicrobial activity of the host lysozyme (Alakomi et al., 2000). The microbiota also stimulates the production of antimicrobial compounds by the host mucosa. Various compounds, including cathelicidins, defensins and C-type lectins are released in the mammalian gastrointestinal tract (GIT) and act by disrupting surfaces of both commensals and pathogenic bacteria (Salzman et al., 2007). In addition to production by microbial cells, microbial metabolites also have potential to lead to expression of antimicrobial peptides in vivo and in several cell lines. Short chain fatty acids led to the production

of LL-37 cathelicidins by human lung epithelial cells (Kida et al., 2006). Therefore, it is likely that the presence of gut commensal bacteria and their metabolites contribute towards expression of antimicrobial peptides thus protecting the host against colonization by pathogens and also preventing an overgrowth of the commensals themselves.

1.3.1 Microbiome and disease

Gut colonization within a critical window period early in life has been shown to have lasting metabolic consequences and can have effects on later immunity (Cox et al., 2014; Nobel et al., 2015). Aberrations in the adult microbiome has also been linked to various diseases and disorders including allergic disease and asthma (Trompette et al., 2014), colon cancer (Scanlan et al., 2008), autoimmune diseases (Candon et al., 2015), obesity (Ley et al., 2005), type 1 diabetes (Scott, 1996), and even progression and severity of HIV (Gori et al., 2008). Disruption in the native microbiome, referred to as dysbiosis is accompanied by an overgrowth of pathogenic organisms and fungi and loss of particular functional groups from the microbial ecosystem (Manichanh et al., 2006). This in turn results in inflammatory responses by the host which contributes to diseases and various disorders (Gori et al., 2008; Stecher et al., 2007). Further, gut dysbiosis has been linked to the imbalance between T helper and anti-inflammatory T regulatory cells in the host. Prolonged overproduction of Th1 and Th17 cytokines has been linked to Inflammatory Bowel Disease (IBD) as well as other autoimmune conditions for example Multiple Sclerosis and Systemic Lupus Erythematosus (Cultural & Lloreda, 2011; Fujino et al., 2003). Consequently, host driven inflammatory responses disrupt the gut microbiota further and permanently eliminate commensal bacteria and allow for colonization by the pathogenic microbes which maintain an inflammatory state (Fujino et al., 2003). Recently, helminth infections have been shown to impact the microbiota and to attenuate pathology associated with allergic asthma in a microbiota dependent manner

(Zaiss et al., 2015). Reynolds and others linked *Heligmosomoides polygyrus* to the expansion of lactobacillus species in murine models (Reynolds et al., 2014), while Broadhurst demonstrated how helminths interact with resident bacteria communities to drive control against diarrhea (Broadhurst et al., 2012). Furthermore, a recent study revealed that enteric helminth infection can have protective antiviral effects in the lung through the induction of a microbiota-dependent type 1 interferon response (McFarlane et al., 2017). Helminths remain endemic in sub Saharan Africa and susceptibility is enhanced during gestation (de Silva et al., 2003; Yatich et al., 2009). Yet whether the interplay between maternal helminth infections and the microbiota impact offspring microbiota as well as immunity is unknown. Importantly, a dysbiotic gut microbiota has a reduced microbial diversity; a characteristic that has been associated with microbiome associated disorders. Reduction in diversity following gut dysbiosis leads to a loss of key bacteria taxa that are butyrate producers. Butyrate is an important gene regulator and serves as an energy source for particular mucosa associated microbial groups (Canani et al., 2011).

1.3.2 Prenatal exposure to microbes and microbial compounds

The fetus has long been believed to be biologically sterile before birth but this dogma has recently been challenged with the discovery of a placenta microbiome (Aagaard et al., 2014; Carmen Collado et al., 2016). Further, reports of bacteria in amniotic fluid suggest that the fetus is continuously exposed to microbes *in utero.* Recent studies have determined that predisposition to disease may at least in part be determined in utero. Maternal exposure to stimuli during pregnancy programs postnatal immunity and susceptibility to allergic conditions (Penders et al., 2006). A large European study revealed that mothers exposed to farm animals during pregnancy were less likely to have children who developed allergies later adulthood (Schaub et al., 2009a). Thus it is plausible that microbiota exposure *in utero* impacted neonatal immune responses and increased

numbers of regulatory T cells dampening TH2 responses in cord blood which drive allergic responses (Fujimura et al., 2010).

Similarly, maternal exposure to pets has also been shown to protect against allergic disease development (De Meer et al., 2005) and this protection is thought to be microbiome dependent. Pet exposure has been associated with reduced cord blood levels of IgE (Niladri et al., 2008), elevated levels leads to development of allergic disorders (Bergmann et al., 1997). Wegienka et al. also demonstrated that exposure of pregnant women to pets is associated with enhanced T regulatory cell numbers (Wegienka et al., 2009) suggesting that the protective role of pet exposure against allergic conditions may in part be mediated by T regulatory cells (Schaub et al., 2009a). The dogma that microbial exposure in utero is central in defining development of immunity postnatally is further supported by a study showing that parental dietary lifestyle during gestation and lactation impacts neonatal microbiome and response to infection (Myles et al., 2013). Parental high fat diet during gestation or nursing resulted in alteration in offspring gut microbiome and poor clinical outcomes when offspring are subjected to bacterial challenge (Myles et al., 2013). However, whether these prenatal exposures to stimuli directly or indirectly impact offspring immunity is not well understood. Further, as to whether alterations in neonatal immunity are mediated by the microbes themselves or their metabolites through other pathways remains to be elucidated.

1.4 Early life microbiota

The first fecal specimen to be produced by the infant is termed the “meconium”. This consists of mainly the amniotic fluid, intestinal epithelial cells and pancreatic secretions (Kumagai et al., 2007). Recent reports have described a meconium microbiome providing strong evidence that neonatal colonization begins long before birth (Hu et al., 2013; Moles et al., 2013). A number of

bacteria phyla including Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria have been detected in meconium (Hu et al., 2013; Moles et al., 2013). Vaginally born infants acquire bacteria communities resembling their mothers vaginal microbiome dominated by *Lactobacillus, Prevotella* or *Sneathia* species while C section infants harbored bacteria similar to those found on the skin surface dominated by *Staphylococcus, Corynebacterium* and *Propionibacterium* species (Dominguez-Bello et al., 2010). Postpartum, diet is the greatest determinant of microbiome colonization. Majority of breast-fed infants have an increased abundance of Bifidobacteria in their fecal microbiome which persists to three months after birth (Turroni et al., 2012). In contrast, formula-fed infants have a reduced abundance of Bifidobacteria, which form approximately 25% of their microbiota composition (Roger & McCartney, 2010).

At weaning, as infants start to feed on solid food, the abundance of Bifidobacteria begins to decrease gradually to around 60% at 4 months, 25% at 6 months and 10% at 2 years (Avershina et al., 2014; Nylund et al., 2013). Simultaneously, the abundance of Lactobacilli decreases while bacteria abundant in the adult microbiota including Bacteroidetes and Clostridium cluster XIVa and XIV increase (Fallani et al., 2011). However, bacterial abundances early in life vary greatly due to interindividual variations and will fluctuate further due to different environmental stimuli including dietary lifestyle and antibiotic exposure (Avershina et al., 2014; Koenig et al., 2011; Palmer et al., 2007). However, considering the physiological processes and changes that the human host undergoes including adolescence and the impact this could have on the developing microbiome, it is plausible that the gut consortia of bacteria continues to change over time and only stabilizes at adulthood.

1.5 Metagenomic technology for microbial community analysis

Sequencing and phylogenetic analysis of 16S rRNA has provided the foundation for modern study of microbial communities (Zhou et al., 2015). PCR based 16S RRNA cloning analysis has driven the explosion of information about community membership and vastly expanded the known diversity of microbial life. Community DNA is extracted from environmental samples using various extraction and purification kits (Hurt et al., 2001). Once high quality DNA is obtained, targeted genes are amplified with conserved primers. Each set of primers is generally barcoded for sample multiplexing (Caporaso et al., 2012). Non target DNA are removed by gel electrophoresis, target DNA quantified, sequenced and analyzed by bioinformatic approaches (Zhou et al., 2015). After quality filtering, sequences are clustered into operational taxonomic units (OTUs, sometimes referred to as phylotypes) which provide a working name for groups or related bacteria. OTUs are based on sequence identity (97%) and the degree of sequence variability will depend on the 16S region in question (Goodrich et al., 2014). The OTUs are then assigned to their corresponding bacteria taxonomy using a reference 16S bacteria data base such as Greengenes (McDonald et al., 2012). Many OTUs will lack a complete taxonomy label, for example an OTUs may be annotated to the family level but lack the genus or species level. Abundances can then either be assessed at higher levels such as order or family by summing up the sequences for all OTUs belonging to the group of interest (collapsing taxonomies) or picking OTUs at a lower threshold (%) which can in turn yield different results (Goodrich et al., 2014).

1.6 The Immune system

The immune system is an organization of cells with specialized roles in defending the body against pathogens including bacteria, viruses, and fungi among others. There are two types of responses following infection: Innate immunity which is historically thought to occur in the same way no matter the number of times the same infection is encountered while the adaptive immune response

improves with repeated exposure to the same infection (Parkin & Cohen, 2001). Recent observations have challenged the notion of innate immunity being nonspecific and lacking in immunological memory (Netea et al., 2011; Stevens et al., 2016). An increasing body of evidence suggests that exposure to pathogens lead not only to specific immunological memory (T and B cells) but also an enhanced innate immune response. Memory properties have been proposed for NK and macrophages, which are prototypical cells for innate immunity following first encounter with antigen which has led to the term "trained immunity" being proposed (Netea et al., 2011). The concept of *trained immunity* is whereby a heightened response to a secondary infection is exerted both towards the same organism and a different one, and therefore in a sense, have "memory" (Netea et al., 2011; Stevens et al., 2016). Phagocytic cells (neutrophils, macrophages and monocytes), and those that release inflammatory mediators (basophils, mast cells and eosinophils) as well as natural killer cells are involved in innate immunity (Delves, 2000). Adaptive immune responses involve the proliferation of antigen specific B and T cells which occurs when the surface receptors of these cells bind to specific antigens. Specialized cells called antigen presenting cells (APCs) process the antigen and present it on their surface in the correct MHC complex to T cells. Therefore, the T-cell receptor (TCR) recognizes an individual's own MHC molecule (self) together with peptides derived from foreign antigen. The adaptive immune response is the hallmark of the immune system in higher animals and has immunological memory which enables the mounting of a stronger immune response although this is not immediate (Delves, 2000).

1.6.1 Innate Immunity

1.6.1.1 Neutrophils

Neutrophil recruitment to the site of infection and phagocytosis and killing of pathogens is central to innate immunity (Janeway, 2001). The same process occurring inappropriately can lead to inflammation and tissue damage and injury. Like most immune cells, neutrophils are not static and travel through the body. They move freely from a marginating pool in response to proinflammatory mediators, chemokines and adhesion molecules (Andrian & Mackay, 2000).

1.6.1.2 Complement

The complement system has several important functions in innate immunity and consists of about 20 serum glycoproteins with some being regulatory (Parkin & Cohen, 2001). These proteins are activated in a cascade sequence with the activation of a single molecule leading to thousands of molecules being generated. There are three pathways involved in the complement system: the classical pathway, activated by antigen antibody complex, alternative pathway by polysaccharides from yeast and gram negative bacteria, and most recently described, the lectin pathway which activates complement by stimulations of mannose containing lectins (Andrian & Mackay, 2000).

1.6.1.3 Innate lymphoid cells (ILCs)

ILCs are a growing family of immune cells that mirror the phenotypes and functions of T cells. They include natural killer (NK) cells which are considered innate counterparts of cytotoxic CD8 T cells and ILC1, ILC2 and ILC3 which may represent the innate counterparts of TH1, TH2 and TH17 respectively (Cherrier et al., 2012). ILCs play fundamental roles in processes such as cytotoxicity, lymphoid organogenesis, intestinal homeostasis and immunity against infections (Hwang & Mckenzie, 2013). However, in contrast to T cells, ILCs do not express antigen receptors or undergo clonal selection and expansion when stimulated (Eberl et al., 2015).

1.6.1.3.1 Natural killer cells

These cells have the morphology of lymphocytes but lack the antigen specific receptor. They recognize abnormal cells in either of two ways: first is by the antibody receptor (Fc) on their surface that enables them to bind to antibody-antigen complexes and drive killing by antibody dependent cellular cytotoxicity (ADCC) or by recognition of antigens presented to them in the MHC class I complex (Parkin & Cohen, 2001). NK cells are activated in the absence of MHC 1 expression (Mandal & Viswanathan, 2015) and tolerized towards normal cells by their expression of germline-encoded inhibitory receptors that recognize MHC I (Campbell, 2013). However, when mature NK cells encounter abnormal cells that do not express MHC I, inhibitory receptors are not engaged which triggers targeted attack of the cell (Campbell, 2013). The principal inhibitor receptors are killer cell Ig-like receptors (KIR) in humans and Ly49 in mice. Activating receptors expressed on NK cells include FcRIIIA, activating forms of KIR (KIR2DS, KIR3DS) and the Natural Cytotoxicity Receptors (NCR) called NKp30, NKp44 AND NKp46 (Campbell, 2013). FcRIIIA triggers ADCC upon encounter with cells opsonized with IgG while NCR are key in stimulating responses against tumor cells (Huntington, 2014).

1.6.1.3.2 ILC1s

ILC1s are largely non cytotoxic lineage negative cells that produce Interferon gamma and tumour necrosis factor and have been implicated in immunity to intracellular bacteria and parasites (Klose et al., 2014).

1.6.1.3.3 ILC2s

ILC2s are derived from the lymphoid lineage and express a combination of hematopoietic (CD45, sca-1) and lymphoid markers (IL-7Rα, ICOS, Thy 1.2 and CD44) (Kabata et al., 2015; Neill et al., 2010). They produce TH2 cell associated cytokines (including IL-4, IL-13, IL-5 and IL-9) and promote type 2 inflammation which is central for helminth immunity, allergic inflammation and

tissue repair (Kabata et al., 2015; Price et al., 2010). Type 2 ILCs do not differentiate into other lineage cell types in the presence of SCF and IL-13 suggesting that they are terminally differentiated (Neill et al., 2010).

1.6.1.3.4 ILC3s

ILC3s produce IL-17A, IL-17F,IL-22, granulocyte macrophage (GM) colony stimulating factor (CSF) and TNF and can promote immunity against bacteria, chronic inflammation and tissue repair (Satoh-Takayama et al., 2008; Sonnenberg et al., 2011). In mice, two subsets can be distinguished on the basis of their expression of the chemokine receptor CCR6 while in humans almost all ILC3s express CCR6 and CD117, and at least two subsets can be distinguished on the basis of the expression of natural cytotoxicity receptor NKp44 (Satoh-Takayama et al., 2008; Sonnenberg et al., 2011).

1.6.1.4 γδ-T cells

γδ- T cells are one of the immune cell types that express antigen receptors that undergo somatic recombination, and they contribute to immune responses to infection, cellular transformation and tissue damage (Vantourout & Hayday, 2013). They develop in the thymus generating their signature receptor via RAG-mediated V (D) J recombination. Key properties that distinguish these T cells from the αβ T cells include their ability to recognize qualitatively distinct antigens, fact that they are suited for protection of defined anatomical sites, are of primary value in young animals and although not invariably important, γδ- T cells mediate critical responses to specific pathogens in a manner similar to NK cells (review (Vantourout & Hayday, 2013).

1.6.1.5 Eosinophils

Eosinophils are innate immune granulocytes best recognized for their cytotoxic effector functions, causing damage to parasitic pathogens in helminth infections, and to host tissues in allergic disease (Shamri et al., 2011). Interleukin-5 is produced by several cell types and is responsible for the maturation and release of eosinophils in the bone marrow (Greenfeder et al., 2001; Nakajima & Takatsu, 2007).

1.6.1.6 Mast cells and basophils

Mast cells and basophils are relatively few in the body, yet are involved in some of the most severe immunological reactions, for example anaphylaxis. These cells have high affinity receptors for Ig E and once bound and crosslinked, there is degranulation and release of preformed mediators such as serotonin, amines and histamines (Delves, 2000).

1.6.2 Adaptive Immunity

The adaptive immune response is characterized using antigen specific receptors on T and B cells to drive targeted effector response in two stages; first the antigen is presented to and recognized by the corresponding T cell leading to cell priming, activation and proliferation which occurs in specific areas of the lymphoid tissue. Second, the effector response occurs either due to the activated T cell leaving the lymphoid organs and homing to the disease site, or the release of antibodies by activated B cells (plasma cells) into blood and tissue fluids (Parkin & Cohen, 2001).

1.6.2.1 T lymphocytes

The major class of T cells is defined by surface expression of αβ T cell receptor (TCR). Individual T cells bear antigen specific TCRs (Chaplin, 2010). This receptor has evolved to enable antigen recognition by both MHC class I and II proteins. Further, these T cells differentiate primarily into T cell subsets that enable killing of cells infected by intracellular pathogens (CD8+ T cells) or those that regulate both cellular and humoral responses (CD4+ T cells) (Chaplin, 2010). T cell development occurs in the thymus which contains three compartments. The first termed the sub capsular zone is where bone marrow derived pro-thymocytes differentiate and rearrange their TCR β chains. In the second, the thymic cortex, there is rearrangement of the α chain eventually forming a functional TCR chain (Janeway, 2001). In addition, the cells test whether these receptors have sufficient affinity to recognize self MHC antigen complex which involves the interaction between the developing lymphocytes and the cortical epithelium (Nitta et al., 2008). Lymphocytes that fail this positive selection process are cleared by thymic cortical macrophages. Finally, cells are screened for potential autoreactivity in the thymic medulla. Cells recognizing self-peptides that are expressed by the thymic epithelial cells are removed by apoptosis. T cells that survive this negative selection are then exported to the circulation which normally constitute less than 5% of the total developing T cells (Chaplin, 2010).

1.6.2.1.1 T cell sub populations

During their migration within the thymus, αβ T cell differentiate into several T cell subsets each with different effector functions. In the thymus, most T cells follow a developmental program where they first express neither CD8 nor CD4 (double negatives) and then express both CD8 and CD4 (double positives) (Chaplin, 2010). These double positives then undergo positive selection and those selected on MHC class I molecules become CD4-CD8+ while those selected on MHC class II molecules become CD4+CD8-. Both CD4+ and CD8+ differentiate into functionally

distinct subsets when they experienced their cognate antigens. CD4 T cells are involved in multiple functions ranging from activation of cells of the innate immune system, B-lymphocytes, cytotoxic T cells and immune regulation. They differentiate into various subsets including TH1, TH2, TH17, TH9 and T regulatory cells. This differentiation is controlled by a complex network of specific cytokine signaling and transcription factors allowed by epigenetic modifications (Luckheeram et al., 2012). IL-12, IFN-gamma and the transcription factor T bet drive the differentiation into TH1 cells (Trinchieri et al., 2003) while IL-4, IL-2 and STAT6 are critical for TH2 differentiation (Kaplan et al., 1996).

1.6.2.2 B lymphocytes

B-lymphocytes constitute about 15% of peripheral leukocytes in blood. B cells produce antibodies which serves a range of functions including: neutralizing toxins, preventing organisms adhering to mucosal surfaces, activating complement, opsonising bacteria, and also antibody dependent cellular cytotoxicity (Parkin & Cohen, 2001). They differentiate from hematopoietic stem cells in the bone marrow where their antigenic receptors are assembled in a RAG-1/RAG-2 mediated process similar to the development of the TCR in T cells (Thomas et al., 2009). Akin to the TCR, the fully mature B lymphocyte also express the transmembrane proteins Igα and Igβ involved in intracellular signaling following antigenic exposure (Khan, 2009). Moreover B cells have a co-receptor complex comprising CD19, CD81 and CD21 which is activated by binding to the activated complement protein C3d (Carroll, 2008).

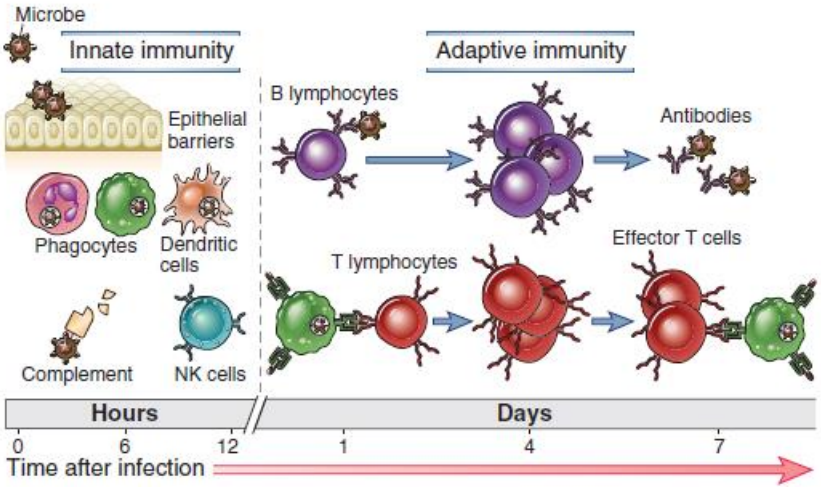


Figure 1.2: Innate and adaptive immunity. Innate immunity provides initial defense against infection. Adaptive immune responses develop later and consist of lymphocyte activation (From the immunology textbook by Abul Abbas, 7th edition).

1.7 Neonatal immunity

The neonatal immune system is considered "immature" and is functionally different from the adult immune system (Basha et al., 2014). Maternal IgG that crosses the placenta barrier contribute to neonatal protection early in life (Virella et al., 1972). However, this protection is short lived, as the amount of antibody wanes after birth. Moreover, IgA antibodies are continuously supplied through breastmilk from the mother and protect the gastrointestinal tract against colonization by pathogens without having an effect on the immune responses (Edwards, 2015; Niewiesk, 2014). Infant cellular immunity develops within the first three months and this process is influenced by multiple factors (Hodgins & Shewen, 2012; Siegrist, 2007).

1.7.1 Neonatal cellular immunity

T cell progenitors that express CD34 receptors migrate to the thymus and differentiate into mature CD4+ and CD8+ cells after 7 weeks of gestation (Borgella et al., 2013). Human studies demonstrate that multi-potent lymphoid progenitors (CD34+CD7+ and CD34+CD10+CD19+)

differentiate to become B cells (Hodgins & Shewen, 2012; Siegrist, 2007). Development of the neonatal lymphoid progenitors is influenced by multiple factors including cytokines, stromal cells, transcription factors and extracellular matrix components (Basha et al., 2014). Moreover, *in utero* exposure to allergens, microbial infections or maternal immune-mediated disease can also impact fetal immunity, postnatal immune development and susceptibility to disease later in life. Earlier studies revealed that maternal infections with malaria in the third trimester of pregnancy are strongly associated with increased neonatal susceptibility to malaria (Borgella et al., 2013). Similarly, maternal filarial infections influence neonatal immunity and drive imbalanced cytokine levels in plasma (Achary et al., 2013). In humans, maternal HIV infection may impact neonatal immunity and susceptibility to infection (Kidzeru et al., 2014). Other factors such as maternal nutritional imbalance, both deficiency or excess also impact neonatal immunity (Fisher et al., 2012).

1.7.2 Neonatal innate immunity

Neonatal innate immune components include granulocytes (mostly neutrophils), antigen presenting cells, natural killer and γδ-T cells (Wynn & Levy., 2014).

1.7.2.1 Pattern recognition receptors (PRRs)

The immune system is equipped with receptors called PRRs to detect pathogens such as bacteria and viruses. PRRs are divided into 4 families: (1) Toll-like receptors (TLRs), Nucleotide oligomerization receptors (NLRs), C-type lectin receptors (CLRs) and Retinoic acid inducible gene-like receptors (RLRs) as reviewed by (Philbin & Levy, 2009). These receptors recognize conserved molecular structures of pathogens called pathogen associated molecular patters (PAMPs). These motifs are specific to the micro-organism and essential for its viability. Activation of PRRs on antigen presenting cells enhances co-stimulatory function, and thus PRRs agonists are

potential vaccine adjuvants (Philbin & Levy, 2009). Toll-like receptors are the most extensively studied and are so far composed of 11 members in mammals named TLR1 to 11 and are specialized in recognition of conserved molecular structures in bacteria, viruses, fungi and parasites (Thompson et al., 2011).

1.7.2.2 Neonatal neutrophils

Neutrophils form a major component of innate immunity and are responsible for engulfing pathogens and killing them by phagocytosis. They form the highest number (70-75%) of immune cells in human blood (Willems et al., 2009). However, neonatal neutrophils have qualitative and quantitative deficiencies. For example, they have a lower surface expression of Toll-like receptor 4 (TLR4) but similar level of expression of TLR2 when compared to adults (Melvan et al., 2011). In addition to a deficits in TLR expression on neonatal neutrophils, they also have deficiencies in downstream signaling via the MyD88 adaptor molecule following stimulation (Melvan et al., 2011).

In addition, neonatal neutrophils respond poorly to Fas-mediated apoptosis and this has been associated with low surface expression of the Fas receptor (Howard & Meyer, 1984). Similarly, these neutrophils are defective in making Neutrophils Extracellular Traps (NETs), which comprise granules that are vital for the killing of extracellular bacteria (Willems et al., 2009). In summary, functional deficits in neonatal neutrophils enhance susceptibility to neonatal sepsis.

1.7.2.3 Neonatal APCs

Neonatal APCs are mainly monocytes and dendritic cells. Neonatal monocytes have a low level of expression of MHC class II which contributes to impaired antigen presentation (Holloway & Warner, 2002).

Toll like receptors (TLRs) expressed on neonatal dendritic cells, monocytes and other immune cells are important in early response to pathogens (Medzhitov & Janeway 2000). However, maturation and functional differences of these TLR mediated signaling and associated effector cytokines are different from adults. In general, neonatal monocytes, conventional dendritic (cDC) cells and plasmacytoid (pDC) dendritic cells exhibit and altered immunological profile with low level expression of MHC II, CD80, CD86, CD40 and ICAM-1, and are biased towards TH2 environment which leaves them susceptible to microbial infections and also results in poor responses to certain vaccines (Basha et al., 2014). Neonatal T cells have a reduced capacity to produce TH1 cytokine (IFN-γ) but this capacity is enhanced when these T cells are cultured with adult, rather than cord, APCs suggesting that APCs lack the capacity to deliver important TH1 polarizing signals (Delespesse et al., 1998). While reports suggest that neonatal and adult peripheral blood mononuclear cells (PBMCs) synthesize equivalent amounts of IL-12p40, secretion of bioactive IL-12p70 by cord blood is generally thought to be defective (reviewed (Upham et al., 2002). Reduction of IL-12 production drives impairment of TH1 responses but this can be corrected by provision of exogenous IL-12 (reviewed (Upham et al., 2002). Recent studies in the neonatal mouse have revealed that lung CD103+ DC are functionally limited in infants and CD11b+ DC are diminished in both numbers and function which affects the cross presentation and the CD8 T cell effector response (Ruckwardt et al., 2014). Further, CD103+ DC in the gut are also

thought to be involved in establishment of oral tolerance as they interact with naïve T cell and drive differentiation into FOXP3+ T regulatory which prevent food allergies (Stern et al., 2013).

1.7.2.4 Neonatal NK cells

Neonatal blood counts of NK cells are higher than adults with a higher expression of the inhibitory receptor CD94/NKG2A (Guilmot et al., 2011). NK cells are activated upon contact with DCs, monocyte/macrophages and cytokines (Guilmot et al., 2011). Activated NK cells drive effector functions by releasing large amounts of IFN-γ (Guilmot et al., 2011). However, the natural killer cytotoxic capability of neonatal NK cells is 3-fold lower than that of adults (Dalle et al., 2005). The capacity of neonatal NK cells to produce effector cytokines is highly reduced when compared to adults. The impaired ability of cord blood to produce IL-12 and IFN-γ may impair NK cell functionality (Suen et al., 1998). Neonatal NK cells are central in the resolution of severe acute respiratory infections such as respiratory syncytial virus and influenza (Culley, 2009).

1.7.2.5 Neonatal γδ-T cells

γδ- T cells represent less than 5% of the total lymphocytes in the peripheral blood and a lower percentage in cord blood. This subset of T cells provides the first response to microbial infections including *Mycobacterium tuberculosis, Listeria monocytogenes* and *Brucella arbutus.* Further, they release substantial amounts of IFN-γ as part of their effector mechanism among other cytotoxic responses. Moreover, human cord blood γδ- T cells have lower levels of perforin and granzymeB effector molecules (Moens et al., 2011). γ/δ T cells do not migrate to the thymus and play a central role in protection from microbial infections in early life. Murine studies reveal that these T cells stimulate dendritic cells to release cytokines thus activating specific immunity (Shi et al., 2011).

1.7.3 Neonatal adaptive immunity

1.7.3.1 Neonatal T cells

The α/β T-cells migrate to the thymus where they undergo various developmental stages and finally become either CD4+ or CD8+ T cells. They then leave the thymus in a phenotypically and functionally immature state called recent thymic emigrants (RTEs) (Fink, 2013).

1.7.3.2 Neonatal CD4 T cells

Murine studies have revealed that CD4+ Recent Thymic Emigrants (RTEs) are biased towards the production of IL-4, IL-13 and IL-5 as compared to their mature naïve counterparts (Hendricks et al., 2011). In neonatal humans CD4+ RTEs proliferate in response to IL-7 in the absence of TCR stimulation (Opiela et al., 2009). The TH2 bias of neonatal CD4+ is revealed at the chromatin level where the TH2 cytokine locus is hypo-methylated and subject to enhanced transcription (Webster et al., 2007). A number of studies have shown that the environment *in utero* could provide some co-stimulatory signals that influence the TH cell differentiation profile and could help establish particular subsets early during immune development (Basha et al., 2014)

Differentiation into either TH17 or Treg populations depends on the local cytokine environment. An abundance of TGF-β, IL-6 and IL-21 drive naïve T cell differentiation into TH17 cells while IL-2 induces TGF-β treated cells to differentiate into T regulatory cells (FOXP3+) (Zheng, 2013).

Tregs are important in maintaining immunologic tolerance and negatively regulate various immune response. Regulatory T cells (CD4+CD25+FOXP3+) encompass two categories of lymphocytes that are distinct in origin, antigen specificity as well as stimuli driving differentiation (Chatenoud, 2011). Natural Tregs are an independent lineage generated in the thymus by high avidity interactions with their T cell receptor and are specific for self-antigens (Chatenoud, 2011; Curotto de Lafaille & Lafaille, 2009). Adaptive or induced Tregs stem from mature CD4+CD25-

FOXP3- precursors under subimmunogenic antigen presentation, during chronic inflammation and during normal gut homeostasis (Curotto de Lafaille & Lafaille, 2009). They are essential in mucosal immune tolerance and in control of chronic allergic inflammation (Chatenoud, 2011). These regulatory cells are present in high numbers at infancy, both in human cord blood and neonatal lymph nodes (Burt, 2013). Fetal naïve T cells demonstrate a greater propensity to differentiate into T regs in response to maternal antigens that cross the placenta (Mold et al., 2008). Further, maternal CD4+CD25+FOXP3+ Tregs control maternal alloreactivity to the developing fetus and help in establishing central tolerance during pregnancy (Schumacher & Zenclussen, 2014).

1.7.3.4 Neonatal CD8 T cells

Human cord blood and neonatal murine studies demonstrate deficiencies in numbers and functionality of the CD8+ response early in life (McCarron & Reen, 2010). Recently, human neonatal CD8+ T cells have been shown to be less toxic than adult cells and do not produce the degranulation molecule, granzyme B (Galindo-Albarran et al., 2016). Murine studies have shown that infantile CD8+ responses are distinct and are influenced by inherent T cell properties in RSV infected mice (Ruckwardt et al., 2014). Impairment of neonatal CD8+ response is also in part due to the decreased APC expression of CD80 and CD86 (Ruckwardt et al., 2014). CD8 T lymphocytes are important in immunity against intracellular pathogens, antiviral and antitumor immunity. Human infants mount adult-like CD8 T cell responses to viral infections and DNA vaccines (Zhang et al., 2002).

1.7.3.5 Neonatal B cells

Neonatal B cells are immature and naïve largely due to lack of antigenic exposure with consequent poorly developed immunoglobulin repertoire on the surface (Walker et al., 2011). Human infants

have high numbers of B cells increasing gradually from the first weeks and remaining high for the first year (Walker et al., 2011). Based on the expression of surface CD5, B cells can be categorized into B-1 and B-2 B cells. B-1 class of B cells differ functionally from the B-2 and has a natural Ig repertoire, and are vital for early immunity against bacterial and viral infections. They express CD11b+, high levels of IgM (sIgM) and low levels of secreted IgD (sIgD), CD21, CD23 and CD45R (B220) (Ghosn et al., 2008). Furthermore, this class has been demonstrated to be involved in T independent antibody production and protect against *Borrelia hermsii* (Alugupalli et al., 2004). Studies on neonatal B cells have shown the deficiencies in antibody production following T independent stimulation with pneumococcal polysaccharides (Klein Klouwenberg & Bont, 2008). Stimulation of human neonatal B cells following the CD40 co-stimulatory signal alongside IL-4 signaling results in increased expression of the B cell receptor (BCR) negative regulator (CD22) and lowers the magnitude of the BCR signaling (Tian et al., 2006). Overall, neonates have underdeveloped germinal centers in the lymph nodes and spleens and low-level expression of B cell receptors including CD40, CD80, and CD86, which result in deficiencies in IgG responses to primary infections and vaccination.

1.8 Microbiome and immunity

The innate and the adaptive arms of the immune system work together to direct appropriate response to invading pathogens and they make up the host immune system (Iwasaki et al., 2015). The immune system and the intestinal microbiota develop and evolve together beginning at birth or possibly start *in utero* (Aagaard et al., 2014). This mutual maturation starting early in life is central to development of tolerance and avoidance of unwanted immune responses to microbial components which could be deleterious to the host (Tomkovich & Jobin, 2015). The concept of the microbiome influencing immunity is better understood in germ free mouse models. Germ free

mice lack intestinal microbiota. These animals have abnormal numbers of immune cells and have deficits in both local and systemic immune structures. For example, their spleens and lymph nodes are poorly formed and they exhibit reduced levels of IgA and cytokine production (Bouskra et al., 2008). The incomplete nature of the immune system in the germ-free mice highlight the importance of gut microbes in immune development. Studies in mouse models show that the presence and the composition of the microbiota are critical in promoting effective immune response to pathogens and maintaining homeostasis (Maynard et al., 2012). However, whether the species of commensal present influence the type of memory T cells that reside there is not known. Although memory T cells specific for commensal bacteria have recently been detected in mice (Hand et al., 2012), they are associated with a pathogenic infection and could be due to a dysregulated immune system. In humans, memory T cells survive longer and are exposed to more antigens during their lifetime, thus commensal specific responses may be part of healthy immune balance between microflora and indigenous T cells (Farber et al., 2014).

1.8.1 Intestinal effects

The rapid early changes in the microbiota coincide with the development of the immune system. Throughout this developmental period, the innate immune system plays a key role in protecting the infants from invasion by pathogens (Tomkovich & Jobin, 2015). IgA present in breast milk prevents immune activation in infants and helps protect against pathogenic microbes. Similarly, this antibody is produced in the intestinal tract and helps in maintaining homeostasis throughout life (Belkaid, 2013). Recently, a study has shown that Proteobacteria; dominant in newborns; trigger the induction of IgA which in mouse models which was central in control of Proteobacteria colonization in adulthood (Mirpuri et al., 2014). In addition, 16S sequencing of the rRNA gene revealed *Sutterella* species to be partly responsible for variable IgA levels probably by degrading

the IgA secretory component (Tomkovich & Jobin, 2015). Evidence shows that the microbiota continues to impact immunity long after attaining stability. For example, antibiotic induced alterations of the gut microbiome which reduces Clostridiales members among others, reduces intestinal T regulatory cells and alter colonic thymic T reg repertoire indicating that microbes are key influencers in the dynamics of T regulatory cells in the gut (Cebula et al., 2013). These findings support previous work where indigenous Clostridia species have been shown to induce colonic T regulatory cells via SCFA production (Atarashi et al., 2011, 2013).

Among the most studied class of microbes are the segmented filamentous bacteria (SFB), which colonise the terminal ileum in mice. SFB, previously known as *Candidatus arthromitus,* are closely related to the genus *Clostridium* and have been shown to induce IgA and expand the T helper 17 immune subset (Ericsson et al., 2014). Goto et al. demonstrated that TH17 cells reside in the ileum rather than peyers patches or the mesenteric lymph node (Goto et al., 2014). These authors also provide evidence that MHCII presentation by innate lymphoid cells may inhibit Th17 differentiation. Important to note is that SFB have not been detected in the human GIT, but there is evidence that human cell lines support its growth (Schnupf et al., 2015). Although majority of the microbiota reside in the gut, the microbiome in extra-intestinal regions also influence local host immunity (Surana & Kasper, 2014).

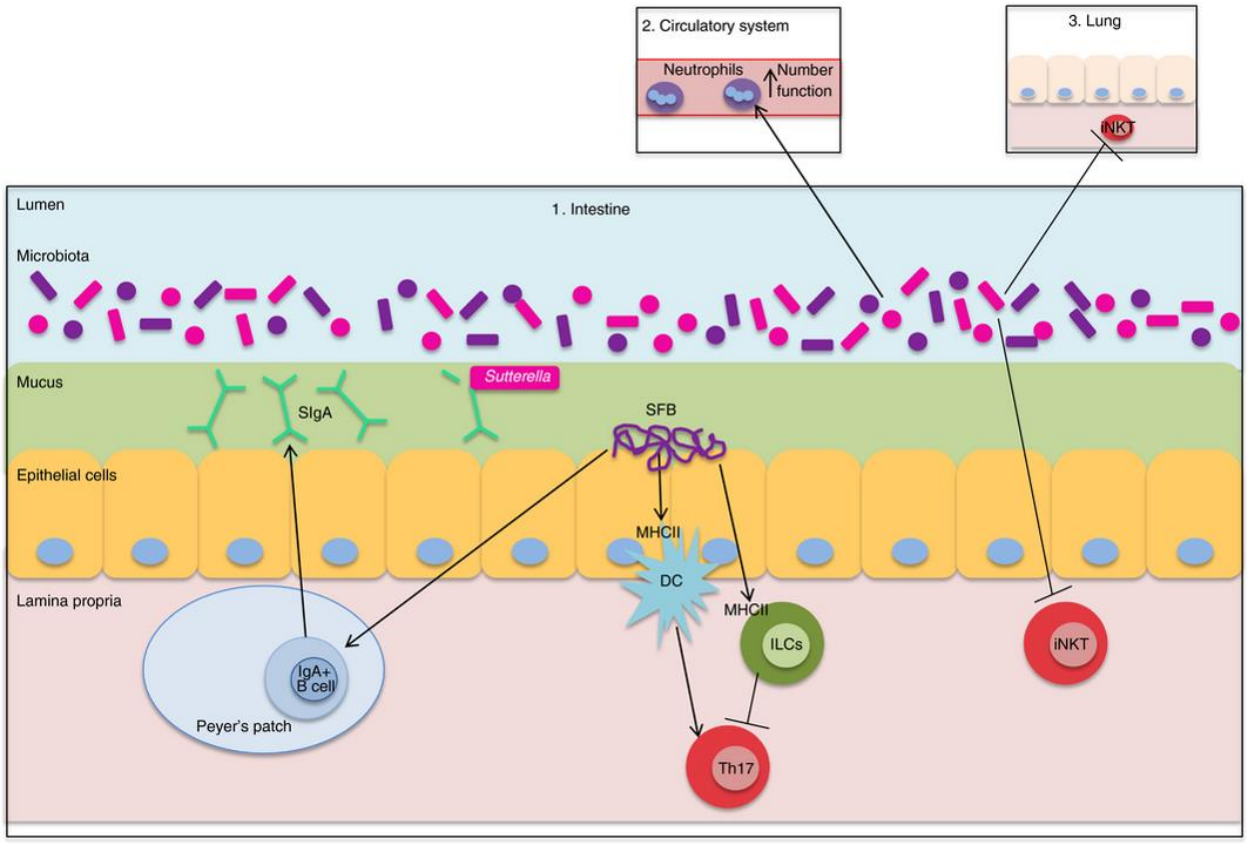


Figure 1.3: The microbiota affects local and systemic immunity. The intestine contains the greatest number of microbiota and diversity. From Tomkovich & Christian (2015).

1.8.2 Bacterial components that effect innate and adaptive immunity

1.8.2.1 Structures detected by pattern recognition receptors

The innate immune system detects microbial components through several pattern recognition receptors that are found on the surface of macrophages, dendritic cells and epithelial cells (Iris K. Pang and Akiko Iwasaki, 2012). *Bacteroides fragilis* polysaccharide A (PSA) is one of the most characterized bacterial immunomodulators. PSA is recognized by toll like receptor-2 (TLR2) and influences T cell development and homeostasis (Troy & Kasper, 2010). TLR5 which is expressed on several intestinal epithelial cells and dendritic cells recognizes bacterial flagellins (Tomkovich & Jobin, 2015). TLR5 signaling influences microbiota composition and host immune response

(Chassaing et al., 2014). Recent work has shown that the microbiota also impacts vaccine responses to inactivated influenza vaccine via TLR5 mediated signaling (Oh et al., 2014).

Nod like receptors (NLRs) also recognize bacterial components particularly the peptidoglycan through muramyl dipeptide. Studies using the $NOD2^{-/-}$ mice reveal reduced intraepithelial lymphocytes (IEL), and administration of muramyl dipeptide to antibiotic treated mice restores the numbers of IEL via the elevation of IL-15, suggesting that Nod2 mediated sensing of the microbiota impacts IEL homeostasis (Jiang et al., 2013).

1.8.2.2 Metabolites

Metabolites are intermediates or by-products of diet dependent commensal bacterial metabolism and are normally present in the millimolar range. These metabolites serve as signaling molecules for interbacteria communication and quorum sensing (Shapiro et al., 2014).

1.8.2.2.1 Short-chain fatty acids

Diet profoundly impacts on the composition and function of the microbiota (David et al., 2014). Components of the human diet include plant-derived polysaccharides such as cellulose, which are only broken down by the resident gut bacteria. Bacterial metabolism of these polysaccharides produces short chain fatty acids (SCFA) which are about 1-6 carbons in length, including acetate, propionate and butyrate (Shapiro et al., 2014). SCFA receptors include GPR41, GPR43 and GPR109A (Shapiro et al., 2014). Recently, SCFA have been described as key regulators of host metabolism and immunity. These metabolites dampen inflammatory responses through GPR43 and studies show that mice deficient in this receptor are susceptible to colitis, arthritis and asthma (Maslowski et al., 2009). Further, SCFA have been shown to expand the number and suppressive function of colonic T regulatory cells through Treg intrinsic expression of GPR43 (Smith et al., 2013). Similarly, propionate has been shown to have the potential to enhance peripheral T

regulatory cell subset (Arpaia et al., 2013). Other SCFA also impact on host immunity. For example butyrate, which acts as a histone deacetylase inhibitor, dampens the production of pro-inflammatory cytokines by intestinal mononuclear phagocytes (Chang et al., 2014). Moreover, butyrate also exerts anti-inflammatory function acting via the GPR109A receptor (Singh et al., 2014).

SCFA have likewise been shown to impact brain development and the central nervous system. Germ free mice have deficits in the microglial maturation as well as poor responses to lipopolysaccharides. However the microglial maturation is restored when a mixture of propionate, butyrate and acetate is administered in drinking water (Erny et al., 2015). Therefore, SCFAs impact both innate and adaptive immunity in the intestines (Fig. 1.4) (Tomkovich & Jobin, 2015). The relevance of other metabolites including serotonin and tryptophan has also been described (Shapiro et al., 2014). Serotonin, a neurotransmitter, is synthesized through the interaction of the host and its microbiome and acts locally to regulate gastrointestinal, cardiac, respiratory and endocrine functions as well as crossing the blood brain barrier (Berger et al., 2009). Serotonin is derived from tryptophan by tryptophan hydroxylases -1 or -2 (Walther, 2003), while tryptophan is obtained from dietary or microbial sources (Young, 2013). Germ free mice have lower levels of serotonin, tryptophan and indole compared to conventionally raised of humanized mice (Marcobal et al., 2013). Bacteria phyla including Proteobacteria, Firmicutes and Actinobacteria have been implicated in tryptophan biosynthesis (Yanofsky, 2004)

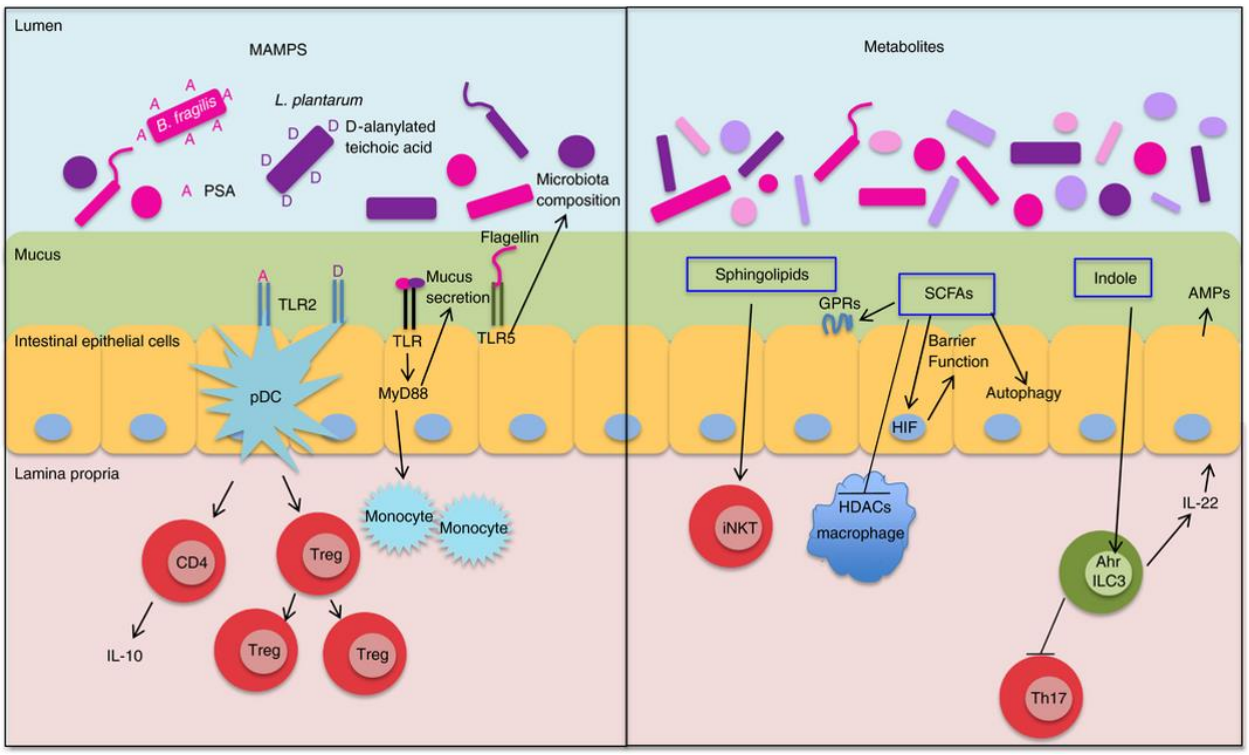


Figure 1.4: Bacteria components that affect innate and adaptive immunity in the intestines. (Tomkovich et al., 2015).

1.8.3 Maternal factors influencing prenatal immune development the neonatal gut.

There is constant cross talk between the mother and fetus which impacts fetal development. Therefore, maternal exposure to environmental stimuli, including microbes, during pregnancy can influence fetal immune development.

1.8.3.1 Maternal diet

Maternal malnutrition can severely compromise fetal growth and maturation of the fetal immune system (Hovdenak & Haram, 2012). Maternal undernutrition leads to deficiencies in important nutrients required by the fetus including minerals and vitamins. In addition, dietary lifestyle during pregnancy impacts on the developing neonatal gut community and immunity. For example, a recent murine study reveal that parental high fat diet during gestation alters infant microbiome and

leads to poor clinical outcomes to bacterial challenge among her offspring (Myles et al., 2013). Similarly, pups born to obese mothers have decreased lymphocyte count and reduced antigen specific antibody production implying that maternal obesity is detrimental to the developing infant immunity (Odaka et al., 2010). Maternal obesity is also associated with abnormal feto-placental function and increased disease risk in general (Kristensen et al, 2005).

1.8.3.2 Animal exposures

As already highlighted, maternal exposure to farm animals impacts neonatal immunity and can protect against the development of allergies and asthma (Ege et al., 2006; Schaub et al., 2009a). These prenatal exposures led to an increase in the numbers of T regulatory cells in cord blood which ameliorated the effects of allergy mediated by Th2 cytokines. Pet exposure is also associated with lower cord blood levels of IgE; which is associated with the development of allergies (Bergmann et al., 1997). Similar findings were reported by Aichbhaumik et al. who found that mothers with either cats or dogs in their home during pregnancy delivered children with lower cord blood IgE compared to mothers who did not live with these pets (Aichbhaumik et al., 2008). Others have shown that protection from allergy when exposed to farm dust is mediated via the induction of an ubiquitin modifying enzyme, A20 (Schuijs et al., 2015). While it is not clear what the mechanism of allergic protection is when infants are exposed to pets or farm animals, it is tempting to speculate that part of the environmental effect can be explained by the modulation of the microbiota composition.

1.8.3.3 Maternal disease and infection status

Maternal disease and infection status prior to conception and during pregnancy can influence neonatal gut microbiome and immune development. Diversified microbiota in the meconium has been reported in infants born to diabetic mothers (Hu et al., 2013). Commensal bacteria from the maternal gut have been isolated from umbilical cord blood of healthy neonates born by caesarean section (Jimenez et al., 2005) and oral bacteria have been found in the placenta and amniotic fluid in healthy term pregnancies(Jimenez et al., 2005). Thus, it is likely that maternal dysbiosis could impact offspring intestinal microbiome. A recent study has also shown that maternal HIV status impact infant gut microbiome (Bender et al., 2016).

1.8.3.4 Effect of antibiotics on gut microbiome and disease outcomes

Antibiotic perturbations of the rapidly developing neonatal gut has consequences on health, as this may contribute towards programming of metabolism and immunity and may dictate future actions of the immune system. The impact of antibiotics on the intestinal microbiome has recently been investigated through a variety of "omic" techniques as has been reviewed (Franzosa et al., 2015). Further, reports show that short-term parental antibiotic intake may influence the composition and type of bacteria colonizing the infant gut. Indeed, it has recently been demonstrated in a mouse models that antibiotic administration during gestation or while nursing has lasting metabolic consequences in the offspring (Cox et al., 2014; Nobel et al., 2015). Here, the authors showed that altering the gut microbiome using low dose penicillin within a critical window period at infancy was sufficient to drive far reaching metabolic consequences that persisted to adulthood. Similarly, Choe et al. have shown that early life exposure of mice to sub-therapeutic doses of antibiotics, alongside the alteration of the intestinal microbiome resulted in increased total and relative body fat mass, bone density and microbiota driven production of short chain fatty acids and an altered hepatic metabolism (Cho et al., 2012). Apart from these obesity related traits, antibiotics have also

been implicated in other conditions. For example, antibiotics have been shown to increase the risk of type 1 diabetes (Candon et al., 2015). Furthermore, early antibiotic exposure reduced infant gut diversity and altered bacteria composition with a reduction of Bifidobacteria and increases in the abundance of Proteobacteria. Untreated infants born to mothers treated with antibiotics prior to delivery revealed the same gut alterations as treated infants (Tanaka et al., 2009).

Antibiotic driven gut alterations can also impact basic immune development and could have other far-reaching long term repercussions. Atopic, inflammatory and autoimmune diseases have been associated with gut dysbiosis and in some cases significant associations have been drawn between development of these conditions and early antibiotic intake (Arrieta et al., 2015; Foliaki et al., 2009; Murk et al., 2011). A recent meta-analysis found that antibiotics exposure during pregnancy may increase the risk of asthma/wheeze in childhood (Zhao et al., 2015). However, the authors noted that availability of various confounding factors, for example, impact of maternal asthma on childhood asthma may influence associations. Therefore, prospective studies with large cohorts should be conducted to assess whether the associations were causal. Consequently, there is still no consensus whether antibiotic use at infancy increase susceptibility to asthma and allergies. There are still gaps in current knowledge and fundamental questions that remain unanswered. The impact of antibiotics on neonatal infections such as respiratory syncytial virus is unclear. How maternal use of broad spectrum antibiotics during gestation or while nursing impact infant disease outcomes remain to be conclusively investigated.

Importantly, most of these studies have used orally bioavailable antibiotics as penicillin in their studies. These antibiotics could therefore potentially have effects beyond just alteration of the gut microbiome. Data are limited on the impact of alteration of maternal gut microbiome in isolation, using broad-spectrum antibiotics that are poorly absorbed from the gut, during gestation and

nursing on infant microbiome, immunity and development. Whether alteration of only the maternal gut bacteria (and not microbiota elsewhere such as vagina or breast milk) during gestation or lactation is sufficient to influence neonatal gut community and immunity is not clear.

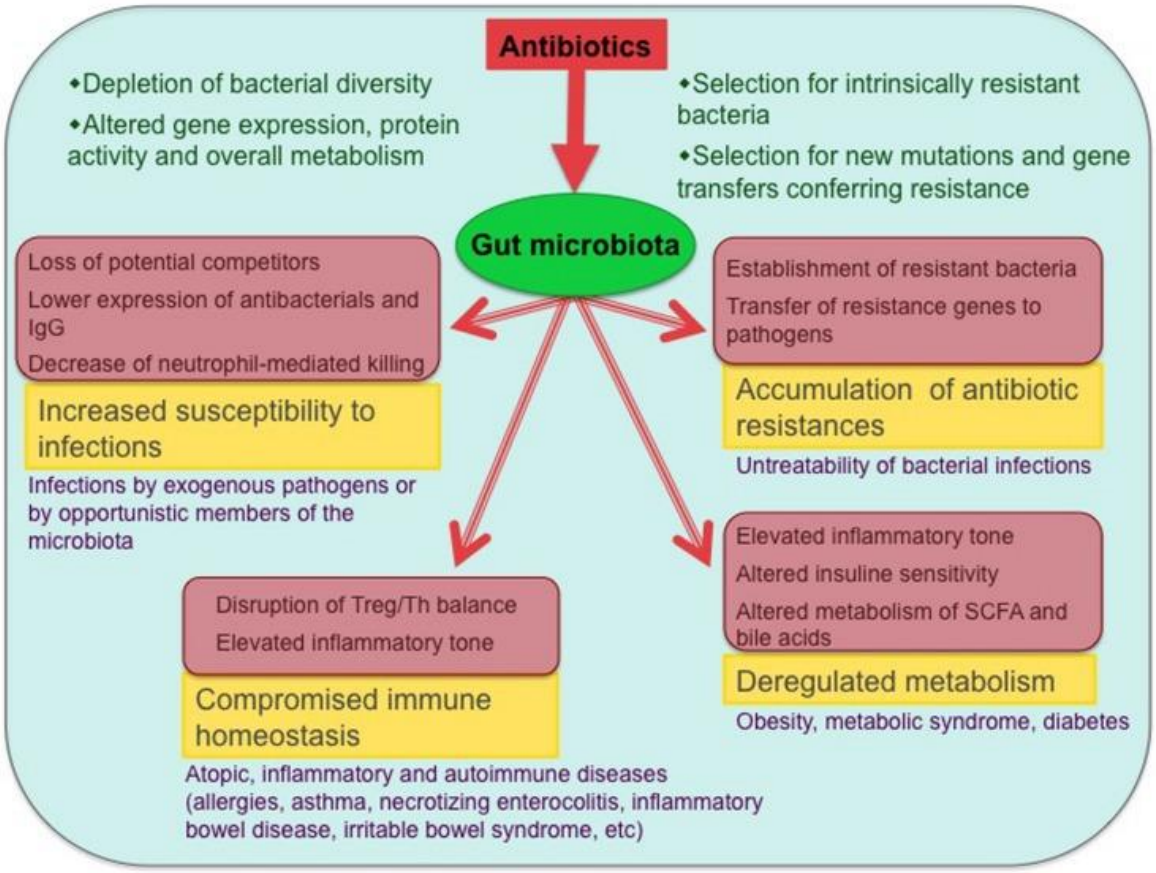


Figure 1.5: Antibiotic effects on the gut microbiome and associated effects (Francino et al., 2016).

1.9 Experimental models of infection

1.9.1 Helminthiasis

Approximately a quarter of the world's population is infected with helminths with majority residing in developing countries (Hotez & Kamath, 2009). 750-1300 million people are infected with hookworms and this is restricted to sub Saharan Africa, Latin America and South East Asia (Hotez & Kamath, 2009). Helminths are largely successive parasites that establish long lasting infections in the definitive host. They compete for nutrients with the host and result in immunological pathology as they migrate through various tissues (Finlay et al., 2014). Children are more vulnerable to infection and harbor greater worm burdens as compared to adults, reviewed in (Hotez et al., 2008). Furthermore, helminth infections have serious effects in children including growth retardation, anemia, impairment in cognitive development and reduced physical fitness (Crompton, 2002). Studies have revealed a strong association between maternal helminth infections and altered offspring health and development. Maternal helminth infections could lead to maternal malnutrition and anemia, which ultimately result in low birth weight among neonates, a risk factor for disease later in life (Gluckman, 2008). In terms of childhood immunizations, children born to mothers in sub-Saharan Africa have been shown to respond poorly to BCG, Typhoid and Measles vaccinations (Labeaud et al., 2009). Protective immunity to helminths is mediated by TH2 cells and the cytokines they produce including IL-4, IL-5, IL-10 and IL-13 (Maizels et al., 2012). These responses rarely kill the parasite but they limit their viability and reproducibility in the human host and contribute to their expulsion from mucosal barriers. Most of the understanding of helminth immunity has been from murine infection models.

1.9.1.1 *Nippostrongylus brasiliensis*

Nippostrongylus brasiliensis (N. brasiliensis) is a rat gastrointestinal nematode that establishes acute infections in wildtype mice. Worms are generally cleared after 9 days leaving behind sustained memory against secondary infections (Finkelman et al., 2004). *N. brasiliensis* is similar biochemically and physiologically to the human hookworms, *Necator americanus* and *Ancylostoma duodenale*. For this reason, this murine infection model has been used to understand the pathogenesis of human hookworm (Else & Finkelman, 1998). It is easy to handle in the laboratory as it does not require an intermediate host and is not fatal to rodents. Also this parasite is not infectious to the human host (Camberis et al., 2003).

1.9.1.1.1 Life cycle of *N. brasiliensis*

Infection occurs by the L3 larvae stage which is injected subcutaneously in animal models. Once under the skin, the larvae migrates in circulation to the lungs where they are trapped in the capillaries, undergo maturation and moulting to the L4 stage, and subsequently enter the pulmonary alveoli within 24-48 hours (Finkelman et al., 2004; Reece et al., 2008). They then move up the trachea and are swallowed back to the gastrointestinal tract by day 4-5 post infection. The L4 stage find their way into the small intestines where they undergo the final moult (L5) into mature adults. Here the adult worms establish themselves and lodge into the intestinal epithelium, mate and produce eggs by day 6 post infection, which then leave the host in feces (Zhao et al., 2007). In BALB/c mice, egg production stops at day 9 post infection when the worms are expelled due to robust immune response from the host (Reece et al., 2008). *N. brasiliensis* induced IL-13 stimulation of the intestinal cells has been demonstrated to be the likely cause of worm expulsion from an immunocompetent host during a primary infection (Finkelman et al., 2004).

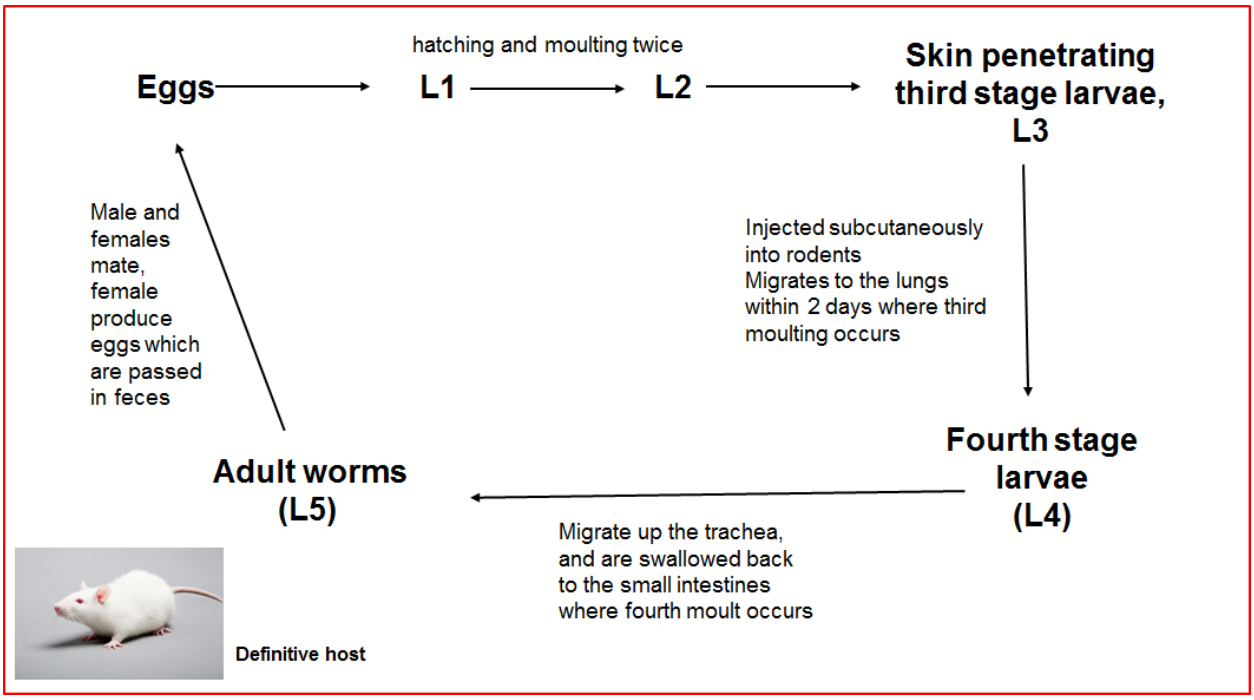


Figure 1.6: Life cycle of primary *N. brasiliensis* infection. There is no intermediate host. Eggs leave the host in feces. L3 larvae is the infective stage.

1.9.1.1.2 Primary immunity to *N. brasiliensis*

TH2 cells orchestrate helminth immunity through the production of signature cytokines that direct and activate other cell types which synergistically act to expel the worm from the host. An effective anti-helminth response includes eosinophilia, basophilia, goblet cell hyperplasia and mucus secretion, CD4 T cell dependent secretion of cytokines (IL-4, IL-13, IL-9 and IL-5) as well as isotype class switching in B cells to produce antigen specific IgG1 (Finkelman et al., 2004; Urban et al., 1998). IL-4 acts by binding both IL-4R and the IL-13R resulting in STAT6 activation. Moreover, both receptors share the IL-4Rα subunit which is a critical receptor for optimal helminth immunity (Finkelman et al., 2004). IL-4 is also key in driving isotype class switching in B cells leading to the production of antigen specific IgG1 (mouse), IgG4 (human) and IgE. *N. brasiliensis* leads to alternative activation of macrophages (M2) which induce fibrosis through the expression

of fibronectin and matrix metalloproteinases (Maizels et al., 2004). Although IL-4 is central in orchestrating TH2 immunity, it is not required for expulsion of *N. brasiliensis* from the host. However, IL-13, IL-4Rα and STAT6 activation are critical for worm clearance indicating that particular facets of TH2 work together to drive optimal helminth immunity (Urban et al., 1998). Overall. IL-4 orchestrates a TH2 immune responses and signals B cells to initiate antibody production, while IL-13 stimulates intestinal cells and smooth muscle expulsion of worms from the intestines. Both cytokines signal through a common receptor, IL-4Rα, which is critical for anti-helminth immunity.

1.9.2 Respiratory syncytial virus

Respiratory syncytial virus (RSV) is the commonest cause of bronchiolitis in children under 5 years and is estimated to cause 3.4 million hospitalization and at least 66,000 deaths annually around the world (Hall et al., 2012). Childhood asthma and wheezing has also been linked to RSV infections in infancy (Welliver, 2003). Immunity to re-infection is partial, as infection with antigenically similar strains occurs throughout life (reviewed in Lambert et al., 2014). Moreover, RSV infection results in production of Th2 cytokine leading to eosinophilic infiltration into the lung which drives lung pathology and airway hypersensitivity (Matsuse et al., 2000). Much of the available information on RSV pathogenesis is derived from animal models. However, it is unclear how the available murine data on RSV accurately represents human disease. In particular, the adult mouse has historically been used to study immunity to RSV. It is likely that some of the discrepancies between human and animal data could be due to the use of adult mice to mimic the immunological environment in human infants. Therefore, a corrective measure could be the use of neonatal or infant mice as a model for human RSV infection in infants. There exists no vaccine

against RSV, which necessitates the need to better understand immunity to RSV as a way of reducing the global mortality and morbidity associated with RSV infections.

1.9.2.1 Virology of RSV

Respiratory syncytial virus is an enveloped, non-segmented, negative strand RNA virus of the order *paramyxoviridae*. It is comprised of a 15.2kb genome with 10 genes encoding a total of 11 proteins as depicted in a recent review (Lambert et al., 2014). Antibody responses to RSV infection target two major proteins: F and the highly glycosylated G protein. Antigenic variability in protein G accounts for the majority of the differences between the two major RSV strains (A and B). The F protein promotes fusion of viral and cell membrane resulting in the transfer of viral genetic material and the fusion of infected and adjacent cell membranes causing the formation of syncytia (Domachowske & Rosenberg, 1999) Moreover, RSV infections are confined to the respiratory mucosa and do not disseminate to other organs or blood.

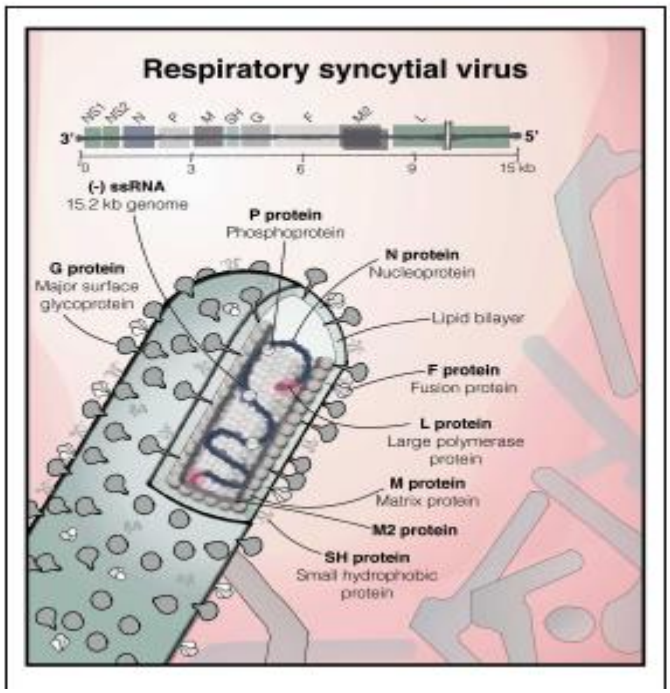


Figure 1.7: Structure of RSV virus. The 15.2kb negative sense
RNA RSV genome consisting of 10 genes encoding 11 proteins.
(Figure from Lambert et al., 2014).

1.9.2.2 Pathogenesis and Pathophysiology

Transmission of RSV occurs through inoculation of the nasopharyngeal mucosa with respiratory secretions from infected individuals. The incubation period ranges from 2-8 days and immunocompetent individuals can shed the virus for up to 3 weeks (Piedimonte & Perez, 2014). Infection starts in the nasopharyngeal epithelium then spreads through the lower airways reaching the terminal bronchioles where replication of the virus is most efficient (Piedimonte & Perez, 2014). Several observations suggest that immunological mechanisms may be the key to the severity of RSV bronchiolitis in infancy. For example, RSV-induced cytokines and chemokines recruit and activate neutrophils, monocytes, eosinophils , dendritic and T cells which in addition to fixed lung macrophages, produce proinflammatory mediators such as TNF-α and cyclooxygenase which contribute to lung damage and pathology (Pribul et al., 2008). This acute

inflammatory changes following RSV replication leads to airway obstruction and air trapping, producing the classic clinical triad of polyphonic wheezing, patchy atelectasis and bilateral hyperinflation (Piedimonte & Perez, 2014). Airway obstruction in infants has a greater clinical significance compared to obstruction in adults. Sloughed necrotic epithelium and excessive mucus production also add to airway obstruction and the formation of mucus plugs (Mcnamara & Smyth, 2002). Therefore, disease severity and duration are primarily a function of the immune response mounted by the host.

1.9.2.3 Mouse RSV model

The most widely used model for human RSV are the inbred laboratory mouse strains because of ease of housing and handling them, availability of wide variety of transgenic and gene-deletion mice as well as immunopathological pathways (Bem et al., 2011). However, they are at best a semi-permissive host, their lung anatomy is much simpler compared to humans and do not mirror clinical manifestations observed in humans as signs of illness include weight loss, ruffled fur and lethargy. In addition, the virus is a human pathogen and replicates poorly in mice thus requiring high inoculums for productive infection and induction of pathology (reviewed (Cormier et al., 2010)). Despite these differences, many of the immunological responses of mice to RSV infection are similar to humans and the use of the mouse model has enhanced understanding of RSV pathogenesis (Openshaw, 1995). Cormier et al. estimated that 77% of RSV published studies have been carried out in mice (Cormier et al., 2010). Majority of studies using animal models have been performed in adult animal models. It can be argued that the use of adult animal models could be a contributing factor to the discrepancies between human and mouse data. Current data suggest

that a more relevant model for the human infant is the neonatal mouse (Culley, 2009; Harker et al, 2014).

1.9.2.4 Immune response to RSV infection

Data are limited on neonatal immune responses to RSV, especially at the site of disease (lung) rather than in peripheral blood; human studies of lung pathology are not feasible. Consequently, much of the available information is derived from animal studies, clinical trial in human adults or very few severe cases in babies in which postmortem analyses were performed. Tissue analysis has revealed that the virus infects the superficial ciliated cells of the upper airway, the epithelium of the bronchioles, and the type 1 pneumocytes (Johnson et al., 2007). Reports suggest that severe disease is characterized by higher viral loads and a paucity of lymphocytes in the lung (Welliver et al., 2007). Maternal RSV specific antibodies generated before or during pregnancy are transferred via the placenta or breastmilk and can influence offspring RSV and other vaccine responses (Guzman Sanchez-Schmitz, 2011). Viral detection occurs through pattern recognition receptors (PRRs) expressed on the surface of fibroblast, epithelial cells and antigen presenting cells of the respiratory tract. Some PRRs that are central to RSV recognition include TLR3, TLR4, TLR 2/6 and TLR7/8 [reviewed in (Kolli, Velayutham, & Casola, 2013)]. Once the host immune system detects the virus, pro-inflammatory responses are initiated leading to production of cytokines which then trigger cell recruitment into the airways. Ruckwardt and colleagues revealed imbalances in particular DC subsets in neonates infected with RSV with an increase of CD103+CD11c+ cells found in the lymph nodes (Ruckwardt et al., 2014). Natural killer cells are also important in RSV control in murine models providing an early source of IFN-γ, which activate DCs and primes T cell response, as well as destroying virus infected cells (Culley, 2009). However,

there is no consensus as to whether NK cells contribute to protection or immunopathology in human infants.

CHAPTER 2
2.0 CURRENT STUDY

2.1 Background

As discussed above, the gut microbiota plays significant roles in maintaining our homeostasis and ability to combat disease (Atarashi et al., 2011; Nistal et al., 2012; Nylund et al., 2014). The composition of bacterial species colonizing our gut influence our immune system (Round, O'Connell, & Mazmanian, 2010). Indeed, recent evidence demonstrates that the immune system and the microbiota evolve concomitantly starting at birth or possibly *in utero* (Aagaard et al., 2014). Infant acquisition of microbiota and how this pioneer microbiota could impact on immune development and disease susceptibility is an area actively under investigation. Until recent work by Aagaard and others showing the placenta to harbor a unique microbiome, the fetus was believed to be sterile and to be only colonized after birth (Aagaard et al., 2014). Various factors impact infant gut colonisation majority of which are derived from the mother. Maternal disease and infections alter infant microbiome. Helminth infections are endemic in sub Saharan Africa and susceptibility has been shown to be enhanced during gestation (de Silva et al., 2003; Yatich et al., 2009). Yet whether, maternal helminth infections impact infant gut microbiome and immunity is unknown.

It is also apparent that antibiotics profoundly impact the microbiota (Cox et al., 2014; Nobel et al., 2015). Generally, antibiotic treatment leads to reduction in diversity and richness in the gut communities (Jernberg et al., 2007). These drugs are widely used during pregnancy in the treatment of respiratory, sexual and urinary tract infections. However, how the unintended gut dysbiosis driven by maternal antibiotic use during either pregnancy or lactation could influence offspring gut microbiota as well as immune development remains to be elucidated. In addition,

whether manipulation of maternal microbiome during pregnancy and/or lactation has consequences on offspring ability to control infections is not clear to us.

2.2 Objectives

Although the fetus has for a long time been considered sterile, recent findings show that colonization may infact begin *in utero*. Therefore, the maternal microbiome during the last trimester of pregnancy could be important in influencing infant gut composition. Maternal helminth infections have been shown to be enhanced during pregnancy with recent data showing the impact of helminths on the microbiota. Further, administration of antibiotics to mothers during pregnancy and lactation are likely to impart on the bacteria that are transferred to her offspring possibly in utero or in breast milk respectively. Our primary objective was to evaluate the effect of maternal gut microbiome manipulation on infant microbiome and consequent immunity. To do this, we utilized two models. First, we utilize oral broad-spectrum antibiotics (vancomycin and polymyxin B) to study the effects of manipulating maternal microbiome during gestation and/or nursing on offspring intestinal microbiota and immunity to various challenge models. Second, we investigate the role of preconception maternal helminth infections on infant microbiome as well as immunity. We had two hypotheses: (1) Antibiotic driven manipulation of maternal gut microbiota during gestation and/or nursing impacts offspring intestinal microbiota and results in increased susceptibility to infections. (2) Preconception helminth infections alter infant microbiome and immunity. The aims of this study were:

1. To investigate the effect of maternal oral broad spectrum antibiotics on offspring intestinal microbiota and immunity
2. To determine the ability of offspring born to antibiotic breeders to control Respiratory Syncytial Virus and *Nippostrongylus brasiliensis* infections.
3. To investigate the impact of preconception helminth infections on infant gut microbiome and immunity

CHAPTER 3.
3.0 MATERIALS AND METHODS

3.1 Maternal oral antibiotics and the impact on infant gut microbiota and immunity

3.1.1 Mice, mating and litters

Female BALB/c mice, 6-8weeks of age, were used for these experiments. For mating, two females and an adult male per group were housed in the same cage for 7 days after which the male was removed. Dams were treated with antibiotics (vancomycin, polymyxin B or both) in drinking water for 5 days prior to giving birth (gestation group), for 14 days after delivery (nursing group), or for 5 days prior to birth until 14 days postpartum (gestation plus nursing group). There were no antibiotics administered to the control dams. We investigated the effect of single antibiotics when administered at various phases as well as a mixture of these antibiotics on offspring growth, gut microbiome and immunity. This timing was informed by our preliminary study where we observed that antibiotic exposure from conception to delivery had no effect on infant body weight and immunity at 14 days. Cox et al found that antibiotic treatment 3 days prior to delivery had lasting metabolic consequences in offspring (Cox et al., 2014), indicating that maternal antibiotics are most consequential when administered in the last trimester of pregnancy.

3.1.2 Experimental design

Two, 6 week old female BALB/c mice were used per group. Controls dams were not subjected to any antibiotic treatment. Offspring from the four groups were then killed day 14 postpartum alongside their mothers for microbiome and immune analysis. We analysed pups at 14 days for two reasons: (1) Pups are still breastfeeding and have not yet begun coprophagic behaviour which allows us to examine impact of an altered maternal microbiome transferred via nursing (2) A neonatal period of 1-2 weeks has been established by previous studies where mice are able to elicit an antibody response suggesting establishment of the adaptive immune response (Brandt et al.,

1997; Siegrist, 2001). This critical period allowed us to examine how the establishment of microbiota and immunity impacted each other. Fecal samples were collected from individual pups from their colons. Spleens were collected for immunological analysis.

3.1.2.1 Antibiotics

Vancomycin and Pomymyxin B (PMB) were used each at a concentration of 1mg/ml and were administered orally in drinking water. Vancomycin is produced by Actinobacteria species and acts by inhibiting proper cell wall synthesis in gram positive bacteria while Polymyxin B is derived from Bacillus and is active against gram negative bacteria. It acts by altering outer membrane permeability in gram negative bacteria leading to cell death

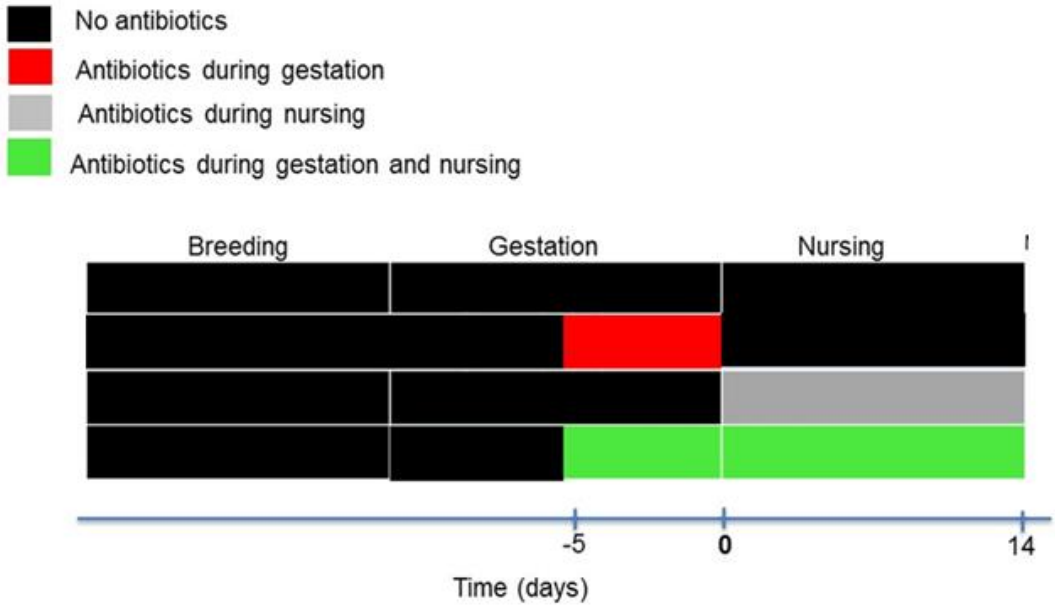


Figure 3.1: Schema of maternal antibiotic experiments. Dams are treated with antibiotics during gestation, nursing, gestation plus nursing or not treated at all. Pups are killed two weeks postpartum for microbiome and immune analysis.

3.1.3 Immunological challenges

3.1.3.1 Respiratory syncytial virus (RSV) infections

RSV A2 viral strain is maintained in the Horsnell's lab in the Division of Immunology, University of Cape Town. Briefly, the virus is maintained by propagating in HEp-2 cells and the viral titer determined by plaque assay or PCR. Viral stocks are frozen down at -80°C until infection. 3-week-old pups were anesthetized and infected intranasally with 8 $X10^5$ PFU in a volume of 40µl. RSV inoculum per g body weight and killed 4 or 8 days post infection.

3.1.3.2 Quantification of viral RNA

Viral RNA in the lungs of RSV infected infants was quantified by PCR according to (Culley et al., 2002). RNA was extracted from the lungs using the RNeasy mini kit (Qiagen, CA) and cDNA generated with random hexamers using the transcription cDNA synthesis kit (Roche). PCR specific for RSV L gene was performed at 95°C for 5 minutes followed by 45 cycles (15s at 95°C, 15s at 60°C, 10s at 72°C and 1s at 68°C) using SYBR green master mix (Roche) and 900nM forward primer (5-GAACTCAGTGTAGGTAGAATGTTTGCA-3), 300nM reverse primer (5-TTCAGCTATCATTTTCTCTGCCAAT-3). We used cDNA obtained from day 4 post-infection to generate standard curves with a 10fold dilution starting at 10^6 copies/µl. Melting curve analysis was performed at the end of the PCR to determine if the SYBG/DNA intercalation assay produced single specific PCR products.

3.2 Preconception helminth infections and influence on infant microbiome

3.2.1 Mice, mating and litters

Female BALB/c mice from University of Cape Town specific pathogen free (SPF) facility were used. Mice were kept under SPF conditions (light/dark cycles of 12h) in ventilated cages equipped with standard bedding, filter tops, environmental enrichment and fed standard diet and autoclaved water *ad libitum.*

3.2.2 Parasite and Infections

Female mice were injected with 500 *Nippostrongylus brasiliensis (Nb)* L3 larvae subcutaneously in 200µl of 0.9% NaCl using a 21G needle ensuring the larvae were in suspension. The infection was then cleared 7 days post infection by treating mice with Ivermectin (10mg/ml, VetServ) in drinking water for 7 days. An immunocompetent host expels the *Nb* adult worms in feces at day 9-12 post infection (Panhuys et al., 2013). Deworming of mice was to ensure we investigate impact of *Nb* associated memory and not *Nb* antigen, prior to conception and how this imprint on the offspring. One week after Ivermectin treatment (three weeks post *N.b* infection), mice were mated. Control mice were not infected but were treated with Ivermectin in the same fashion as infected mice.

3.2.3 Experimental Design

6-week-old BALB/c female mice housed in separate cages were infected subcutaneously with 500 Nb L3. Mice were dewormed by Ivermectin (VetServ) treatment 7 days postinfection and mated three weeks post infection. Control mice were not infected but were treated with ivermectin and mated simultaneously alongside the experimental mice. Male BALB/c were used for mating. For mating, two, 6-week-old female mice were housed in a single cage with a single male for 8 days after which the male was removed. Females gave birth 21 days post fertilization and the pups were

culled to 6 pups per mother and monitored daily for 14 days. Maternal fecal samples for microbiome analysis were collected between day 16 and 19 of gestation. Pups were sacrificed by cervical dislocation after inhalation of halothane at day 14 postpartum and fecal samples collected from their colons for microbiome analysis. Breast milk samples from pups' stomach were also collected for microbiome analysis. Spleens and mesenteric lymph nodes were collected for immune analysis.

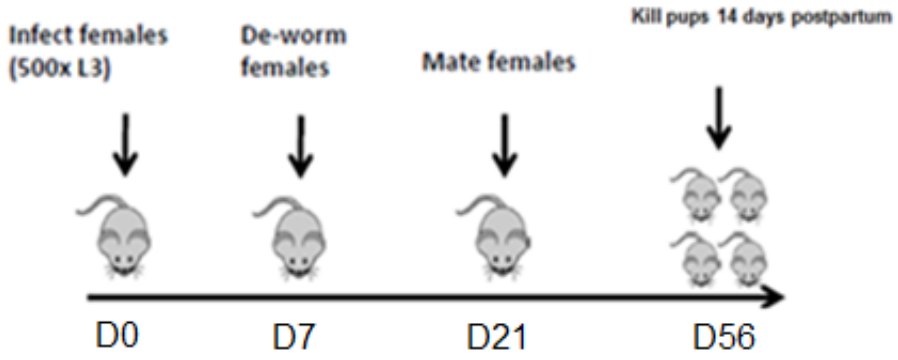


Figure 3.2: Schema of the maternal helminth experiments. 6-week-old female mice are infected with 500Nb L3 three weeks prior to mating. Pups are killed 14 days postpartum

3.2.4 *Nippostrongylus brasiliensis* (*Nb*)

3.2.4.1 Maintenance of *Nb*

Nb is maintained by Horsnell's lab at the Division of Immunology, University of Cape Town. Briefly, naïve rats are infected subcutaneously with 5000*Nb* L3 per rat every two weeks. Rat fecal pellets are collected on day 6, 7 and 8-post infection and liquefied in 5µg/ml Fungizone then incubated in moist filter paper in petri dishes at room temperature. The larvae hatch after 7 days

and migrate to the edge of the filter paper where they are harvested at the L3 stage and used for future infections.

3.2.4.2 Infections with *Nb*

For infections, the L3 larvae from the filter papers are washed in 0.9% NaCl and the larvae counted under a dissecting microscope to establish a concentration of 2500L3/ml. Infections are performed at the Research Animal Facilities (BSL 2) and each mouse is injected subcutaneously with 500Nb L3 in 200 µl of 0.9% NaCl using 21G needles. Pups receive half the adult dosage (250Nb L3) and are killed 5 days post infection.

3.2.4.3 Intestinal worm counts

Small intestines were harvested from mice at the termination of the experiment. Mice were incised longitudinally to expose the abdominal lumen and intestines collected in 0.9% NaCl. The tissue was then incubated at 37° C for 4 hours which induces the migration of the worms from tissue to the supernatants. Afterwards, the supernatant was poured off into a large graded petri-dish and the total worms per mouse counted under a dissecting microscope.

3.2.5 Sample preparations and DNA extractions

Fecal samples from colons and breast milk from pups' stomach were collected and stored at -20º C. After defrosting,1 ml of sterile PBS was added to the breast milk pellet and the suspension homogenized. Samples were then spun at 3500rpm for 10 minutes and the aqueous phase isolated which was used for further downstream analysis. We included an additional enzymatic lysis procedure (Yuan et al., 2012) before using the Powersoil Isolation Kit (Mo Bio Laboratories) for DNA extractions from both stool and breast milk. Briefly, 50µl lysozyme (10mg/ml, Sigma-Aldrich), 6µl mutanolysin (25KU/ml, Sigma-Aldrich), and 3µl lysostaphin (4000U/ml, Sigma-Aldrich) were added to 100µl aliquot of cell suspension followed by incubation for 1 hour at 37º

C. The lysate was then subjected to further DNA isolation and purification using the Powersoil DNA Isolation Kit (Mo Bio Laboratories) as per the manufacturers instructions. The final DNA concentration was determined by the Quanti-It Picogreen dsDNA HS assay kit (Invitrogen, UK).

3.2.5.1 16S rRNA Next Generation sequencing

16S rRNA sequencing was performed on fecal and breast milk samples. Extracted DNA was used for library construction according to (Arthur et al., 2012). Two sets of primers were used (Table 1). The first set were the MF/MR; V6 universal primers modified by adding barcodes 4 to 6 nucleotides long to allow for multiplexing and the Illumina paired-end sequencing adapters (Caporaso et al., 2011). A total of 12 different barcodes were generated for each end, allowing for 144 combinations. The second set was designed to have the Illumina paired-end sequencing adapters and the flow-cell adapters. The hypervariable V6 region of the 16S rRNA gene was amplified via PCR in two steps: step one used the first set of primers allowing for a unique combination of barcodes for individual samples. 50ng of template DNA was used per PCR reaction. Cycling was done at 98º C for 30 s, followed by a touch down protocol consisting of 15 cycles with a denaturation step of 98º C for 5 s, annealing at 61º C for 15 s with a 1º C drop in each cyle until the temperatures reached 51º C, and an extension at 72º C for 15 s. PCR was then terminated with a final elongation step of 72º C for 5 min. In the 2nd PCR, 10µl of 1st PCR products were used per reaction with the second set of primers. The concentration of all other reagents remained the same. Cycling was done at 98º C for 30 s and amplification proceeded for 15 cycles with a denaturation step of 98º C for 30 s, annealing at 65º C for 15 s, extension at 72º C for 15 s and a final elongation step of 72º C for 5 mins. The PCR amplicons were purified using the Qiagen 96 well purification kit (Qiagen, CA). DNA concentations per reaction were then determined using the Quanti-It ds DNA BR assay (Invitrogen, UK) and 50ng from each reaction pooled into a single

tube. Pooled DNA was run on a 1.5% agarose gel and visualised using blue light. The 330bp band was carefully excised from the gel and purified using a gel purification kit (Qiagen, CA). The final DNA concentration was determined and the library sequenced at the Centre for Applied Genomics at the Hospital for Sick Children in Toronto, Canada on the Illumina HiSeq 2000 platform. Sequencing reads were 2x100bp in length.

Table 1 Primer sequences used for preparing the V6 Illumina library. Underlined nucleotides represent barcodes used for sample multiplexing

Primer	Primer sequence
MF1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATAGCGAAACTCAAAKGAATTGACGG
MF2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGGTAAACTCAAAKGAATTGACGG
MF3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCATAAACTCAAAKGAATTGACGG
MF4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCGTAAACTCAAAKGAATTGACGG
MF5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCCGTAAACTCAAAKGAATTGACGG
MF6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGTCAAACTCAAAKGAATTGACGG
MF7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCACGTAAACTCAAAKGAATTGACGG
MF8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTACGAAACTCAAAKGAATTGACGG
MF9	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGACAAACTCAAAKGAATTGACGG
MF10	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGAAAACTCAAAKGAATTGACGG
MF11	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCATAAACTCAAAKGAATTGACGG
MF12	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTTAAACTCAAAKGAATTGACGG
MR1	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTATAGCGACGAGCTGACGACARCCATG
MR2	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGGGTACGAGCTGACGACARCCATG
MR3	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTCATACGAGCTGACGACARCCATG
MR4	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTGATCGTACGAGCTGACGACARCCATG
MR5	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTGCCCGTACGAGCTGACGACARCCATG
MR6	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTGTCACGAGCTGACGACARCCATG
MR7	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCACGTACGAGCTGACGACARCCATG
MR8	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCGTACGACGAGCTGACGACARCCATG
MR9	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTGGACACGAGCTGACGACARCCATG
MR10	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTAGAACGAGCTGACGACARCCATG
MR11	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCATACGAGCTGACGACARCCATG
MR12	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTACTTACGAGCTGACGACARCCATG
PCRFWD1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCRRVS1	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT

3.2.6 Microbiome analysis

Sequence data was pre-processed in QIIME and UPARSE with support of our bioinformaticians. Briefly sequences lacking barcodes were removed and samples with less than 100,000 reads discarded. PCR errors were removed by SeqNoise (Quince et al., 2011). Primers and barcodes were removed from de-noised sequences. Consequently, de-noised sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using *de novo* picking. OTUs appearing in less than 10% of the samples were eliminated from downstream analysis. Taxonomic assignment was done by RDP classifier using the Green genes data base. Statistical analysis was done using the Calypso software version 6.4 using default parameters. Analyses were performed by ANOVA or t test and p-values were corrected for multiple testing by false discovery rate (FDR). Differences in abundances of various OTUs was assessed using the anova plots with p-value correction by FDR. Principal Coordinate analysis (PCA, Bray curtis distance) and alpha diversity (Shannon or Chao1) were run in Calypso with default parameters (Zakrzewski et al., 2016). Corrected p values less than 0.05 were considered statistically significant.

3.2.7 Cell and Tissue processing

Spleens and mesenteric lymph nodes were isolated aseptically and single cell suspensions made in complete media comprising Iscove's Modified Eagle Medium (IMDM) (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100U/ml penicillin G and 100µg/ml streptomycin. Lymph nodes were pooled due to few cells in pups from these organs. Single cell suspension was achieved by passing the organs through a 40µm nylon cell strainer (Becton Dickson, NJ) using a 2ml syringe plunger. Cells were then spun at 1200rpm for five minutes, media discarded, and red blood cells lysed by resuspending in 1 ml RBC lysis buffer for 1 minute. Cells were pelleted again and resuspended in complete media. Viability was determined by using

trypan blue and cells counted using the Neubaeur chamber under the microscope. Cells were then constituted to a working concentration of 10^7 cells/ml and were used for culture and flow cytometry. Cells from both organs were plated in a 96 well plate and stimulated with PMA (50ng/ml) and Ionomycin (500ng/ml) for 4 hr at 37º C in the presence of brefeldin A for detection of intracellular proteins.

3.2.7.1 Flow cytometry

Splenic and lymph node lymphocytes were analyzed for different surface and intracellular markers by flow cytometry. For extracellular markers, single cells were stained at 2 x10^6 cells per well in a 96 well V bottomed plate. In particular, cells were stained for B and T cells with anti-CD3 Alexa 700, anti-CD4 PerCP, anti-CD8 V500, anti- CD19 PEcy7, anti-CD21 APC, anti-CD23 PE, anti-CD80 V450, anti-CD44 FITC, anti-CD62L V450 and anti-FOXP3 APC. All antibodies were from BD, Becton Dickinson. We also stained for innate cells in the RSV models. These were anti-SIGLECF PE, anti-CD11c APC, anti-Ly6G APCcy7 and anti MHC II FITC. For intracellular cytokine staining, 2 x10^6 cells were plated on a flat bottomed 96 well plate, stimulated with PMA (50ng/ml) and Ionomycin (500ng/ml) for 4 hr at 37º C before being transferred into a V bottomed plate for staining. Cells were fixed using the BD cytofix/cytoperm (Becton Dickinson, NJ), washed with MACS buffer and stained with anti-CD3, anti-CD4, anti-IL 13 PEcy 7 and anti-IFN-gamma V500. 50µl of the antibody master mix prepared in MACS buffer was added per well in all staining procedures. Cells were acquired on an LSRII (Becton Dickinson) and analyzed by FlowJo version 9.8 (Tree Star, Ashland) software.

Flow Cytometry gating strategies for different cell populations in spleen and lung

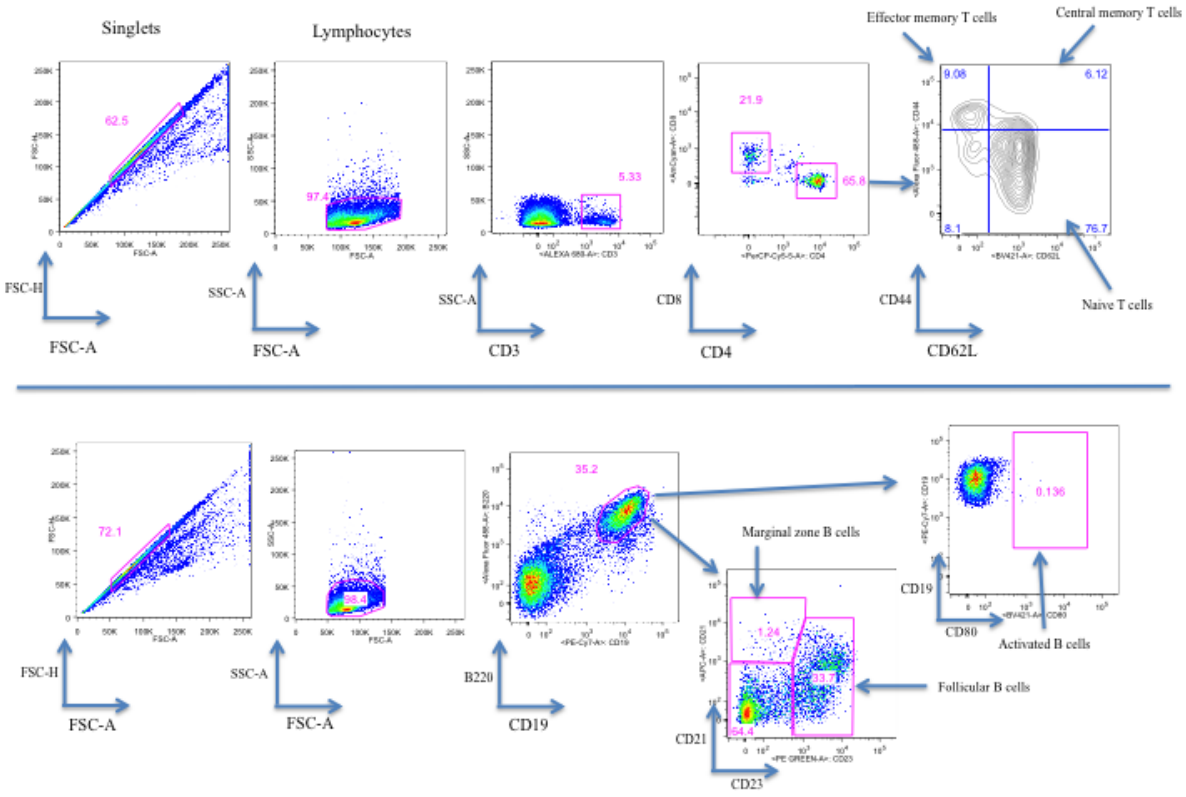


Figure 3.3: T cell gating strategy (top panel) and B cell gating strategies (bottom panel). First, doublets were excluded, then lymphocytes were gated. Thereafter, T cells are gated by CD3 expression while B cells are identified via CD19 and B220 on their surface. CD3+ cells are then stratified into CD4+ or CD8+cells. Within the CD4+ cells, we used CD62L and CD44 expression to define the memory and activated phenotype which we expressed as proportions of CD4+ cells. Within the CD19+B220+ cells, we used CD23 and CD21 to define Follicular and Marginal Zone B cells respectively. CD80+ was used to identify activated B cells.

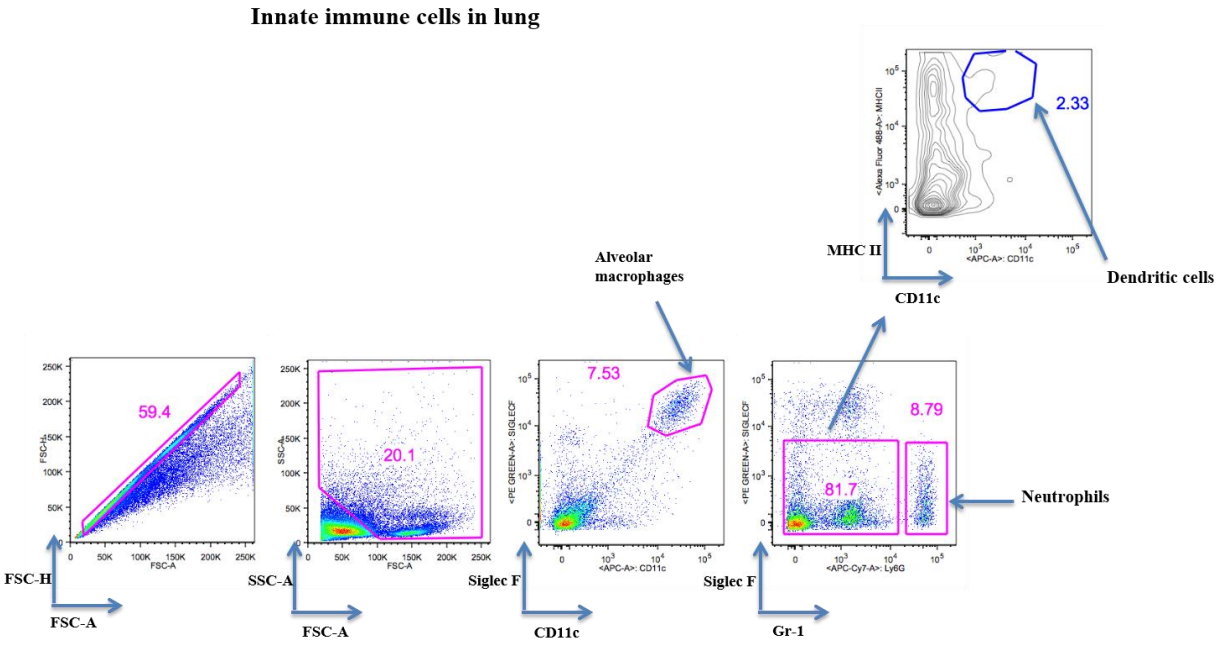


Figure 3.4: Flow cytometry gating of innate immune cells in the lung. First, doublets were excluded then we gated on total cells. Thereafter, Alveolar macrophages were characterized as Siglec F+ CD11c+. Neutrophils were defined by Gr-1 expression. Within the SiglecF- Gr-1- cells, we gated on MHCII+CD11c+ to identify immature dendritic cells (DCs).

Antibodies used in this study alongside their clones, isotype controls and working concentrations are shown in Table 2.

Antibody	Fluorophore	Clone	Rationale	Isotype Control	Working Concent
CD3	Alexa 700	500A2	Lineage, T cell	IgG2	1/400
CD4	PerCP	RM4-5	Lineage	IgG2a	1/500
CD8	V500	53-6.7	Lineage	IgG2a	1/400
CD44	FITC	IM7	Activation	IgG2b	1/320
CD62L	V450	MEL-14	Homing marker	IgG2a	1/640
CD19	PEcy7	6D5	Lineage, B cell	IgG2a	1/800
B220	FITC	RA3-6B2	Lineage	IgG2a	1/400
CD21	APC	7G6	Marginal B	IgG2b	1/500
CD23	PE	B3B4	Follicular B	IgG2a	1/500
CD80	V450	16-10A1	Activation	IgG2	1/320
SIGLEC F	PE	E50-2440	Eosinophils	IgG2a	1/320
CD11c	APC	HL3	Macrophages	IgG1	1/320
Gr-1	APCcy7	RB6-8C5	Neutrophils	IgG2b	1/640
MHC II	FITC	NIMR-4	Dendritic cells	IgG2b	1/320
CD49b	PEcy7	DX5	NK cell	IgM	1/320
IL-13	PE	eBio13A	Cytokine	IgG1	1/100
IFN-γ	V500	XMG1.2	Cytokine	IgG1	1/100
FOXP3	Alexa Fluor 647	MF23	T regulatory	IgG2b	1/200

Table 2: Monoclonal antibodies used for analysis of immune populations by flow cytometry

3.2.7.2 Preparation of compensation controls

In multicolor flowcytometry it is necessary to acquire individually stained controls to compensate for spectral overlap in every channel. Spectral overlap occurs when fluorochrome signals are detected in more than one detector (Roederer, 2001). Failure to compensate may lead to false positives or false negatives events. Therefore, we prepared a compensation control for each fluorochrome used alongside appropriate negative controls. Briefly, a single drop of antimouse or antirat BD CompBeads (BD Bioscience, USA) was added to a labeled falcon tube and correct calculated volume of each fluorochrome conjugated antibody added in single tubes. Individual tubes were then incubated at 4°C for 15 minutes in the dark to allow for the binding of the antibody to the beads. After incubation, 300 µl of PBS was added to all the compensation tubes to stop the binding reaction.

3.2.7.3 Fluorescence Minus One (FMO) controls

FMO controls are important to identify positive events in a sample and to assist with identifying gating boundaries. A FMO control is a sample stained with all fluorochromes used in the panel except for the one of interest. We used FMO controls in our intracellular cytokine staining to identify the true positive events. At least 100,000 events were acquired on the LSR II flow cytometer.

3.2.7.4 BD LSRII configurations

The LSR II flow cytometer (BD Bioscience) was used in this study. The cytometer has four lasers (violet laser, 405 nm; green laser, 543 nm; blue laser, 488 nm; red laser, 633 nm) enabling the detection of more than 18 colors. Acquisition was done on the BD Diva software and data further analyzed in FlowJo version 9.8 (Tree Star, Ashland) software. Figure 2.4 shows the fluorochromes, which can be detected, and the longpass and bandpass filters used for each detector.

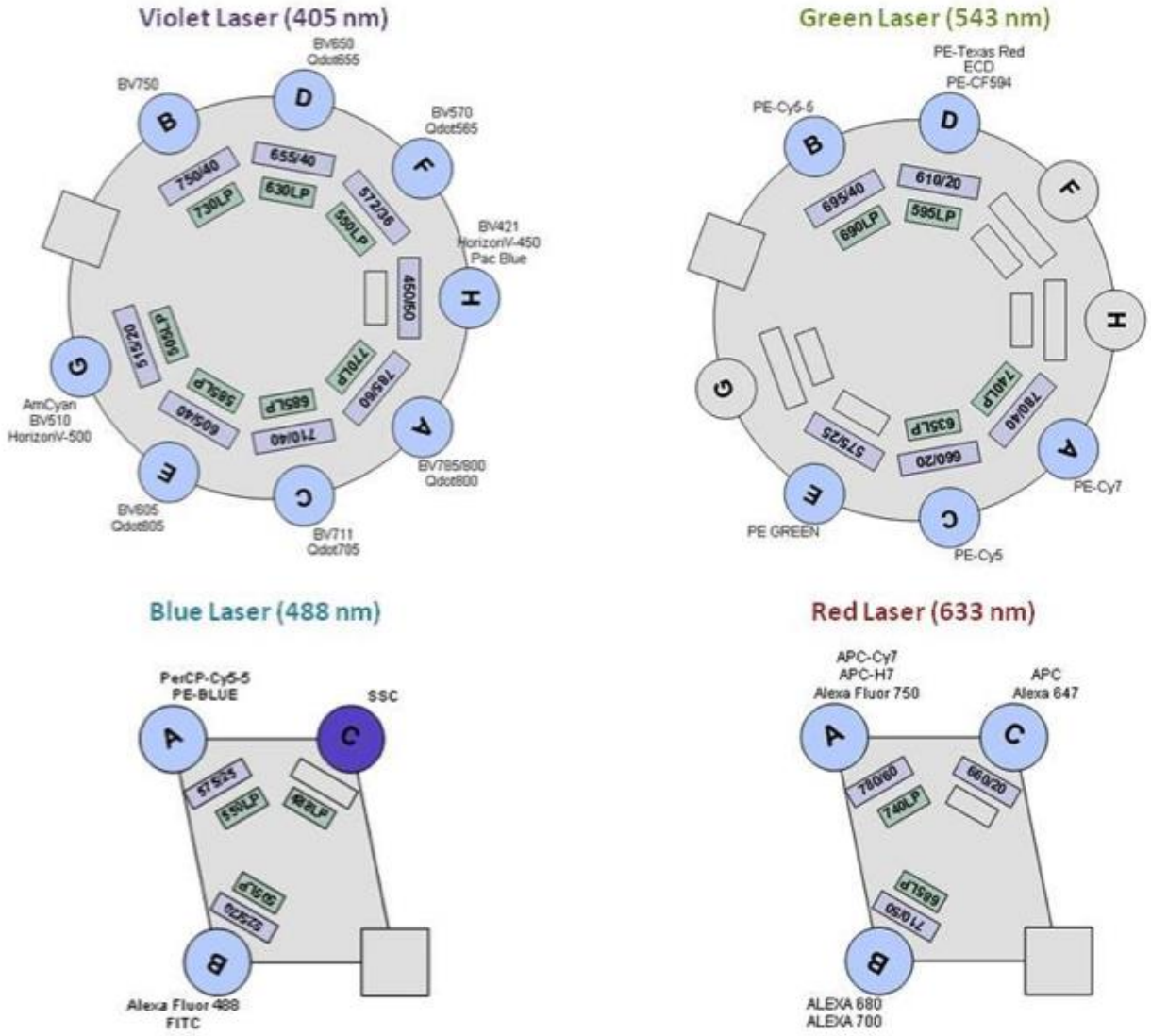


Figure 3.5: BD LSR II configuration for different lasers. Courtesy of the Agano, Erica and Gwen who maintain the LSR II facility.

3.2.8 Enzyme-linked Immunosorbent Assay (ELISA)

3.2.8.1 Antibody ELISA

Relative antigen-specific serum antibody levels were determined by the antibody ELISA. Briefly, flat bottomed 96 well plates (Nunc, Maxisorp) were coated with the *N. brasiliensis* or RSV antigen (Horsnell lab) at optimal concentrations (5-10µg/ml) diluted in carbonate buffer at a volume of 50µl/well overnight at 4° C. The next day, plates were blocked with 200µl/well of 4% BSA in PBS (Appendix) for 3 hours at 37° C. After washing three times, samples were added. Serum samples were diluted to a starting concentartion of 1:3 in dilution buffer and serially diluted across six wells. Samples were left overnight. Plates were washed and AP conjugated secondary antibody (IgG, IgG1, IgG2a) added at a dilution of 1:1000 (Southern Biotech) at 50µl/well and incubated at 37° C for 3 hours. Plates were then washed and the signal detected using the substrate p-Nitrophenylphosphate (PNP) (Sigma-Aldrich) powder at 1mg/ml in substrate buffer (Appendix). Plates were incubated at 37° C until the desired coloration was obtained after which they were read at a wavelength of 405nm using the Softmax Pro Program. Relative antibody titres were plotted as dilution graphs versus optical densities.

3.2.8.2 Cytokine ELISA

Cytokines were quantified from supernatants obtained from tissue homogenates. Briefly, IL-10, IFN-γ, and IL-13 levels were quantified by coating 96 well plates (Nunc, Maxisorp) with appropriate coating antibody in 1 X PBS overnight at 4° C. Plates were then washed three times and blocked with 200µl/well of blocking buffer (Appendix) for 3hours at 37° C. Samples were then added starting at a neat concentration, 1:2 then a 1:4 dilution. Recombinant protein standards were used to generate standard curves and were diluted serially at 1:2 from 100ng across 15 wells. Plates were incubated overnight after samples and standards were added. Plates were washed and biotinylated secondary antibody added in dilution buffer and incubated at 37°C for 3 hours.

Streptavidin-linked horseradish peroxidase or alkaline phosphatase was then added at a 1:5000 dilution for an hour at 37°C. Plates were again washed and developed with 50µl/well. TMB microwell peroxidase substrate system or by p-Nitophenylphosphate until desired coloration was obtained after which the reaction was stopped (mainly for the TMB detection system, stopped by adding 25µl/well of 1M phosphoric acid). Plates were then read at a wavelength of 450nm against a reference filter of wavelength 540nm (TMB detection system) or at a wavelength of 405nm against a refernce filter of wavelength 492nm (AP detection system). All antibodies were from BD Pharmingen.

3.2.9 Statistical analysis

Data was summarized using routine methods. Kruskal Wallis test was used to compare more than two means. Two-way associations were explored using the Mann-Whitney test for non-parametric data. All statistical analysis were two tailed and p values less than 0.05 were considered statistically significant. Microbiome analysis was performed by QIIME, UPARSE and Calypso version 6.4 as described in section 3.2.6.

CHAPTER 4.
4.0 RESULTS SECTION I

4.1 Influence of maternal antibiotics during pregnancy and/or nursing on infant immunity and intestinal gut microbiome

4.1.1 Introduction

Prenatal microbial exposure has been shown to influence postnatal immune development (Schaub et al., 2009b). Also, the gut microbiota has been shown to program immunity, a process that is thought to begin at birth as the microbiota and the immune system are simultaneously establishing in the host. Historically, the mantra has been that the fetus is sterile and that gut colonization only begins at birth (Ley, Peterson, & Gordon, 2006a). However emerging evidence has revealed a unique microbiome within the placenta suggesting that neonatal colonization may begin *in utero* (Aagaard et al., 2014). A relationship between mode of delivery and infant gut microbiome has been described, and thought to be due to exposure to vaginal microbiota during vaginal delivery, versus skin and environmental microbiota during C section (Dominguez-Bello et al., 2010). Moreover, exposure to maternal fecal microbiota during vaginal delivery may also occur (Backhed et al., 2015; Khoruts, 2016). The impact of maternal microbiome during pregnancy on infant microbiome has not been investigated. Data from large cohorts suggest that the developmental origins of health and disease begin *in utero*, or even prior to conception (Wadhwa et al., 2009), but the relationship between these findings and maternal microbiome have not been explored. Furthermore, due to the relative vulnerability to infections during pregnancy, and the increase in sexually transmitted infections worldwide, antibiotics have become widely used during gestation. In this chapter, we investigate how the unintended dysbiosis of maternal microbiota during pregnancy and/or lactation impacts infant immunity and gut microbiota. We utilized vancomycin (active against gram positive) or polymyxin B (active against gram negative) as a means of

manipulating maternal microbiome. These broad-spectrum antibiotics were used as monotherapy or in combination during gestation, during nursing, or both and we investigated the overall impact on offspring fecal microbiome, growth and immunity 14 days postpartum.

4.1.2 RESULTS

4.1.2.1 Maternal oral antibiotics during gestation and/or lactation profoundly impacts infant intestinal microbiota

At day 14 of life, pups were killed from the vancomycin alone and polymyxin B alone experiments. Pups born to control dams are designated (C), those born to dams treated with antibiotics during gestation only (G), those born to dams treated with antibiotics while nursing (N) and pups born to dams treated with antibiotics during gestation plus nursing (GN). We collected fecal samples from their colons and extracted fecal DNA. We sequenced the V6 region across all groups to analyze infant microbiome. Treatment of dams with either vancomycin or polymyxin B during gestation, nursing or both profoundly impacted the gut community in her offspring.

4.1.2.2 Vancomycin mediated alterations of maternal microbiome significantly impacts infant gut microbiome.

We measured vancomycin levels in maternal and infant serum samples. Dams treated with vancomycin 5 days prior to delivery were sacrificed 2 days after delivery and serum samples collected. Additional maternal serum samples were collected from dams treated with vancomycin 5 days prior to delivery through 14 days postpartum. Infant serum samples were collected 14 days postpartum. There were undetectable vancomycin levels in all serum samples as determined by Abbott Architect ELISA (detection limit 3mg/L) **(Appendix A, Table A1)**. Therefore, the effect of vancomycin was intraabdominal only, and any alterations in microbiome elsewhere in mothers or pups would therefore have been mediated by changes in maternal gut microbiome only. Although we did not measure polymyxin B levels in serum, we do not expect to detect any levels

in circulation. We analysed infant fecal microbiome fourteen days postpartum by sequencing the V6 region of the 16S gene. We had a total of 32,470,176 reads; a minimum of 4542 reads and a maximum of 1683427 when we looked at all samples. 104 OTUs were identified in the sequencing run. We found a significant reduction in microbial diversity by the shannon index when comparing control pups versus pups born to vancomycin breeders regardless of antibiotic timing ($p<0.0001$, ANOVA) **(Fig. 4.1A)**. Furthermore, principal coordinate analysis revealed distinct infant microbiome when we compared antibiotic versus control pups. There was strong clustering based on grouping with the largest variation of 41% being explained on PC1 and associated with maternal vancomycin treatment **(Fig. 4.1B)**. Pups born to mothers treated during gestation only had a microbiome distinct from the control group **(Fig. 4.1B)** which supports recent findings that fetus may not be sterile (Aagaard et al., 2014). Together, our data shows that maternal vancomycin treatment in dams resulted in profound alterations of offspring gut microbial communities.

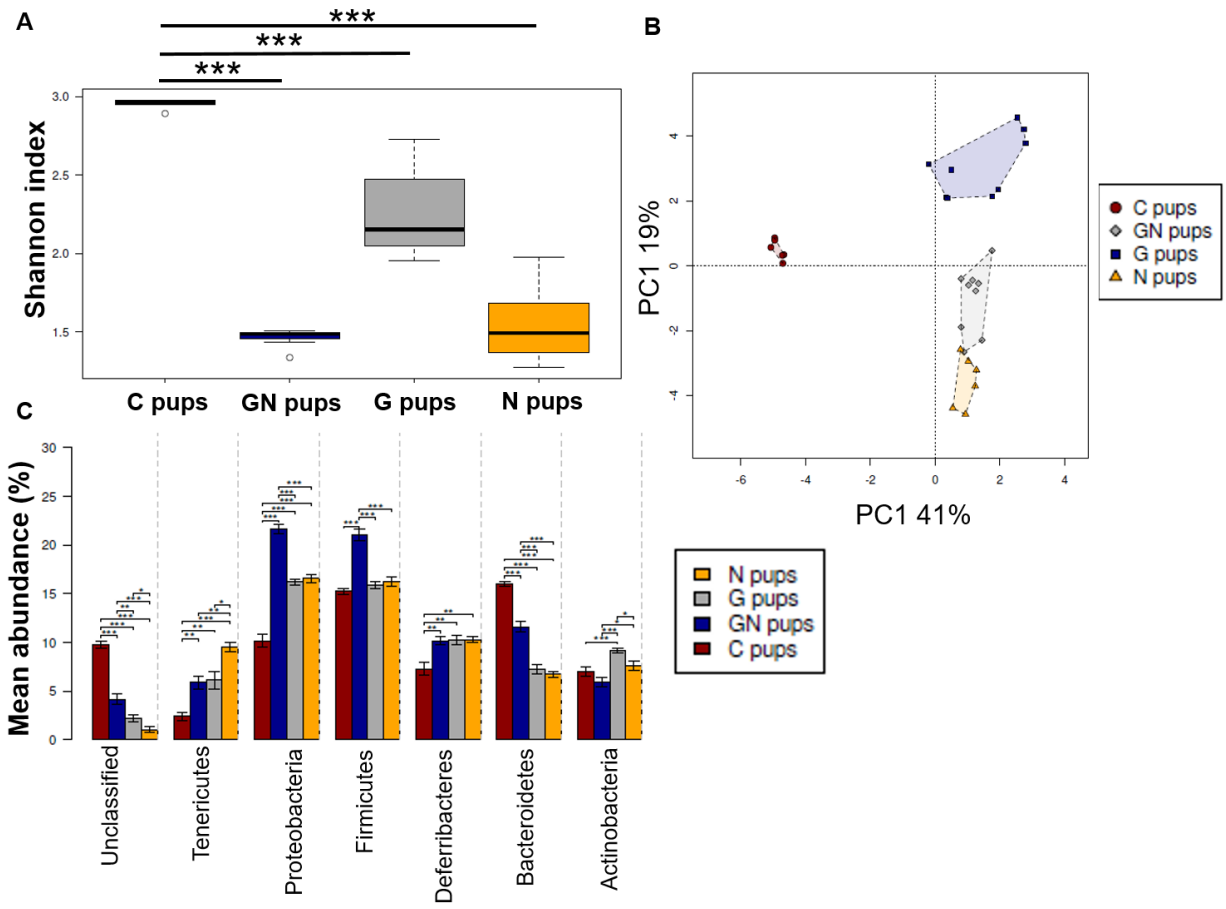


Figure 4.1: Maternal vancomycin treatment impacts offspring gut bacterial diversity and taxonomic abundance at phylum level. Female BALB/c mice were treated with vancomycin orally 5 days prior to delivery (gestation), 14 days after birth (nursing), 5 days prior to delivery through 14 days of nursing (gestation plus nursing) or untreated throughout the experiment (controls). Pups were killed 14d postpartum and fecal microbiota analyzed and are represented as G, N, GN and C pups respectively. **(A)** Alpha diversity of offspring microbiota. **(B)** Principal coordinate analysis of offspring microbiota by bray Curtis distance representing 60% of total variation. PC1 represents 41% while PC2 represents 19%. **(C)** Significantly different bacteria taxa at the phylum level across all infant groups (ANOVA with FDR correction). Error bars show SEM. Data are representative of two independent experiments. n=6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

When we analyzed abundance at the phylum level, pups born to vancomycin breeders displayed significantly higher abundance of Tenericutes, Proteobacteria and Deferribacteres ($p<0.001$, FDR=0.000029, 0.00042, 0.00029 respectively) regardless of antibiotic timing in their mothers **(Fig. 4.1C)**. Firmicutes were significantly increased in gestation plus nursing pups compared to controls **(Fig. 4.1C)**. Moreover, we observed a significantly greater abundance of Actinobacteria in gestation pups while the difference did not reach statistical significance for other groups compared to controls **(Fig. 4.1C)**. In stark contrast, Bacteroidetes were significantly reduced in all vancomycin infant groups compared to pups born to untreated mothers ($p<0.001$, FDR=0.000012) **(Fig. 4.1C)**. Although the spectral activity of vancomycin is limited to gram positive bacteria and only in the maternal gut, it is interesting to note that Bacteriodetes (gram negatives) were significantly lower among all offspring born to antibiotic dams.

At the class level, Mollicutes, Gammaproteobacteria, Deferribacteres were prevalent in vancomycin treated pups **(Fig. 4.2A)**. However, Coriobacteria and Bacteroidia were significantly reduced in the vancomycin groups **(Fig. 4.2A)**. Nursing only pups had significantly lower abundance of Erysipelotrichi compared to controls while there was no difference in the abundance of Bacilli and Actinobacteria in the gestation plus nursing and gestation only pups respectively compared to control pups **(Fig. 4.2A).** We observed similar trends at order level with a preponderance of Pasteurellales, Mycoplasmatales and Enterobacteriales in offspring born to vancomycin breeders **(Fig. 4.2B)**. Clostridiales, Coriobacteriales and Bacteroidales were significantly reduced in these offspring **(Fig. 4.2B).** Finally, we identified genera that were distinct to various infant groups. *Adlercreutzia, Streptococcus, Bacteroides, Prevotella* and *Parabacteroides* were significantly elevated in control pups **(Fig. 4.2C)**. However, *Allobaculum* and *Ruminococcus* were abundant in gestation pups, *Actinobacillus* and *Lactobacillus* in gestation

plus nursing pups while *Staphylococcus* were predominant in nursing pups **(Fig. 4.2C)**. All three of the vancomycin infant groups had distinct microbiota, suggesting that the influence of maternal gut microbiota during gestation is distinct from that during breastfeeding. Taken together, our data demonstrates that maternal vancomycin treatment during gestation, nursing or gestation plus nursing has a profound impact on the establishment of the gut flora in her offspring.

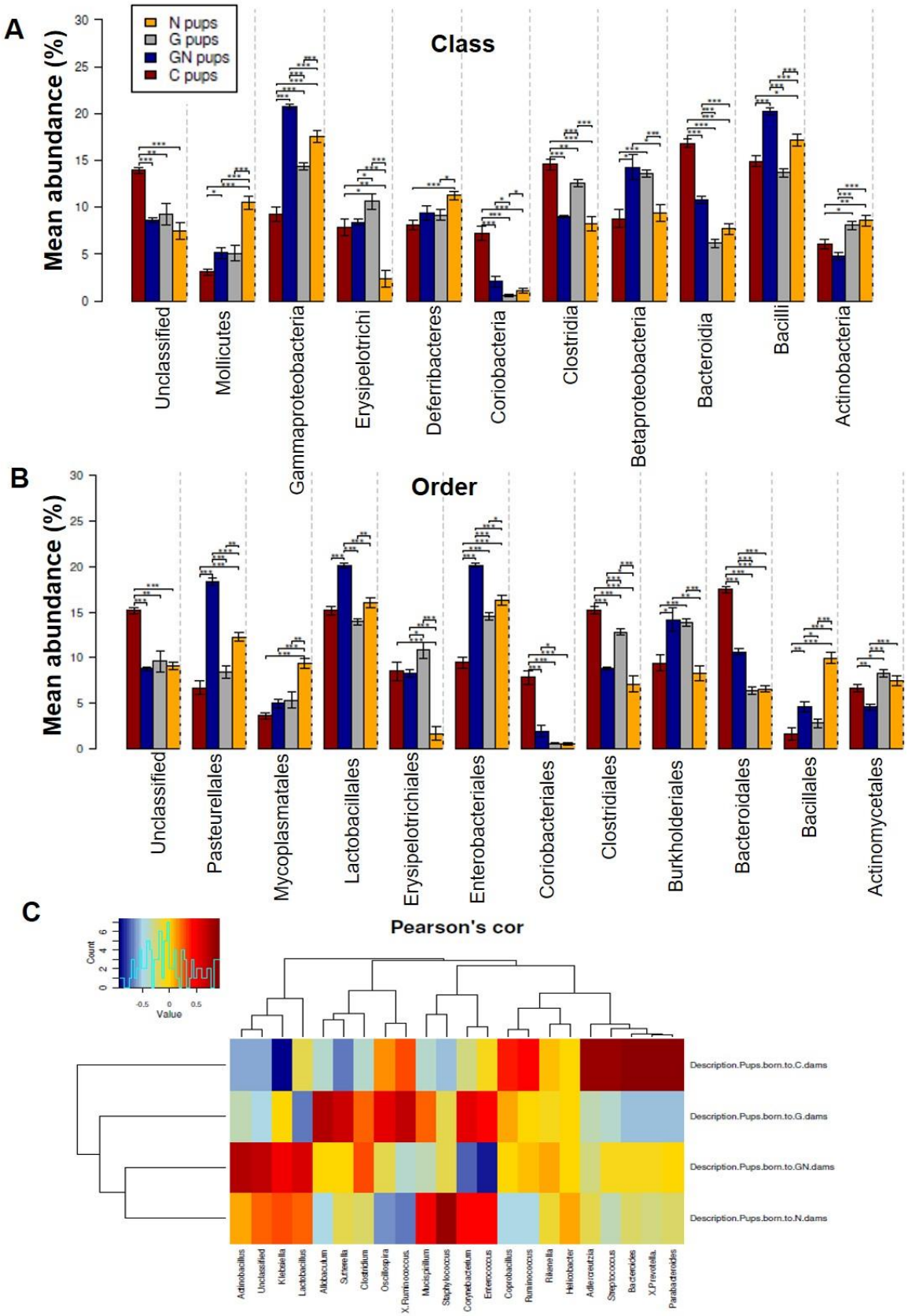

A
Class
Mean abundance (%)
N pups
G pups
GN pups
C pups
Unclassified
Mollicutes
Gammaproteobacteria
Erysipelotrichi
Deferribacteres
Coriobacteria
Clostridia
Betaproteobacteria
Bacteroidia
Bacilli
Actinobacteria
B
Order
Mean abundance (%)
Unclassified
Pasteurellales
Mycoplasmatales
Lactobacillales
Erysipelotrichiales
Enterobacteriales
Coriobacteriales
Clostridiales
Burkholderiales
Bacteroidales
Bacillales
Actinomycetales
C
Pearson's cor
Count
Value
Description.Pups.born.to.C.dams
Description.Pups.born.to.G.dams
Description.Pups.born.to.GN.dams
Description.Pups.born.to.N.dams
Actinobacillus
Unclassified
Klebsiella
Lactobacillus
Alkbaculum
Sutterella
Clostridium
Oscillospira
X.Ruminococcus
Mucispirillum
Staphylococcus
Corynebacterium
Enterococcus
Coprobacillus
Ruminococcus
Rikenella
Helicobacter
Adlercreutzia
Streptococcus
Bacteroides
X.Prevotella
Parabacteroides

Figure 4.2: Maternal vancomycin treatment alters offspring intestinal microbiota at class, order and genus level. **(A)** Significantly altered infant bacteria taxa at class level **(B)** Significantly altered infant bacteria taxa at order level. **(C)** Heatmap constructed by unsupervised clustering using bray curtis distance showing bacteria taxa at genus level significantly differentially abundant in various infant groups. Analysis by ANOVA with FDR correction for multiple comparisons. Error bars show SEM. Data are representative of two independent experiments. n=6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

4.2 Impact of maternal oral Polymyxin B on infant microbiome

We sequenced 29 offspring samples and found 134 operational taxonomic units (OTUs), a minimum of 377,208 reads and a maximum of 1,385,245 reads. Pups born to polymyxin B (PMB) treated mothers, regardless of antibiotic timing exhibited a trend towards reduced alpha diversity compared to controls (p=0.598, ANOVA) **(Fig. 4.3A)**.

Similar to the maternal oral vancomycin data, principal coordinate analysis of OTUs among offspring by bray curtis distances revealed distinct clusters based on grouping **(Fig. 4.3B)**. 26% of total variability was explained by PC1, which seemed to be largely determined by the additive effect of PMB administration during gestation and nursing **(Fig. 4.3B)**. Control pups clustered distinctly from PMB infant groups. Consistent with our vancomycin data, pups exposed to polymyxin B only during gestation revealed a distinct cluster from the controls indicating profound differences in their microbiome. This provides evidence to further support and extend emerging paradigm that neonatal gut colonization could begin *in utero.*

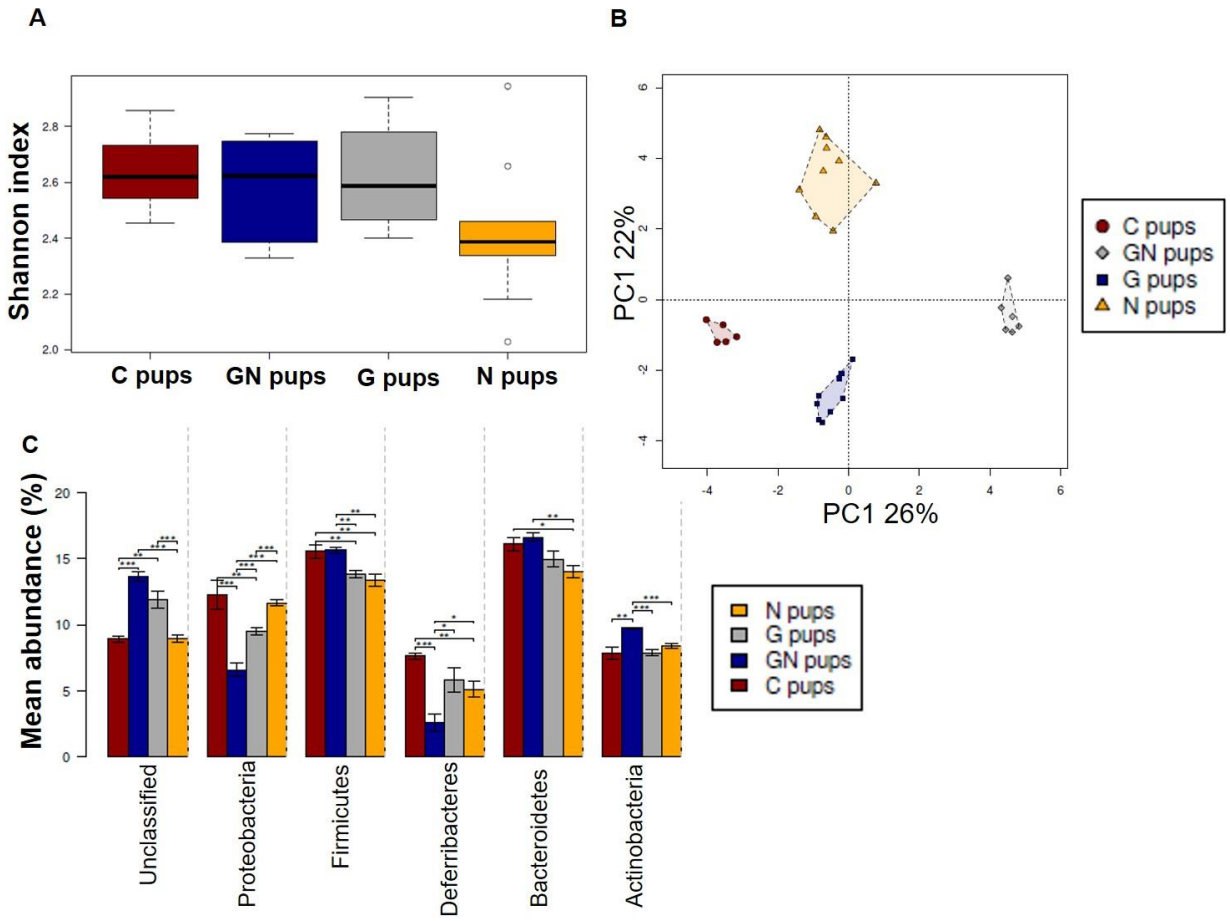


Figure 4.3: Maternal polymyxin B treatment impacts offspring gut bacterial diversity and taxonomic abundance at phylum level. Female BALB/c mice were treated with polymyxinB orally 5 days prior to delivery (gestation), 14 days after birth (nursing), 5 days prior to delivery through 14 days of nursing (gestation plus nursing) or untreated throughout the experiment (controls). Pups were killed 14d postpartum and fecal microbiota analyzed and are represented as G, N, GN and C pups respectively. **(A)** Alpha diversity of offspring microbiota. **(B)** Principal coordinate analysis of offspring microbiota by bray Curtis distance representing 48% of total variation. PC1 represents 26% while PC2 represents 22%. **(C)** Significantly different bacteria taxa at the phylum level across all infant groups (ANOVA with FDR correction). Error bars show SEM. Data are representative of two independent experiments. n=6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

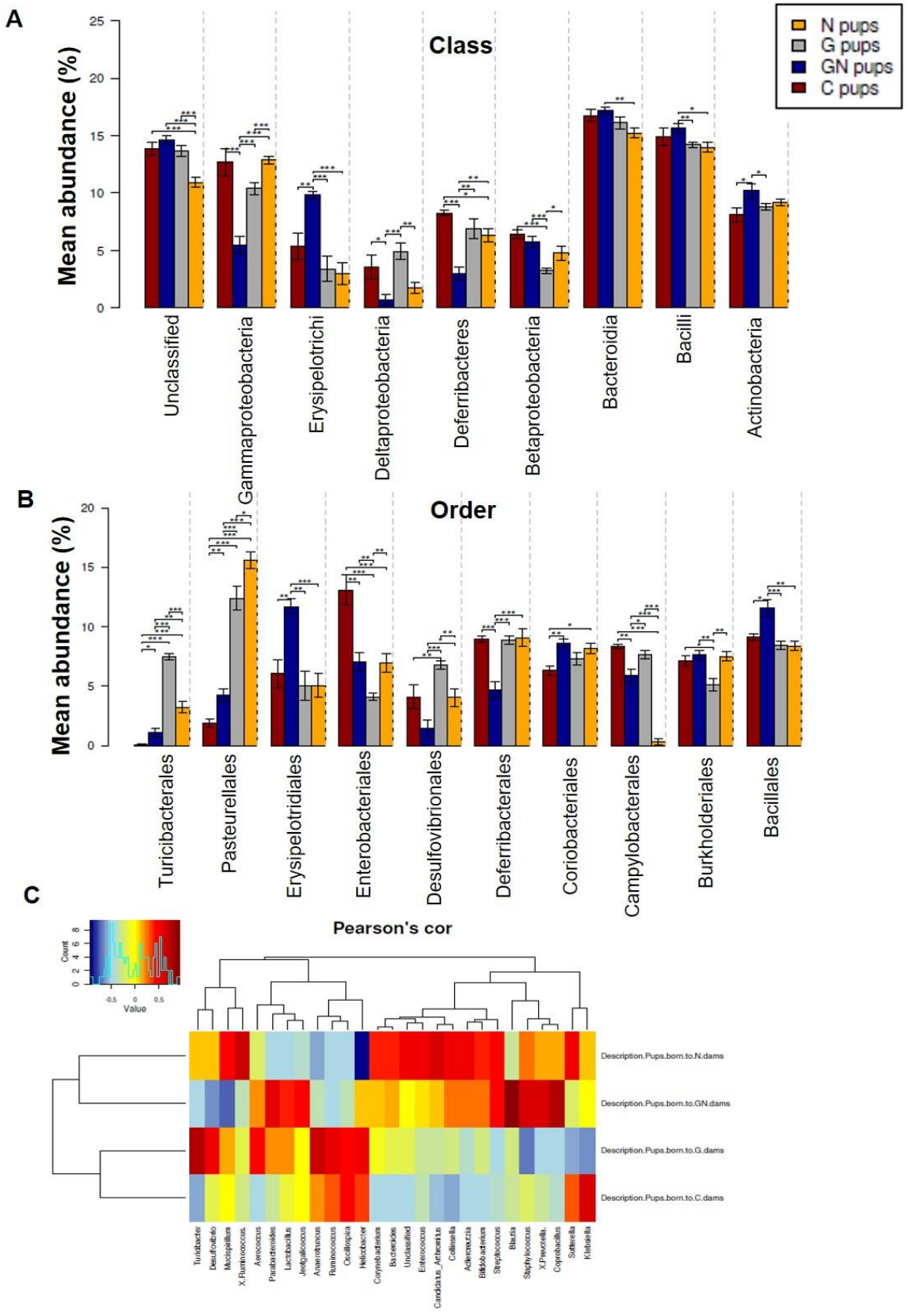

A
Class
Mean abundance (%)
N pups
G pups
GN pups
C pups
Unclassified
Gammaproteobacteria
Erysipelotrichi
Deltaproteobacteria
Deferribacteres
Betaproteobacteria
Bacteroidia
Bacilli
Actinobacteria
B
Order
Mean abundance (%)
Turicibacterales
Pasteurellales
Erysipelotridiales
Enterobacteriales
Desulfovibrionales
Deferribacterales
Coriobacteriales
Campylobacterales
Burkholderiales
Bacillales
C
Pearson's cor
Count
Value
Description.Pups.born.to.N.dams
Description.Pups.born.to.GN.dams
Description.Pups.born.to.G.dams
Description.Pups.born.to.C.dams
Turicibacter
Desulfovibrio
Mucispirillum
X.Ruminococcus.
Anaerotruncus
Ruminococcus
Oscillospira
Helicobacter
Corynebacterium
Bacteroides
Unclassified
Enterococcus
Candidatus_Arthromitus
Collinsella
Adlercreutzia
Bifidobacterium
Streptococcus
Blautia
Staphylococcus
X.Prevotella.
Coprobacillus
Sutterella
Klebsiella

Figure 4.4: Maternal polymyxin B treatment alters offspring intestinal microbiota at class, order and genus level. **(A)** Significantly altered infant bacteria taxa at class level **(B)** Significantly altered infant bacteria taxa at order level. **(C)** Heatmap constructed by unsupervised clustering using bray Curtis distance showing bacteria taxa at genus level significantly differentially abundant in various infant groups. Analysis by ANOVA with FDR correction for multiple comparisons. Error bars show SEM. Data are representative of two independent experiments. n=6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

We also observed differences in microbiome comparing control pups versus pups born to PMB breeders at all taxonomic levels **(Fig. 4.3C and 4.4)**. At the phylum level, we found significantly lower abundance of Proteobacteria in offspring born to dams treated with PMB during gestation or gestation plus nursing ($p<0.001$, FDR=0.00078, **Fig. 4.3C**). Firmicutes were significantly reduced in gestation or nursing pups compared to controls while Deferribacteres were less abundant in gestation plus nursing or nursing only pups when compared to littermate controls **(Fig. 4.3C)**. Bacteroidetes were significantly reduced in gestation plus nursing pups while Actinobacteria were significantly abundant only in the gestation plus nursing pups **(Fig. 4.3C).**

In addition, Gammaproteobacteria were significantly less abundant in infants born to PMB breeders compared to controls **(Fig. 4.4A)**. Erysipelotrichi were prevalent in gestation plus nursing pups while there were no significant differences in abundance of Bacteroidia and Bacilli in infants from PMB treated dams compared controls **(Fig. 4.4A)**. At the order level, Turicibacterales and Pasteurellales were predominant in pups from antibiotic treated dams while Enterobacteriales and Campylobacterales were significantly reduced in infants born to PMB breeders compared to those born to untreated mothers **(Fig. 4.4B)**. Furthermore, certain genera were enriched among the infant groups. *Klebsiella* were preponderant in control pups, *Turicibacter* in gestation pups, *Blautia* and *Sutterella* in gestation plus nursing pups while *Ruminococcus* were predominant in the nursing group **(Fig. 4.4C)**.

Whether these changes in microbiota could be associated with observed phenotypes in the offspring or other biological functions is the topic of discussion in the next chapter. Altogether, these data demonstrate that control pups had a distinct microbiota from PMB infant pups. Similar to observations we made with the vancomycin data above, we show existence of differences in microbiota within the PMB infant groups emphasizing independent effects of maternal microbiome during pregnancy as well as nursing. We conclude that maternal oral polymyxin B administration during pregnancy, lactation or both drives drastic alterations in infant intestinal microbiome 14 days postpartum.

4.3 Maternal antibiotics treatment during gestation or lactation significantly impacts infant growth and development.

Dams were treated with vancomycin, polymyxin B or a mixture of the two 5 for days prior to giving birth (gestation), 14 days postpartum (nursing) or 5 days prior to giving birth through nursing (gestation plus nursing), alongside control animals. Pups were born on the same day from mothers that were same age. Pups body weight; spleen weight and spleen cell counts were measured 14 days postpartum across all experiments.

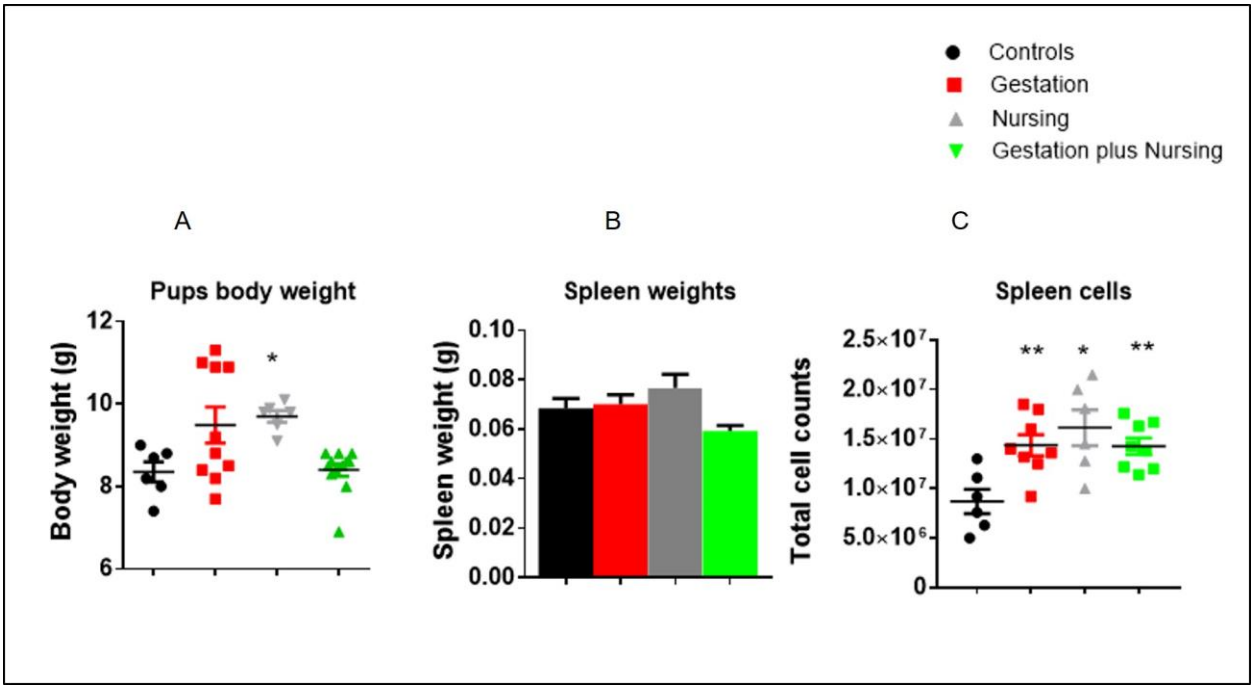


Figure 4.5: **Maternal oral vancomycin impacts infant growth and development**. Female BALB/c mice were treated with vancomycin orally 5 days prior to delivery (gestation), 14 days after birth (nursing) or 5 days prior to delivery through 14 days of nursing (gestation plus nursing). The body and spleen weights alongside the spleen cell counts of pups born to vancomycin breeders were measured 14 days postpartum. **(A)** Body weight of pups. **(B)** Spleen weights and **(C)** spleen cell counts. Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann Whitney U test. Data are representative of two independent experiments n= 6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

Maternal oral vancomycin treatment while nursing led to significant increase in body weight among pups **(Fig. 4.5A)**. We observed a trend towards increased body weight in pups born to mothers treated with vancomycin during gestation when compared to controls **(Fig. 4.5A).** Oral polymyxin B or mixed antibiotics in dams led to significantly higher body weights in pups born to mothers treated during gestation or while nursing **(Fig. 4.6A and 4.7A).** However, across all antibiotic regimens, we found no association between prolonged antibiotic treatment in mothers (gestation plus nursing group) with weight gain among her offspring **(Fig. 4.5A, 4.6A and 4.7A)**. Maternal antibiotics had no effect on spleen weights in pups **(Fig. 4.5B, 4.6B and 4.7B).** Although

there were no differences in spleen weights, we observed substantial antibiotic dependent effects on the total spleen cell counts.

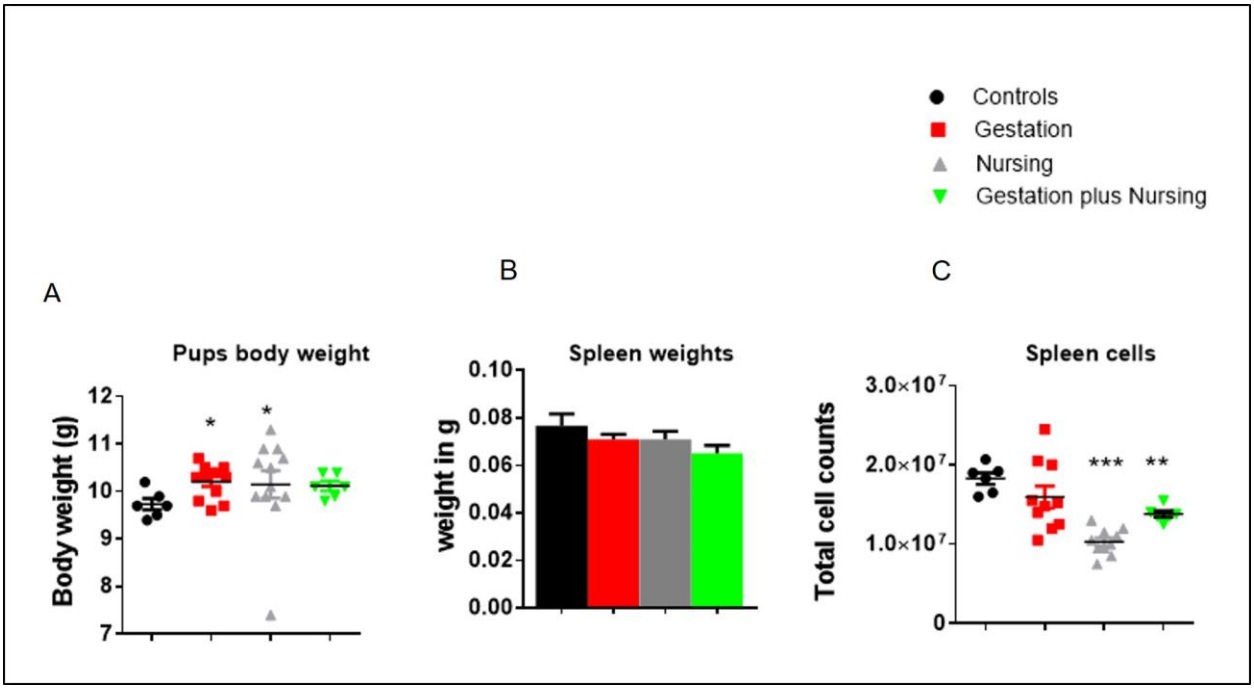


Figure 4.6: Maternal oral polymyxin B impacts infant growth and development. Female BALB/c mice were treated with polymyxin B orally 5 days prior to delivery (gestation), 14 days after birth (nursing) or 5 days prior to delivery through 14 days of nursing (gestation plus nursing). Pup body weight, spleen weights and spleen cell counts were measured in pups born to polymyxin B breeders. **(A)** Pups body weight. **(B)** Spleen weights. **(C)** Spleen counts. Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann Whitney U test. Data are representative of two independent experiments n= 6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

Maternal oral vancomycin treatment led to significantly higher total spleen counts in the infant spleen in all antibiotic groups of pups compared to control pup group **(Fig. 4.5C).** In contrast, maternal polymyxin B led to significant decrease in total cell counts in the spleen **(Fig. 4.6C).** Surprisingly, administering a mixture of both vancomycin and polymyxin B to dams also led to significant increase in total spleen cells among pups born to antibiotic treated mothers, mimicking that of vancomycin alone **(Fig. 4.7C).** Overall, maternal oral antibiotics regardless of

regimen impacted infant growth and spleen cellularity. Polymyxin B and vancomycin had contrasting impacts on total spleen cell counts.

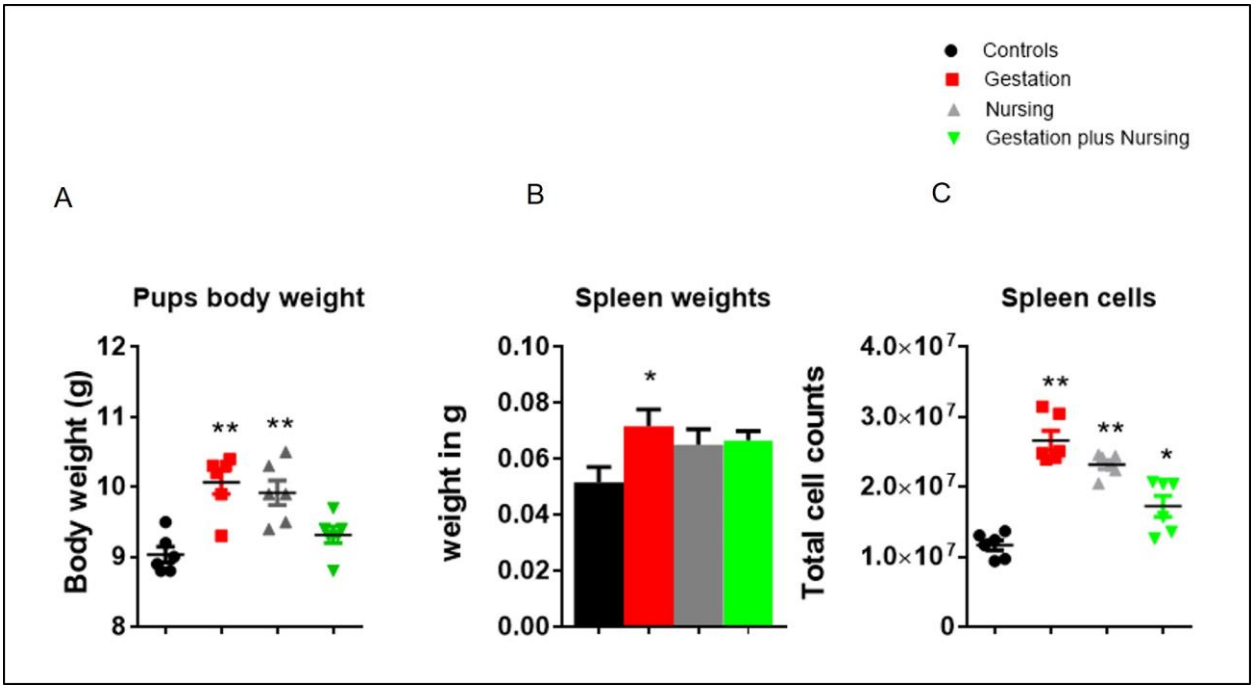


Figure 4.7: Maternal broad spectrum antibiotics (vancomycin and polymyxin B) impacts infant growth and development. Female BALB/c mice were treated with mixed antibiotics orally 5 days prior to delivery (gestation), 14 days after birth (nursing) or 5 days prior to delivery through 14 days of nursing (gestation plus nursing). We measured pup body weight, spleen weights and spleen cell counts. **(A)** Pups body weight. **(B)** Pups spleen weights. **(C)** Pups spleen cell counts. Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann Whitney U test. Data are representative of two independent experiments n= 6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

4.4 Antibiotic driven alteration of maternal microbiome alters infant T cell compartment

We next characterized infant T cell subsets in the spleen. Maternal vancomycin regardless of timing did not impact proportions of infant CD4 nor CD8 T cells **(Fig. 4.8A and B)**. Similarly, there was no difference in proportions of Effector CD4 T cells comparing antibiotic exposed pups versus control pups **(Fig. 4.8C)**. However, offspring born to vancomycin breeders had significantly lower proportions of central memory CD4 T cells compared to controls regardless of antibiotic

timing in mothers **(Fig. 4.8D).** In addition, there was no difference in proportions of T regulatory cells across all groups **(Fig. 4.8E)** as well as IL-10, measured as a proxy for T regulatory cells, concentrations in both small intestines and colons **(Fig. 4.8F)**.

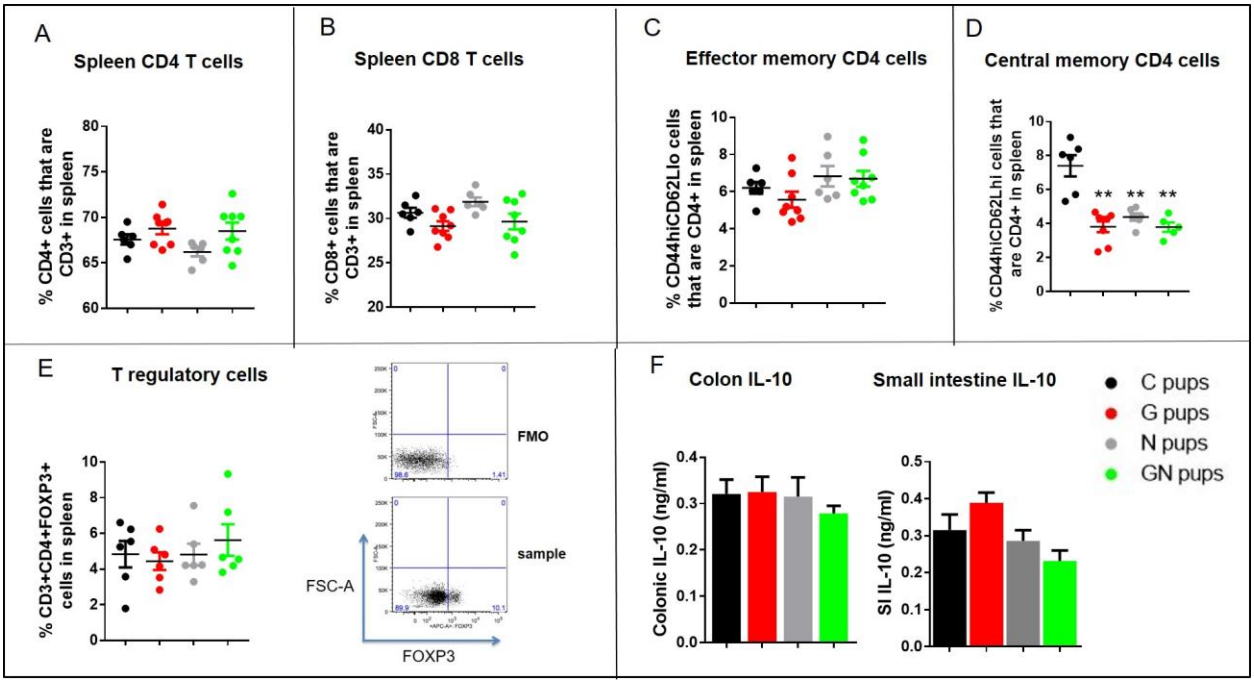


Figure 4.8: Maternal oral vancomycin drives subtle changes in T cell compartment in offspring. Female BALB/c mice were treated with vancomycin orally 5 days prior to delivery (gestation), 14 days after birth (nursing) or 5 days prior to delivery through 14 days of nursing (gestation plus nursing). Pups were killed 14d postpartum and spleen immunity analyzed. Flow cytometry was used to characterize different subsets of T cells. Proportions of **(A)** Infant CD4 T cells (CD3+CD4+), **(B)** Infant CD8 T cells **(C)** Effector memory CD4 T cells (CD4+CD44hi CD62Llo), **(D)** Central memory CD4 T cells (CD4+CD44hiCD62Lhi), **(E)** T regulatory cells (CD4+FOXP3+) and **(E)** IL-10 concentrations in colons and small intestines. Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann Whitney U test. Data are representative of two independent experiments n= 6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

Polmyxin B (PMB) treatment of dams similarly impacted infant T cells. Here, we observed significantly lower proportions of CD3+CD4+ T cells in pups born to dams treated with PMB during gestation compared to control pups **(Fig. 4.9A).** CD8 T cells exhibited a trend towards an increase in gestation pups **(Fig. 4.9B)**. Furthermore, proportions of effector CD4+ T cells were significantly reduced in nursing versus control pups while other PMB infant groups exhibited a trend towards decreased proportions **(Fig. 4.9C)**. In contrast, proportions of central memory CD4 T cells were significantly increased in pups born to dams treated with PMB during gestation or gestation plus nursing **(Fig. 4.9D)**. Akin to trends observed on central memory T cells, we noted significant increase in proportions of regulatory T cells in G and GN pups versus control pups **(Fig. 4.9E).**

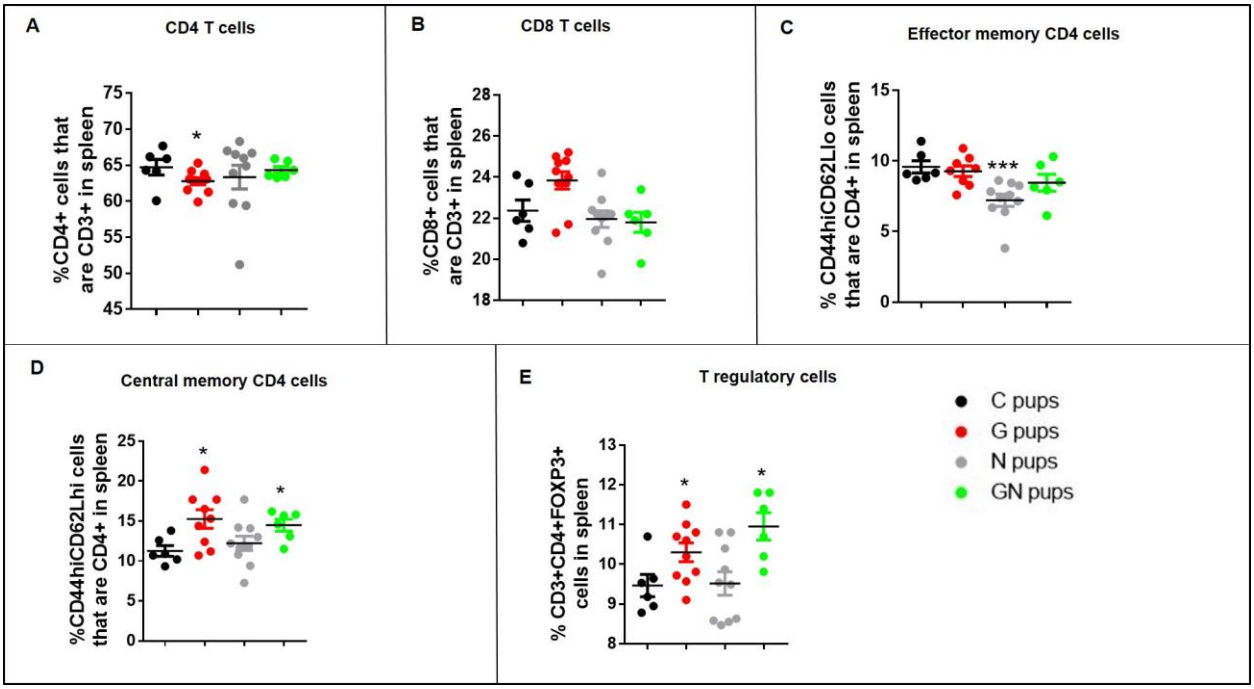


Figure 4.9: Impact of maternal oral polymyxin B on infant T cells in spleen. Female BALB/c mice were treated with polymyxin B orally 5 days prior to delivery (gestation), 14 days after birth (nursing) or 5 days prior to delivery through 14 days of nursing (gestation plus nursing). Pups were killed 14d postpartum and spleen immunity analyzed. Flow cytometry was used to characterize different subsets of T cells. T cells subsets were phenotyped based on their surface marker or transcription factor expression. **(A)** CD4 T cells (CD3+ CD4+), **(B)** CD8 T cells (CD3+CD8+), **(C)** Effector memory CD4 T cells (CD4+CD44hiCD62Llo), (**D)** Central memory CD4 T cells (CD4+CD44hi CD62Lhi) and **(E)** T regulatory cells (CD4+FOXP3+). Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann Whitney U test. Data are representative of two independent experiments n= 6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

When mothers are treated with a mixture of both vancomycin and PMB, we also observed significant alterations in T cells among the offspring. We found no difference in proportions of CD4 T cells **(Fig. 4.10A).** However, CD3+CD8+ T cells were significantly reduced in offspring born to antibiotic treated mothers regardless of timing compared to those born to control mothers **(Fig. 4.10B)**. Furthermore, effector CD4 T cells were significantly increased across all pups born to antibiotics treated dams versus control pups **(Fig. 4.10C)**. We found no difference in both

frequencies of central memory CD4 T cells and T regulatory cells across all infant groups **(Fig. 4.10D and E).**

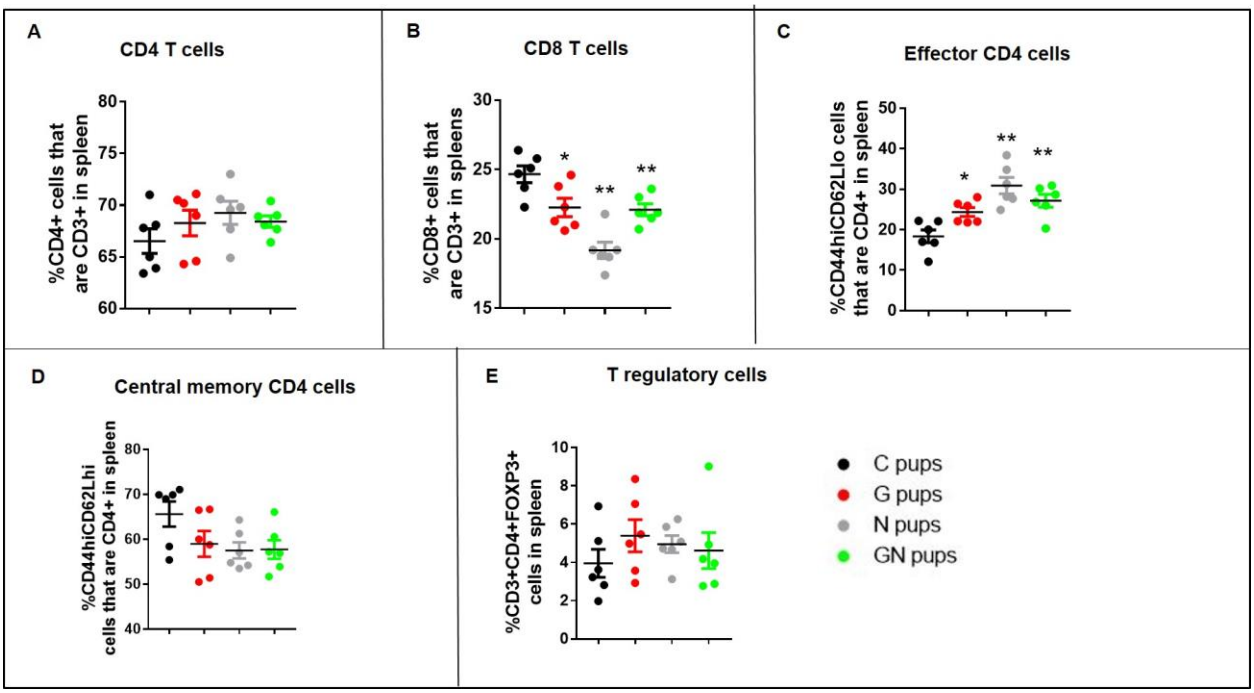


Figure 4.10: Maternal mixed antibiotics (vancomycin and polymyxin B) alter infant CD8 and effector memory T cells. Female BALB/c mice were treated with a mixture of vancomycin and PMB orally 5 days prior to delivery (gestation), 14 days after birth (nursing) or 5 days prior to delivery through 14 days of nursing (gestation plus nursing). Pups were killed 14d postpartum and spleen immunity analyzed. Flow cytometry was used to characterize different subsets of T cells. **(A)** Spleen CD4 T cells (CD3+CD4+), **(B)** CD8 T cells (CD3+CD8+), **(C)** Effector memory CD4 T cells (CD4+CD44+CD62L-), **(D)** Central memory CD4 T cells (CD4+CD44+CD62L+) T cells (**E)** T regulatory cells (CD4+FOXP3+). Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann Whitney U test. n= 6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

Maternal antibiotics during gestation, nursing or both impacts infant B cells

Prenatal and postnatal antibiotics also had an impact on the infant B cell compartment. Pups born to mothers treated with vancomycin during nursing or gestation plus nursing had significantly lower proportions total B cells (CD19+B220+), follicular B cells (CD19+CD23+) and marginal zone B (CD19+CD21+) compared to control pups **(Fig. 4.11A,B,C)**. There was no association between maternal PMB treatment and proportions of B cells (CD19+B220+) in her offspring **(Fig. 4.12A)**. However, we observed significant increase in proportions of follicular B cells in nursing and gestation plus nursing pups versus controls **(Fig. 4.12B).** Our analysis revealed no difference in proportions of marginal zone B cell subset across all groups **(Fig. 4.12C).** Apart from the independent effects of these antibiotics, administration of a mixture of both vancomycin and PMB also impacted B cells in infant mice **(Fig. 4.13)**. We found significantly lower proportions of total B cells in pup groups when mothers were treated with a mixture of both vancomycin and PMB regardless of timing **(Fig. 4.13A).** Akin to total B cells, and similar to maternal vancomycin treatment alone, proportions of follicular B cells were also significantly lower in antibiotic pups when compared to controls **(Fig. 4.13B)**. However, we observed no difference in proportions of marginal zone B cells in the spleen among infant groups born to dams treated with mix antibiotics **(Fig. 4.13C).**

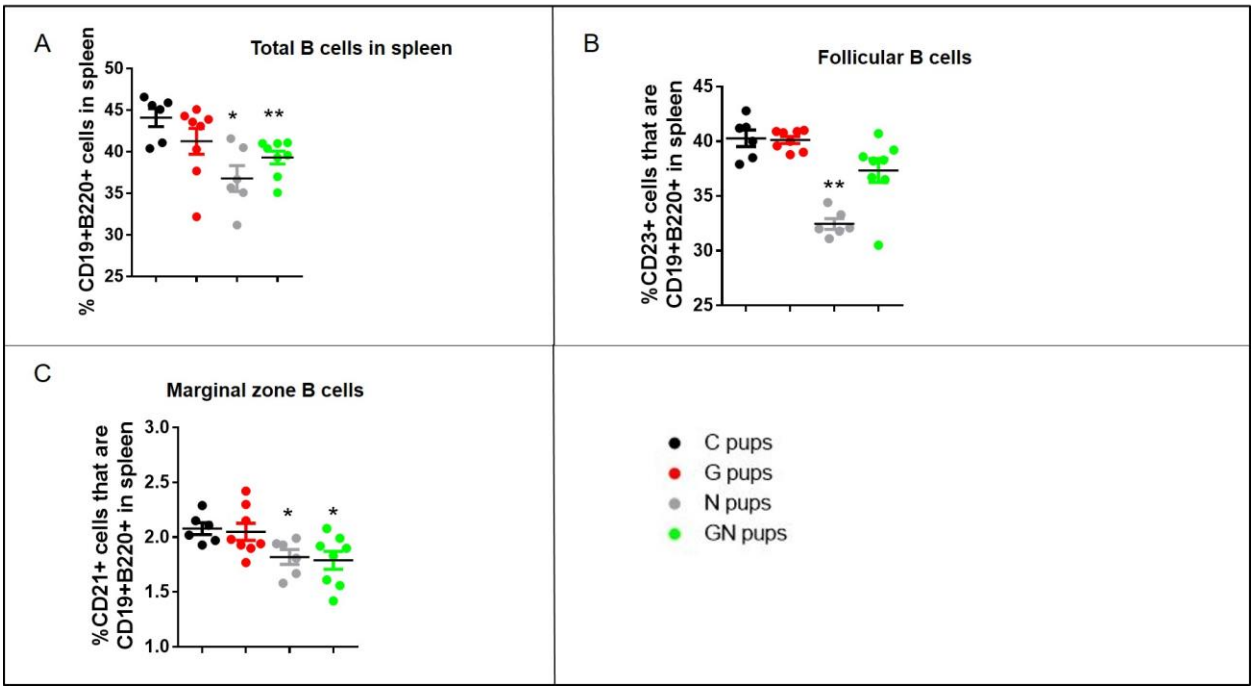


Figure 4.11: Maternal vancomycin impacts offspring B cell development in spleen. Infant B cells were analyzed in the spleen 14 days postpartum. **(A)** Total Infant B cells (CD19+B220+), **(B)** Follicular B cells (CD19+B220+CD23+) and **(C)** Marginal zone B cells (CD19+B220+CD21+). Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann Whitney U test. Data are representative of two independent experiments n= 6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

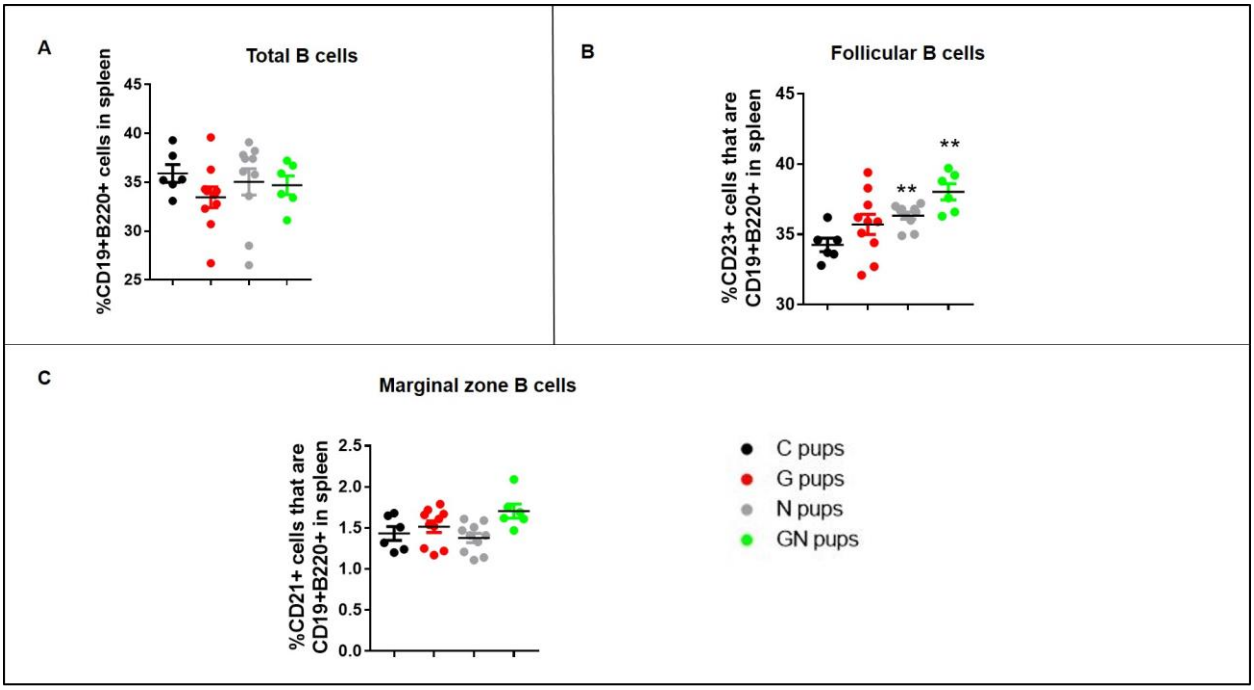


Figure 4.12: Maternal polymyxin B impacts infant B cells in spleen. Spleen B cells in pups were characterized 14 days postpartum by FACS. **(A)** Infant B cells in spleen (CD19+B220+), **(B)** Spleen B cells stratified into Follicular B cells (CD19+B220+CD23+) and **(C)** Marginal zone B cells (CD19+B220+CD21+). Graphs are shown as mean ± SEM and data analyzed Kruskal-Wallis test followed by Mann Whitney U test. Data are representative of two independent experiments n= 6-10 per group. *p<0.05, **p<0.01, ***p<0.001.

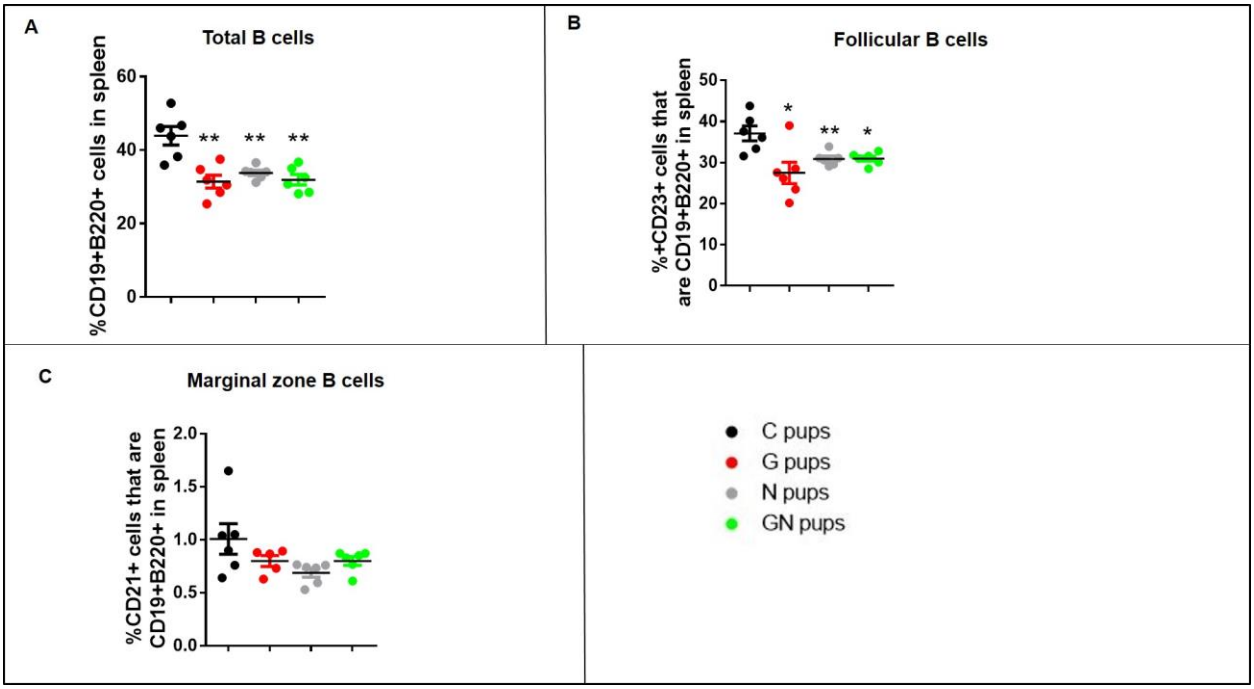


Figure 4.13: Maternal oral mixed antibiotics (vancomycin and polymyxin B) influences offspring B cells in spleen. Infant B cells in the spleen were characterized by FACS 14 days postpartum. **(A)** Total spleen B cells (CD19+B220+), **(B)** Follicular B cells (CD19+B220+CD23+) and **(C)** Marginal zone B cells (CD19+B220+CD21+). Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann Whitney U test. n= 6-10 per group. Data are representative of two independent experiments n= 6-10 per group *p<0.05, **p<0.01, ***p<0.001.

4.5 Discussion

In this chapter, we explore the impact of maternal oral antibiotics during pregnancy and lactation on infant growth, intestinal microbiota and immunity. We find that regardless of timing of antibiotic administration, alteration of maternal gut microbiome has profound influence on the offspring. These data are highly relevant for two reasons: 1. Antibiotic use during these periods in humans is a regular occurrence as a means of treating urinary, genital, and respiratory tract infections (Hauth et al., 1995; Jaeger et al., 2013). Penicillins, the most common antibiotic class administered during pregnancy are delivered intravenously in the form of Ampicillin for *Streptococcus agalactiae* carriage, and therefore would have effects not only on maternal gut microbiome, but on the other maternal body sites and potentially even direct effects on fetal or infant microbiome. 2. Most Importantly, our study shows, through the use of oral antibiotics that are not absorbed from the gut, that an altered maternal gut microbiome alone, during pregnancy and lactation, has these profound downstream effects. Although the antibiotic candidates we used here are not administered orally in humans, our findings are critical in informing the effects of alteration of maternal gut microbiome (through any means) during this period on infant development. We developed a murine model of maternal antibiotic treatment during gestation and/or nursing to investigate the impact on the establishment of the infant intestinal microbiome, body composition and the developing immune system. We selected vancomycin and polymyxin B that are active against gram positive and gram-negative bacteria respectively because these antibiotics are poorly orally bioavailable.

4.5.1 Impact on infant intestinal microbiota

It is apparent that observed changes in infant growth and immunity were largely driven by alterations in gut bacterial composition in the mother, underpinning the importance of maternal gut microbes on infant health. We observed profound differences in offspring microbiota following maternal oral polymyxin B or vancomycin treatment. Treatment of dams with antibiotics five days prior to delivery resulted in distinct offspring microbiome compared to controls, suggesting that maternal gut composition during gestation influences the type of bacteria colonizing the infant gut. Although we did not investigate the mechanisms behind these biological phenomena, it is likely that bacterial translocation occurs from the maternal gut to the placenta and/or maternal breast milk or antibiotics alter maternal metabolism, which could begin to impact the fetal microbiome *in utero.* A bi-directional relationship has previously been described between pregnancy and periodontal disease, and that periodontal infections at least in some populations can increase the risk of adverse pregnancy outcomes (Armitage, 2013). Recently, Aagaard et al. described a placental microbiome that was most akin to human oral microbiome (Aagaard et al., 2014). Therefore, it is tempting to speculate that there is a bi-directional flow of blood between the mouth and uterus producing a similar microbiome in these sites. This blood flow also interacts with the GIT where dendritic cells which interact with commensal bacteria squeeze through the lamina propria and travel via lymph and circulatory system to mammary glands (Fernandez et al., 2013). Using 16S rRNA profiling, we observed significant decrease in abundance of Proteobacteria and Deferribacteres in offspring following Polymyxin B treatment regardless of timing. This was expected since this antibiotic has spectrum activity against the gram-negative bacteria. Therefore, it is reasonable that targeting these bacteria in the maternal gut impacted transfer to offspring. However, we also noted a significant decrease in abundance of Firmicutes in gestation or nursing pups despite being gram positive bacteria suggesting that PMB treatment could be having indirect

impact on gram positive bacteria thus influencing their acquisition in infants. In addition, Firmicutes alongside Bacteroidetes are the main bacteria that have been linked to metabolism of undigested food remnants (Tsuji et al., 2008). It is possible that increased body weight in gestation as well as nursing pups could be attributed to differences in abundance of Firmicutes which may have driven metabolic alterations in offspring. Bacteroidetes (gram negatives) were not different comparing control against gestation plus nursing as well as gestation pups suggesting significant alterations in this phylum in offspring are probably determined by maternal microbiota while nursing. It is interesting that offspring born to PMB treated mothers had high abundance of Bacteroidetes although this phylum was absent in maternal gut. This indicates the presence of others sources of Bacteroidetes aside from the mother's gut. We found significantly higher abundance of Actinobacteria in offspring born to dams treated with PMB during gestation plus nursing compared to controls. Members of this phylum have previously been associated with immunity. For example, Bifidobacteria has been shown to influence immune development in young rats (Dong et al., 2010) as well as aging mice (Fu et al., 2010). It is likely that the preponderance of Actinobacteria in the gestation plus nursing group was associated with profound changes observed both in offspring B and T cell compartment. Furthermore, Turicibacterales were significantly increased in all PMB pups compared to controls. Member of this order have been shown to have an indirect relationship with CD8 T cells. When CD8 T cells are deficient, *Turicibacter* blooms (Presley et al., 2010). Therefore, it is possible that increased abundance of Turicibacter among PMB infants was associated with reduced populations of CD8 T cells. Although we did not investigate a direct causal effect and the underlying mechanism, it is likely that signaling via microbial compounds by members of this order is critical for immune development. In the present dysbiotic state, the possibility that immune cells can have impaired

functional capability cannot be ruled out. Although we observed increased proportions of certain immune cell types, we are not clear yet if functionality of these cells was affected. Whether these alterations in intestinal microbiota translated to improved or impaired capability of offspring to control pathogenic infections is explored further in the next chapter. Oral vancomycin in dams also impacted infant microbiome. We observed distinct beta diversity clusters indicating inherent differences in bacteria composition. Akin to the polymyxin B effects, we observed reduced bacterial diversity in pups born to vancomycin treated dams, which emphasizes the role of antibiotics in reducing microbial diversity. In contrast to PMB data, Proteobacteria and Deferribacteres were predominant in vancomycin infant groups regardless of vancomycin timing indicating that the antibiotics indeed had different spectra of activity and impacted microbiota distinctly. We also observed a high abundance of Enterobacteriales and Pasteurellales in vancomycin pups. Others have reported an increase in abundance of Enterobacteriaceae in murine models treated with vancomycin (Ubeda et al., 2010). This can be explained by the fact that vancomycin has spectral activity against the gram-positive bacteria which prompted an outgrowth of compensatory bacteria in the gram-negative family which were possibly transferred to her offspring. We also drew associations between the bacterial communities observed in the vancomycin study with observed immunological outcomes in offspring. Although all vancomycin infants had significantly lower abundance of Clostridia which has been associated with induction of T regulatory cells (Atarashi et al., 2011, 2013), we did not find any differences in proportions of T regulatory subset in our model. We think the effect on regulatory T cells could be localized and not systemic which could explain our inability to detect differences in the spleen. We found significantly lower proportions of total B cells, marginal zone and follicular B cells in offspring born to dams treated with vancomycin during nursing or gestation plus nursing compared to the

control group. These two groups had significantly higher abundance of bacillales compared to gestation or control pups. Although we did not investigate the direct role of microbes on systemic immunity, it is possible that these Firmicutes are associated with observed immunological outcomes especially on the B cell compartment and merits further investigation potentially using gnotobiotic mouse models. Moreover, Erysipelotrichiales which are often mentioned in passing but are highly immunogenic and thrive post antibiotic treatment (reviewed in (Kaakoush, 2015)) were significantly reduced in the nursing group but not in other vancomycin infants. Members of this taxa have been found to be enriched in colorectal cancer although evidence has not been consistent (Chen et al., 2012; Palm et al., 2014). Others have shown an association between Erysipelotrichaceae and TNF levels (Dey et al, 2013). In our model, the near complete ablation of this order in nursing pups could be indicative of an altered innate and adaptive immune profile which we did not investigate in our study but would be intriguing to see how these infant pups respond to pathogenic challenge in our next chapter. We observed significantly lower proportions of central memory CD4 T cells (CD4+CD44hiCD62Lhi) while B cells revealed a trend toward decreasing proportions in pups born to dams treated with vancomycin only during gestation. These data conflicts recent work by Gomez and colleagues who showed no impact of maternal microbiome during pregnancy on infant T cell activation status (Gomez de Agüero et al., 2016). The authors utilized germ free maternal neonatal model where mothers were colonized transiently during gestation; an effect that was short-lived as by the time of delivery, the birth canal was sterile and neonates remained germ free. This could explain differences in our results as we applied a different approach where the possibility of infants being colonized *in utero* cannot be ruled out.

4.5.2 Impact on infant growth and development

Pups born to dams with gut dysbiosis induced by either antibiotic alone or in combination during gestation or nursing, had significantly higher body weight compared to littermate controls. Changes in offspring body weight are likely due to changes in maternal metabolism, which could begin to impact the fetus *in utero*. Our data shows pulsed antibiotic treatment (gestation or nursing only) has a significant impact on weight rather than continuous treatment (gestation plus nursing) suggesting that certain organisms may compensate metabolically overtime. Indeed, previous studies have implicated the gut microbiota in control of metabolism. Wikoff and colleagues demonstrated large effects of the gut microflora on mammalian blood metabolites (Wikoff et al., 2009). Similarly, Vrieze and others showed oral vancomycin to significantly impact host physiology, bile acid metabolism and insulin sensitivity in patients with metabolic syndrome (Vrieze et al., 2014). These changes in maternal metabolism are largely due to perturbation in the resident bacterial community. Maternal microbiome could therefore be acting either directly or indirectly to impact adiposity among the offspring. Further, it is also plausible that offspring inherit a unique "obesogenic" microbiome within a critical window period, which begins to influence metabolic activity early in life. Our data corroborates the findings of others who reported an impact of early life antibiotic exposure on accelerating growth and altering body composition (Cox et al., 2014; Nobel et al., 2015). Moreover, our findings expand current knowledge in demonstrating that metabolic programming begins *in utero*.

4.5.3 Impact on Infant immunity

We observed a significant impact of maternal oral antibiotics on infant immunity. Firstly, we observed differences in total immune cells in spleens that were antibiotic specific. Maternal oral vancomycin treatment led to significantly increased spleen cellularity among offspring while polymyxin B treatment had contrasting effects. Interestingly, we found a significantly higher total

spleen cells numbers in pups born to mothers treated with mixed antibiotics (vancomycin and polymyxin B) regardless of phase of treatment. Spleen weights did not correlate with total cell numbers. This indicates that effects of antibiotic exposure although sufficient to alter cell counts were not matched to those otherwise induced by infections that would induce splenomegaly. However, it is possible there was increased blood flow to the spleen albeit not significant to alter organ weight. Recent studies have revealed an important programming role of gut microbiota on immunity. For example, particular clostridia clusters have been shown to induce T regulatory cells in the gut (Atarashi et al., 2011, 2013) while segmented filamentous bacteria induce TH17 subsets (Ivanov et al., 2009). Interestingly, maternal oral vancomycin led to significant reduction in central memory CD4+ T cells, and a trend towards an increase in effector CD4+ T cells among her offspring regardless of phase of treatment. Polymyxin B treatment in mothers while nursing led to a reduction in proportions of central memory CD4+ T cells in her offspring. Finally, mixed antibiotics, which although had no effect on CD4 T cells, resulted in significantly reduced proportions of CD8+ T cells. This implies that the effects of vancomycin and polymyxin B treatment on CD4+ T cells may cancel each other out. Although we did not explore the effect of vancomycin on CD8+ T cells, the finding that these were reduced in both the polymyxin B and mixed antibiotic treated pups implies that polymyxin B sensitive bacteria (gram negatives) are the major taxa driving immunological changes for this subset.

Tormo-Badia et al. demonstrated using non-obese diabetic mice that maternal antibiotic treatment during pregnancy led to alterations of neonatal gut T cell compartment. In particular, they reported an increase in CD8 T cells and T regulatory cells (Tormo-Badia et al., 2014). Our polymyxin B and mixed antibiotics data is consistent with these findings, and show that this phenomenon occurs at other immunological sites. Related studies have revealed that maternal diet during pregnancy

can impact development and immune function of the fetus (Hoyo et al., 2011; Myles et al., 2013). We also noted discrepancies between frequencies and absolute counts in various immune subsets we analyzed. It is possible that although we have increased total counts, the effector functions of these cells in response to antigenic challenge may be impaired. However, we will address the overall impact of an altered infant immune system on ability to control pathogenic infections when we challenge these pups born to antibiotic breeders with various infections models in the next chapter. To our knowledge, this is among the very few reports characterizing infant immunity 14 days post-partum in pups born to dams with an aberrant microbiome. Together, these data reveal an indispensable role for maternal microbiome during gestation or breastfeeding on offspring immune development.

Our study had a number of limitations. First, we utilized only two mothers per group in part because we get 6-10 litters per mother and we cull to 6 per dam to ensure the mother has enough resources for the offspring during breastfeeding. However, in terms of studying the impact the antibiotics had on maternal gut microbiome, the numbers were not sufficient. Our main focus was largely on infants, an objective we conclusively addressed but we were not able to do the same in terms of studying the maternal-infant microbiome transfer and the dynamics involved. Secondly and tied to the first limitation is the cage effect which cannot be overlooked. We housed two dams per group together and treated them with antibiotics in drinking water. While we expect these dams to have a similar microbiome having gone through the same treatment, the microbiota may not necessary be exact and given the approach we used in our model, that allowed the two mothers in the same group to be cohoused, it is likely we may not have been able to pick up these differences albeit subtle. In addition, we did not study the impact of maternal antibiotics on microbiota in extra intestinal sites. Although we verified that vancomycin mediated changes were only restricted to

the gut, we did not investigate if these antibiotics could be acting indirectly to impact microbiota in other sites such as vagina and breastmilk which obviously contribute in shaping offspring microbiota early in life. Our immediate future experiments will address this question.

Nonetheless our data demonstrates that inherited gut dysbiosis very early in life which coincides with the developing immune system at infancy impacts inherent adaptive immunity. This to our knowledge is one of the very few reports to specifically characterize baseline immunity in infants born to mothers with an altered gut microbiome. In the next chapter, we will subject these offspring to various challenge models and test their immunity further. Taken together, antibiotic treatment in dams during gestation, nursing or both significantly altered infant microbiome and profoundly impacted the developing immature immune system.

CHAPTER 5
5.0 RESULTS SECTION II

5.1 Impact of maternal antibiotics during gestation or lactation on offspring immunological ability to control infections and respond to vaccine challenge

5.1.1 Introduction

Newborn humans and animals have an impaired ability to mount an immune response to certain stimuli. Consequently, they are frequently subject to overwhelming infections (Morein, Blomqvist, & Hu, 2007). Stimulation of the neonatal immune system by vaccination is therefore necessary to protect infants against some infections. However, some of the neonatal vaccines are not as effective owing to various phenomena, including TH2 biased cellular immunity along with transferred maternal antibodies that can potentially attenuate neonatal responses (Siegrist, 2007). For example, RSV is a significant infection early in life and is the major cause for hospitalizations in infants under 3 months (Hall et al., 2012). Although passive immunization exists for this infection, there remains no active vaccine for RSV but even in the presence of one, it is likely that booster doses will be required for adequate protection, which will then fail to protect infants in the early period when they are most vulnerable. Apart from RSV infections, other infections such as helminths may also pose an immunological burden to infants. Studies show that *in utero* helminth exposure may influence susceptibility to the same helminths later in life (reviewed (Mpairwe et al, 2014)) as well as to unrelated antigens (Gebreegziabiher et al., 2014). Furthermore, lack of gut microbiota has been shown to influence host immunity and accelerate susceptibility to helminth induced pathology (Holzscheiter et al., 2014). Yet whether maternal gut microbiota during pregnancy or breastfeeding impacts infant immunity to helminths is unknown.

Considering the challenges of neonatal vaccination and the high risk of overwhelming infections in early life, the role of other immune-modulatory factors for example, microbiota in enhancing neonatal immunity is not well understood. The gut microbiota coevolves with the immune system, and they each in turn influence the establishment of the other. Recent evidence demonstrates that microbiota mediated programming of neonatal immunity may begin *in utero*, underscoring the importance of maternal microbiome during pregnancy (Aagaard et al., 2014). In the previous chapter **(CHAPTER 4)**, we have shown that antibiotic driven manipulation of maternal gut microbiota during gestation, lactation or both phases has profound consequences on infant immune cell subsets and inherent activation levels and intestinal microbiota at 14 days postpartum. However, what an altered infant immunity translates to in the context of live infections is not well understood. In this chapter, we utilized infant RSV and *N. brasiliensis* challenge models to further our understanding on the role of maternal microbiota on infant immunity.

5.2 Maternal oral vancomycin and the infant RSV model

Here, we mated dams (2 dams per group) and exposed them to vancomycin in drinking water (1mg/ml) 5 days prior to delivery (gestation), for 14 days after delivery (nursing) or both 5 days prior to delivery through 14 days of nursing (gestation plus nursing) as shown in the methods chapter **(Fig. 3.1).** Control dams were not treated with antibiotics. Pups were culled to 6 per mother. Maternal antibiotic treatment was stopped 14 days after delivery in the nursing or gestation plus nursing group. Pups were then each infected intranasally with $8x10^5$ PFUs in 40µl DMEM at 21 days of life. Severity of RSV was assessed by daily weight monitoring and mice were killed at day 4 or 8 post RSV infection and lung immunity analysed.

5.2.1 RESULTS

5.2.1.1 Maternal oral vancomycin treatment increased offspring susceptibility to RSV in the early onset of disease

We challenged offspring with $8x10^5$ PFUs of RSV A2 strain intranasally at 21 days of age. Weight was monitored daily, and pups were killed at day 4 or 8 post infection **(Fig. 5.1a).** Weaned RSV infected pups born to vancomycin bredeers had a significant growth delay versus control pups but they did not lose weight **(Fig. 5.1b).** RSV infected pups born to dams treated only during gestation begun to catch up with control pups weight by day 5. However, the pups whose mothers had received vancomycin during nursing were susceptible to RSV throughout the 8 days post-infection **(Fig. 5.1b)**. To tease out the effect that maternal antibiotics could be having on pup weight and clearly examine if RSV infection was impacting weight gain in the pups, we examined the effect of RSV challenge on all pups against their uninfected controls **(Fig. 5.1 c, d, e, f).** In control pups, we observed that RSV infection caused a significant delay in growth compared to uninfected pups for the first few days post-infection, but then had a rapid growth spurt that surpassed that of the uninfected pups **(Fig 5.1c)**. RSV infected gestation pups exhibited a similar trend to control pups, they surpassed their uninfected littermate controls at day 6 post RSV challenge **(Fig. 5.1d).** However, the nursing or gestation plus nursing pups did not catch up with the weights of their control peers after infection **(Fig. 5.1e, f).** We observed a trend towards increased viral burden in the lung among infant antibiotic groups at day 4 post RSV challenge compared to control group **(Fig. 5.1g).**

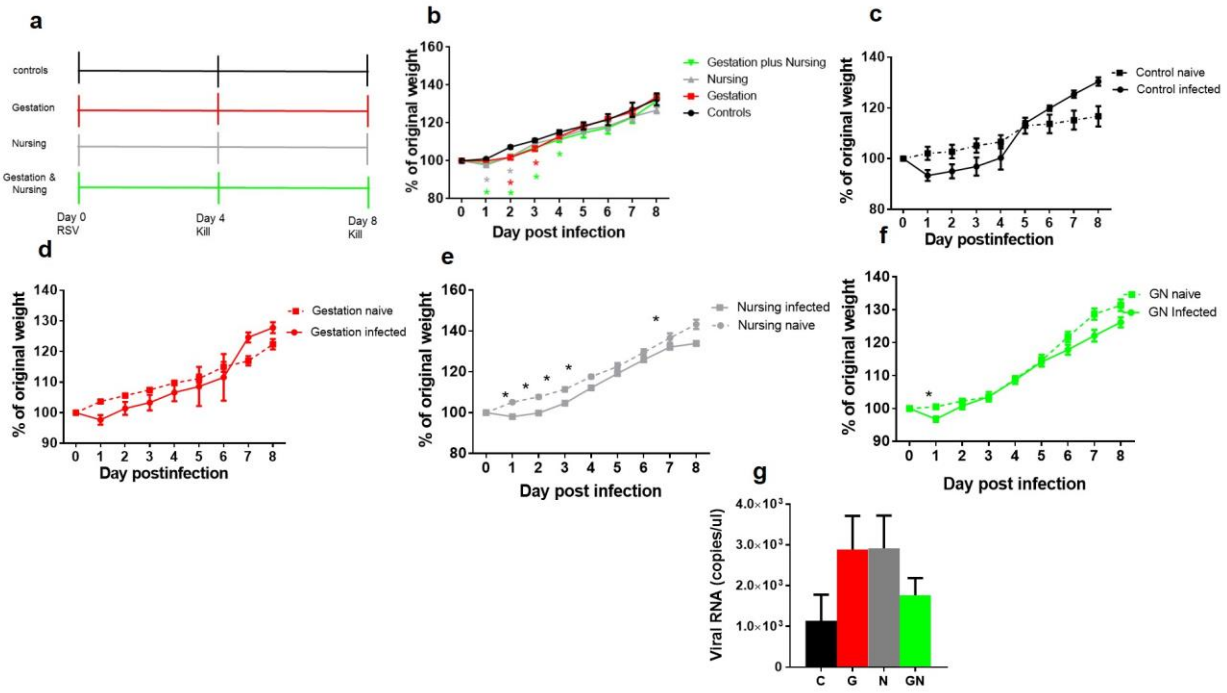


Figure 5.1: **Maternal oral vancomycin impacts infant immunity to RSV**. **(a)** Pups born to vancomycin breeders treated during gestation, nursing or gestation plus nursing were challenged intranasally at 3 weeks with RSV and killed 4 or 8 days post infection. **(b)** Daily percentage weight gain among RSV infected pups born to vancomycin treated or untreated mothers. **(c)** Percentage weight gain in RSV infected or naïve control pups. **(d)** Percentage weight gain in RSV infected or naïve pups born to dams treated with vancomycin during gestation **(e)** Percentage weight gain in RSV infected or naïve pups born to mothers treated with vancomycin during nursing. **(f)** Percentage weight gain in RSV infected or naïve pups born to mothers treated with vancomycin during gestation plus nursing. **(g)** Viral RNA copies at day 4 post RSV infection. Graphs are represented as mean ± SEM and data are representative of at least three independent experiments. Weight data was analyzed by multiple t test with Holm Sidak multiple comparisons adjustment (b-f). n=8-12 per group. $^{*}p<0.05$.

5.2.1.2 Maternal vancomycin during pregnancy and/or lactation impacted infant lung immunity.

We next analysed local lung immunity in the infant following RSV challenge at day 4 and 8 post infection. RSV infected pups born to vancomycin breeders had reduced total cell counts at both time points compared to control group (**Fig. 5.2A, Fig. 5.4A**). At day 4 post infection, total numbers of natural killer cells (CD3-CD49b+), considered among the first immune cells to be recruited to the lung following an RSV infection providing an initial source of interferon gamma (Openshaw et al., 1995), were significantly lower in offspring born to dams treated with vancomycin regardless of timing compared to littermate controls **(Fig. 5.2B)**. Similarly, proportions of activated NK cells (CD3-CD49b+CD69+) were significantly lower in the gestation plus nursing pups (mean 60.7 versus 41.2, p=0.0022) with a trend towards decreasing proportions in other antibiotic groups **(Fig. 5.2D)**. Our analyses of the total numbers revealed significant reduction in activated NK cells in gestation and gestation plus nursing pups compared to controls. Although total numbers of activated NK cells were reduced for the nursing group, the difference did not reach statistical significance **(Fig. 5.2D)**. IFN-gamma concentrations were reduced in gestation or gestation plus nursing groups but these were not significant **(Fig. 5.2C)**. Although we had NK cell recruitment to the lungs in these groups, we noted discrepancies in levels of interferon gamma. Alveolar macrophages have previously been shown to be key mediators of neonatal RSV immunity (Eichinger et al., 2015; Empey et al., 2012). There were no differences in proportions of alveolar macrophages in our model but total numbers were significantly reduced in gestation plus nursing pups compared to control pups **(Fig. 5.2E)**. The proportions and total numbers of CD8 T lymphocytes, thought to mediate pathology in RSV infection, were similar across all pup groups **(Fig. 5.2F)**. In contrast, we observed significantly increased proportions of CD4 T cells in nursing and the gestation plus nursing pups **(Fig. 5.3A).** Moreover, proportions of effector memory CD4+

T cells were significantly lower in nursing and gestation plus nursing pups **(Fig. 5.3B)** while those of central memory CD4+ T cells were significantly increased in aforementioned infant groups **(Fig. 5.3C)**. We found no difference in levels of serum RSV specific IgG **(Fig. 5.3D)**.

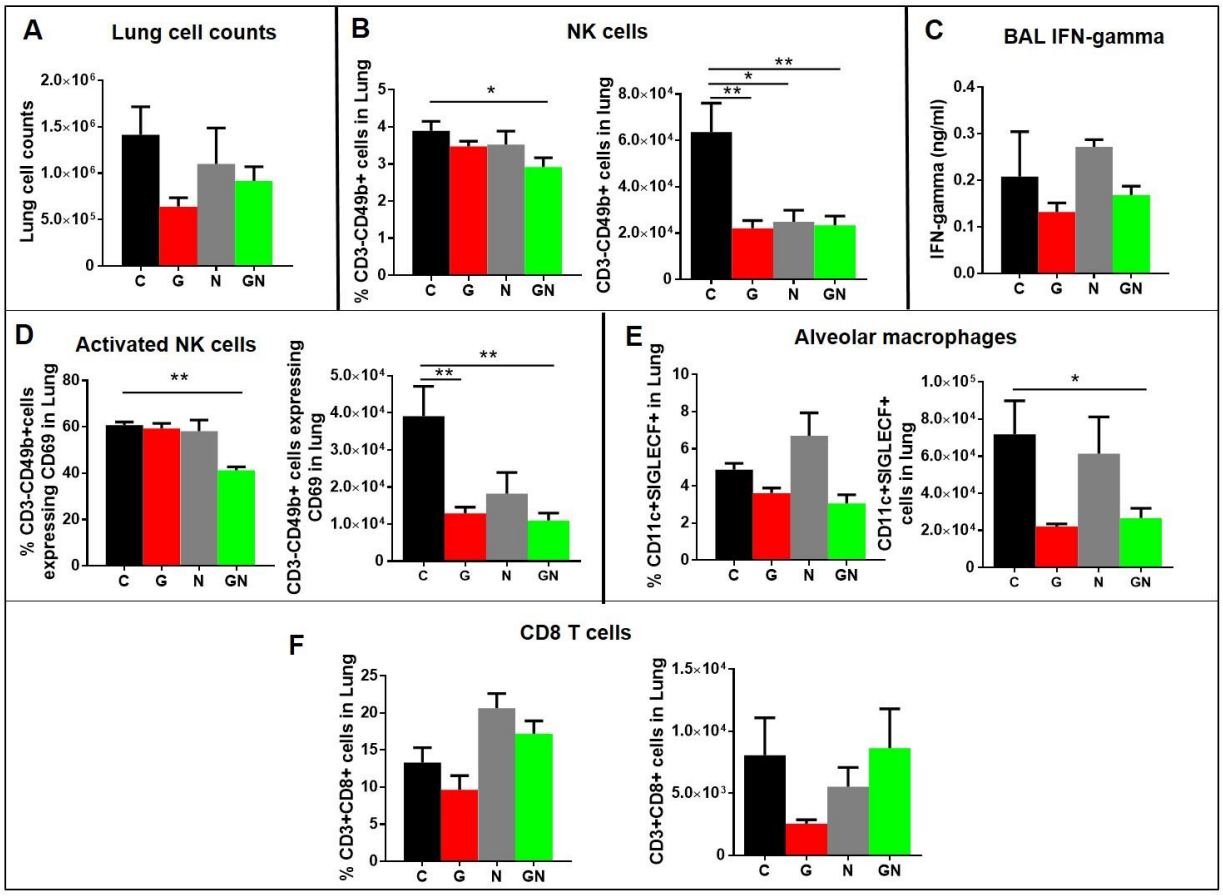


Figure 5.2: Maternal oral vancomycin impacts offspring NK cells day 4 post RSV infection. Pups born to vancomycin breeders were infected intranasally with RSV at 21 days and killed at day 4-post infection. Lung samples were harvested and analyzed. Innate immune cells and CD8 T cells were characterized by FACS. **(A)** Total lung counts, **(B)** NK cells (CD3-CD49b+), **(C)** BAL IFN- gamma levels, **(D)** Activated NK cells (CD3-CD49b+CD69+), **(E)** Alveolar macrophages (SIGLECF+CD11c+), **(F)** CD8 T lymphocytes (CD3+CD8+). Data are shown as mean ± SEM. Statistical analysis was performed by Kruskal-Wallis test followed by Mann-Whitney U test. Data are representative of two independent experiments. n=4-6 per group. *p<0.05 and **p<0.01.

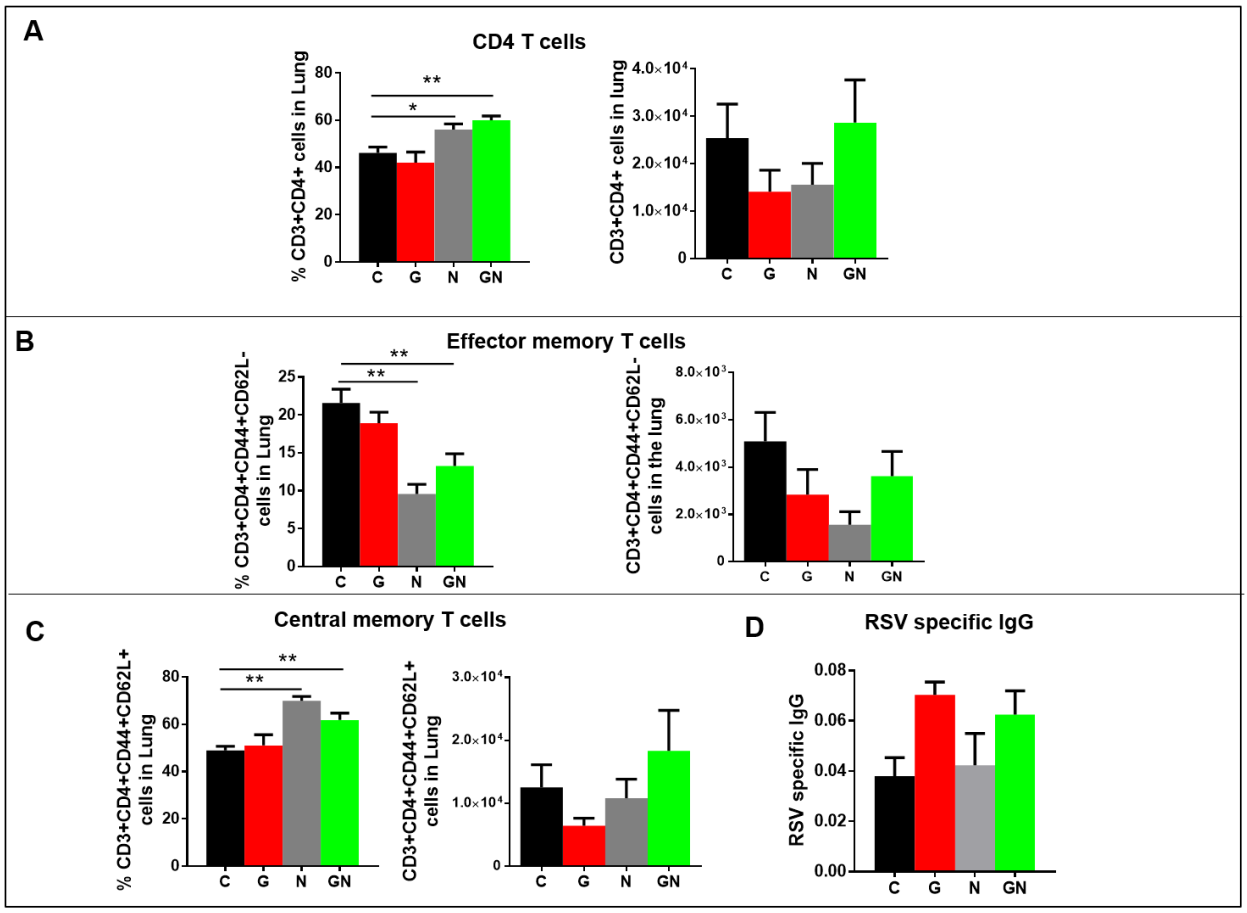


Figure 5.3: Maternal oral vancomycin impacts effector memory CD4 T cells in offspring day 4 post RSV infection. Pups born to vancomycin breeders were infected intranasally with RSV at 21 days and killed at day 4-post infection. Lung samples were harvested and analyzed. CD4 T lymphocytes were characterized by FACS. **(A)** CD4 T cells (CD3+CD4+), **(B)** Effector memory CD4 T cells (CD3+CD4+CD44+CD62L-) and **(C)** Central memory CD4 T (CD4+CD44+ CD62L+) cells respectively. **(D)** Serum RSV specific IgG. Data are shown as mean ± SEM. Statistical analysis was performed by Kruskal-Wallis test followed by Mann-Whitney U test. Data are representative of two independent experiments. n=4-6 per group. *p<0.05 and **p<0.01.

At day 8 post infection, when the adaptive immune response peaks and cellular infiltration is thought to exacerbate symptoms, we observed a trend towards an increase in proportions of NK cells in gestation and nursing pups but not gestation plus nursing. The total number of NK cells were significantly reduced in gestation plus nursing pups compared to control pups **(Fig 5.4B)**. Proportions of NK cells were significantly reduced in gestation plus nursing pups compared to nursing only pups. There were no differences in proportions of activated NK cells **(Fig 5.4D).** Furthermore, IFN-gamma levels in bronchoalveolar lavage exhibited a trend towards decreasing concentrations in all offspring born to vancomycin breeders but this was not statistically significant **(Fig. 5.4C).** Similarly, we found no significant differences in proportions of alveolar macrophages while the numbers displayed a trend towards a decrease comparing vancomycin infants with control infants **(Fig. 5.4E)**. There was no difference in frequencies and numbers of infant CD8 T cells **(Fig. 5.4F).** When we compared proportions of CD8 T cells between day 4 and day 8, we noted an increase in frequencies of this subset at day 8 across all groups **(Appendix B, Fig B1)**. There was a significant increase in CD8 frequencies for gestation and gestation plus nursing pups, where mean proportions increased from 9.626% in day 4 to 23.02% in day 8 **(Appendix B, Fig B1)**. Similarly, proportions increased from 17.2% in day 4 to 22.52% in gestation plus nursing **(Appendix B, Fig B1)**. Although control and nursing groups also displayed an increase in the CD8 frequencies, these were not significant. Proportions of CD4 T cells were significantly reduced in gestation plus nursing pups compared to controls **(Fig. 5.5A)**. We also found significantly reduced total number of effector memory **(Fig. 5.5B)** and central memory CD4 T cells **(Fig. 5.5C)** in this group. Taken together, our data demonstrates that vancomycin driven modulation of maternal gut microbiome at various phases during gestation and lactation profoundly impacts lung immunity in infants and influences immune response and clinical outcomes to RSV infections.

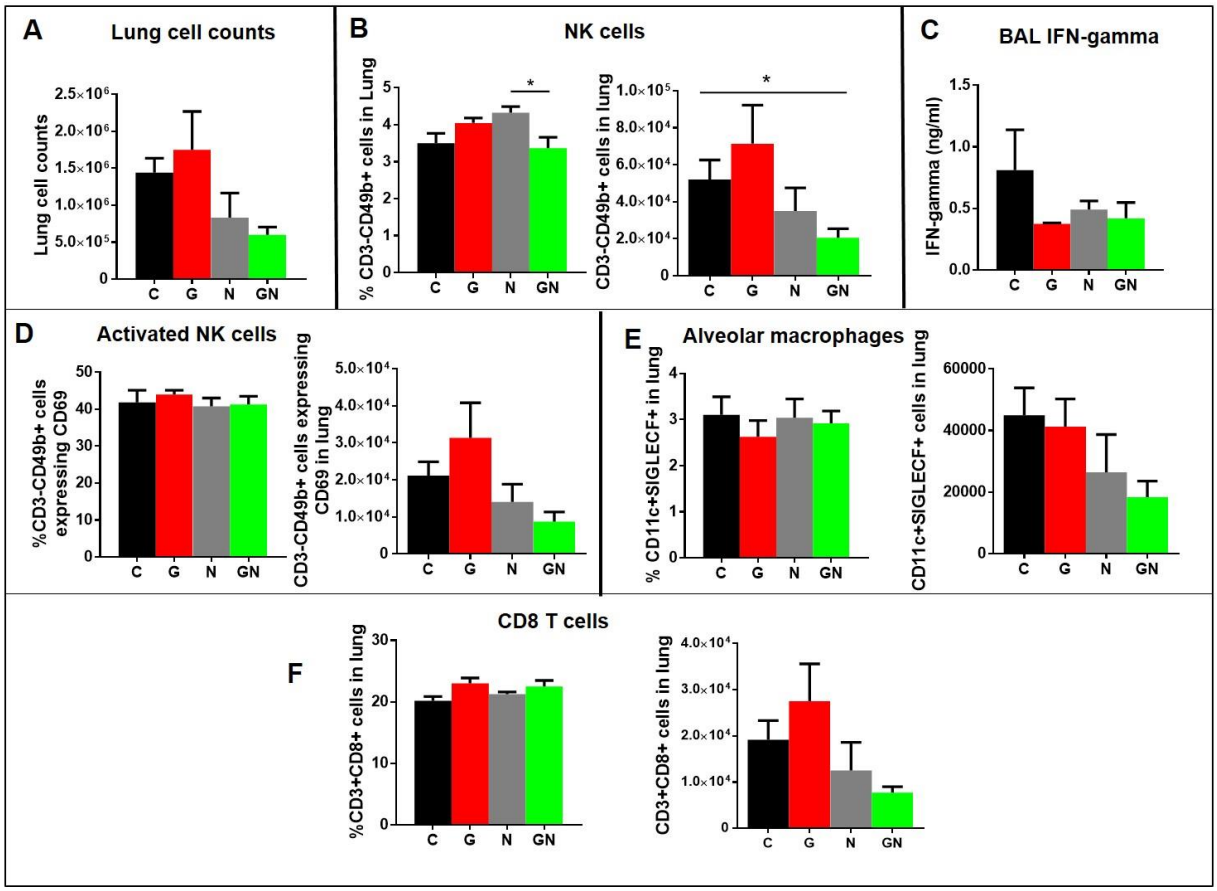


Figure 5.4: Maternal oral vancomycin has no effect on most innate cells in infant lung day 8 post RSV infection. Pups born to vancomycin breeders were infected intranasally with RSV at 21 days and killed at day 8-post infection. Lung samples were harvested and analyzed. Innate immune cells and CD8 T cells were characterized by FACS. **(A)** Total lung counts, **(B)** NK cells (CD3-CD49b+), **(C)** BAL IFN- gamma levels, **(D)** Activated NK cells (CD3-CD49b+CD69+), **(E)** Alveolar macrophages (SIGLECF+CD11c+), **(F)** CD8 T lymphocytes (CD3+CD8+). Data are shown as mean ± SEM. Statistical analysis was performed by Kruskal-Wallis test followed by Mann-Whitney U test. Data are representative of two independent experiments. n=4-6 per group. *p<0.05 and **p<0.01.

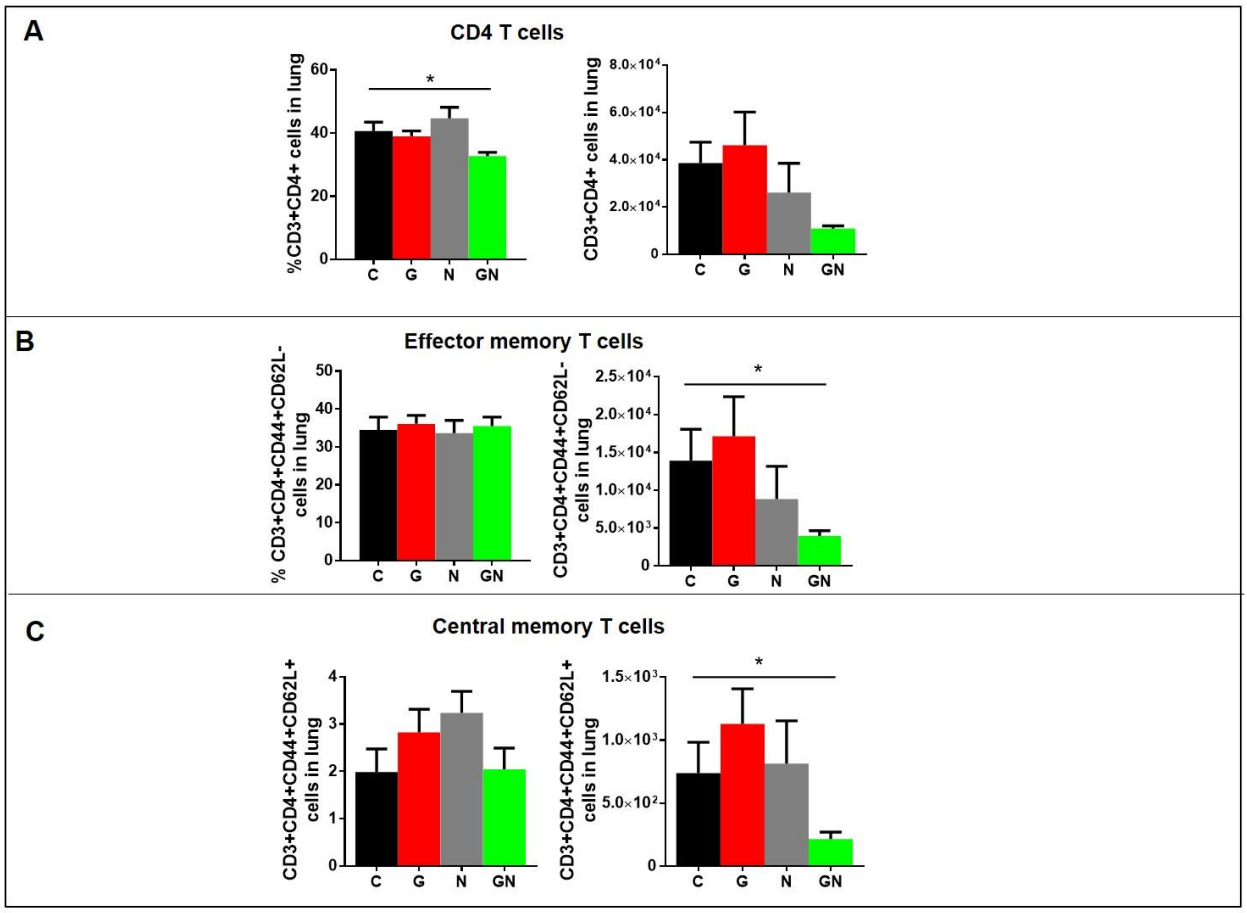


Figure 5.2: **Maternal oral vancomycin induces subtle changes in infant CD4 T cells day 8 post RSV infection**. Pups born to vancomycin breeders were infected intranasally with RSV at 21 days and killed at day 8-post infection. Lung samples were harvested and analyzed. CD4 T lymphocytes were characterized by FACS. **(A)** CD4 T cells (CD3+CD4+), **(B)** Effector memory CD4 T cells (CD3+CD4+CD44+CD62L-) and **(C)** Central memory CD4 T (CD4+CD44+ CD62L+) cells respectively. Data are shown as mean ± SEM. Statistical analysis was performed by Kruskal-Wallis test followed by Mann-Whitney U test. Data are representative of two independent experiments. n=4-6 per group. *p<0.05 and **p<0.01.

5.3 Maternal oral polymyxin B and the infant *N. brasiliensis* model

In this study, we investigated the impact of targeting gram-negative commensals in the maternal gut during gestation and/or lactation on infant ability to control helminth infections. We utilized the *Nippostrongylus brasiliensis* model, which is well developed, in the lab of our collaborator and cosupervisor, Dr Horsnell. In chapter 4, we showed that maternal PMB treatment significantly impacted infant growth, intestinal microbiota and immunity. Here, we interrogated the Th2 arm of

infant adaptive immunity by challenging pups born to polymyxin B breeders at 21 days of age with 250Nb L3 of *N. brasiliensis* and killing the pups 5 days post-infection **(Fig. 5.6A).**

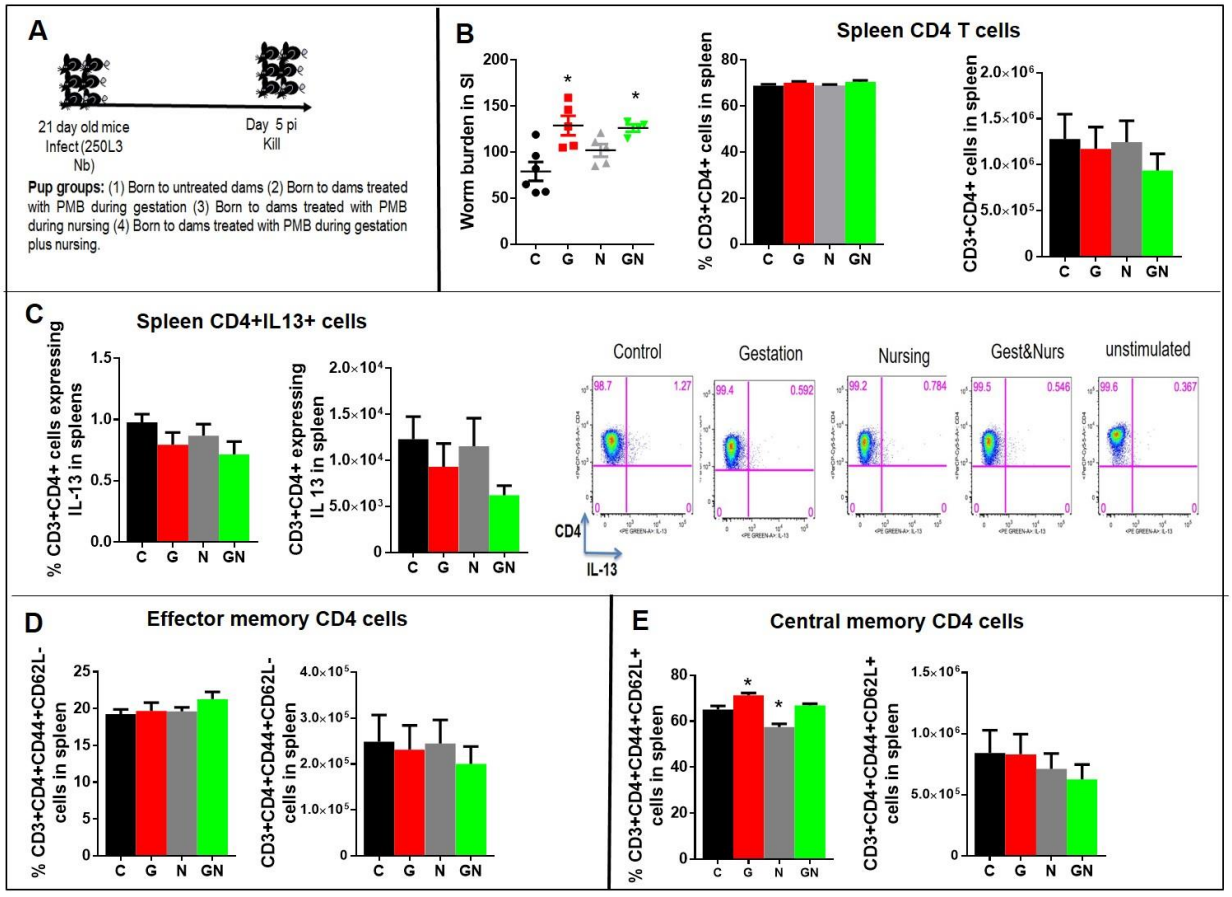


Figure 5.6: Polymyxin B modulation of maternal microbiome impacts offspring immunity to *Nippostrongylus brasiliensis*. (A) Pups born to polymyxin B breeders were infected subcutaneously with 250Nb L3 at 3 weeks of age and killed 5 days post infection, **(B)** Small intestinal worm burden and CD3+CD4+ T cells respectively, **(C)** CD4 T cells expressing IL-13 following PMA/Ionomycin stimulation and representative flow plot showing intracellular staining for IL-13, **(D)** Effector memory CD4 T cells (CD3+CD4+CD44+CD62L-) and **(E)** Central memory CD4 T cells (CD3+CD4+CD44+CD62L-). Intracellular IL-13 production by CD4 T cells following stimulation by PMA (50ng) and Ionomycin (500ng) for 4 hours in the presence of brefeldin A. Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann-Whitney U test. Data are representative of two independent experiments. n=4-8 per group. *p<0.05.

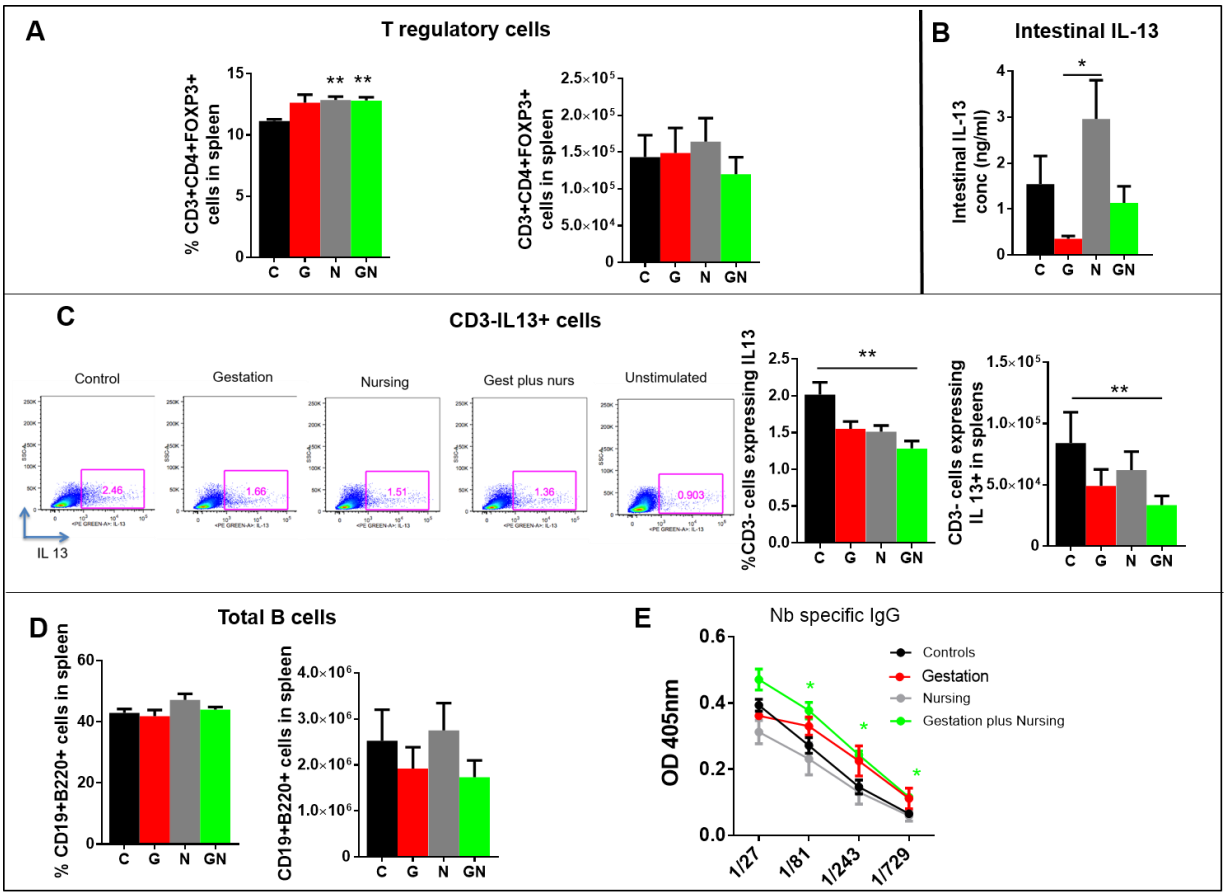


Figure 5.7 Polymyxin B modulation of maternal microbiome impacts IL-13 production in offspring following infection with *Nippostrongylus brasiliensis.* Pups born to polymyxin B breeders were infected subcutaneously with 250Nb L3 at 3 weeks of age and killed 5 days post infection. **(A)** T regulatory cells (CD3+CD4+FOXP3+), **(B)** Intestinal IL-13 concentrations in homogenates, **(C)** CD3- cells expressing IL-13 after PMA/Ionomycin stimulation and representative flow plot for all infant groups and **(D)** Total B cells (CD19+B220+). Graphs are shown as mean ± SEM and data analyzed by Kruskal-Wallis test followed by Mann-Whitney U test. **(E)** Serum Nb specific IgG. Data are representative of two independent experiments. n=4-8 per group. *p<0.05.

Pups born to dams treated with PMB during gestation or gestation plus nursing had significantly higher worm burden in the small intestines compared to infected controls **(Fig. 5.6B).** We found no difference in splenic cellularity across all infected pups. We found no significant difference in both proportions and total cell numbers of CD4+ T cells **(Fig. 5.6B)**, IL-13 producing CD4+ T cells **(Fig. 5.6C)** and effector CD4+ T cells **(Fig. 5.6D)** across all infected pups. Proportions of central memory CD4+ T cells were significantly higher in gestation pups and lower in nursing pups. Although not significant, gestation plus nursing pups had a trend towards increased frequencies of this subset **(Fig. 5.6E).** In addition, proportions of T regulatory cells were significantly increased in nursing and gestation plus nursing pups but there was no difference in total numbers across all groups **(Fig. 5.7A).** Interestingly, we observed substantially reduced intestinal concentrations of IL-13 in gestation and gestation plus nursing pups (susceptible groups) compared to control pups **(Fig. 5.7B).** IL-13 concentrations were significantly lower in gestation pups compared to their nursing peers **(Fig. 5.7B)**. Analysis of IL-13 production by CD3- cells following PMA/Ionomycin stimulation revealed significantly reduced proportions and numbers in gestation plus nursing compared to infected controls. We observed a trend towards a reduction in proportions as well as numbers of CD3-IL-13+ cells in other PMB infant groups **(Fig. 5.7C)**. Finally, we found no difference in proportions and numbers of B cells across all infected pups **(Fig. 5.7D)**. However, we found significantly higher levels of antigen specific IgG in gestation plus nursing pups versus control pups **(Fig. 5.7E)**. Taken together, we show that PMB mediated alterations in maternal microbiota during gestation or the additive antibiotic effect of gestation plus nursing profoundly impacts offspring ability to control *Nb* infections largely by impairing IL-13 production which is central in parasite clearance.

5.4 Discussion

5.4.1 Maternal vancomycin and infant RSV model

Toll like receptor 2 has been shown to control murine immune response to RSV (Murawski et al., 2009) and is activated by soluble peptidoglycans and lipoteichocic acids, main stimulatory components in gram positive bacteria (Schwandner et al., 1999). We tested if depletion of gram positive bacteria (TLR2 activators) in mothers impacted RSV recognition and immunity in her offspring. Pups born to vancomycin treated mothers are susceptible to RSV infections in the early stage of the disease and this phenomenon is maintained throughout the viral pathogenesis in offspring born to dams treated with vancomycin during nursing. Unlike in adult mice, where RSV infection leads to dramatic weight loss, we and others describe a decline in rate of growth in RSV infected pups (Culley et al., 2002). This is expected since healthy pups should gain weight daily. Gestation plus nursing pups revealed a trend towards increased susceptibility similar to the nursing group but recovered day 7-post RSV challenge. When comparing the antibiotic groups, maternal antibiotic exposure during nursing (N, GN pups) led to enhanced susceptibility in offspring as these pups did not catch up with growth patterns of their uninfected controls. Exposure during gestation only (G pups) resulted in a similar phenotype as pups born to mothers who were not treated with vancomycin (C pups). RSV infected pups from these groups began to catch up with growth patterns of their uninfected controls from day 4 onwards. Together, our data reveals that maternal vancomycin exposure during nursing could accelerate RSV-associated failure to thrive in infants. These findings are in agreement with recently published work which demonstrated that maternal antibiotics during gestation and nursing accelerated mortality associated with vaccinia virus infection (Gonzalez-Perez et al., 2016). However, our study is fundamentally different from Gonzalez-Perez's, since our antibiotic intervention was only limited to maternal gut and vancomycin is poorly absorbed and was not detected either in maternal or offspring sera**.** In their

study, they used a mix of clindamycin and penicillin, both of which are absorbed, raising the possibility of these antibiotics being transferred to offspring. Furthermore, in their experiments, antibiotics were administered antepartum and throughout nursing to all dams, therefore, the effect could have been purely via breastmilk antibiotic transfer.

Progressive intestinal colonization in infants has been shown to influence development of mucosal and systemic immune tissues and immune cell populations (Maynard et al., 2012). It is thought that NK cells are central to RSV immunity in the early onset while CD8 T cells seem to enhance RSV pathology (Lambert et al., 2014). Antibiotic groups had significantly lower numbers of total and activated NK cells compared to controls at day 4 post-infection. Consequently, these mice were susceptible to RSV and had an impaired ability to clear the virus. Data shows that mice lacking in microbiota early in life accumulate an invariant natural killer T cell population in the lungs and intestines which increases their susceptibility to IBD and asthma (Olszak et al., 2012). Moreover, antibiotic treatment in adult mice has been shown to provide anti-inflammatory signals by dampening proinflammatory cytokine production (Culic et al., 2001), impairing neutrophil recruitment (Sugihara et al., 1997) and function and altering dendritic cell function (Ishida et al., 2007). Consequently, it is reasonable that antibiotic driven changes in maternal microbiota impacted both numbers and function of infant NK cells following RSV challenge which led to poor viral control in our model. It is possible that reduction of microbial density and diversity among the offspring interfered with the viral recognition process by impairing early innate processes and dendritic cells critical for T cell priming. Importantly, we noted significantly lower microbial diversity (described in chapter 4) in the nursing group which may be a strong contributing factor to RSV susceptibility. Considering that an aberrant microbiome has been shown to impact on DC function at least in adult mice (Ishida et al., 2007), it is likely that infant

DC ability to process RSV was hindered. In our next experiments, we will co-culture lung DCs isolated from control or vancomycin-infant groups with T cells and monitor proliferation as well as ability of the DCs to deliver important TH1 polarizing signal necessary for RSV immunity. Collectively, these events may potentially lead to an impaired antigen presentation to T cells. Our study was tailored towards examining the impact of the intestinal gut commensals on RSV immunity. The microbiota at the site of infection, the lung, would most likely be critical in influencing host immune responses to RSV. The study of the human lung microbiome and the context of pulmonary disease and health is an area of emerging research and it is not clear when the respiratory microbiome is established. A recent report in humans found a diverse airway microbiome at birth regardless of gestational age but older preterm infants with chronic lung disease had a less diverse microbiota, suggesting that dysbiosis in the airway microbiome may set the stage for subsequent lung disease (Lal et al., 2016). A related murine study reported the ability of lung microbiota to promote tolerance to allergens in neonates via PD-1 (Gollwitzer et al., 2014). Therefore, it is conceivable that the lung microbiota may have an immuno-modulatory potential and dictate immunological responses, at least in the lung. Although most bacteria populating infant lung are derived from contact with the mother's flora during delivery, the ability of bacteria to translocate from the infant's own gut to the lung in a gut-lung axis cannot be ruled out. This implies that the less diverse microbiota of infants born to vancomycin treated dams may potentially find their way to the lung. Here, there is a possibility that the microbiota host cross talk makes the lung more susceptible to injury and inflammation and may provide justification for susceptibility to RSV in these pups. Host-microbiota interactions may not be the only factor driving immune development in infants. However, they appear to play an essential role.

At the later stage of RSV, day 8, we think CD8 T cells and not NK cells contributed to viral control in the lung. When we analyzed only the vancomycin infant groups, we noted significant increases in proportions of CD8 T cells in both gestation and gestation plus nursing comparing means at day 4 and day 8. However, the increase in nursing group was not significant which suggests a limited expansion of CD8 T cells in nursing group which are critical for viral control. Beyond expansion in the CD8 pool, neonatal CD8 T cells may already be biased in terms of phenotype differentiation and have been shown to expand rapidly and quickly become terminally differentiated (Smith et al., 2014). Gut dysbiosis in infants is likely to skew this CD8 phenomenon further. Although we did not find any differences in proportions of CD8 T cells at day 8 post RSV, It is likely that intestinal dysbiosis may have led to alteration of immune function in CD8 cells delaying viral clearance in susceptible infant pups. The modulatory effect of gut dysbiosis on the central and effector memory T cells could have critical implications. Others have shown alveolar macrophages to be indispensable in RSV immunity and that interferon gamma stimulation of these cells attenuated weight loss and led to enhanced viral immunity (Eichinger et al., 2015; Empey et al., 2012). We found no significant differences in both alveolar macrophages and concentrations of interferon gamma in our model on day 8 post challenge. The profound differences in immune response to RSV in maternal vancomycin-infants is associated with reduction of bacteria quantity and diversity as well as alteration of taxonomic composition in the gut.

Furthermore, microbial derived metabolites have been shown to attenuate pathology associated with allergic airway indirectly by expanding the T regulatory pool (Zaiss et al., 2015). Infant dysbiotic microbiota in our model may have altered systemic metabolite profile which led to a diminished ability of infants to mount protective and regulatory immune responses to RSV. In addition, we observed microbiota changes unique to the nursing group that may have contributed

to the underlying immunological phenomena. For example, Erysipelotrichiales which have been shown to be highly immunogenic (Kaakoush, 2015) were significantly less abundant only in the nursing pups. In addition, *Staphylococcus* and *Mucispirillum* OTUs which are potentially pathogenic were predominant in the nursing pups at genus level **(chapter 4).** Although we did not investigate the direct effect of these taxa on immunity, the idea that they could be contributing to changes in immunity in these infants permitting effective RSV pathogenesis cannot be ruled out. Furthermore, functional changes because of these microbial biomarkers in this group could also be associated with observed phenotype.

Our data introduce a new paradigm of the relationship between maternal gut microbiota during lactation and offspring immunity to RSV. This to our knowledge is the first time this kind of relationship has been demonstrated using experimental RSV infection in a maternal infant model. We are convinced that disrupting maternal gut microbiota has consequences on infant immunity to RSV. Most studies of this nature have largely been based on association in humans. Our data could provide some useful insights into how alteration of gut composition in human mothers could potentially impact immunity in infants. Future work will evaluate how maternal gut microbiota during pregnancy programs infant lung microbiota and how these microbiota influences infant immunity to RSV.

5.4.2 Maternal PMB and infant *N. brasiliensis* model

In this model, we show offspring born to mothers treated with PMB during gestation or gestation plus nursing to be susceptible to primary *N. brasiliensis* infection. Pups were infected at 21 days of age with *Nb* and killed 5 days after. It has previously been shown that intestinal nematode infection in mice is associated with increase in gram negative bacteria (Enterobacteriaceae and *Prevotella*) in the small intestinal tract as well as the parasite-free large intestines (Rausch et al.,

2013). Moreover, Ramanan et al. showed that helminth infection protect against Crohn's disease in mice by promoting colonization resistance by gram negative (*Bacteroides vulgatus*) bacteria (Ramanan et al., 2016). These studies guided our approach to test the *Nb* model on pups born to dams treated with PMB as the direct role of gram negative bacteria on host immunity to helminths, particularly in infants is not clear. We observed significantly higher worm burden in the gestation or gestation plus nursing pups compared to infected controls. *N. brasiliensis* is shortlived and induce a strong and protective TH2 response which is critical for parasite clearance (Lawrence et al., 1996; Mohrs et al., 2001). IL-4 and IL- 13 are signature cytokines involved in establishment of TH2 immune responses (McKenzie et al., 1998). Early reports revealed that IL-4Rα, IL-13, Stat6 but not IL-4 are required for host protection against Nb (Urban et al., 1998). These cytokines have been shown to act on non-bone marrow derived cells effectively orchestrating helminth immunity (Madden et al., 2002; Shea-Donohue et al., 2001). Importantly, IL-13 has been linked to mucus production which has been positively associated with enhanced expulsion of Nb (McKenzie et al., 1998). When we analyzed the proportions of CD4 T cells capable of producing IL-13 after non-specific stimulation, we observed a trend towards decreasing proportions of CD4 T cells producing IL-13 in pups born to PMB treated dams. Similarly, we found significantly reduced numbers of CD3- cells that expressed IL-13 in gestation plus nursing pups with a trend towards a decrease in gestation pups compared to control pups. Furthermore, we noted significantly reduced concentrations of IL-13 in small intestinal homogenates in gestation or gestation plus nursing pups. Together, our IL-13 data corroborated the intestinal worm burden data, depicting why these infant groups were susceptible to Nb; they were unable to mount an effective TH2 immune response that's imperative for parasite control. This to our knowledge is the first time the role of maternal microbiota during gestation in establishment of TH2 immunity

in infants has been defined. Analysis of taxonomic profiles across all groups showed Enterobacteriales to be significantly less abundant in gestation and gestation plus nursing groups (susceptible to *Nb*). Enterobacteriales have been shown to increase following helminth infection in murine models and to be positively associated with TH2 immunity (Rausch et al., 2013). Reduction in abundance of members in this taxon in susceptible groups could be associated with impaired TH2 immunity. In addition, *Lactobacillus* were significantly increased in gestation and gestation plus nursing pups (susceptible groups). Bacteria members in this genus have previously been shown to exhibit a mutualistic relationship with helminths where they support greater establishment of helminths in the gut (Reynolds et al., 2014). Therefore, it is likely that the preponderance of *Lactobacillus* in the gut of susceptible offspring groups led to a receptive intestinal environment, further supporting pathogenesis of *N. brasiliensis*. Hayes et al described a similar phenomenon where bacteria taxa including *Escherichia coli*, *Staphylococcus* and *Pseudomonas* promoted hatching of *Trichuris muris* eggs (Hayes et al., 2010). Others have demonstrated a central role of TH2 immunity in promoting colonization resistance and altering resident gut composition (Fricke et al., 2015; Ramanan et al., 2016). Our study adopted a different approach where changes in maternal gut bacteria were antibiotic mediated but not helminth driven. Secondly, we utilized a maternal infant model to examine the impact of an altered maternal gut on helminth immunity in offspring making our findings novel as far as infant TH2 immunity is concerned. Future experiments will focus on testing members of order Enterobacteriales in both mothers and offspring to farther our understanding of microbe host crosstalk that drive TH2 immunity in infants. We will also investigate the role of the lung microbiota on helminth immunity and how this may impact other immune populations which are central to helminth immunity such as the innate lymphoid cells 2 (ILC2s) and alveolar macrophages

CHAPTER 6

6.0 RESULTS SECTION III

6.1 Influence of preconception helminth infection on infant gut microbiota and immunity

6.1.1 Introduction

Various factors that influence early gut colonization are derived from the mother (Koenig et al., 2011). Maternal disease and infection status may impact offspring microbiota. For example, taxonomy analysis suggested that the overall bacteria content significantly differed between meconium from babies born to mothers with diabetes versus controls, where Bacteroidetes and *Parabacteroides* were enriched in meconium from diabetes group (Hu et al., 2013). Recently, maternal HIV infections have been shown to influence microbiota in HIV exposed but uninfected infants (Bender et al., 2016). Other maternal infections such as helminths may potentially have a similar impact on offspring gut microbial composition. Helminths are estimated to infect 2 billion people worldwide and susceptibility is increased during pregnancy (Woodburn et al., 2009; Yatich et al., 2009). Furthermore, helminth infections impact the microbiota. For instance, *Heligmosomoides polygyrus (H. polygyrus)* infection in mice led to an increased abundance of *Lactobacillus* and Enterobacterial loads in the gut (Rausch et al., 2013; Reynolds et al., 2014). Interestingly, a mutualistic relationship has been described between commensals and helminths. *H. polygyrus* infection led to an expansion of *Lactobacillus taiwanensis* which allowed for establishment of the parasite and survival in the gut (Reynolds et al., 2014). In addition, the microbiota has been shown to be essential in hatching of *T. muris* eggs (Hayes et al., 2010). Yet whether the interaction between maternal gut bacteria and the intestinal helminths influences gut microbiota of the progeny remains unknown. Aside from maternal infection and disease status, diet is a key determinant of infant gut colonization postpartum (Mackie et al., 1999). Breastmilk

provides the first source of nutrition in infants and leads to transfer of bacteria which are thought to be key in the development of intestinal homeostasis (Beattie & Weaver, 2011). Furthermore, breastmilk helps in shaping the immune response in the progeny. For example, its role in prevention of diseases such as asthma has been elucidated (Verhasselt et al., 2008). Other components such as lactoferrins and defensins inhibit pathogens and further contribute to microbiota colonization. (Morrow & Rangel, 2004). Despite this, our understanding of the impact of maternal helminth infections on the microbial composition in breastmilk is not well developed. In this chapter, we explore the effect of preconception maternal helminth infection on maternal and infant gut microbiota using a mouse model of *Nippostrongylus brasiliensis (Nb).* We also examine how maternal helminth infections impact breastmilk microbiome

6.1.2 RESULTS

6.1.2.1 Preconception *Nippostrongylus brasiliensis* infection alters maternal intestinal microbiome during pregnancy.

We infected seven 6-week-old female mice per group with 500*Nb* L3 subcutaneously, or left them uninfected. Seven days later, all mice were treated with Ivermectin for 7 days to clear the infection (although naturally an immunocompetent host clears Nb infections by day 9). Three weeks post infection, dams were mated and males removed from the cages 8 days after introduction. Eight days after males were isolated from the cages, maternal fecal microbiome during pregnancy was analyzed **(Fig. 6.1A).** We found significantly higher microbial diversity in pregnant previously infected (PI) dams compared to pregnant naïve dams (p=0.0185, ANOVA, **Fig. 6.1B).** Principal coordinate analysis of all samples revealed distinct clustering based on grouping. The largest variation was explained on PC1 (37%) and appeared to be driven by previous *Nb* infection **(Fig. 6.1C).**

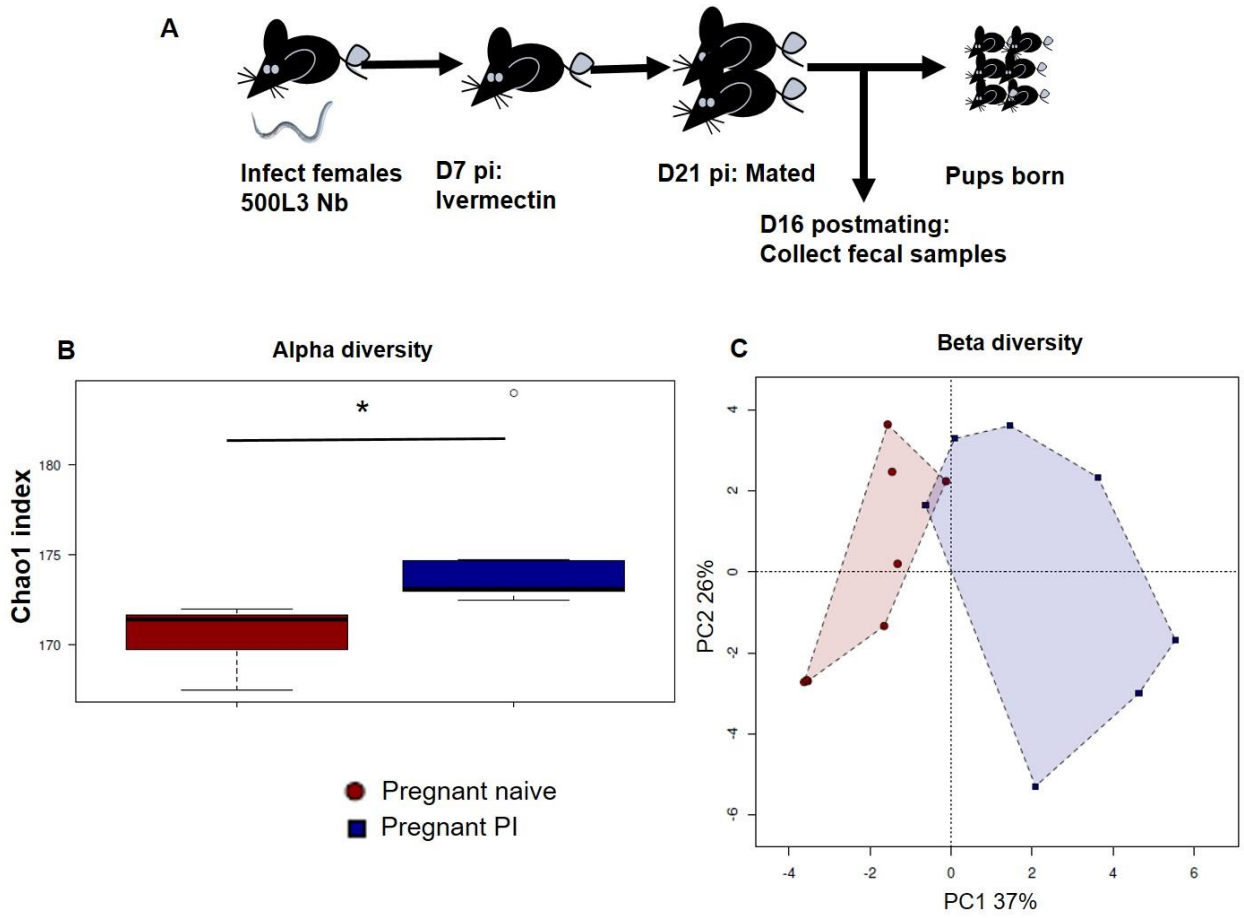


Figure 6.1: **Preconception *Nippostrongylus brasiliensis* infection significantly increases microbial diversity in stool during pregnancy and result in distinct clustering according to beta diversity**. **(A)** Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. Fecal samples were collected from pregnant dams approximately 5 days prior to delivery for microbiome analysis. (B) Alpha diversity as measured by chao1. (C) Principal coordinate analysis showing bray curtis distances of maternal microbial communities. Data are representative of two independent experiments. PI – Previously infected. n=6-7 per group. *p<0.05, **p<0.01, ***p<0.001.

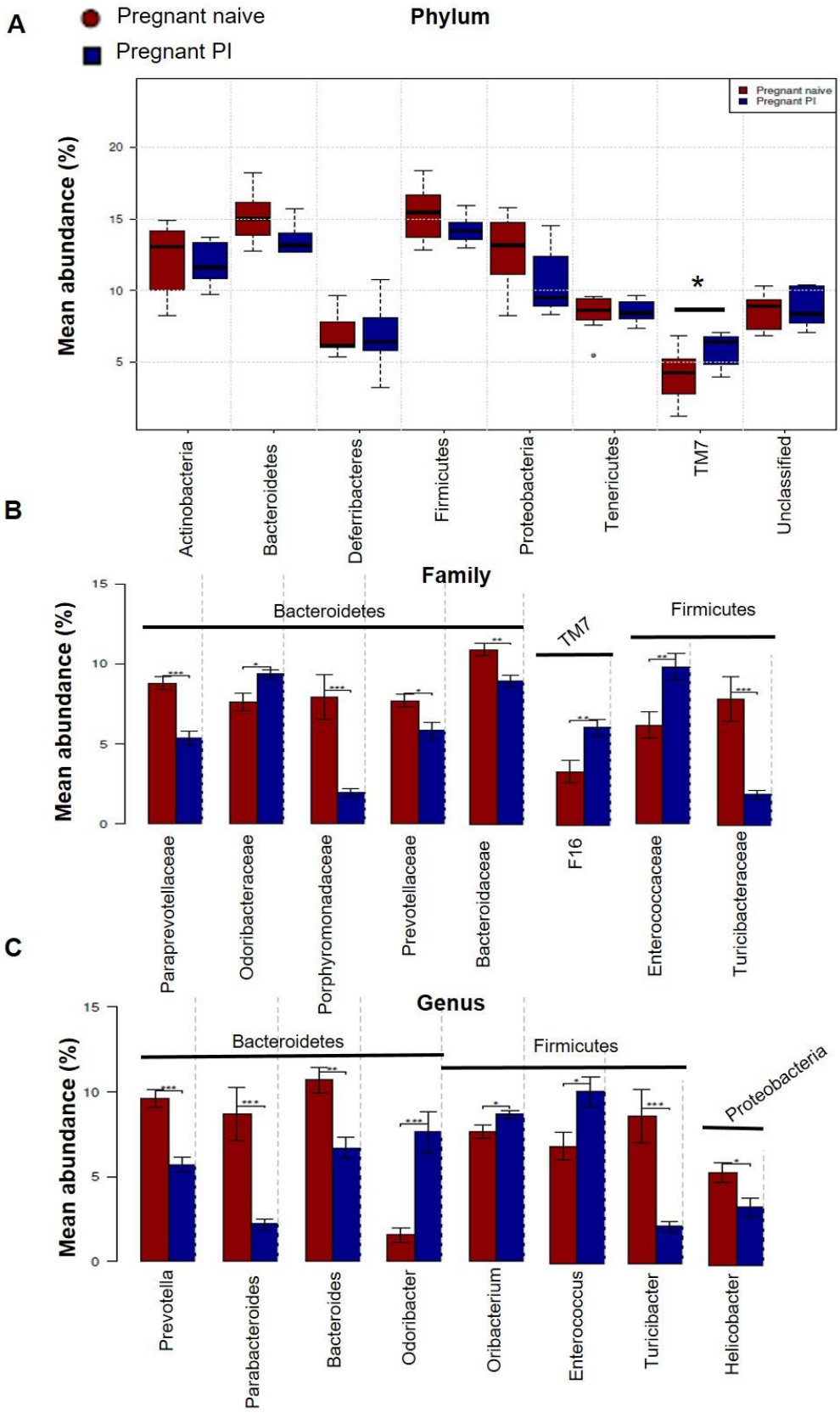

A
Pregnant naive
Pregnant PI
Phylum
Mean abundance (%)
Actinobacteria
Bacteroidetes
Deferribacteres
Firmicutes
Proteobacteria
Tenericutes
TM7
Unclassified
B
Family
Bacteroidetes
TM7
Firmicutes
Paraprevotellaceae
Odoribacteraceae
Porphyromonadaceae
Prevotellaceae
Bacteroidaceae
F16
Enterococcaceae
Turicibacteraceae
C
Genus
Bacteroidetes
Firmicutes
Proteobacteria
Prevotella
Parabacteroides
Bacteroides
Odoribacter
Oribacterium
Enterococcus
Turicibacter
Helicobacter

Figure 6.2: Preconception *Nippostrongylus brasiliensis* infection significantly impacts maternal stool microbiota during pregnancy. Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. Fecal samples were collected from pregnant dams approximately 5 days prior to delivery for microbiome analysis. Mean bacteria abundance at **(A)** Phylum level, **(B**) Class level and **(C)** Genus level. Analysis by ANOVA with FDR adjustment for multiple comparisons. Error bars show SEM. Data are representative of two independent experiments. PI – Previously infected. n=7-8 per group. *p<0.05, **p<0.01, ***p<0.001.

At the phylum level, we reported significantly higher abundance of TM7 in pregnant PI dams compared to naïve pregnant dams (p=0.043, FDR=0.22). There was no difference in abundance of Bacteroidetes between the two dam groups (p=0.054, FDR=0.22, **Fig. 6.2A**). Our analyses at the family level showed Odoribacteraceae, F16 and Enterococcaceae to be significantly increased in pregnant PI dams compared to pregnant naïve dams (p=0.0092, 0.0055 and 0.0056, FDR=0.034, 0.024 and 0.024 respectively). Prevotellaceae, Turicibacteraceae and Bacteroidaceae were predominant in pregnant naïve dams **(Fig. 6.2B).** At genus level, *Oribacterium*, *Odoribacter* and *Enterococcus* dominated fecal microbiota of pregnant PI dams while *Turicibacter*, *Prevotella*, *Helicobacter* and *Bacteroides* were enriched in naïve controls **(Fig. 6.2C).** Together, these data demonstrated that maternal *N. brasiliensis* infections alters maternal gut microbiota during pregnancy. This raises questions as to whether helminth driven modulations of intestinal microbiota during pregnancy potentially have repercussions on the offspring gut microbial colonization and immunity.

6.2 Influence of *Nippostrongylus brasiliensis* on breastmilk microbiota

The human milk provides the first source of nutrition for the infant and contains optimal ingredients required for growth and development. In addition, breastmilk educates the developing immune system and confers a certain degree of protection against gastrointestinal and other infections. Therefore, we next asked if the impact of preconception helminth infections on the microbiota goes beyond the gut by examining the impact of helminth infections prior to pregnancy on breast milk microbiome. Given the difficulty in obtaining milk from female dams, we collected breast milk from the pup's stomachs 14 days post-partum **(Fig. 6.3A).** At this time point, pups are still exclusively breastfeeding and are not taking any other solid food or liquids. When we analyzed breastmilk microbiota, we found no significant difference in alpha diversity between breastmilk obtained from naïve versus PI dams (p=0.62, ANOVA, **Fig. 6.3B**). However, there was distinct clustering of the OTUs in breast milk based on grouping. The highest variability of 42% was explained on PC1 and was associated with previous *Nb* infection in mothers **(Fig. 6.3C)**. Proteobacteria were increased in milk from naïve dams compared to PI dams but the difference did not reach statistical significance (p=0.094, FDR=0.22). There was no significant difference in abundance of Actinobacteria, Bacteroidetes and Firmicutes **(Fig. 6.4A).**

At the family level, we noted a significant increase in abundance of Paraprevotellaceae, S24-7, Rikenellaceae, Prevotellaceae, Erysipelotrichaceae, Enterobacteriaceae and Coriobacteriaceae in breastmilk obtained from PI dams compared to naïve dams. Other families including Corynebacteriaceae and Porphyromonadaceae were abundant in breastmilk from naïve dams **(Fig. 6.4B)**. Similarly, at genus level, *Prevotella, Parabacteroides, Collinsella, CF231, Klebsiella* and *Allobaculum* dominated breastmilk microbiome in PI dams while *Sporosarcina, Porphyromonas* and *Corynebacterium* were enriched in breastmilk from naïve **(Fig. 6.4C)**. Altogether, we show

here that preconception *Nb* infections impact breastmilk microbiota and that these microbiota is unique from fecal microbiota.

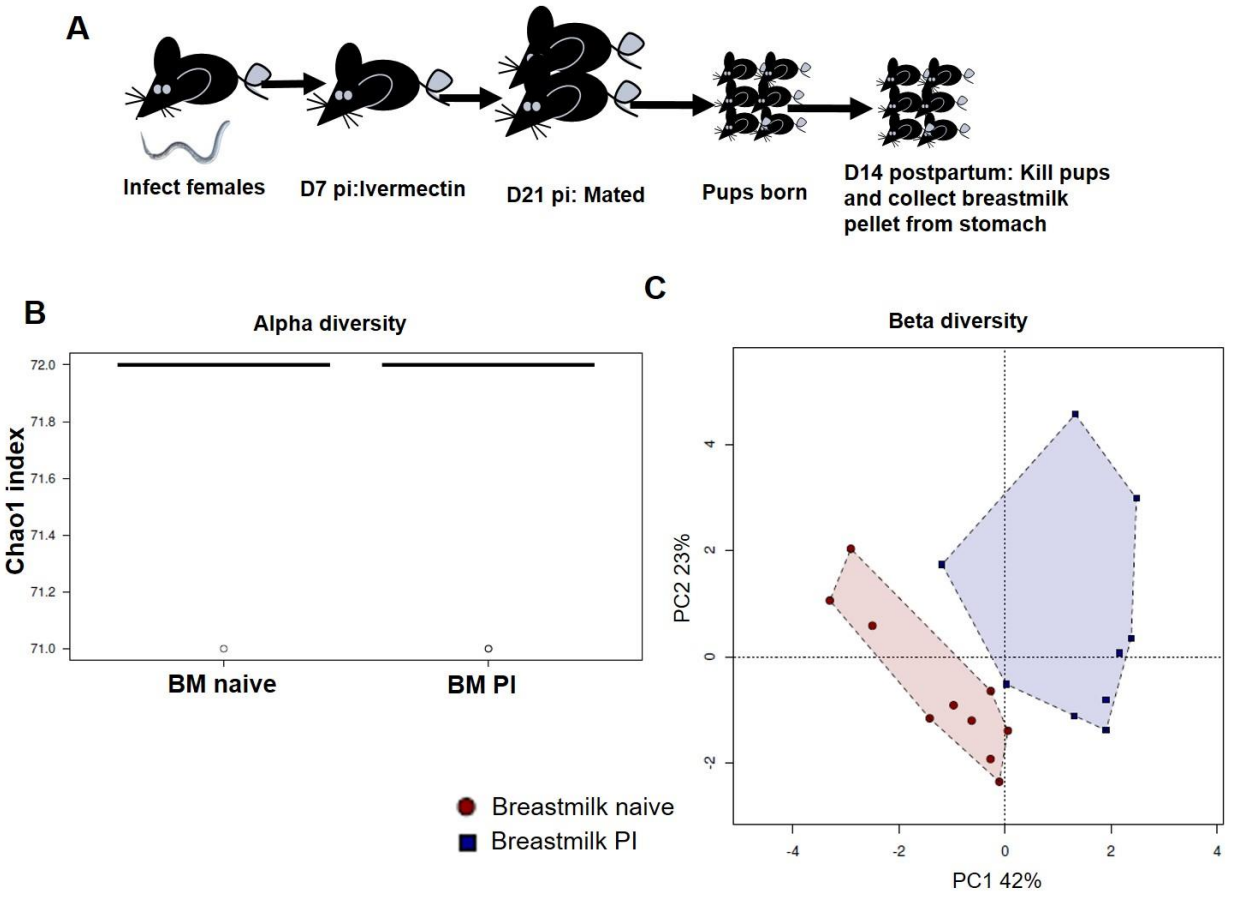


Figure 6.3: Preconception *Nippostrongylus brasiliensis* infection has no impact on microbial diversity in breastmilk but results in distinct clustering according to beta diversity. (A) Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. 14days postpartum, pups were killed and breastmilk pellet collected from their stomach and used for microbiome analysis. **(B)** Alpha diversity in breastmilk microbiota measured by chao1. **(C)** Principal coordinate analysis by bray Curtis distance of breastmilk microbiota. Data representative of two independent experiments. PI – Previously infected. n=7-10 per group. *p<0.05, **p<0.01, ***p<0.001.

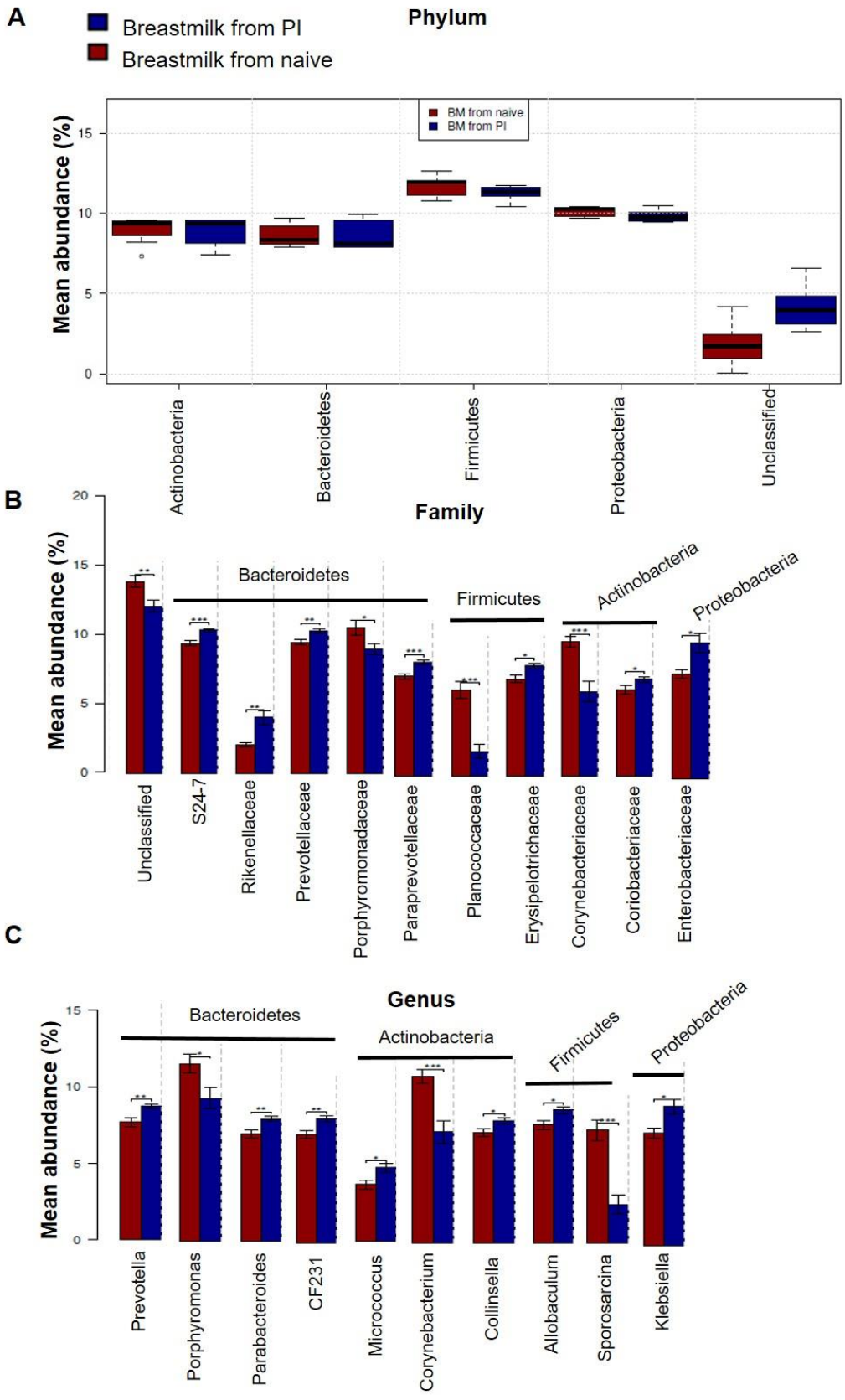

A
Breastmilk from PI
Breastmilk from naive
Phylum
BM from naive
BM from PI
Mean abundance (%)
0
5
10
15
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Unclassified
B
Family
Mean abundance (%)
0
5
10
15
20
Bacteroidetes
Firmicutes
Actinobacteria
Proteobacteria
Unclassified
S24-7
Rikenellaceae
Prevotellaceae
Porphyromonadaceae
Paraprevotellaceae
Planococcaceae
Erysipelotrichaceae
Corynebacteriaceae
Coriobacteriaceae
Enterobacteriaceae
C
Genus
Mean abundance (%)
0
5
10
15
Bacteroidetes
Actinobacteria
Firmicutes
Proteobacteria
Prevotella
Porphyromonas
Parabacteroides
CF231
Micrococcus
Corynebacterium
Collinsella
Allobaculum
Sporosarcina
Klebsiella

Figure 6.4: Preconception *Nippostrongylus brasiliensis* infection impacts breastmilk microbiota. Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. 14days postpartum, pups were killed and breastmilk pellet collected from their stomach and used for microbiome analysis. Mean bacteria abundance at **(A)** Phylum level, **(B**) Class level and **(C)** Genus level. Analysis by ANOVA with FDR adjustment for multiple comparisons. Error bars show SEM. Data are representative of two independent experiments. PI – Previously infected. n=7-8 per group. *p<0.05, **p<0.01, ***p<0.001.

6.3 Preconception *Nippostrongylus brasiliensis* infections influence offspring intestinal microbiota and immunity

Having established that prior *Nb* infections alter both maternal intestinal microbiota during pregnancy as well as breast milk microbiota and that alterations were not similar in these sites, we sought to investigate whether changes in maternal microbiome in the two sites, influence gut colonization in her offspring. As before, female mice were infected with 500Nb L3. The control group was not infected. A week after the infection, all mice (infected and controls) were treated with Ivermectin in drinking water for 7 days to clear the infection (10mg/ml). Mice were rested for a week post Ivermectin treatment, after which they were mated. We analyzed fecal microbiota in pups born to previously helminth infected or naïve dams at day 14 of life **(Fig. 6.5A).** Fecal samples were collected from the colons of individual pups so as not to confuse maternal and pup stool, and microbiome analysis conducted using the 16S DNA sequencing approach as before. Throughout these experiments, parents were maintained on the same diet of normal chow. Hence any changes observed in offspring intestinal microbiota or immunity were solely because of preconception Nb infections in dams. We observed no significant difference in alpha diversity in pups born to previously infected (PI) mothers compared to controls (**Fig. 6.5B**). However, we noted distinct clusters between the two groups as revealed by principal coordinate analysis of infant fecal microbiota at day 14 suggesting profound differences in the microbiota **(Fig. 6.5C).** Indeed 37% of total variability was explained by PC1 and could be attributed partly to previous

maternal infections. At phylum level, we found a trend towards higher abundance of Bacteroidetes in offspring born to PI dams while Firmicutes were reduced but these differences did not reach statistical significance **(Fig. 6.6A)**. Analysis of predominant bacteria taxa at family displayed significantly higher abundance of Mycoplasmataceae, Micrococcaceae and Coriobacteriaceae in pups born to PI dams (p=0.035, 0.0012 and 0.046, FDR=0.091, 0.016 and 0.11 respectively, **Fig. 6.6B**). At genus level, *Adlercreutzia* and *Arthrobacter* were enriched in pups born to PI dams while *Streptococcus, Ruminococcus, Blautia* and *Corynebacterium* were predominant in pups born to naïve dams **(Fig. 6.6C)**. In a separate experiment to investigate the period in which microbiota transfer occurs from mother to offspring, we transferred pups born to PI dams to be nursed by lactating naïve mothers and those born to naïve dams to lactating PI mothers, 3 days after delivery. We analyzed stool microbiota at 14 days as before. Principal coordinate analysis demonstrated that microbiota from PI dams to their offspring were transferred both transplacentally and while breastfeeding **(Appendix C, Fig. C1)**. The key microbiota altered in maternal gut, breastmilk and offspring following maternal *Nb* infection are summarized in **table C2, Appendix C**. Taken together, we show that preconception *N. brasiliensis* infections impacted composition of bacteria colonizing the infant gut. This can be attributed at least, in part to the impact of helminth infection on both maternal gut and breastmilk microbiome. To our knowledge, this is the first time the relationship between preconception helminth infections and offspring gut microbiota has been described.

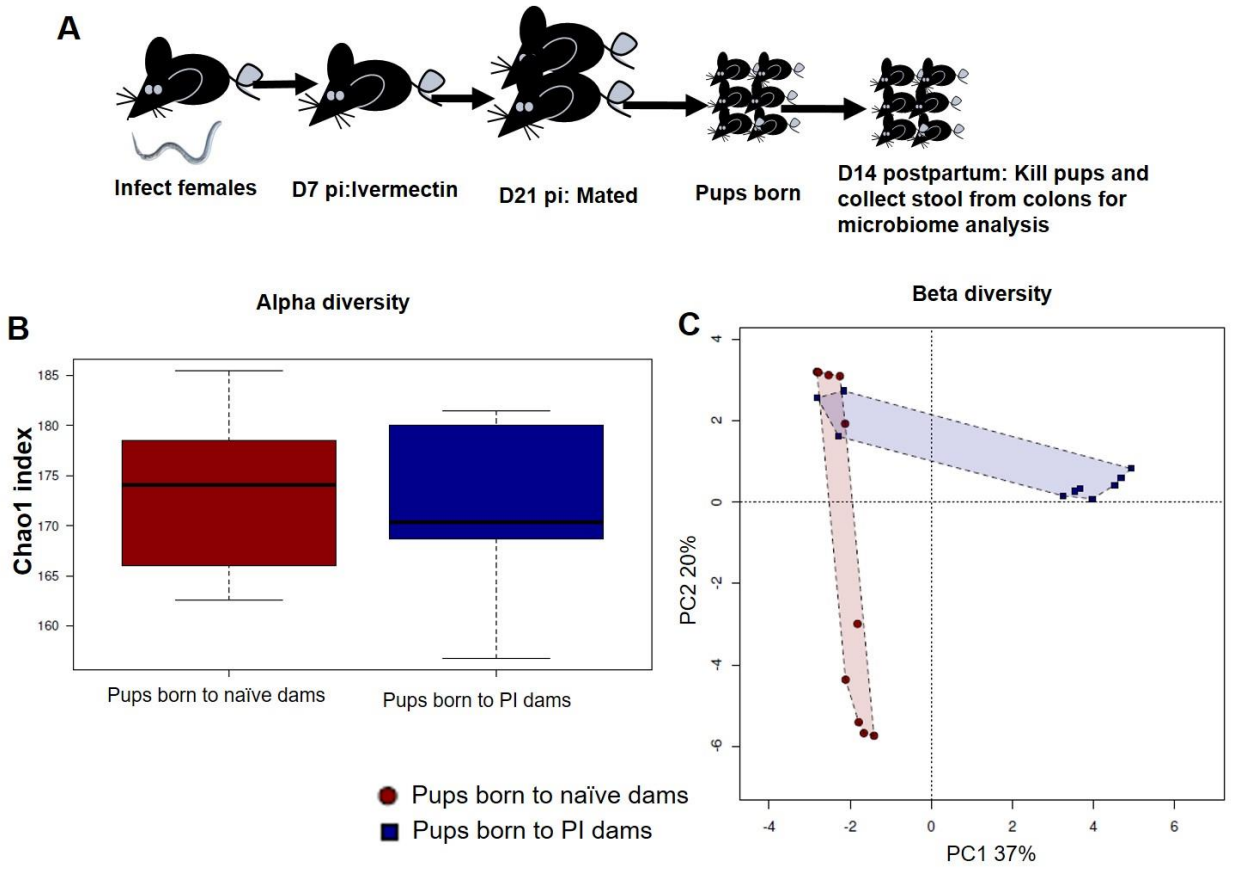
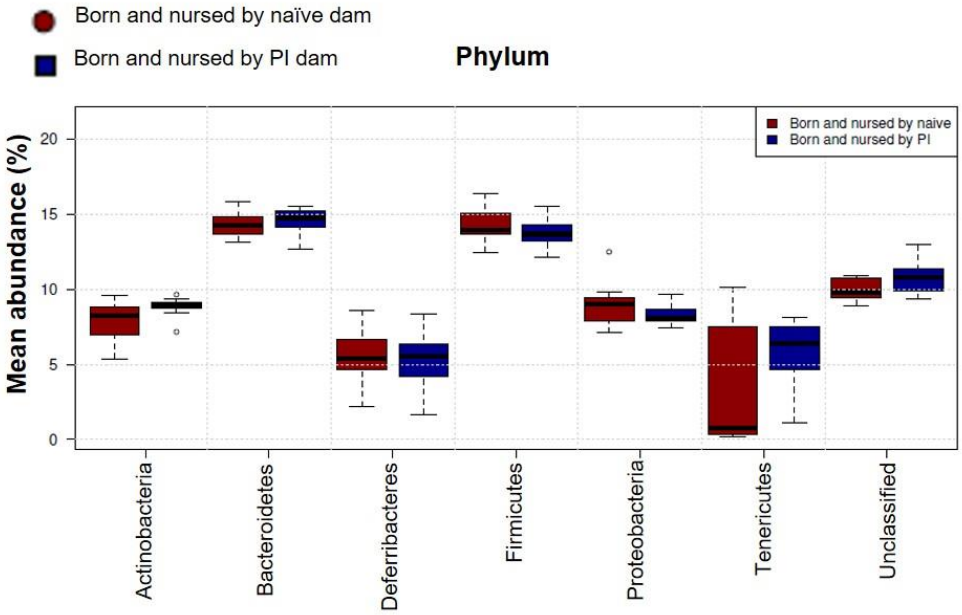
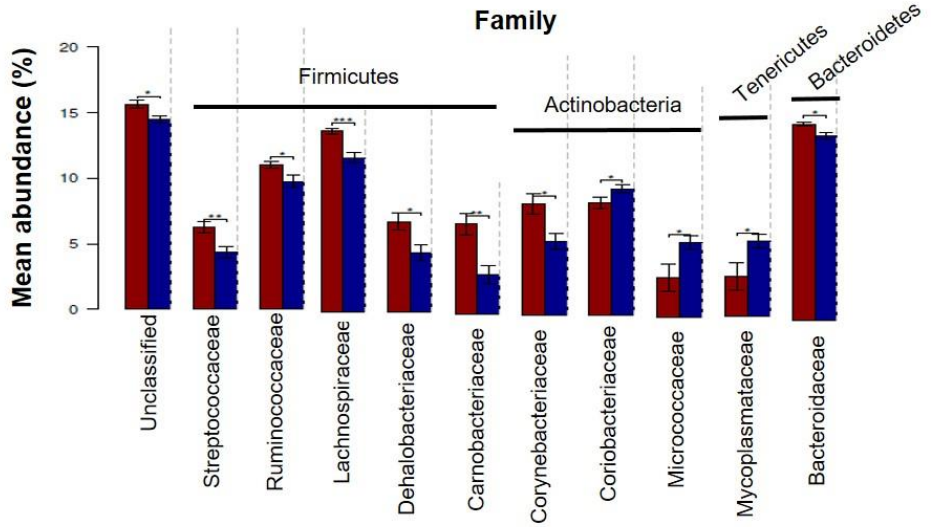


Figure 6.5: Preconception *Nippostrongylus brasiliensis* infection has no impact on offspring stool microbial diversity but leads to distinct clustering by grouping according to beta diversity. (A) Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. 14days postpartum, pups were killed and fecal samples collected from their colons for microbiome analysis. **(B)** Chao1 alpha diversity of infant microbiota. **(C)** Principal coordinate analysis of infant microbiota by bray curtis distance. Data are representative of two independent experiments. PI-Previously infected. n=10 per group. *p<0.05, **p<0.01.

A


Born and nursed by naïve dam
Born and nursed by PI dam
Phylum
Born and nursed by naive
Born and nursed by PI
Mean abundance (%)
0
5
10
15
20
Actinobacteria
Bacteroidetes
Deferribacteres
Firmicutes
Proteobacteria
Tenericutes
Unclassified

B


Family
Mean abundance (%)
0
5
10
15
20
Firmicutes
Actinobacteria
Tenericutes
Bacteroidetes
Unclassified
Streptococcaceae
Ruminococcaceae
Lachnospiraceae
Dehalobacteriaceae
Carnobacteriaceae
Corynebacteriaceae
Coriobacteriaceae
Micrococcaceae
Mycoplasmataceae
Bacteroidaceae

C

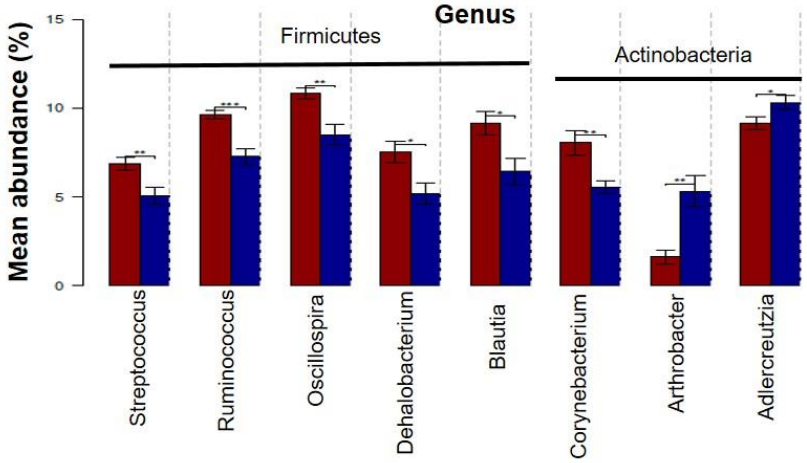

Genus
Mean abundance (%)
0
5
10
15
Firmicutes
Actinobacteria
Streptococcus
Ruminococcus
Oscillospira
Dehalobacterium
Blautia
Corynebacterium
Arthrobacter
Adlercreutzia

Figure 6.6: Preconception *Nippostrongylus brasiliensis* infection impacts gut microbiota in infant 14 days postpartum. Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. 14days postpartum, pups were killed and fecal samples collected from their colons for microbiome analysis. Mean bacteria abundance at **(A)** Phylum level, **(B)** Family level and **(C)** Genus level. Analysis by ANOVA with FDR adjustment for multiple comparisons. Error bars show SEM. Data are representative of two independent experiments. PI – Previously infected. n=10 per group. *p<0.05, **p<0.01, ***p<0.001.

Since microbial colonization of the infant gut is crucial for immune development, we also analyzed pup immunity. We set up the experiment as before and examined cells in the spleen and mesenteric lymph node (MLNs) 14 days postpartum **(Fig. 6.7A)**. There were significantly higher total cell numbers in spleens of pups born to PI dams **(Fig. 6.7B).** Moreover, we found significantly higher total numbers of CD3+CD4 cells, activated CD4 T (CD3+CD4+CD44hi) cells as well as T regulatory cells (CD3+CD4+FoxP3+) **(Fig. 6.7C, D and E)**. When we stimulated total cells with PMA and Ionomycin, we noted a significantly higher proportion of CD4+ T cells expressing IL-13 in offspring born to PI mothers when compared to those born to naïve mothers **(Fig. 6.7F).** Similarly, total B cells were significantly higher in offspring born to PI dams when compared to naïve dams **(Fig. 6.7G)**. Unexpectedly, we did not find any difference in the proportion and total numbers of cellular populations in the MLNs **(Fig. 6.8)**. Altogether, our immunological data provides evidence of early activation of adaptive immunity in the spleens of pups born to PI dams. These pups also have unique microbiota compared to those born to naive dams which raises the possibility of microbiome involvement either directly or indirectly in the early changes in immunity.

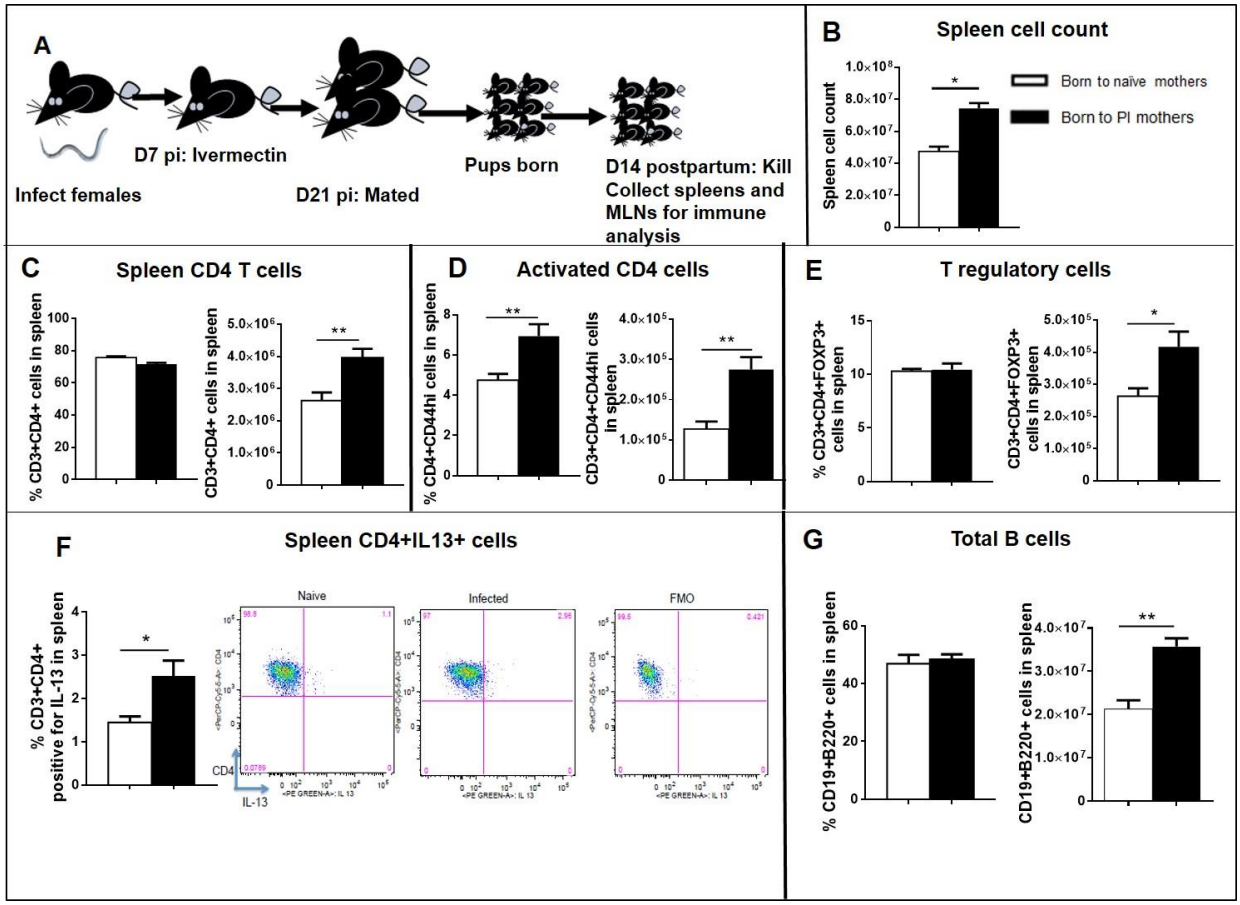


Figure 6.7: Preconception *Nippostrongylus brasiliensis* infection alter spleen immune populations in infants. (A) Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. 14days postpartum, pups were killed. Spleens were collected and immune populations characterized by FACS. **(B)** Total cell counts in spleen, **(C)** CD4 T cells (CD3+CD4+), **(D)** Activated CD4 T cells (CD4+CD44hi), **(E)** T regulatory cells (CD3+CD4+FOXP3+), **(F)** CD4 T cells expressing IL-13 following PMA/Ionomycin stimulation for 4 hours and **(G)** Total B cells (CD19+B220+). Graphs are shown as mean ± SEM and analyzed by Mann Whitney U test. Data are representative of two independent experiments. n=5-6 per group. PI- previously infected. *p<0.05, **p<0.01.

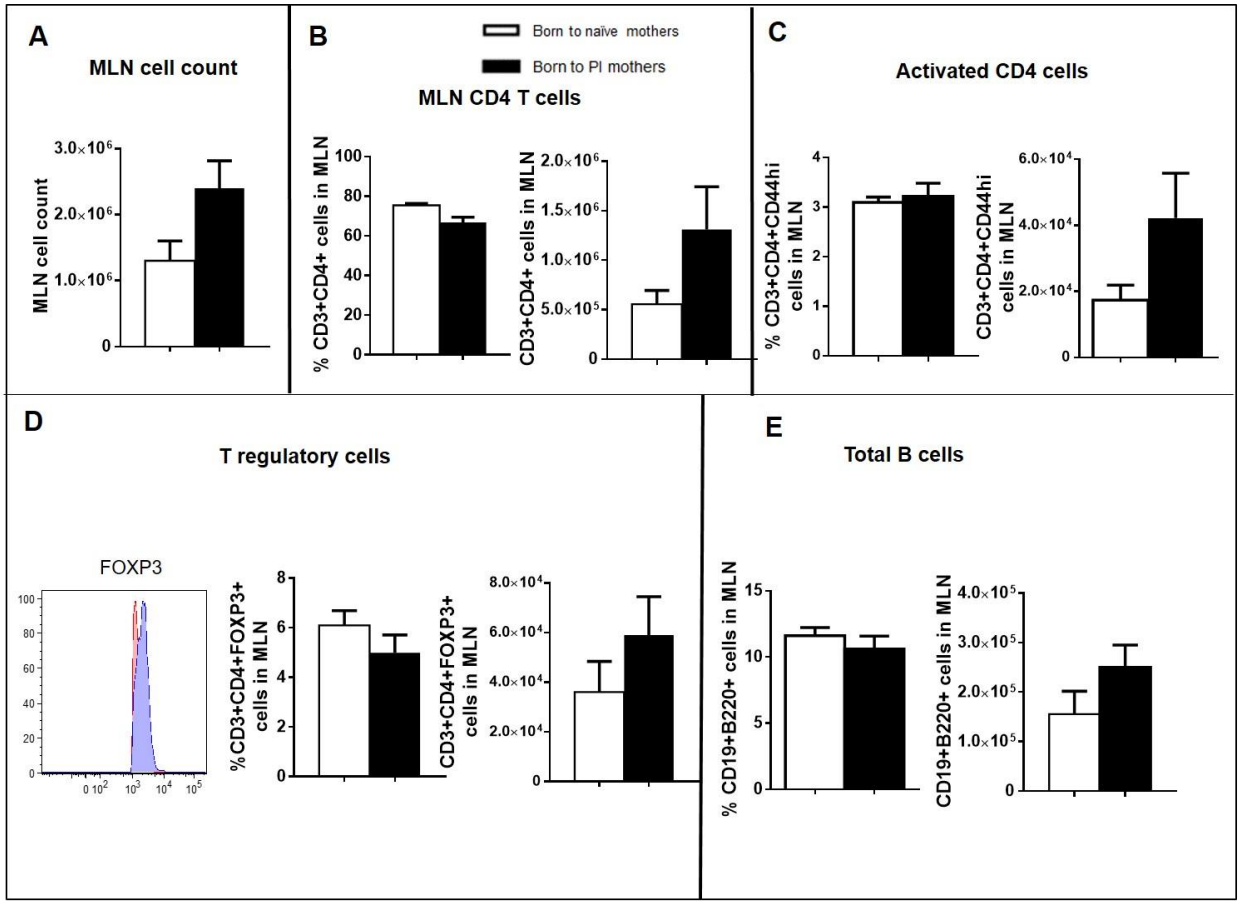


Figure 6.8: Preconception *Nippostrongylus brasiliensis* infections does not significantly alter immune populations in MLNs in the offspring. Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. 14days postpartum, pups were killed. Mesenteric lymph nodes were collected and immune populations characterized by FACS. **(A)** Total cell counts in MLNs, **(B)** CD4 T cells (CD3+CD4+), **(C)** Activated CD4 T cells (CD4+CD44hi), **(D)** T regulatory cells (CD3+CD4+FOXP3+), **(E)** Total B cells (CD19+B220+). Graphs are shown as mean ± SEM and analyzed by Mann Whitney U test. Data are representative of two independent experiments. n=5-6 per group. PI- previously infected.

6.4 Discussion

In this chapter, we have characterized how preconception helminth infections impact intestinal microbiota of dams and pups using a murine model of *Nippostrongylus brasiliensis*. Although we treated both control and experimental mice with Ivermectin 7 days post infection, we were still able to pick up differences in maternal microbiota during pregnancy suggesting that helminths induce immediate and lasting changes in resident bacteria community in the gut. By the time of mating, dams presumably had no worms residing in their gut, yet likely had Th2 immune memory to Nb infection. Previous studies have reported an impact of helminth infections on microbiota in both murine and human subjects (Cooper et al., 2013; Fricke et al., 2015; Lee et al., 2014; Rausch et al., 2013; Reynolds et al., 2014).

However, to our knowledge, this is the first report that investigates the impact of helminth infections on microbiota during pregnancy. In terms of compositional changes, we observed trends towards a decrease in abundance of Firmicutes, Bacteroidetes and Actinobacteria in pregnant PI dams. These findings contradict those of Fricke and colleagues who observed an increased abundance of Bacteroidetes and Actinobacteria in *N. brasiliensis* infected mice (Fricke et al., 2015). Considering that *N. brasiliensis* was used in both studies, it is possible that the timing of microbiota analysis could have driven these changes. While we analyzed the microbiota in pregnant dams, Fricke et al. monitored microbiota in non-pregnant mice. Indeed data shows that the microbiota undergoes profound changes during pregnancy (Koren et al., 2012). It is possible that changes in PI dams occurred as a result of hormonal changes during pregnancy.

When we analyzed breast milk microbiota, we noted a significantly higher abundance of Paraprevotellaceae and Prevotellaceae in dams that were PI compared to naïve dams. However, these families were significantly reduced in the gut of these dams during pregnancy. This suggests that the gut microbiota during pregnancy does not necessarily mirror breastmilk microbiota and that some *Nb* driven changes could be partly site specific. Conversely, we noted significantly less abundance of Porphyromonadaceae in both the gut during pregnancy of PI dams and breastmilk from the same group indicating that some taxa were present in similar levels at both sites. This could further point to the existence of a gut-breastmilk microbiome axis, whereby alterations in the gut lead to alterations in breastmilk microbiome. Breastfeeding has been shown to be critical in primary prevention of asthma and allergic diseases (Friedman & Zeiger, 2005). In addition, helminth infections modulate allergic inflammation by regulating Th2 responses (Zaiss et al., 2015). It is possible that helminth driven changes of breastmilk microbiota lead to transfer of regulatory immune components which dampens excessive Th2 responses associated with allergy in infants.

There is growing appreciation that commensal microbes may contribute fundamentally to the growth, development and immune maturation of infants and may have lasting consequences (Cox et al., 2014; Hong et al., 2010; Nobel et al., 2015; Sjögren et al., 2009). Emerging studies have challenged the dogma of a sterile uterine environment and demonstrated presence of bacteria in fetal membranes (Jones et al., 2009), amniotic fluid (DiGiulio et al., 2008), meconium (Hu et al., 2013) and recently the placenta (Aagaard et al., 2014; Collado et al., 2016) suggesting that microbial colonization may occur prior to delivery. Previous reports revealed that maternal diabetes impacted the microbial composition in meconium, suggesting that maternal disease and infection could impact bacterial composition in her offspring (Hu et al., 2013). We used a similar

approach to analyze the impact of helminth infections prior to pregnancy on offspring gut microbiota. We noted dramatic differences in offspring gut microbiota in infants born to PI dams compared to uninfected dams. *Arthrobacter* and *Adlercreutzia* were significantly elevated in pups born to PI dams. These genera were not detected in maternal gut or breastmilk indicating the existence of other sources of microbiota colonisation in infants. Moreover, *Odoribacter, Oribacterium* and *Enterococcus* were predominant in the maternal gut of PI dams but these were undetected in the offspring gut. We observed a similar phenomenon with *Klebsiella, Collinsella* and *Allobaculum* which were enriched in breastmilk microbiota of PI dams. These trends and associations suggest that gut colonization in offspring following preconception helminth infections does not necessarily mirror maternal gut or breast milk microbiome. Our microbiome data in **chapter 4** where we looked at trends in gut bacteria in offspring having treated parents with antibiotics also revealed a consistent phenomenon. When we analyzed the period in which most microbiota are transferred to offspring, PCA analysis revealed that both *in utero* and nursing were important phases for microbiome transfer suggesting that microbiota-dependent helminth immune modulation may begin *in utero* and continue while breastfeeding. Our next experiments will investigate whether *Nb* induced changes in maternal microbiome can be transferred by cohousing and if this *Nb*-induced microbiota has a role in infant immunity to helminths. Others have shown that *H. polygyrus i*nduced changes in intestinal microbiota protect against allergy and transfer of *H. polygyrus*-modified microbiota alone by cohousing was sufficient to mediate protection against allergic asthma (Zaiss et al., 2015). Analysis of offspring immunity in the spleens revealed early activation of adaptive immune system in offspring born to infected dams. We noted significantly increased total numbers of CD4+ T cells, activated CD4+ T cells (CD4+CD44hiCD62Llo), T regulatory cells (CD4+FoxP3+) as well as B cells in spleens. Our data is consistent with

unpublished work from the Horsnell's lab that has observed a similar phenomenon in offspring born to *Nb* infected mothers. Although our analysis is based on association, we think that *Nb* driven changes in maternal microbiota, which are transferred to offspring during delivery and lactation, could be involved in early induction of adaptive immunity and aid in establishment of anti-helminth immunity among her progeny. It is also possible that microbiota changes in offspring as a result of maternal helminth infections could be involved in early immune programming. Our future studies will investigate this hypothesis further in causality models. We will utilize gnotobiotic models to examine the immune impact of *Adlercreutzia* and *Arthrobacter* which we have identified as microbial markers for offspring born to PI dams.

Overall, our data describes in detail for the first time, changes in gut microbiota in dams during pregnancy and associated outcomes in offspring microbiota. We show conclusively that maternal infections with *Nb* prior to pregnancy alters gut microbiome and diversity in dams. Beyond the gut, *Nb* infections also impact breast milk microbiota. Moreover, preconception *Nb* infections influence gut composition in offspring at 14 days of life at least in murine hosts.

CHAPTER 7

7.0 Conclusion and future work

7.1 Summary of results

The gut microbiota plays a significant role in maintaining immune homeostasis and ability to combat disease by programming the immune system (Atarashi et al., 2011; Nistal et al., 2012; Nylund et al., 2014). The composition of early bacteria colonizing the infant gut is largely dependent on the maternal-offspring transfer of microbiota. It has historically been assumed that the fetus is sterile and that colonization of the infant gut only begins after delivery. However, recent studies have identified bacteria in fetal membranes (Jones et al., 2009) and placenta of healthy pregnancies (Aagaard et al., 2014) suggesting that gut colonization could begin prior to delivery. Regardless of *in utero* exposure, massive bacterial colonization begin at birth upon exposure to vaginal, skin or environmental microbiota (Dominguez-Bello et al., 2010). Some of the factors that drive profound shifts in offspring microbiota include C-section delivery, formula feeding and antibiotic use.

Antibiotic driven insults on the maternal microbiota during pregnancy may increase offspring risk of obesity (Mueller et al., 2015) and asthma (Metsälä et al., 2015). Other factors that may alter bacteria composition in offspring include maternal infections or disease status. For example, maternal diabetes leads to diversified microbiota in the meconium (Hu et al., 2013). Furthermore, maternal infections, such as HIV, alter infant gut microbiota (Bender et al., 2016). In our first set of experiments, we investigated the impact of maternal intestinal microbiota during gestation or lactation on offspring gut colonization and immune development. The second aspect of our work delved into the influence of preconception helminth infections on offspring gut bacteria as well as immunity. These aspects were independent of each other but both examined the effects of maternal

events on infant gut microbial colonization and added novel insights to the existing body of work on this topic.

Treatment of dams with vancomycin during gestation or lactation led to profound differences in offspring growth, microbiota and immunity. Pups born to dams treated with vancomycin only while nursing had significantly higher body weight, and although not significant, vancomycin treatment during gestation also led to increased weight in pups. Administration of vancomycin 5 days prior to delivery through 14 days of nursing did not cause alterations in pup weight. Moreover, offspring born to antibiotic treated dams regardless of timing of treatment had significantly higher total spleen cells but the splenic weights were not different. Polymyxin B maternal treatment, also impacted offspring body weight. Pups born to dams treated with polymyxin B during gestation or nursing had significantly higher body weight while prolonged polymyxin B modulation of maternal microbiota had a non-significant impact on offspring weights. There were no differences in the spleen weights but total immune cells were significantly lower in all antibiotic infant groups. When the two antibiotics were used in combination, we observed trends similar to effects driven by vancomycin only administration in terms of offspring body weights, spleen weights and total cell numbers. Although we did not examine maternal glucose tolerance or other metabolic consequences in the mother, these data have implications on potential alteration of maternal microbiota on infant growth.

Manipulating maternal gut microbiome using vancomycin significantly impacted the gut microbial community in offspring. Treating dams with vancomycin 5 days prior to delivery led to a distinct microbiota in the offspring, which was unique from control pups and from the nursing treatment pups, indicating that colonization begins prior to delivery and that maternal microbiota during pregnancy influences early infant gut composition. Further, offspring born to vancomycin breeders

had reduced microbial diversity in their gut and an abundance of Proteobacteria, which are features of chronic gut inflammation. Moreover, Bacteroidetes were significantly less abundant in the antibiotic groups versus control pups. Since Vancomycin has no spectral activity against Bacteroidetes, this supports existence of an indirect mechanism by which vancomycin treatment in dams mediates gut microbial alterations in offspring. This was confirmed by the lack of detectable Vancomycin in the plasma of dams and pups, and will be a subject for our future studies. Polymyxin B driven insults on gut microbiota in dams led to reduced abundance of Proteobacteria and Deferribacteres across all antibiotic infant groups. Principal component analysis revealed unique clusters in the various groups suggesting distinct microbiomes are developed during different periods of maternal exposure, although the impact was not as drastic as what we observed by vancomycin treatment and there was some overlap between G and GN groups. Interestingly, although maternal microbiota is critical in determining the initial bacteria colonizing the offspring, our data shows that offspring microbiota in all groups was distinct from that of the mother.

Administration of vancomycin, polymyxin B or a mixture of the two antibiotics profoundly impacted on offspring peripheral immunity in the spleens in terms of proportions of T and B cells and their subsets. Maternal oral vancomycin led to significant reduction in infant central memory CD4+ T cells (CD3+CD4+CD44hiCD62Lhi) regardless of antibiotic timing. We observed significantly lower proportions of CD4+ T cells in offspring born to dams treated with polymyxin B. Administering polymyxin B to mothers while nursing led to significantly reduced proportions of effector memory CD4+ T cells (CD3+CD4+CD44hiCD62Llo) while treatment during gestation or gestation plus nursing significantly increased proportions of central memory CD4+ T cells in offspring. Beyond T cells, we also reported significant alterations in offspring B cell compartment. Pups born to dams treated with vancomycin while nursing or gestation plus nursing had

significantly reduced proportions of B cells (CD19+B220+), follicular B cells (CD19+CD23+) and marginal zone B cells (CD19+CD21+) compared to control pups. Maternal PMB treatment during nursing or gestation plus nursing led to a significant increase in proportions of follicular B cells among her offspring. Administering a mixture of vancomycin and PMB also impacted B cells in a manner similar to vancomycin alone.

Upon infection with RSV intranasally at 3 weeks of age, offspring born to vancomycin breeders were more susceptible to disease in the early stage of the infection (day 4 post RSV). This was demonstrated by significantly reduced rate of growth and significantly lower weights versus controls in the early stage of the disease. Further, offspring born to vancomycin treated dams had higher viral load burdens in their lungs compared to controls, indicating the microbiota had a role to play in viral immunity. Indeed, vancomycin treatment only while nursing resulted in a RSV susceptible phenotype throughout the experiments whereas the other antibiotic groups recovered between day 5 and 7. Our helminth model also supports these results where we subjected offspring born to polymyxin B breeders to *Nippostrongylus brasiliensis* infection to test the Th2 arm of immunity. We found significantly higher worm burden in the intestines in offspring born to dams treated with polymyxin B during gestation or gestation plus nursing compared to controls. These data are corroborated by the IL-13 cytokine levels, which were significantly lower in the aforementioned groups, which explains why these pups were unable to clear the infection. Similar to the effect we observed of microbiota on RSV immunity, our helminth data extends this dogma and shows that maternal microbiota during pregnancy has a role to play in the establishment of optimal Th2 immunity in offspring.

In the final results chapter, we show that preconception infection with the rodent nematode; *N. brasiliensis* impacts maternal microbiota during pregnancy. Preconception *Nb* infections significantly increased microbial diversity during pregnancy. In addition, PI dams had significantly higher abundance of Enterococcaceae, F16 and Odoribacteraceae during pregnancy. Further, preconception Nb infections altered breastmilk microbiota. At the family level, there was an increase in Paraprevotellaceae, Prevotellaceae, Enterobacteriaceae, Coriobacteriaceae and S24-7 families in the milk of previously infected dams. However, *Nb* infections significantly reduced Porphyromonadaceae and Corynebacteriaceae in breastmilk. Changes in microbiota were also reflected in their offspring and these did not always mirror maternal microbiota. We found dramatic differences in gut microbiota in offspring born to previously *Nb* infected dams compared to offspring born to naïve dams.

Taken together, our data shows that manipulating maternal gut microbiota during gestation and or lactation profoundly impacts offspring growth, intestinal microbiota and peripheral immunity. Moreover, offspring born to antibiotic breeders are susceptible to both RSV and *Nb* infections indicating a critical role of maternal microbiota during pregnancy and lactation in influencing immune development in her young ones. Further, helminth infections prior to pregnancy impact murine gut microbiota during pregnancy and consequently alter offspring microbiota, which may be associated with early immune activation in infant mice.

7.2 Future work

While we have generated a significant body of work in the field of infant microbiome and immunity as well as the role of maternal gut microbiome in programming offspring immunity, several questions remain unanswered. We have shown from our maternal antibiotics experiments that altering the maternal gut during gestation or lactation profoundly impacts body weight and development in offspring. While it is clear, the microbiome is involved in driving metabolic changes in the mothers, which then impact the offspring, the exact mechanism of these biological occurrences is unknown. Various scenarios are likely including epigenetic changes that could begin *in utero* or transfer of microbiota with an altered energy harvest capability. An additional explanation could be local generation of bacterial metabolites which translocate to the offspring *in utero* or during breastfeeding. To further delineate this, follow-up experiments could include determination of breastmilk and serum levels of short chain fatty acids in mothers and offspring respectively. We will utilize mouse models deficient in short chain fatty acid receptors: Free Fatty Acid receptor 2 and 3 (FFA2 and FFA3) to further understand the role of SCFAs in conferring metabolic phenotypes in offspring.

Moreover, we observed profound changes in offspring gut microbiota in pups born to vancomycin breeders. These changes were only as a result of direct intervention in maternal gut since vancomycin was not absorbed and we did not detect it in maternal and offspring serum. Part of the mechanism which drove changes in offspring intestinal microbiota could include bacteria translocation to the placenta. However, it is also possible that vancomycin in dams could indirectly alter microbiota in other sites such as breastmilk and the genital tract which consequently would influence infant microbiota. In our next experiments, we will analyze breastmilk as well as genital tract microbiome in dams fed on vancomycin versus controls. This will further our understanding

of how vancomycin impacts maternal microbiota and shed light on mechanisms by which alterations in infant microbiota occur.

Most importantly, we present findings that an altered maternal microbiota during gestation or lactation has health consequences for the offspring, including altered immune development. This indicates that microbial signals from the dams during this unique period are critical for optimal development of offspring immunity. We observed drastic changes both T and B cell compartments when pups are born to dams treated with antibiotics. Next, we will investigate functionality of these cells (T and B), studying the cytokine profiles and ability to produce antibody. In addition, for maternal vancomycin experiments will analyze maternal microbiota during pregnancy. We will then gavage fecal material obtained from SPF mice during pregnancy to pregnant germ free mice and investigate the effect on inherent B cells in offspring. Furthermore, we will isolate individual bacteria from the fecal material and mono-colonize pregnant germ free mice to determine individual bacteria with a significant effect on offspring B cell compartment.

Moreover, when we challenged offspring born to antibiotic breeders either with RSV or *Nb*, we noted that antibiotic treatment in dams rendered the offspring susceptible. This introduced a novel conceptual framework showing rather unappreciated players in shaping both Th1 and Th2 immunity in infants. While our findings clearly show that microbes were necessary for optimal immunity to these pathogens which tested various arms of cellular immunity, we were not able to link specific microbes to these functions. Our next experiments will test individual effects of the abundant bacterial taxa on immunity using germ free models. Further, although our intervention was directed at the maternal gut, we showed that these changes altered respiratory immunity in offspring which raises interesting questions as to how the gut could be interacting with immune cells in the lung of the offspring which will also be a subject of further investigation.

Our findings in the last chapter show that preconception *Nb* infections alter not only maternal gut microbiota during pregnancy but also the offspring microbiota. Although we treated dams with Ivermectin to clear the *Nb* antigen from the host, we detected changes in microbiota during pregnancy suggesting that *Nb* modulated alterations of microbiota were lasting beyond the active infection. Alterations in the gut community were also revealed in their offspring at 14 days of life, implying long-lasting effects on pup gut microbiota, likely mediated by alterations in breastmilk microbial makeup Apart from the changes in microbiota, we also noted early activation of the adaptive immune system in offspring born to infected dams. Although we show that changes in offspring microbiota are associated with changes in the immune system, our data does not prove a causal relationship. Having shown that maternal antibiotics during pregnancy altered offspring immunity to *Nb* whether administered during gestation or gestation plus nursing, we are also convinced that the *Nb* driven changes in maternal microbiota during pregnancy could be associated with protection in offspring. Our collaborators (Horsnell lab) have shown that pups born to *Nb* infected dams are protected against the infection as revealed by lower worm burdens compared to controls. We are currently conducting these experiments with the aim of investigating whether the changes introduced by *Nb* during pregnancy have any role to play in protecting their offspring from *Nb* infections.

Overall, our data reveals the impact of maternal antibiotics prior to delivery and during lactation on offspring intestinal microbiota and immunity to various pathogens. Also, we show that maternal infections impact offspring microbiota. Combining the maternal infant model with 16S rRNA profiling, we have identified key microbiota altered in offspring following antibiotic treatment or helminth infections in their mothers. However, there exist several factors that differentiate mice and humans that could contribute to different microbiota profiles. Experimental mice have

restricted diet and are of an inbred strain minimizing any genetic factors that may impact microbiota while humans have greater dietary and lifestyle variations. Together, these factors limit our ability to generalize the relevance of these data in a clinical setting. Further studies are needed to determine specific microbiota impacted early in life following maternal antibiotic exposure or maternal infections and the relevance on infant immunity and response to vaccines.

Appendices

Appendix A: Influence of maternal antibiotics on offspring gut microbiome and immunity

Results List Report
Unreleased

Operator ID:
System serial number: 34517

C / P	Module	SID	Name	Assay	Result	Flags	Code	Date / Time
V888/1	2	01		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:44
V888/2	2	02		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:44
V888/3	2	03		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:45
V888/4	2	04		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:46
V888/5	2	05		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:47
V889/1	2	06		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:54
V889/2	2	07		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:55
V889/3	2	08		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:55
V889/4	2	09		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:56
V889/5	2	10		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:56
V890/1	2	11		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:57
V890/2	2	12		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:58
V890/3	2	13		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:58
V890/4	2	14		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:59
V890/5	2	15		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 15:59
V891/1	2	16		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 16:00
V891/2	2	17		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 16:01
V891/3	2	18		iVanco	< 3.0 mg/L	CNTL, <		01.12.2015 16:01

Table A1: **Vancomycin levels in serum**. Vancomycin levels were measured in serum collected from both dams treated with vancomycin or their offspring. Levels were measured using the Abbott Architect ELISA technique and levels were all below the detection limit of 3mg/L.

Appendix B: CD8 T lymphocytes pool expands following RSV infection in infant pups

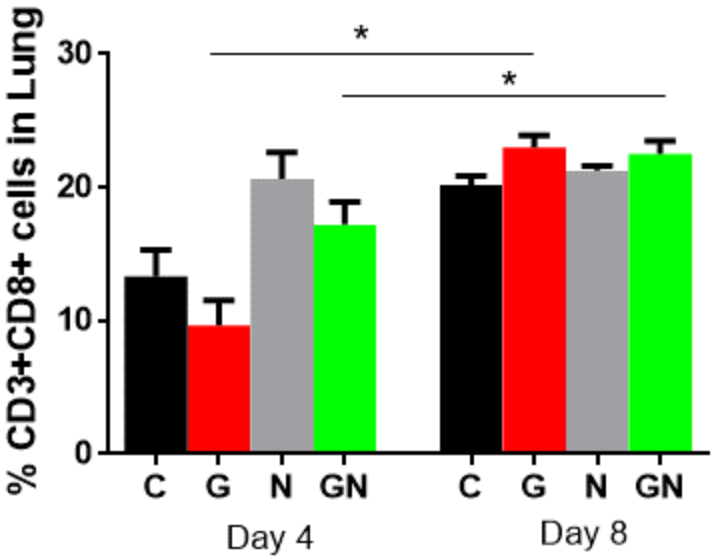


Figure B1: CD8 T cells significantly increase at day 8 post RSV infection in infants among gestation and gestation plus nursing pups. Data are representative of two experiments. n=4-6 pups per group per time point.

Appendix C: Microbiota transfer to offspring following maternal *Nb* exposure occurs in utero and while breastfeeding

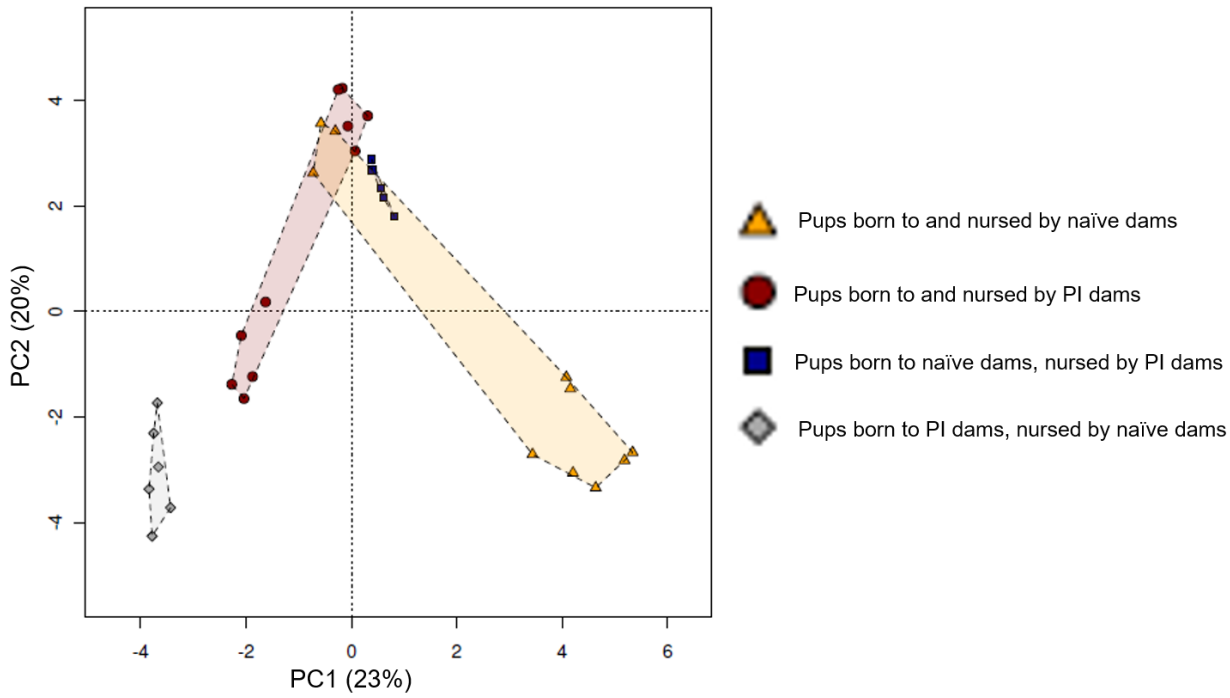


Figure C1: Microbiota transfer to pups after maternal *Nb* infection occurs both in utero and while nursing. Female BALB/c mice were infected with 500Nb L3 and infection cleared 7 days post infection (p.i) by oral Ivermectin treatment for 7 days. 21d post infection, mice were mated. 3 days after delivery, pups born to previously infected (PI) dams were transferred and nursed by naïve lactating mothers while those born to naïve dams were transferred and nursed by lactating PI mothers. Two weeks postpartum, infant stool microbiota was analyzed. Principal coordinate analysis showing bray-curtis distance of infant stool microbial communities. PI-Previously infected. n= 6-10 per group.

Table C2: Summary table showing changes in microbiota in both dams (stool and breastmilk) and offspring (stool 14 days postpartum) following preconception *N. brasiliensis* infection.

Bacteria (Taxa)	**Significantly differentially abundant in stool from pregnant PI dams**	**Significantly differentially abundant in breastmilk from PI dams**	**Significantly differentially abundant in pups born and breastfed by PI dams**
Phylum	TM7	-	-
Family	Odoribacteraceae F16 Enterococcaceae	S24-7 Rikenellaceae Prevotellaceae Erysipelotrichaceae Enterobacteriaceae Coriobacteriaceae	Mycoplasmataceae Micrococcaceae Coriobacteriaceae
Genus	Oribacterium Odoribacter Enterococcus	Prevotella Parabacteroides Micrococcus Klebsiella Collinsella CF231 Allobaculum	Arthrobacter Adlercreutzia

General buffer recipes

ELISA Blocking buffer

40g BSA

Dissolve in a final volume of 1000ml 1XPBS and store at 4° C

ELISA Dilution Buffer

10g BSA

Dissolve in a final volume of 1000ml of 1XPBS and store at 4° C

ELISA Washing Buffer

20g KCI

20g $KH_2HPO_4.2H_2O$

800g NaCl

50ml Tween-20

100ml 10% NaN_3

Make up to 5L with ddH20 and store at room temperature. Use at 1.20 in ddH20

ELISA Substrate Buffer (horseradish peroxidase conjugates)

Peroxidase Substrate Solution B (Roche Diagnostics GmbH, Mannheim, Germany)

TMB Peroxidase Substrate Solution A (Roche Diagnostics GmbH)

Before use, mix equal volumes of TMB Peroxidase Substrate (Soln A) with Peroxidase Substrate (Soln B)

Complete media (Iscove's Modified Dulbeco's Medium, IMDM)

1 tube IMDM (Gibco)

50ml Fetal Bovine Serum (10% FBS)

5ml Penicillin/Streptomycin (1X) (5% PenStrep).

Mix contents and filter sterilize and store at 4° C

MACS Buffer

2mM EDTA

0.5% BSA

Dissolve contents in a final volume of 1000ml of 1XPBS and store at 4° C

Red Cell Lysis Buffer

8.34g NH_4Cl

0.037g EDTA

1g $NAHCO_3$

Dissolve reagents in 1000ml ddH20. Filter sterilize (0.22μm) and store at 4° C or 25° C

Lung Digestion Buffer

0.002g DNase I (Roche Germany)

0.02g Collagenase Type I (Gibco-Invitrogen)

Dissolve reagents in 150ml DMEM (containing 100U/ml penicillin G, 100μg/ml streptomycin).

Filter sterilize with a 0.22μm filter and store at 4° C for up to 7 days.

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