NEONATAL IMMUNE RESPONSES TO *BORDETELLA PERTUSSIS* AND DISRUPTION BY PERTUSSIS TOXIN

by

COLLEEN J. SEDNEY

(Under the Direction of ERIC T. HARVILL)

ABSTRACT

The increased susceptibility of neonates to specific pathogens has previously been attributed to an underdeveloped immune system. More recent data suggest neonates have effective protection against most pathogens but are particularly susceptible to those that target immune functions specific to neonates. *Bordetella pertussis* (*Bp*), the causative agent of "whooping cough", causes more serious disease in infants attributed to its production of pertussis toxin (PTx), although the neonate-specific immune functions it targets remain unknown. Problematically, the rapid development of adult immunity in mice has confounded our ability to study mechanisms of the neonatal immune system. Here, we define a period of five- to eight-days-old during which mice are much more susceptible to *Bp* than mice even a couple of days older. These more narrowly defined "neonatal" mice effectively respond to and control *Bp*Δ*ptx*, more rapidly even than adult mice. This clearance correlates with the early accumulation of neutrophils and T cells and suggests a role for PTx in disrupting their accumulation. Depleting neutrophils inhibited pups' ability to rapidly clear *Bp*Δ*ptx*, revealing a critical role for neutrophils. Pups deficient in complement ($C3^{-/-}$) failed to recruit neutrophils and did not efficiently clear *Bp*Δ*ptx,* revealing a critical role for complement. Delivering C3a largely ameliorated the defect of $C3^{-/-}$, indicating that immune cell recruitment is more important than either direct complement-mediated lysis or opsonization, both of which are mediated by C3b. The transition from neonatal to perinatal immune responses, driven in large part by thymus development and the changing repertoire of T cells it generates, is also poorly understood. Here, we utilize two complementary models of disrupted thymic development to assess the effects of different thymic development stages on responses to *Bp*. Utilizing a genetic model of disrupted thymic development, we show that post-birth thymus development is required to respond to and control *Bp.* With a complementary surgical model, mice thymectomized at P0 and P7 had substantially different T cell responses to *Bp*, with the later demonstrating an early CD8 response associated with more rapid control. Together, these results suggest the neonatal immune system can be highly effective at controlling respiratory infections, but these responses are severely disrupted by PTx and undergo dramatic development very early in life.

INDEX WORDS: neonatal, bacteria, neutrophils, T cells, immunity

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DEDICATION

This dissertation is dedicated to my teachers and mentors who inspired and encouraged me to pursue science, whom I work to make proud, specifically Terry Grant and Dr. Amy Beumer.

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CHAPTER 1

THE NEONATAL IMMUNE SYSTEM AND *BORDETELLA PERTUSSIS*[1,2]

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ABSTRACT

Neonates are more susceptible to some pathogens, particularly those that cause infection in the respiratory tract. This is often attributed to an incompletely developed immune system, but recent work demonstrates effective neonatal immune responses to some infection. The emerging view is that neonates have a distinctly different immune response that is well-adapted to deal with unique immunological challenges of the transition from a relatively sterile uterus to a microbe-rich world, tending to suppress potentially dangerous inflammatory responses. Problematically, few animal models allow a mechanistic examination of the roles and effects of various immune functions in this critical transition period. This limits our understanding of neonatal immunity, and therefore our ability to rationally design and develop vaccines and therapeutics to best protect newborns. This review summarizes what is known of the neonatal immune system, focusing on protection against the respiratory pathogen *Bordetella pertussis* (*Bp*). We will also discuss current knowledge on the immune responses to *Bp* and how the disease process differs in adult and neonatal models, with a specific focus on the unique challenge of microbiota acquisition. Highlighting recent advances in the mouse model, we identify knowledge gaps to be addressed.

INTRODUCTION

Infants and neonates have long been considered to have an underdeveloped and ineffective immune system. This view emerged from the observed susceptibility to adverse outcomes of infection with particular pathogens that are more severe in infants than in adults. Infants also have distinct immune components and functions, which when evaluated against adult immune standards, were found to be defective in their activities[3,4]. For example, infants generate primarily anti-inflammatory TH2 responses that likely help maintain fetal–maternal tolerance and reduce the risk of damaging inflammatory responses against the many new commensal organisms encountered after birth[5,6]. This results in limited and/or delayed responses against some harmful pathogens, that can grow to cause more severe disease. However, increasing evidence suggests that newborns and neonates have a quite effective immune system that is very different from that of adults[7]. This includes populations of unique cells and subsets of immune cells that specifically make up the neonatal immune system (NIS). These neonatal cells also have differential expression of genes and factors which regulate the anti-inflammatory response towards pathogens. Pathogens which primarily cause severe disease in infants, such as *Bp*, influenza, and RSV, mainly infect and cause disease in the respiratory tract, suggesting a unique interaction between the neonatal pulmonary immune environment and these particular pathogens. However, we still have limited knowledge regarding how the differences between the neonatal and adult immune components affect their responses to pathogens, and how we can best utilize the unique capabilities of neonatal immune cells to develop targeted vaccines and treatments against neonate-specific pathogens.

The limited understanding of the NIS is in large part due to the lack of appropriate animal models to study the neonatal response to infection. Immunology has leaned heavily on the murine model, due to its low cost, reproducibility, and extensive library of immunological tools. Mice mature extremely rapidly, which facilitates studies of adult immunity, but greatly reduces the timeframe of the neonatal period from months in humans to only days in mice[8]. Although there is no discrete cutoff point, the general consensus of the field is that the neonatal stage of immune development in mice lasts only until ~7–10 days post birth (P7-P10), based on the metric utilized. However, infections can progress over periods of days, weeks, or longer, so experimental infections initiated in neonates have ranged from 2 to 21 days post-birth (P2–P21), during which there are significant developmental shifts in the immune system. This lack of consistency with the use of neonatal murine models has resulted in some confusion that further frustrates a comprehensive understanding of the NIS.

One such area which suffers is our understanding of the neonatal immune response to infectious diseases, especially those which appear to specifically target unique aspects of the NIS. An important pathogen included in this group is *Bp*, the causative agent of whooping cough. Infants under 2 months old account for 29.3% of all pertussis cases, with approximately 60% of these infected infants requiring hospitalization[9]. While much is known about the disease process of *Bp* in adult murine models, very little work has been completed in neonatal models, limiting our knowledge about the disease process in this most susceptible group. The well-appreciated bacterial factor which affects disease severity in both adults and neonates is pertussis toxin (PTx), an AB_5 exotoxin which disrupts cell communication. However, the mechanisms by which PTx specifically disrupts neonatal immune responses are not well known, confounding the generation of effective vaccines and treatments.

In this review, we will discuss current knowledge of the development of the neonatal pulmonary immune environment, the rapid shifts in immune components, and striking differences in techniques used to assess them. The differences between the adult and neonatal immune systems will also be reviewed as a consequence as the unique challenge of complete holobiont acquisition by neonates. We will also specifically address the literature regarding the adult and neonatal murine immune response to *Bp* and specifically in response to PTx. We will also highlight gaps in knowledge of the important differences between the neonatal and adult immune systems that limit optimal applications of vaccines and other approaches to protect highly sensitive newborns from respiratory disease, leading to recent rises in infant infections[10].

NEONATAL PULMONARY IMMUNE SYSTEM

The immune system is a complex network of cells and signals which regulate the host's response to self and foreign antigens. This delicate system requires substantial regulation to prevent severe damage to the host but is also balanced against the potential damage that could be inflicted if a pathogen is not controlled. Thus, responses are primarily dependent on the needs of the hosts and the nature of the signal. In the case of immune stimulation by pathogens, the primary goal of the immune system is to mediate clearance of the pathogen while minimizing damage inflicted to the host by the immune response itself. The adult immune system (AIS), which is better studied and understood than is the neonatal, efficiently generates pro-inflammatory responses which mediate the efficient control of most pathogens. The NIS, however, is well evolved to respond to the unique challenges of the rapid transition from the near-sterile womb to the microbe-rich world beyond. This suddenly introduces millions of new antigens for potential immune recognition and responding to those as the AIS does would be both difficult and dangerous. The NIS appear to instead respond to novel antigens primarily with anti-inflammatory TH2 responses to prevent unnecessary inflammation which can severely harm the infant (**Figure 1.1**). However, the increase in TH2-skewed responses may result in a delayed response to some dangerous pathogens, leading to the interpretation that the NIS is defective in general, compared to the AIS. However, the NIS can be very effective when the unique cellular components are appropriately stimulated.

Understanding the differences between the AIS and NIS can inform our ability to properly stimulate the NIS to make an effective response and lead to new approaches to specifically treat neonatal infections. This begins with the optimization of neonatal mouse models that can allow us to better understand how NIS responds to various infections (**Figure 1.2**). Here, we describe current knowledge of the cellular components of the murine NIS, how they are regulated, and the wide range of ages which have been used and labeled as "neonatal".

Innate Immune System

1. Neutrophils

Neutrophils are white blood cells that rapidly respond to pathogens, particularly in the lungs. They release granules, reactive oxygen species (ROS), and ultimately neutrophil extracellular traps (NETs) that can trap and kill pathogens, but also can damage host cells. In adult mice, neutrophils develop from precursors in the bone marrow before migrating to the periphery[11]. Neutrophils are derived from the granulocyte-macrophage progenitor (GMP) and proliferative neutrophil precursor (preNeu)[11]. Their short half-life requires that they be consistently replenished in the periphery[11]. While few requirements are known for neutrophil development in murine models, iron regulatory protein (IRP) has been observed to be critical for neutrophil development and differentiation due to its role in iron homeostasis[12]. Together, the development and activities of neutrophils aid in the control and clearance of pathogens in the lungs; however, the specific roles of neutrophils in the neonatal lungs are unclear.

In adults, lung infection results in the rapid recruitment of neutrophils, via L-selectin-dependent migration[13]. Many studies have observed that neonatal neutrophils are deficient in numerous activities compared to those of adults, resulting in increased susceptibility to pathogens. For example, one-week-old mice inoculated with RSV had less neutrophil recruitment to lungs compared to adults after seven days of infection (P14)[14]. After inoculation with *Escherichia coli*, both P1 neonatal and adult mice were neutropenic at 1 day post-inoculation (dpi) (P2). However, the neonatal mice took longer to recover than did adults and the former did not experience the increases in neutrophils, GM-CSF, and IL-6 production that were observed in adults[15]. Multiple studies have suggested that this reduction in neutrophil recruitment may be affected by the production of IL-10 from B cells and dendritic cells. Compared to wildtype pups, TLR2-deficient pups inoculated with Group B *Streptococcus* at P2 were not susceptible to sepsis and did not produce increased amounts of IL-10 associated with damaging inflammation in wildtype pups at 24 hrs after infection (P3). Importantly, it was also observed that in the absence of TLR2 and the resulting IL-10, neonatal neutrophils were able to migrate to infected lungs and control infection[16]. This suggests that neonatal neutrophils have the inherent ability to migrate to infected tissues and stimulate appropriate immune responses, but that the anti-inflammatory regulation of the NIS modulates this ability.

There is significant evidence that neonatal neutrophils fundamentally have the same capabilities as adult neutrophils do to control pulmonary infections. Neonatal mice (P2) inoculated with influenza had a slightly slower accumulation of neutrophils to the lungs compared to adults at 7 dpi (P9) but had higher amounts of neutrophils than adults did at 10 dpi (P12). These neonatal neutrophils were also successful in infiltrating the alveolar spaces, demonstrating the ability of neonatal neutrophils to migrate to the site of infection[17]. Neonatal mice (P5) inoculated with a mutant of *Bp* lacking the pertussis toxin demonstrated neutrophil accumulation to the lungs as early as 2 hrs after inoculation, also demonstrating that neonatal neutrophils can respond very rapidly to the lungs. A subsequent increase in T cells in the lungs indicates that neonatal neutrophils can also efficiently recruit T cells. These responses, however, were not observed in P5 pups inoculated with wildtype *Bp*, suggesting that pertussis toxin disrupts neutrophil accumulation in the lungs[18]. Similarly, a mutant of *E. coli* lacking Lpp was successfully controlled in mice and rats by P5–P9. This control required NADPH oxidase-generated ROS from neutrophils. However, this effect was not observed with wildtype *E. coli*, suggesting that lipoprotein (Lpp) suppresses neutrophil killing to promote disease in neonates[18]. This suggests that pathogens produce specific factors which disrupt neonatal neutrophils and prevent control of infection from neonatal lungs.

The immunosuppressive effects of pathogens on neonatal neutrophils, however, can be curtailed via pre-treatment with drugs that stimulate the NIS. Pretreating neonatal mice (P5–P7) with alum 24 hrs before the induction of polymicrobial sepsis improved phagocytosis by peritoneal neutrophils resulting in decreased pathogen numbers by 24 hrs post-challenge (P6–P8). Alum pretreatment also increased numbers of NET-positive neutrophils and the expression of co-stimulatory molecules (CD80 and CD86)[19]. This suggests that peripheral neonatal neutrophils can successfully respond to microbial infection but require differential stimulation to generate a protective response. This process is likely applicable to neutrophils in neonatal murine lungs; however, further work is required to confirm additional applications. Similarly, when treated with memantine and inoculated with *P. aeruginosa*, P7–P8 Sprague Dawley (SD) rats had significantly reduced bacterial loads in the lungs, blood, liver, and spleen, accompanied by the inhibition of IL-6 production[20]. This evidence suggests that stimulation of neonatal neutrophils can result in increased anti-microbial efficiency and successful control.

Though neutrophil activity is typically associated with a protective response, this activity can also cause tissue damage, threatening the health of neonates. The primarily anti-microbial activities of neutrophils include the release of granules which results in bacterial killing, regulation of cytokine signaling, and stimulation of NETs and ROS by neutrophils in an autocrine/paracrine manner. These anti-microbial functions, however, can cause serious damage in neonatal lungs and can exacerbate infections. This is due to the fact that NETs can cause non-specific inflammation which allows pathogens to escape the immune response and generate severe pulmonary and systemic disease[21–23]. It has also been observed that NETosis can be regulated via cytokines, with

IFN-γ signaling limiting NETosis and resulting in better outcomes in pups with viral bronchitis[24]. The production of ROS by neutrophils has also been implicated in acute lung injury of neonatal mice[25,26]. Therefore, while neonatal neutrophils have the ability to respond similarly to adult neutrophils, the specific challenges of neonates require these activities to be modulated to prevent unnecessary damage, presenting an opportunity to some pathogens.

2. NK Cells

Natural killer (NK) cells are lymphocytes of the innate immune system that derive from the same precursors as T and B cells and belong to the Group 1 innate lymphoid cells (see below). Amongst their known functions is the ability to release granules to kill pathogens and signal the immune system. NK cells are first detected in the lungs as immature $CD27^{+}CD11b^{lo}$ cells. Mature $CD27^{lo}CD11b^{+}$ NK cells first appear in the lungs at 3 weeks post birth and are the primary NK cell population by 8 weeks[27,28]. Mice lacking FcRn have low levels of NK cells, indicating that FcRn plays a role in their development. These NK cells which are first observed in the neonatal lungs have an immature phenotype and express lower levels of CD107a, which suggests reduced degranulation, and produced smaller amounts of IFN-γ, though their cytotoxicity was similar to that of mature adult NK cells[29]. However, there is also evidence that neonatal NK cells can function to control pulmonary infections. P2 pups inoculated with *Chlamydia muridarum* had significant accumulation of NK cells in the lungs by 3 dpi (P5), accompanied by decreased bacteria in the lung[30]. Additionally, the ablation of erythroid suppressor cells in P6 pups inoculated with *Bp* resulted in increased NK cell accumulation in the lungs and increased protection against *Bp* by 4 dpi (P10)[31]. This evidence suggests that there is a protective role of neonatal NK cells in controlling infections in the neonatal lung.

NK cells are a major producer of IFN-γ. Though neonates primarily rely on the anti-inflammatory TH2 response, IFN-γ may play a role in protecting neonates from infections. IFN-γ-deficient mice inoculated at P2 with the measles virus suffered increased lethality compared to wildtype pups at 6 dpi (P8)[32]. Additionally, IFN-γ can play a unique role in the neonatal immune response by preventing ROS and NETosis by neutrophils, thereby preventing inflammation of the lungs in neonates[24]. Conversely, IFN-γ can also suppress production of antibodies[33,34].

3. Dendritic Cells

Dendritic cells are antigen-presenting cells which can phagocytose pathogens and present antigens to activate T cells. There are two primary categories of DCs, plasmacytoid (pDC) and conventional (cDC). pDCs specialize in secreting type I interferons while cDCs (further separated into groups 1 and 2) specialize in antigen presentation to activate TH1 and TH17 responses. Though most DCs fall into these categories, there are numerous additional subsets which exist, particularly in neonates during development. Single cell sequencing analyses of C57Bl/6 pups at various stages of development have revealed that neonatal murine lungs contain cDCs, as well as an additional subset characterized by the expression of melanoregulin (*Mreg*)[34]. cDC1 is the most abundant subset in the lungs from P1–7, with cDC1 and cDC2 being in the lungs in equal amounts by P21. Additionally, the expression of *Itgae* and *Cd209a* by neonatal DCs suggests that they can induce T cell immunity upon birth to generate an effective immune response[34]. Interestingly, the *mreg*-expressing DCs were not detected prior to birth but were present in high numbers at P7 before ultimately decreasing by P21. This suggests that they are a unique, transient DC subset in neonatal murine lungs[34]. Similarly, two subsets of migratory $CD103^+$ DCs were observed in the lungs of neonatal mice inoculated at P7 with RSV. These subsets ($CD103^{lo}$ and $CD103^{hi}$) were observed starting at 1 dpi (P8) and during infection with influenza. These two subsets were also found to have distinct functional characteristics, including the presence of co-stimulatory molecules and ability to stimulate specific responses. Though the CD103^{hi} DC subset was found to have superior function and increased amounts of co-stimulatory molecules, CD103^{lo} DCs were more prominent in neonatal lungs[35]. The different functions and characteristics of these DC subsets may contribute to the decreased TH1 activation of neonatal T cells.

DC populations in the neonatal thymus are similar to those of adults by P7, though they proved to be less efficient at antigen processing and presentation at this stage[36]. After egress from the thymus, they shift greatly during mouse development. P7 neonatal mice had low levels of GM-CSF compared to naïve adults, which is believed to limit the development of CD103$^+$ DCs. However, P7 neonatal mice inoculated with RSV produced a CD103$^+$ DC response at 7 dpi (P14)[35,36]. CD11b$^+$ DC populations were low at P6 but increased greatly during the first 3 weeks of life. Additionally, DCs from P7 pups were unable to transport antigens from the lung to the lymph nodes at the same efficiency as adults were able to[36]. It was also observed that neonatal CD103$^+$ DCs stimulate CD8$^+$ T cells differently than do adult CD103$^+$ DCs. While neonatal DCs can present antigens at the same efficiency as adult DCs can, they have decreased expression of the costimulatory molecules CD28, CD80, and CD86, resulting in a failure to stimulate CD8$^+$ T cell proliferation and a limited CD8$^+$ T cell response[35–37]. Neonatal lungs had more cDC1 subsets; however, these proportions shift during development, with adults having more cDC2s in the lungs[36,38,39]. Surprisingly, neonatal DCs also have differential cytokine production, with DCs isolated from the spleen displaying slightly increased production of IL-12p40, an important inducer of TH1 responses[37,38]. This was accompanied by increased production of IL-10, further repressing activation of CD8$^+$ T cell responses[37].

One of the major issues with the NIS is the apparent inability to generate TH1 responses, as these are considered the most effective against some pathogens. The development of this response requires early exposure of immune cells to IFN-γ followed by priming with IL-12[40]. However, the development of this response is impeded by the production and actions of IL-10, of which neonatal immune cells are major producers. It has been observed that neonatal DCs have the capacity to generate TH1 responses by producing IL-12 and stimulating T cells; however, this process is affected by IL-10 production, particularly from neonatal B cells[40]. The shift in the ability to produce IL-12 and generate a TH1 response occurs around P6 in mice, when the DC populations increase and subsets shift away from the neonatal pattern and become more adult-like, causing a shift toward primarily TH1 responses[41].

4. Macrophages

Macrophages are immune cells that phagocytose pathogens and are derived from the same progenitor as neutrophils[42]. Single-cell sequencing identified unique clusters of macrophages that shift from fetal development to post-birth[34]. Prior to birth, the primary cluster of macrophages (Mac I) in the lung are highly proliferative and localize to small vessels, likely to promote growth and remodeling. A new cluster (Mac II) arises the day after birth at P1 with possible roles in immune regulation and tissue remodeling[34]. This subset is of particular importance as it is detected at the beginning stages of postnatal alveolarization, a process which begins after birth and peaks in adulthood (~P39)[43,44]. Another cluster of alveolar macrophages (Mac III) was also observed at P1 and P7. These clusters of macrophages (Mac I, II, and III) express genes that promote bacterial killing but suppress inflammation, likely contributing to the anti-inflammatory response in the lungs at P1 and P7. Surprisingly, macrophages with proinflammatory signatures (Mac IV), likely contributing to cytokine production and leukocyte chemotaxis, were also identified in P1 and P7 mice. Domingo-Gonzalez et al. suggested that the Mac II cluster of macrophages are a transitory subset, due to the overlap of expression with Mac I and Mac III clusters[34]. This work displays the dramatic shifts in macrophage populations and phenotypes that occur during neonatal lung development. One of the most important subsets in the neonatal lung are alveolar macrophages due to their tendency to prevent damaging inflammation. Fetal monocytes develop into alveolar macrophages by P7 and persist for approximately 3 months[45]. Differentiation of monocytes into alveolar macrophages is also aided by lung basophils[46]. The persistence and maintenance of alveolar macrophages requires GM-CSF and neonatal neutrophil-derived 12-hete[45,47]. An additional subset of macrophages which exist in the lungs are M2 macrophages. These are in lungs at highest numbers from P14–P21 and have roles in tissue remodeling and immunosuppression[48].

Neonatal macrophages are generally believed to have similar functions and capabilities as adult macrophages. However, neonatal macrophages adhere, spread, and phagocytose in a CR3-dependent manner while adult macrophages complete the same activity in a CR3-independent manner[49]. Similar to other immune cells, neonatal macrophage functions are often affected and modulated by the neonatal pulmonary environment. The production of IL-6 and IL-10, and lack of production of IL-12, by macrophages prevents the production of IL-1β and TNF-α via stimulation with LPS[50]. The reduction of IL-10 allows for the increased activation of neonatal macrophages[51]. The regulation of macrophages by IL-10 can also be modulated via the pretreatment of neonatal mice with alum, which increased phagocytosis and costimulatory markers on neonatal macrophages[19]. The response of neonatal alveolar macrophages in pups inoculated with RSV was also improved by the addition of IFN-γ[52]. Therefore, neonatal macrophages can play important roles in protecting neonates from pulmonary infections; however, their function can be greatly improved via TH1 skewing.

5. Innate Lymphoid Cells

Innate lymphoid cells (ILCs) have important roles in cytokine production and similar functions to T cells but lack the ability to display antigens and subsequently activate B cells. There are three groups of ILCs that are differentiated by the cytokines they produce. Group 1 ILCs (ILC1s), which includes NK cells, primarily produce IFN-γ and TNF-α and are involved in immunity to bacteria, viruses, and cancer, while Group 3 ILCs (ILC3s) produce IL-22, IL-17A, and IFN-γ and are involved in immunity to bacteria, chronic inflammation, and lymphoid development. While ILC1s and ILC3s can be recruited to the lungs, Group 2 ILCs (ILC2s) are naturally resident in the lungs[53,54]. These cells make IL-5 and IL-17 in response to stimulation with IL-33 and IL-25. In neonatal IL-33-deficient mice, ILC2s are still observed; however, they are not activated[55]. Pulmonary ILCs descend from ILC precursors that populate a niche defined by fibroblasts in the developing lung. The fibroblasts make insulin-like factor 1 and this instructs the expansion and maturation of pulmonary ILC precursors. Depleting IGF-1 prevented ILC3 development which led to increased susceptibility of neonatal mice to pneumonia[56]. Lung ILC2s are found starting at P4 and peak at P14, then decrease as the lungs mature[57]. ILC development is also dependent on the transcription factor RORα, an important factor for the preservation of the ILC phenotype[58,59]. In P12 lungs, three populations of ILCs are found. One is a progenitor population similar to that in adults and the two others differentially produce TH2 cytokines and amphiregulin. Together, these subsets have distinct proinflammatory and tissue-repairing functions[58]. ILC2s increase after birth and peak at P10, where they are found at three-fold higher levels than those in adult lungs. At P11, ILC2s uniquely express IL-5 and IL-13, proliferate via IL-33 signaling, and promote TH2 immunity[60].

The production of cytokines from ILCs generates and maintains the unique neonatal immune environment. Specifically, the production of IL-13 by neonatal ILCs maintains the M2 status of macrophages[61]. An important mediator of this response is IL-33, produced by epithelial cells and associated with alveolarization and tissue remodeling of the lungs, along with acute TH2 responses[43,60]. However, ILCs can also induce damaging inflammation. P5 pups inoculated with RSV had increased IL-33 expression and increase in ILC2 numbers in lungs at 1 dpi (P6), a response which was not observed in adults[43,62]. Additionally, P6 neonatal mice with rhinovirus demonstrated increased IL-13 and IL-25 as early as 1 dpi (P7) with suppressed IFN-γ, IL-12p40, and TNF-α expression while IL-33-producing ILC2 populations were expanded. This response was attenuated by IL-25-neutralizing antibodies, implicating IL-25 as an additional mediator of ILC activity[63].

6. Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are similar to neutrophils and monocytes; however, they express potent immunosuppressive abilities, primarily of T cell responses. They are activated by T cell-derived IFN-γ but then suppress T cells via the expression of iNOS and arginase 1, which generate NO and urea, respectively[64,65]. While they suppress α/β T cells, they also promote the development of Tregs in an IL-10-dependent manner[66]. Neonatal mice specifically have a large transitory population of MDSCs in the lungs in the first few weeks of life, while adult mice have reduced populations throughout life. Their potent antimicrobial activities aid in the protection of neonates from infection in these first few weeks[65]. While MDSCs support and protect the NIS such that it reduces inflammation, they can also cause reduced responses to pathogens.

Adaptive Immune System

1. T Cells

T cells are lymphocytes that are developed in the thymus and participate in immune responses via the regulation and production of cytokines which mediate the responses of other cells. T cells can be activated by antigen-presenting cells (APCs) that present their specific antigen. After activation, T cells then expand and produce cytokines to promote additional responses from lymphocytes. There are two main populations of T cells that are defined by the type of T cell receptor (TCR), α/β or γ/δ, that they express. While γ/δ T cells are the first to exit the thymus during neonatal development, most T cells in the neonatal lung are α/β[34]. γ/δ T cells participate in mediating responses to influenza and generate TH2 responses in the lung[67]. T cells also have functions in cytokine production, B cell activation, and phagocytosis. T cells with specific functions are classified into different subsets which mediate different responses. Most neonatal T cells are activated to generate TH2 responses, defined by the production of IL-4 and IL-13. These cytokines promote the differentiation of B cells to produce IgE antibodies and the M2 status of macrophages, and repress the production of IFN-γ. To generate these responses, T cells need to be activated via the presentation of their specific antigen by antigen-presenting cells. Once activated, T cells increase production of cytokines and often migrate to sites of infection. While neonatal T cells can be successfully activated by DCs, the lack of costimulatory molecules on DCs reduces this efficiency[36,37]. Additionally, $CD62L^+$ T cells which can migrate to sites of infection are in low numbers in neonatal lungs[68].

While traditional α/β T cells are prominent in adult lungs, neonatal lungs have specific subsets, notably virtual memory T cells (T_{VM}), which are classified by their naïve status with memory-like markers which indicate that this subset can rapidly respond to antigens for which they have not previously encountered. Additionally, they can be activated independently of antigens, instead rapidly expanding after cytokine signaling[69,70]. These are observed in highest amount at P8 and are greatly reduced by P10, suggesting they are a unique component of the NIS[71]. Neonatal mice also have populations of regulatory T cells (Tregs) which are primarily anti-inflammatory and produce TH2 cytokines and responses. Neonatal Tregs have also been observed to regulate inducible bronchus associated lymphoid tissue (iBALT)[72]. This is in contrast to Tregs in adult lungs, which interfere with BALT development[73]. Importantly, P2 neonatal T cells are more likely to develop into Tregs than adult T cells, but this ability is diminished by P14[74]. Additionally, infection of P2 neonatal mice with influenza required Tregs for clearance at 6–10 dpi (P8–P12)[75]. Tregs were also required for neonatal responses (P5-P8) to LPS, though this shifted after the neonatal stage (P12–P20)[76]. Additionally, beginning at P14, Tregs require PG-L1 for development, indicating a shift in immune system maturation[77].

T cell activity, specifically activation and expansion, can also be significantly affected by innate immune cells. MDSCs (discussed above) have a primary role in T cell suppression[65]. Despite this, it was observed that P5 mice inoculated with a mutant of *Bp* had an influx of neutrophils into the lungs, followed by T cells at 1 dpi (P6)[71]. However, it has also been observed that T cells are defective in migrating to neonatal alveolar spaces in lungs[17]. This evidence suggests an important role of neutrophils in mediating subsequent T cell responses in neonatal lungs. Additionally, the depletion of alveolar macrophages can significantly reduce neonatal T cell populations in the lungs[65,78].

2. B Cells

B cells are lymphocytes developed in the bone marrow that rearrange immunoglobulin genes to produce a surface antibody, can present peptides from recognized antigens, and with or without T cell help, can develop into different types of antibody-secreting plasma cells. While neonatal mice have high B cell numbers in the lungs, they do not proliferate similar to adults[20]. In addition to having capabilities different than those in adult mice, the composition of the B cell subsets in neonates differs greatly[79]. One particular subset in neonatal lungs is that of regulatory B cells (Bregs), differentiated by their production of IL-10. They colonize the lungs in the first week of life but are found in small numbers in adult lungs. The production of IL-10 has numerous effects on the NIS, including dysregulated neutrophil migration, T cell activation, and macrophage activation[16,19,37,40,80]. This can limit the neonatal immune response to pathogens. In fact, pups inoculated with RSV demonstrated IFN-γ production by alveolar macrophages, but this process was then repressed by IL-10 from Bregs[80].

3. Erythroid Suppressor Cells

$CD45^+CD71^+$ erythroid cells (CECs) are generated in the bone marrow and are strong regulators of the neonatal immune response. Their main functions include suppression of T cell immunity and production of ROS[81,82]. Neonatal mice (P3) had significantly more expansion of CECs than adult mice had, and this resulted in the increased suppression of T cell activation[80]. P6 neonatal mice are replete with $CD71^+$ CECs and highly susceptible to *Bp*, resulting in increased mortality by 8 dpi (P14)[81]. The depletion of CECs in neonates resulted in decreased susceptibility to *Bp* in the lungs. It was also observed that the impaired phagocytic ability of $CD11b^+$ cells contributed to increased susceptibility and was mediated by CEC-derived arginase II[31,81–83]. P6 mice were also found to be susceptible to *Listeria monocytogenes* and *E. coli*. Inoculation of mice at P15, however, resulted in 100-fold less bacteria in the lungs than that in those inoculated at P6. These older mice also had 60% fewer CECs than the younger mice. This suggests a relationship between the relative abundance of CECs in neonatal/juvenile mice and the ability to control bacterial infections[83].

Immune Proteins in Neonatal Lungs

1. Antibodies

In order to develop into antibody-secreting plasma cells, B cells are typically activated by antigens and stimulated by helper T cells. This process often takes place in secondary lymphoid organs, most notably the lymph nodes. However, naïve neonatal mice have poorly organized lymph nodes and low T and B cell numbers, resulting in low antibody titers[84]. Antibodies are produced as various isotypes (IgM, IgD, IgE, IgA, and IgG) which serve various roles in different stages of immune responses. The first isotype produced is IgM, followed by a process called class switching in which the isotype heavy chain is changed based on immune signals. While neonatal B cells can make antigen-specific antibodies and produce similar amounts of IgM to that of adults, neonatal B cells have low activation-induced cytidine deaminase (AID) expression[84]. This enzyme is responsible for isotype and class switching, and thus neonatal mice have reduced IgG titers compared to adults. However, early exposure to adjuvants or pretreatment with IFN-α can induce improved antibody responses[84,85]. Vaccination with Titermax Gold adjuvants at the day of birth (P0) has been observed to enhance the maturation of neonatal lymph nodes as early as at P1. These mice also generated antigen-specific IgG within 21 days after vaccination (P21)[84]. Additionally, P4 mice pretreated with IFN-α and then inoculated with RSV at P5 had increased B cell numbers and B cell activation at 14 dpi (P19). This treatment also increased RSV-specific IgA at 7 dpi (P12) and induced the expression of B cell-activating factor (BAFF) in the nasal associated lymphoid tissue (NALT)[85]. Thereby, neonatal mice are limited in their ability to naturally produce antibodies but also demonstrate an ability to produce antibodies and class switching upon stimulation.

IgG and other antibody isotypes play important roles in the neonatal response to pathogens, and much of the early neonatal IgG titer is transferred from the mother. Multiple studies have demonstrated the importance of maternal antibody transfer, a process mediated by a specialized receptor, the neonatal Fc receptor (FcRn). P6–P7 neonatal mice are protected from *E. coli* infection by maternal IgG obtained via the placenta or breast milk. This transfer of protection requires FcRn to transport IgG from a dam's milk to the serum of neonates[86]. Maternal antibodies also protected P7 and P14 pups from lethal challenge with herpes simplex virus (HSV). Importantly, optimal protection requires the transfer of antibodies via the milk and placenta. Additionally, pups inoculated at P7 and assessed at 14 dpi (P21) specifically require antibodies that activate Fcγ receptor 4 (FcγR4), suggesting an important role for antibody-dependent cellular cytotoxicity (ADCC)[87]. However, this protection conferred by maternal antibodies can also impede the primary generation of antibodies by neonates. Maternal antibodies can prevent B cells at neonatal germinal centers from developing into plasma cells and memory B cells, as well as prevent the isotype switching of antibodies. They also limit T follicular helper T cell expansion, thus affecting B cell activation and differentiation into plasma cells[88]. Therefore, maternal antibodies provide critical protection to newborns, but can also impede the neonatal primary development of antibodies.

As previously discussed, there is increasing evidence suggesting that neonates may be more susceptible to specific pathogens via their modulation of neonatal-specific immune features. As there are currently limited therapeutics to effectively treat these infections, there is considerable interest in proactively protecting neonates via maternal vaccination. This strategy has been demonstrated to be effective against fungal pathogens, with maternal vaccination against *Candida albicans* resulting in P3 pups being protected against infection by 3 dpi (P6). The transplacental transfer of antibodies was found to be critical for the protection and adoptive transfer of serum from vaccinated dams similarly protected pups[89]. Additionally, pups born to an antigen-vaccinated mother had high titers of antigen-specific antibodies on the day of birth (P0)[84]. Experiments have also been conducted to assess the feasibility of the primary vaccination of neonatal pups. P7 pups vaccinated with an outer membrane formulation from *Bp*, followed by a booster at P21 and challenge with *Bp* at P35 demonstrated greatly reduced bacterial numbers in the lungs compared to unvaccinated mice at 7 dpi. Additionally, pups vaccinated with the outer membrane vaccine had high titers of anti-pertussis toxin antibodies, an important marker of immunity, 14 days after the last booster (P35)[90]. However, with the immunization and inoculation course completed at 42 days post-birth, the information gained about the neonatal response is limited. Similarly, Noh et al. vaccinated P7 pups with an RSV glycoprotein core fragment followed by a booster at P21. At P35, while mice had RSV-specific antibodies, this humoral response was determined to be Th2-skewed due to the lower IgG1/IgG2 ratio observed compared to that in RSV-convalescent mice. When inoculated 4–5 weeks after the booster (P49–P56), immunized mice had a significant reduction in viral titers at 4 dpi (P53–P60)[91]. This suggests that responses developed in the neonatal period can have effects that extend into the juvenile and adult period. However, additional experiments originating and terminating in the neonatal period are required to better understand these effects. To this end, mice injected with vaccinia virus at P7–P8 had strong polyclonal B cell activation and IgG secretion 6 days after infection (P13–P14). However, when injected with alum to test responses to hapten carrier conjugates, P8 mice did not generate antibodies by 6 dpi (P14). By 10 dpi (P18), antibodies were detected, though at significantly lower titers than those observed in adults and the neonatal B cells were defective in their ability to differentiate into IgM- and IgG-secreting plasma cells[92]. P1 pups administered monoclonal antibodies specific for the SpA protein of *Staphylococcus aureus* and then challenged 24 hrs later to assess the efficacy of antibody treatment (P2) survived better than control pups did, suggesting antibodies alone are able to protect neonates[93]. This evidence illustrates the limited ability of neonatal B cells to produce effective antibody titers, but also the important role of IgG in neonatal responses.

2. Complement System

The complement system is a cascade of enzymatic components which rapidly amplify the highly localized release of anti-microbial and pro-inflammatory signals that enhance the functions of antibodies and phagocytes. However, few studies have assessed the role of the complement system in neonatal mice in the control of pathogens. The critical C3 protein, central to the complement cascade, is not transferred from the mother, but produced by the infant and is present at low levels in their serum[94]. Importantly, these low levels do not rapidly increase upon neonatal stimulation with tetanus toxoid, as they do in adults. Neonatal macrophages also have a limited capacity to synthesize C3 upon lipopolysaccharide (LPS) exposure[95]. Despite the low levels of complement observed in neonatal mice, this system can play an important role in responses to infection. In experiments by Wessels et al., $C3^{-/-}$ and $C4^{-/-}$ dams were vaccinated against Group B *Streptococcus* and the resulting pups were inoculated with Group B *Streptococcus* at P2. While pups born to $C4^{-/-}$ dams were able to control infection, a majority of the $C3^{-/-}$ pups succumbed to infection by 2 dpi (P4). This suggests that C3 is required for the generation of appropriate responses against bacterial infection[96]. These results suggest that the complement system may play an important, though poorly understood, role in mediating the neonatal response to pathogens.

ACTIVE SELECTION OF SYMBIONTS AND REJECTION OF PATHOGENS BY THE NEONATAL IMMUNE SYSTEM

Neonates/infants and adults have vastly different immune systems, with a growing list of profound differences in immune cells and their functions that leave newborns highly susceptible to certain pathogens. This apparent failure, and the later transition to a more adult-like immune system, support a common view that the NIS is an incompletely developed version of the AIS. However, newer work suggests that rather than being simply deficient, the NIS has different, and in some cases more effective, responses than the AIS, with the potential for vigorous responses being more tightly controlled[7,71]. These neonatal-specific responses, believed to prevent damaging inflammation, appear to contribute to the increased susceptibility of neonates to a subset of pathogens.

Contrary to the current dogma, neonates are not highly susceptible to all pathogens; the NIS can protect the host against most pathogens and therefore must possess some competent, albeit different, immune defenses[1]. To understand the important differences between the NIS and AIS, it may be useful to consider the very different challenges that are unique to the NIS. Specifically, the NIS has an additional responsibility beyond simply identifying and rejecting pathogens, which is the acquisition of a multitude of commensals that make up the complete microbiota. This is done at several body surfaces simultaneously, beginning immediately at birth and evolving rapidly based on the status of the microbiota and the associated host tissues. Performing this critical function while also surveilling for pathogens is a challenge particular to the NIS, which may explain some or all of its differences from the AIS[97,98]. This viewpoint may also contribute a new perspective on why the NIS appears to be less effective against particular pathogens.

Others have proposed that the NIS must be less inflammatory to deal with the onslaught of new organisms at birth, taking a more passive approach that avoids unnecessary inflammatory responses[99]. This perspective remains firmly focused on the role of the immune system as being the control/elimination of pathogens, which is understandable considering the profound burden of infectious disease. However, we have previously proposed that the immune system may be viewed more broadly as a system for managing the incredibly important microbiota that, together with the host, make up a healthy holobiont[97]. Our novel perspective here is an extension of that view, being that the NIS is not simply avoiding unnecessary damaging responses to the many new microbes and antigens encountered after birth but has <u>as its primary function</u> the healthy assimilation of a complete microbiota and is actively involved in that process. The critical transition from near sterile fetus *in utero* to a complete holobiont, the aggregate of host and its microbiota, occurs in the days and weeks after birth, requiring an immune system that functions very differently from that of an adult holobiont with a complete, stable and healthy microbiota. Here we will contrast the common view of the NIS as an incompletely developed AIS, with this alternative, and not necessarily mutually exclusive, view of the NIS as a highly evolved system for actively acquiring the complex microbiota to achieve a healthy holobiont.

We review a subset of recent experimental animal studies of early neonatal interactions with microbes that mostly focus on limitations and weaknesses of the NIS. We reinterpret these findings in light of the view that the NIS at birth is well evolved for the unique challenges of acquiring a healthy microbiota (**Figure 1.3**). Observations such as the relative tolerance to many MAMPs and PAMPs, which could be considered a weakness in defense against pathogens, are reconsidered as critical for the healthy assimilation of microbiota. One key concept that derives from this perspective is that the NIS may simultaneously select <u>for</u> harmless and potentially co-evolved commensal organisms and <u>against</u> pathogens to establish a protective microbiota. Additionally, apparent failures of the NIS to deal with some pathogens are discussed in the context of the prevailing need to rapidly acquire microbiota. The aim of this perspective is to reinterpret recent data concerning the NIS as a highly sophisticated system which efficiently meets the critical and urgent need for the host to acquire a healthy microbiota, with the hope that this perspective leads to reinterpretation of some studies and new considerations and experiments.

<u>Skin</u>

The first area to be exposed to the outside world, and thus develop a microbiota, is the skin. It is also considered to be the first line of defense against all future pathogens, making acquiring healthy skin microbiota crucial. Importantly, commensals of the skin can cause disease in other host sites, such as the nasal cavity, lungs, or GI tract, therefore the NIS must allow for their growth on the skin while preventing colonization/growth in other areas. The skin of newborn mice has been shown to have increased production of antimicrobial peptides (AMP) compared to older mice, primarily of the cathelicidin and B-defensin families; expression of mouse cathelicidin (CRAMP) was 10- to 100-fold higher in perinatal skin than adult murine skin[100]. Importantly, commensals of the skin, such as *S. epidermidis,* induce the production of, and are thus resistant to, AMPs which kill pathogenic group B *Streptococcus* (GBS) and *S. aureus*[101,102]. This suggests a highly evolved synergistic relationship between the NIS and skin commensals which promotes their colonization and a protective response against pathogenic microbes prior to cellular intervention[100].

While the roles of cellular immune components in the skin of neonates are not as well understood as that of other body areas, several studies have identified important contributions of cell-mediated regulation of skin inflammation and bacterial colonization. Tregs that are uniquely prevalent in the NIS contribute to tightly controlled inflammatory responses. A recent study demonstrated that neonates have a crucial window of colonization that is characterized by an abrupt influx of highly activated Tregs[103]. These Tregs are important for the establishment of immune tolerance to commensal microorganisms and inhibition of these cells abrogated tolerance and resulted in disease[103]. Neonatal mice also have a transient reduction of Tregs in the skin which causes an inflammation-mediated dysregulation of subcutaneous tissue in skin, which primes the skin for altered reparative responses to wounding[104]. This suggests that populations of Tregs in the skin shift in response to bacterial colonization. While these studies suggest a role for Tregs in regulating inflammation, they also demonstrate a role in selecting for healthy commensal skin microorganisms, with knock on effects when this role is disrupted. Leech et al. 2019 demonstrated that neonatal exposure to the commensal *S. epidermidis* facilitates specific Treg tolerance, while a toxin produced by the pathogenic species *S. aureus* mediates influxes of effector T cells and subsequent inflammation[98]. Tregs in adults are known to have anti-pathogen mechanisms, however this has not been fully investigated in neonatal models. Considering a critical role in mediating microbiota acquisition suggests that in addition to selecting <u>against</u> pathogens, the NIS may select <u>for</u> healthy, co-evolved commensals that have acquired resistance to skin antimicrobials and may interact with Tregs to reduce inflammation. Further studies contrasting the different interactions of commensals and pathogens with neonatal Tregs might reveal important mechanisms by which they are distinguished and encouraged or discouraged.

<u>Lungs</u>

The lungs are known to be one of the most disease-susceptible organs in adults and newborns. To function properly, their vast surface area must remain nearly sterile despite the repeated introduction of microbe-rich air. Inflammatory responses can impede respiratory function and may be more disastrous than the infection itself, particularly in infants. While a protective microbiota is an important factor for preventing pathogenic colonization, its establishment may be particularly challenging. This suggests that a complex and delicate system must exist which allows the deposition and colonization of commensal organisms, while preventing pathogen colonization with minimal inflammation. Similar to the skin, neonatal mice have high amounts of $CD4^{+}FoxP3^{+}CD25^{+}Helios^{+}$ Tregs in the lungs which play a large role in managing inflammation. Importantly, the diversity of the microbiota in the lungs at birth is fairly low and increases significantly during the first 2 weeks after birth. This shift in microbiota is associated with a shift from $CD4^+FoxP3^+CD25^+Helios^+$ Tregs being the primary Treg subset to the emergence of $Helios^-$ Tregs that require interaction with PD-L1[77]. While this work supports the premise that the lung microbiota induces regulatory cells in early life, it is also plausible that the Treg populations mediate the development of the microbiota at each stage.

An interesting phenomenon that has gained increasing attention is the gut-lung axis, of which the microbiota and inflammatory signals from each can have a large impact on the other. A recent study determined that maternal-derived γ/δ T cells have an important role in shaping offspring TH2 immune responses in a microbiota-dependent manner[105]. Specifically, offspring of $TCR\gamma/\delta^{-/-}$ dams acquired distinct intestinal microbiota which had decreased production of short chain fatty acids (SCFAs), often the end products of bacterial metabolism and a key mediator of inflammatory responses in the neonatal period. This led to increased type 2 inflammation in the lung, demonstrating a microbiota-dependent gut-lung inflammatory axis[105]. This work suggests that γ/δ^+ T cells are an important immune component for the selection of gut microbiota that reduce or prevent inflammation in the lung.

The healthy assimilation of respiratory microbiota can be severely disrupted by administration of antibiotics to neonates. In experiments with rhesus macaques, antibiotic administration after birth resulted in delayed maturation of intestinal microbiota, which was associated with reduced hematopoietic cytokines and decreased neutrophil populations. Challenging such animals with *S. pneumoniae* revealed that antibiotics treatment increased inflammatory reactions in the lungs, mainly from neutrophils and AMs[106], suggesting substantially more cross-talk with the microbiota than would be predicted by the conventional view of the NIS as incompletely developed. If a primary role of the NIS is mediating acquisition of a healthy microbiota, then such cross-talk might be an expected outcome of the antibiotic's disruption of the development of the holobiont. Indeed, the NIS of the respiratory tract may require certain signals from resident microbiota to efficiently develop into an AIS. Understanding those signals might be critical to guiding the treatment of neonatal respiratory infections, which currently center on antibiotics that may complicate AIS development in this critical organ.

Gastrointestinal Tract

The most diverse and widely appreciated microbiota is that of the gastrointestinal tract (GI). The mammalian GI microbiota is substantially affected by antibodies from mother's breast colostrum/milk. Despite the protection granted by maternal-derived factors, neonates are highly susceptible to bacterial meningitis, specifically with GBS which typically originates in the GI tract. However, recent research suggests that this susceptibility is not completely dependent on the microbiota, but the structure of the GI tract and immune system[107]. The Wnt signaling pathway, which regulates cellular calcium levels, has been recently observed to be age-dependent, not strictly microbiota dependent as previously thought. This pathway in the neonatal gut is increased, leading to decreased cell-cell junction polarization[107]. Recent work also indicates that postnatal replenishment of enteric glial cells (EGCs) is dependent on the microbiota[108]. However, Myd88$^{-/-}$ mice that are defective in signaling pathways involved in detecting microbes lack several EGC markers[108]. These observations suggest that the replenishment of EGCs is dependent on both the intestinal microbiota and the Myd88 pathway for detecting them, supporting the view of substantial crosstalk in development. Observation of age-dependent expression of the flagellin receptor TLR5 in the mouse gut epithelium, which mediates REG3γ production, critical for the counter-selection of colonizing flagellated bacteria, further supports this view. Thus, neonatal

TLR5 expression influences the composition of the microbiota throughout life and potentially selects for unflagellated commensals and against possible pathogenic flagellated bacteria[109].

In addition to substantial differences in receptors and pathways, the neonatal GI system includes unique T cell populations and functions which can affect microbiota development. A commensal *Propionibacterium* strain, P. UF1 isolated from the gut of newborn mice caused increased numbers of Th17 cells and maintained IL-10$^+$ Tregs and protected them from *Listeria monocytogenes.* P. UF1 was also associated with expression of neonatal murine genes that regulate TH17 cell differentiation, suggesting that this intestinal commensal regulates T cell-mediated immunity[110]. However, it is also possible that the increase in TH17 cells regulates the populations of P. UF1. Additional work has observed that specific components of the neonatal intestinal microbiota are required for effective protection against pathogens[111]. Neonatal mice lacking *Clostridailes* ssp. were unable to prevent colonization by *Salmonella enterica* serovar Typhimurium or *C. rodentium*[111]. This suggests that the NIS has co-evolved with commensals such as *Clostridailes* ssp. to allow, and perhaps encourage, their colonization, which then acts as a competitive force to prevent pathogenic colonization.

Implications

We have previously argued that, in addition to protecting against pathogens, a critical role of the mammalian AIS is in mediating the complex interactions with the very diverse set of symbionts and commensals that constitute our healthy microbiota[97]. The NIS undoubtedly must deal with occasional pathogens, and it is reasonable to consider that this process is greatly complicated by the onslaught of new microorganisms and antigens first encountered after birth, requiring some modulation of response. But it may be myopic to view non-pathogens as simply distracting the NIS from its primary role of repelling pathogens. Viewing the immune system in general as a complex system of microbiota management changes the perspective on the unique challenges of the NIS. The NIS mediates interactions with microorganisms during the transition from near sterile infant in uterus to microbiota-rich healthy holobiont. There may be substantial insight from the broader perspective of the NIS as uniquely evolved to not just survive this onslaught, but to mediate the acquisition of those that can potentially contribute to a healthy holobiont. Hosts as diverse as plants, fungi, algae, insects, and cephalopods have evolved complex mechanisms to acquire and maintain their symbionts. Indeed, there is increasing evidence of the many effects our microbiota can have on our health. Considering a, or perhaps <u>the</u>, primary role of the NIS to be mediating the acquisition of a healthy microbiota leads to very different interpretation of its differences to the AIS, focusing on the potential evolutionary advantages of some of these profound differences. We propose that this alternative perspective may generate more questions, hypotheses, and ultimately better understanding of the NIS.

IMMUNITY TO *BORDETELLA PERTUSSIS*

<u>Microbial Characteristics</u>

Bp is a strict aerobic, gram-negative coccobacillis (0.2-0.3 μm X 0.5-0.8 μm)[112]. Its genome is 4.806 Mbp and contains 3576 genes[113]. Growth is fastidious, with strict growth conditions required, including incubation at 37° C for 4-5 days on Bordet Gengou blood agar plates, on which colonies appear as small, gray/white and beta hemolytic. Liquid culture is achieved with Stainer Scholte medium, which contains necessary amino acids and growth factors (glutamic acid, proline, cysteine, nicotinamide, and glutathione)[114]. *Bp* is typically regarded as a human-restricted pathogen, though inoculation of rodents, non-human primates, and other mammals have been successful via washing large numbers of bacteria deep into the respiratory tract[115–117].

BVG Regulation System

The host immune system is affected by a slew of virulence factors expressed by *Bp*. Many of these virulence factors are expressed under the control of the two component Bordetella virulence gene (BvgAS) system. BvgS is a sensor kinase that is activated under several environmental conditions, the most well-known/described being temperature (37° C) and the presence of certain salts. Once activated, BvgS phosphorylates BvgA, which directly activates expression of virulence-associated genes (vags). Below, we discuss several vags which are expressed in Bvg^+ phase[118].

Virulence Factors

1. Pertussis toxin

Pertussis toxin (PTx) is an exotoxin produced exclusively by *Bp*. It is an AB_5 toxin, composed of a catalytic A domain and B binding domain, the latter of which is made of 5 subunits. Binding is mediated by sialic acid-containing glycoproteins on the B domain to a variety of glycosylated molecules on mammalian cells. The toxin enters the cells via endocytosis then undergoes retrograde transport from the Golgi to the endoplasmic reticulum. This process results in the B domain undergoing a conformational change that releases the A domain into the cytosol[119]. Once released from the B domain complex, the A domain functions by catalyzing the ADP-ribosylation of a specific subset of membranous G proteins. This occurs via hydrolysis of cellular NAD and the resulting ADP-ribose is transferred to a specific cysteine residue within C terminal end of the target G protein[119]. This activity locks G proteins into an inactive state, unable to couple to the cognate G-protein coupled receptors. This uncoupling disrupts communication between the receptor and adenylyl cyclase, resulting in unregulated conversion of ATP to cAMP which disrupts cellular signaling[119,120].

The role of PTx in *Bp* disease process has been assessed through the use of a PTx-deficient mutant, generated via allelic exchange of the plasmid pJ-ΔPT. This plasmid was generated via amplification of 2 fragments from the *ptx* region, an upstream fragment from the S1 signal peptide and a downstream fragment from the S3 subunit. This resulted in confirmed deletion of the 5 structural genes for PTx (*ptxS1, ptxS2, ptxS4, ptxS5, ptxS3*). The lack of PTx production was confirmed by Western blot and Chinese hamster ovary cell (CHO) assays. The mutant was constructed from the *Bp* strain in the Tohama I background and there were no observed differences in growth conditions between the WT and Δptx strains[121]. Utilization of this mutant in mouse models has resulted in several significant findings regarding the role of PTx in the disease process of *Bp.* This includes early colonization of the respiratory tract, delay of lymphocyte recruitment, disrupted cytokine signaling, and overall disease severity[68,71,121–137]. These effects, however, vary by age of the mouse, with more severe effects being observed in neonatal and juvenile mouse models, suggesting there are specific interactions between PTx and age-related immune features.

2. Lipopolysaccharide

Lipopolysaccharide (LPS) is a molecule produced by all gram-negative bacteria. It is typically composed of three domains: (A) lipid A, (B) oligosaccharide core, and (C) O antigen. However, *Bp* is unique in that it lacks the O antigen, thus being referred to as lipooligosaccharide (LOS)[138–140]. A major component of the outer membrane, the loss of the O antigen domain renders *Bp* more susceptible to host complement. A variety of additional bacterial factors, however, make up for the loss of O antigen[139,141,142].

3. Adenylate cyclase toxin

Adenylate cyclase toxin (ACT) is an RTX toxin composed of 5 domains and exported by the type I secretion system. ACT oligomers form small pores in their target cells, typically $CD11b^+$

phagocytes[143]. This results in the killing of macrophages and impaired DC maturation and cytokine secretion, leading to reduction in IL-1β polarization of T cells[144,145].

4. Fimbriae

Fimbriae are long hair-like appendages composed of chains of subunits, either Fim2 or Fim3, which assists in binding of bacteria to host cells[146]. This activity also interrupts the complement cascade via binding of the FimD subunit to monocytes. Fimbriae typically work in combination with filamentous hemagglutinin to attach to host immune cells[147].

5. Filamentous hemagglutinin

Filamentous hemagglutinin (FHA) is an adhesin which interacts with host epithelial and immune cells[148]. Its binding to macrophages results in disrupted complement activities and IL-12 production, all of which leads to delayed activation of the adaptive immune response, allowing for bacterial persistence[149,150].

<u>Disease Progress/Pathogenesis</u>

Transmission of *Bp* typically occurs via droplet exposure, though there is some evidence which suggests it can be transmitted by aerosol[151–153]. Disease progress is divided into four distinct stages. The bacteria typically first colonizes the nasal cavity, where an incubation period typically lasts 7-10 days. The following catarrhal phase lasts ~1-2 weeks, includes nonspecific respiratory symptoms, and is considered the most contagious stage. However, this stage can also present asymptomatically. The third stage is the paroxysmal phase and lasts ~3-8 weeks. This stage is characterized by the paroxysmal cough ("whooping" cough) symptom. While adults may never display the paroxysmal cough, this is typically the most severe stage of disease for infants and results in numerous complications, such as pneumonia, pulmonary hypertension, and encephalopathy, among others. The final stage of *Bp* disease is the convalescent phase, in which disease subsides and the bacteria are ultimately cleared. Due to the nonspecific symptoms which occur early in infection, and difficulties culturing, PCR testing is the primary method of diagnosing *Bp* disease[152,154].

<u>Adult Immunity to *Bordetella pertussis*</u>

The host immune response to *Bp* has primarily been assessed in adult mouse models and involves a high bacterial inoculum ($5x10^5$ CFU) that is delivered to the lungs, either via intranasal inoculation to the external nares or aerosolization[117,155,156]. The typical progression of disease in mice includes significant growth of CFU in the lungs from time of inoculation to 3 days post inoculation (dpi), after which there is a steady reduction in CFU. Beginning at approximately 7 dpi, there is significant inflammation of the lungs due to rapid infiltration of lymphocytes. This inflammation continues until approximately 28 dpi, with the peak of inflammation occurring at 14 dpi[121,127,157]. Resolution of disease occurs at ~56 dpi and results in robust convalescent immunity.

It is understood that one mechanism by which *Bp* causes such severe disease in the adult model is due to its ability to modulate host innate immune responses. Though typically able to respond to infection within hours of inoculation, neutrophil recruitment is significantly affected by *Bp* virulence factors, which delay recruitment until 5-7 dpi, allowing *Bp* to grow to large numbers resulting in eventual recruitment that peaks around 10-14 dpi[127]. Associated with significant lung inflammation, this disruption of neutrophil arrival is typically attributed to the effects of PTx and ACT[26,127,158]. Importantly, neutrophils also require antibody assistance to mediate uptake of bacteria, resulting in further delay of clearance[127]. *Bp*, however, produces factors to protect against neutrophil-mediated phagocytosis and alternative complement-mediated killing[124,140,141,143,145,159–162]. Despite this, *Bp* is susceptible to the classical complement cascade, a process which also requires antibodies[161–163]. Macrophages are another important immune factor, mediating clearance via phagocytosis, a function assisted by IL-17 and IFN-γ[126,144,164,165]. The latter is primarily generated by NK cells early in infection, which promotes a TH1 response to *Bp*, without which disease severity greatly increases and can expand beyond the lungs[157,166–168].

One of the most well-appreciated immune factors to contribute to control of *Bp* in adults is T cells. T cells responses are generated approximately 3 weeks after inoculation and are typically CD4-skewed[157,168]. Specifically, IL-17 and IFN-γ secreting $CD4^+$ T cells are required for control in order to stimulate and magnify innate immune mechanisms of control, as previously described[130,157,169,170]. The generation of TH1 and TH17 responses are vital to control, as IFN-$\gamma^{-/-}$ and IL-$17^{-/-}$ mice fail to clear *Bp*[165,171,172]. Contrastingly, IL-$4^{-/-}$ mice have no significant failure to clear *Bp,* demonstrating a minimal role for TH2 responses[167]. γ/δ T cells are recruited to the lungs early after inoculation, at ~3 dpi. However, their role is not as clear as traditional α/β T cells, as $\gamma/\delta^{-/-}$ mice have less CFU in the lungs than immunocompetent mice but have significantly more lung pathology[168,173]. The wide use of murine models has provided invaluable information on the adult immune response to *Bp,* however immune responses and susceptibility vary greatly by age and must therefore also be assessed.

<u>Neonatal Immunity to *Bordetella pertussis*</u>

While adults typically either experience asymptotic infections or mild, cold-like symptoms, infants are more susceptible to deeper respiratory infections with more serious symptoms, such as an intense paroxysmal cough, leukocytosis, and pneumonia[174]. Briefly, ~33% of *Bp* infections in children under 1 year old require hospitalization and infants under 6 months old make up ~90% of deaths[175,176]. While vaccines against *Bp* are available and widely utilized, they are not administered to infants under 2 months old[177]. These vaccines in adults also do not prevent asymptomatic nasal carriage and transmission, and therefore, infants are often infected by asymptomatic caregivers[178,179]. This likely stems from the vaccines being developed for and evaluated in children older than the most susceptible group (>6 months)[180]. Additionally, the efficacy of current and potential vaccines are often tested with adult animal models[181–184]. In addition to imperfect vaccines, antibiotic treatments have limited effect after the presentation of diagnostic symptoms[185]. Therefore, more information on the effects of *Bp* on the NIS is required to develop the most appropriate treatments and vaccines.

Some experiments have used neonatal mice to assess the specific response against *Bp*[68,71,90]. These experiments suggest that, like human infants, neonatal mice are more susceptible to *Bp* than adult mice are. Neonatal mice and humans fail to control *Bp* infection and suffer increased mortality and lung inflammation[68,71]. Neonatal mice also develop pulmonary hypertension, one of the primary causes of death of human infants with *Bp*[129,186]. Recent work suggests that this increased susceptibility is due to the dysregulated activity of neonatal immune cells caused by bacterial factors. For example, the reduction in CEC populations in P7 neonates resulted in reduced susceptibility to *Bp*[31]. A possible mechanism for this increased susceptibility is the production of arginase II, which impairs the phagocytic ability of $CD11b^+$ cells[31]. Neonatal mice also have a subset of cells called virtual memory T cells (T_{VM}) (see above)[71,187]. It was observed that as mice aged, their T_{VM} populations and susceptibility to *Bp* greatly decreased[71]. Experiments aimed at assessing neonatal vaccination against *Bp* utilized P7 neonatal mice immunized with an outer membrane protein. Vaccination greatly reduced bacterial numbers in the lungs and elicited high levels of anti-PTx antibodies[88]. These results suggest anti-PTx antibodies can provide some level of protection to neonates.

Currently, the primary mechanism with which to assess clinical protection against *Bp* is to measure serum levels of anti-PTx antibodies, though the true protection provided by these titers is debated[188–190]. The primary effects of PTx on *Bp* disease in adult mice include delayed lymphocyte accumulation to the site of infection, the suppression of serum antibody responses and airway inflammation, in addition to important roles in early colonization[121,122,125,127,134,191]. The largest contribution of PTx to *Bp* infections in adults was observed by Kirimanjeswara *et al.*, in which transferred antibodies rapidly cleared a mutant of *Bp* lacking PTx (*Bp*Δ*ptx*) but not the wildtype *Bp.* The ~10,000-fold reduction observed with the PTx mutant indicates that PTx effectively blocks rapid antibody-mediated clearance[127], allowing *Bp* to persist in immune hosts. More recently, PTx was shown to have greater effects on *Bp* infections in neonatal mice than those in adults[68,71,121]. P7 mice inoculated with *Bp*Δ*ptx* had a ~100-fold reduction in bacterial numbers in the lungs at 14 dpi (P21) compared to wildtype *Bp*[68]. In a similar model, P7 neonatal mice inoculated with wildtype *Bp* had symptoms of pulmonary hypertension, while those inoculated with *Bp*Δ*ptx* lacked these symptoms[129]. However, an even greater effect was observed in mice inoculated at P5 and assessed at P8, a period more completely dominated by neonatal immunity, in which *Bp*Δ*ptx* had a >10,000-fold reduction in bacterial numbers compared to wildtype *Bp.* This effect, much greater than that observed in adult mice, was accompanied by neutrophil accumulation as early as 2 hrs post inoculation with *Bp*Δ*ptx*, followed by increased T cell populations at 1 dpi (P6). Inoculation with the wildtype *Bp*, however, did not cause any increases in immune cell populations until 3 dpi (P8). Importantly, these younger mice displayed a more purely neonatal immune environment, with substantial populations of T_{VM} at P8, and modeled the increased susceptibility of human infants to *Bp*[71]. These experiments indicate that the NIS could effectively control *Bp* infections, but some key functions are blocked by PTx. Further study of these phenomena will likely reveal important immune protection mechanisms in neonates.

CONCLUSIONS

The NIS is a complex network with a primary goal of ensuring the survival of the host via protection against dangerous pathogens. Compared to the well-studied AIS, it may appear deficient in some respects, but viewing the NIS as an incomplete version of the adult's is missing its critical and unique challenges. The most immediate challenge is the exit from the near-sterile uterus, which represents a unique transition which suddenly bombards the immune system with huge numbers and a diversity of microbes, each expressing thousands of antigens and many of which immediately begin to establish permanent residence in intimate contact with all body surfaces. We have promoted the view of the mammalian immune system as a form of microbiome management[2,97]. From this standpoint, birth initiates a sudden and intense period of rapid assimilation of novel commensals and symbionts. While the AIS benefits from prior "knowledge" of these organisms, mostly dealing with a small number of new potential pathogens at any given time, the NIS must rapidly and efficiently determine the benefits and risks of reaction to many microorganisms nearly simultaneously. We propose a view of neonatal immunity as not a flawed version of adult immunity, but as a system evolved to face this particular challenge, very different from that of an adult. With this perspective, and a widening array of experimental tools, a deeper understanding of the workings of the NIS should emerge that will better inform our approaches to protect newborns from disease.

FIGURES

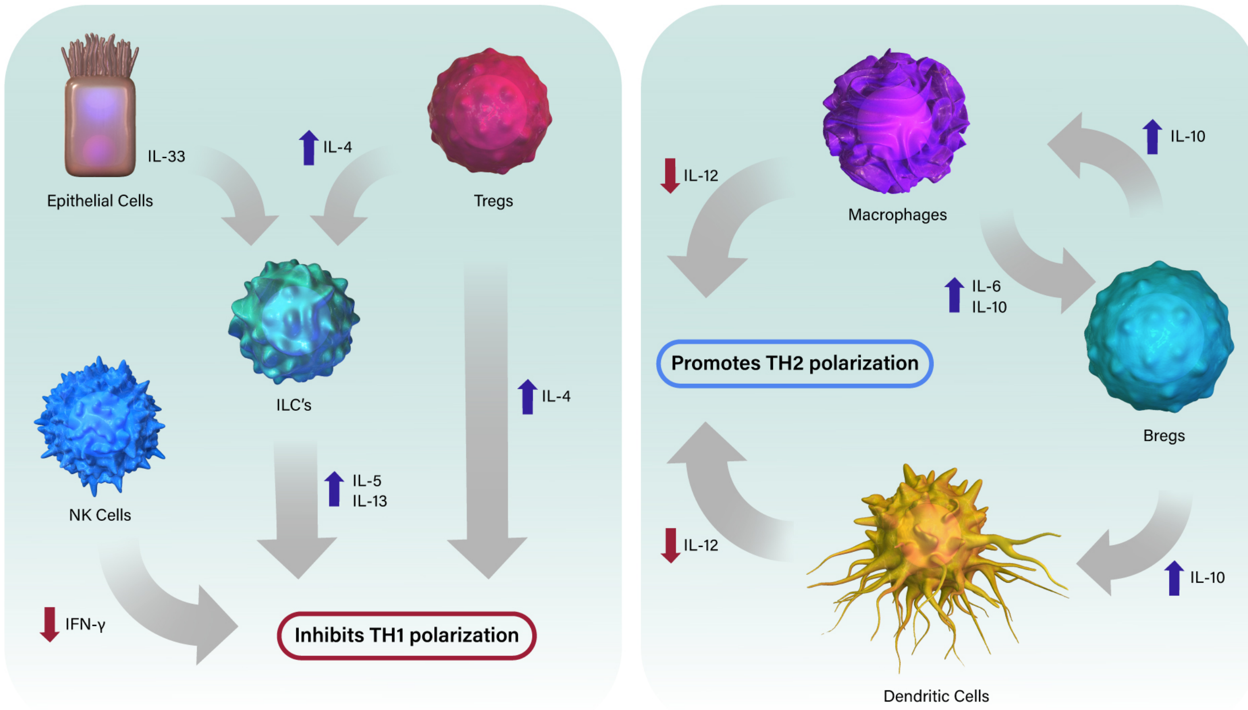

Figure 1.1. Schematic view of factors contributing to the inhibition of TH1 polarization and promotion of TH2 polarization in the neonatal pulmonary environment. Red arrows indicate decreased production and blue arrows indicate increased production. Illustration by Sofia Nahman, Educational Resources, University of Georgia College of Veterinary Medicine.

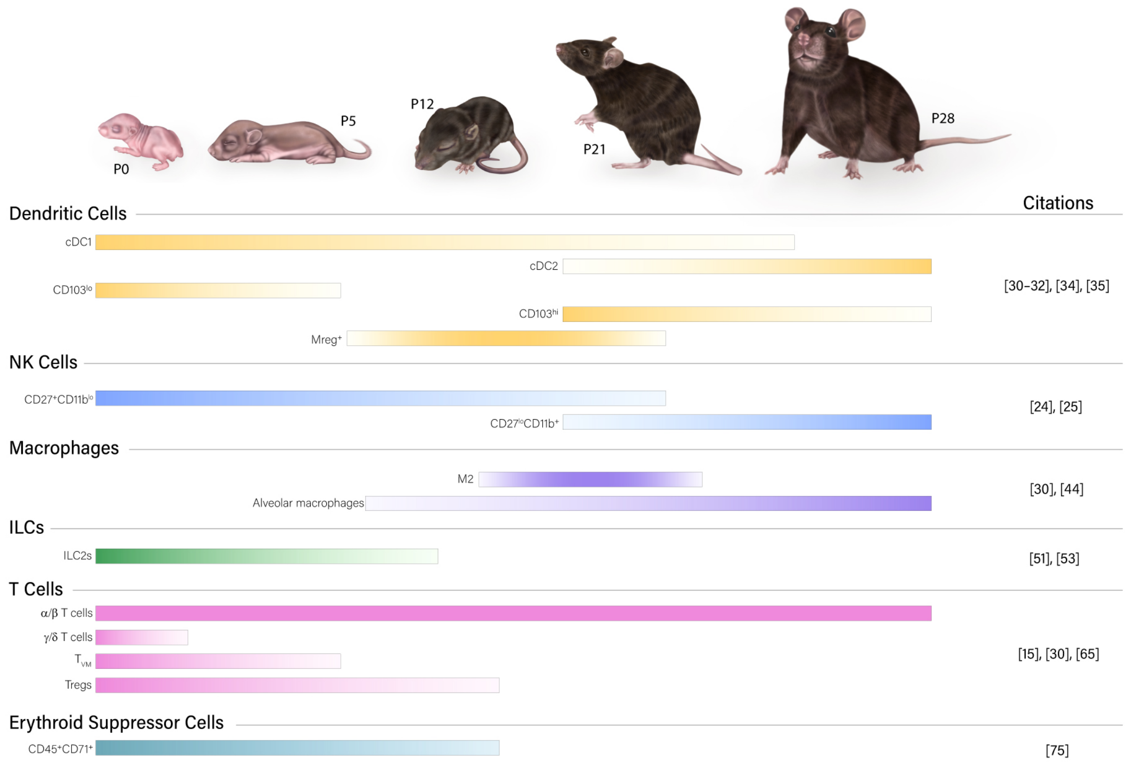

Figure 1.2. Relative frequency of immune cell types/subsets in the neonatal pulmonary immune system. Illustration by Sofia Nahman, Educational Resources, University of Georgia College of Veterinary Medicine.

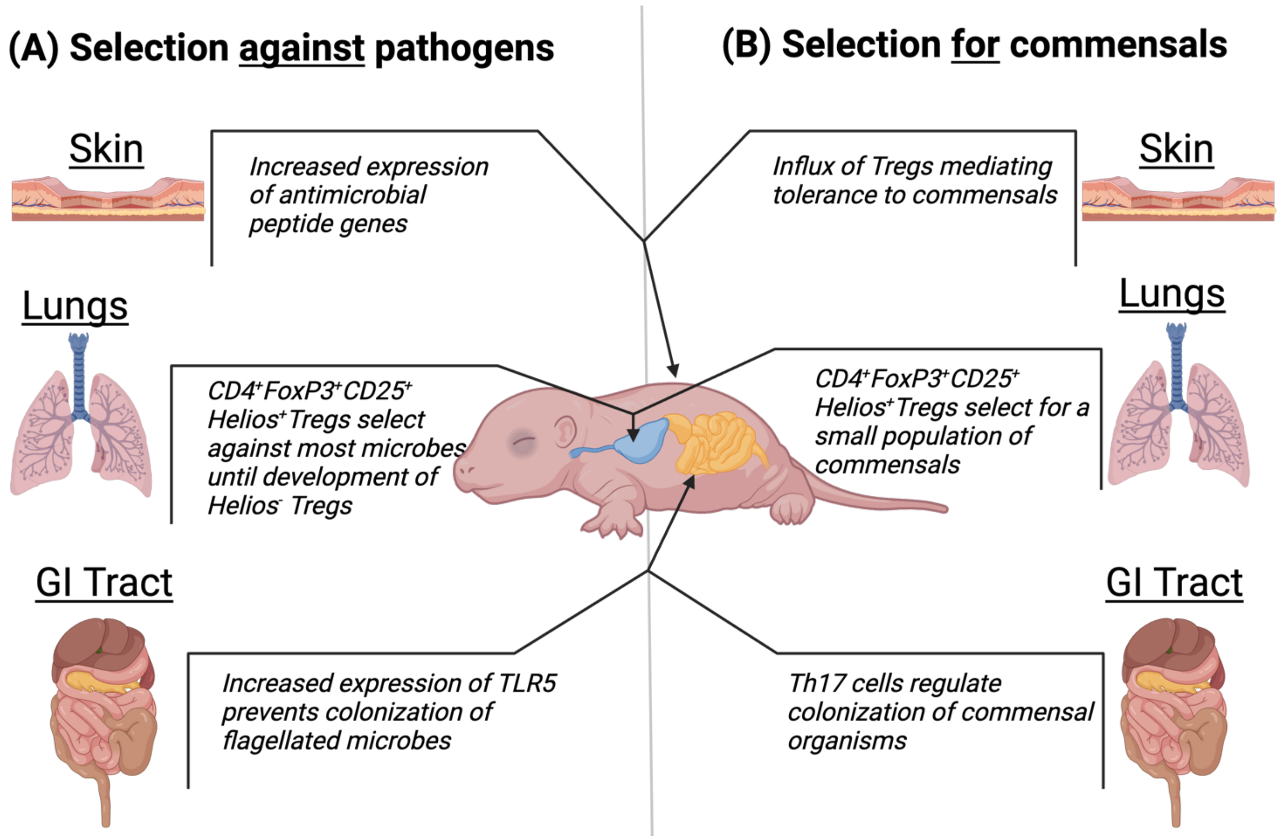

Figure 1.3. Traditional and alternative view of neonatal microbiota development. Mechanisms at the skin, lungs, and GI tract body sights which suggest a role of the neonatal microbiota to select against pathogenic colonization (**A**). Mechanisms at the skin, lungs, and GI tract body sights which suggest a role of the neonatal microbiota to select for colonization of healthy commensals (**B**). Created with Biorender.

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CHAPTER 2

NOVEL MURINE MODEL REVEALS AN EARLY ROLE FOR PERTUSSIS TOXIN IN DISRUPTING NEONATAL IMMUNITY TO *BORDETELLA PERTUSS*[1]

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ABSTRACT

The increased susceptibility of neonates to specific pathogens has previously been attributed to an underdeveloped immune system. More recent data suggests that neonates have effective protection against most pathogens but are particularly susceptible to those that target immune functions specific to neonates. *Bordetella pertussis* (*Bp*), the causative agent of "whooping cough", causes more serious disease in infants attributed to its production of pertussis toxin (PTx), although the neonate-specific immune functions it targets remain unknown. Problematically, the rapid development of adult immunity in mice has confounded our ability to study interactions of the neonatal immune system and its components, such as virtual memory T cells which are prominent prior to the maturation of the thymus. Here, we examine the rapid change in susceptibility of young mice and define a period from five- to eight-days-old during which mice are much more susceptible to *Bp* than mice even a couple days older. These more narrowly defined "neonatal" mice display significantly increased susceptibility to wild type *Bp* but very rapidly and effectively respond to and control *Bp* lacking PTx, more rapidly even than adult mice. Thus, PTx efficiently blocks some very effective form(s) of neonatal protective immunity, potentially providing a tool to better understand the neonatal immune system. The rapid clearance of the PTx mutant correlates with the early accumulation of neutrophils and T cells and suggests a role for PTx in disrupting their accumulation. These results demonstrate a striking age-dependent response to *Bp*, define an early age of extreme susceptibility to *Bp*, and demonstrate that the neonatal response can be more efficient than the adult response in eliminating bacteria from the lungs, but these neonatal functions are substantially blocked by PTx. This refined definition of "neonatal" mice may be useful in the study of other pathogens that primarily infect neonates, and PTx may prove a particularly valuable tool for probing the poorly understood neonatal immune system.

INTRODUCTION

Newborns and young children are highly susceptible to some infectious diseases, such as measles, respiratory syncytial virus (RSV), and whooping cough[2–4]. This has been attributed to aspects of fetal-maternal tolerance and/or an "immature" immune system which is believed to generate weaker, less inflammatory responses relative to adults[5–7]. However, neonates are not extraordinarily vulnerable to all infections, indicating that infants and young children are capable of mounting very effective, protective immune responses against most pathogens, even prior to maturation of the thymus.

The classical adult immune system that is most well-known and studied is comprised of conventional T cells which develop from bone marrow hematopoietic stem cells and mature in a fully differentiated thymus. These T cells undergo a complex selection process to generate antigen-specific immune responses to many pathogens[8]. Prior to the establishment of the classical adult immune system, neonates are replete with distinct populations of immune cells, including myeloid-deriver suppressor cells, $CD71^+$ erythroid cells, and neonatal T cells[7,9–13]. Prominent amongst these fetal liver hematopoietic stem cell-derived T cells are those that display a virtual memory phenotype (T_{VM}) ($CD3^+CD44^{hi}CD49d^{lo}CXCR3^+Eomes^+$) and have a broadly reactive T cell receptor[11,14–16]. Importantly, these cells generate rapid and robust responses to early infection with various pathogens, which differs greatly from the typical delay in adaptive immune responses facilitated by conventional T cells[6]. Recent studies have demonstrated that neonatal T cells can effectively expand and respond to viral and bacterial infections[17–20]. While T_{VM} and other neonatal T cell subsets are prominent in neonates, most are gradually outnumbered and displaced by conventional adult T cells derived from bone marrow-derived progenitors matured in the thymus during the transition from neonate to adult.

Our ability to study neonatal immunity using the mouse model has been confounded by the very rapid thymic maturation in mice that begins to generate adult immune cells in the first week after birth. Due to this, experiments that involve mice older than one week (7 days) cannot clearly distinguish the effects of neonatal and adult T cells[10,21–24]. This is in contrast to human children, in whom thymic development is much slower, providing a wider window of vulnerability to some pathogens that appear to target this particular stage. One such pathogen is *Bordetella pertussis* (*Bp*), the etiologic agent of whooping cough, an internationally recognized re-emerging infectious disease that is highly virulent in neonates. It is estimated that there are over 5.1 million cases of whooping cough in children under 1-year-old annually, with nearly 86,000 of these cases resulting in infant mortality[4]. Additionally, infection with *Bp* is associated with a number of complications in neonates and young children, including pneumonia, seizures, pulmonary hypertension, and encephalopathy[25]. Extensive work has greatly informed our understanding of how this pathogen interacts with the adult immune system, leading to the development of vaccines capable of preventing disease in children, adolescents, and adults. However, due to the limitations of available models, our understanding of the neonatal response to *Bp* has resulted in few preventative options in the very young. Thus, extraordinary lengths are often taken to prevent infant exposure, for example by booster vaccinating all their potential contacts, a practice referred to as "cocooning"[22,23,26].

One of the major virulence factors that contributes to pertussis disease is pertussis toxin (PTx), an AB_5 toxin that disrupts G protein coupled receptor signaling in various cell types[27,28]. PTx also interacts with T cell receptors to initiate signaling events and causes desensitization to signals such as chemokines[29,30]. PTx has also been demonstrated to inhibit neutrophil recruitment in early infection, resulting in delayed control of *Bp*[31]. One of the most notable published effects of PTx on young mice was observed with animals that were challenged with *Bp* at 7-days-old (P7) then evaluated 7 days later in mice that were 14-days-old (P14), which suggested a special interaction between PTx and the "neonatal" murine immune system[24]. However, the rapid development of the murine thymus begins well before P14, so the relative contributions of neonatal and adult-like immune cells are difficult to distinguish in this experimental setup.

Here we demonstrate that five- to eight-day-old mice (P5-P8) have substantial numbers of T_{VM} in the lungs during the first week of life, but by ten to fourteen-days-old (P10-P14), T_{VM} numbers in the lungs are already largely eclipsed by numbers of conventional T cells. To more clearly separate the interactions of *Bp* with neonatal immunity, we present a novel model that focuses on the period before the introduction of substantial conventional T cells. We demonstrate significantly increased sensitivity to *Bp* growth and expansion in P5 mice, relative to mice even two days older (P7). Although highly susceptible to wild type (WT) *Bp*, P5 mice rapidly controlled and eliminated a PTx-deficient mutant of *Bp.* Efficient neonatal immune-mediated clearance was associated with rapid accumulation of neutrophils within 2 hrs post inoculation and T cells within 1 day post inoculation. This experimental system more completely focuses on neonatal immunity, demonstrating that it can be highly effective against respiratory infections. Importantly, our data indicate that PTx specifically disrupts these neonatal-specific functions, potentially explaining the extraordinary sensitivity of newborns to *Bp* and not all other pathogens.

MATERALS AND METHODS

Bacterial Strains and Growth

The *Bp* strains Tohama 1 (WT *Bp*) and BPH101, an isogenic pertussis toxin-deficient derivative (*Bp*Δ*ptx*) have been previously described[24,32–34]. Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% defibrinated sheep blood (Hemostat Laboratories) and either 20 μg/ml streptomycin (Sigma-Aldrich) for *B. pertussis*Δ*ptx* (Sigma-Aldrich) or 20 μg/ml gentamycin (Sigma-Aldrich) for WT *Bp*. Liquid cultures were grown overnight in Stainer-Scholte broth at 37° C to mid-log phase then maintained in 20% glycerol stocks at -80° C for use as inoculum. Purified pertussis toxin (PTx) was obtained from Sigma-Aldrich as a lyophilized powder and resuspended with 500 μl PBS (P7208-5OUG). The purified PTx was added to the described inoculum at a concentration of 10 ng/μl.

<u>Mouse Experiments</u>

Six- to eight-week old female and male C57Bl/6J (00664), B6.129P2-*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom}/J (T cell$^{-/-}$) (002122), and B6(Cg)-*Il15*$^{tm1.2Nsl}$/J (IL-15$^{-/-}$) (034239) mice were procured from the Jackson Laboratory (Bar Harbour, ME) and bred in the Harvill mouse colony (University of Georgia, GA). All mice were maintained in specific pathogen-free facilities, and all experiments were conducted following institutional guidelines and IACUC approvals. Pups were utilized at the indicated ages (five-, seven-, eleven-, fourteen-, twenty-five-, and twenty-eight-days-old) and six- to eight-week old mice were utilized for adult experiments. Mice were lightly sedated with 5% isoflurane (Pivetal) and inoculated (10^4 CFU suspended in either 15 μl PBS for neonates and 50 μl PBS for adults) by pipetting the inoculum as droplets into their external nares to be inhaled. At the indicated timepoints mice were euthanized via CO_2 inhalation and/or decapitation. Organs were excised and homogenized in 1 ml PBS, serially diluted, and plated on BG agar to quantify bacterial numbers. Colonies were counted following incubation for five days at 37° C.

<u>Flow Cytometry</u>

Lungs were processed and stained as previously described[35]. Viable cells were identified with Zombie Aqua (Biolegend). 0.35 μl of each extracellular antibody and 1 μl of each intracellular antibody was added to each sample. Antibodies to identify T and B cell populations included anti-CD45 AF700 (clone: 30-F11, Biolegend), anti-CD3 APC (clone:17A2, Biolegend), anti-CD4 VF450 (clone:RM4-5, Tondo Biosciences), anti-CD8 APC Fire 750 (clone:53-6.7, Biolegend), and anti-CD19 PerCP/Cy5.5 (clone:1D3/CD19, Biolegend). Antibodies to identify virtual memory T cells (T_{VM}) [36] included anti-CD8 BV650 (clone: 53-6.7, BD Biosciences), anti-CD4 AF700 (clone:RM4-55, Biolegend), anti-CD44 PE/Cy7 (clone: IM7, Biolegend), anti-CD49d APC Fire 750 (clone:R1-2, Biolegend), anti-CD3 FITC (clone: 17A2, Biolegend), anti-TCRg/d PE/Cy5 (clone: GL-3, Invitrogen), anti-NK1.1 PE (clone:PK136, Biolegend), anti-Eomes APC (clone: Dan11mag, Invitrogen), and anti-CXCR3 BV750 (clone: CXCR3-173, BD Biosciences). Intracellular staining for Eomes was performed as previously described [37]. To identify neutrophils ($CD45^+Ly6G^+CD11b^+$) and macrophages ($CD45^+Ly6G^-$ Siglec-F^-MHCII^+), the gating strategy from Misharin *et al.* and the following antibodies were utilized; anti-CD45 AF 700 (clone: 30-F11, Biolegend), anti-MCHCII VF450 (clone:M5/114.15.2, Tonbo Biosciences), anti-CD11b PE (clone: M1/70, Tonbo Biosciences), anti-CD11c BV605 (clone: N418, Biolegend), anti-CD69 BV711 (clone: H1.2F3, Biolegend), anti-Ly6G FITC (clone: RB6-8C5, Tonbo Biosciences), and anti-Siglec F BV650 (clone: E50-2440, BD Biosciences). The acquisition of the data was performed using the Quanteon (Agilent) and analysis was performed with NovoExpress (Agilent) following the gating strategies in **Figures S2.2 and S2.3** and Misharin *et al.*[38]

Preparation of Histopathological Samples

Twelve, 5-day-old, female, C57Bl/6J mice were randomly divided into 3 groups of 4 mice each. Mice received PBS (control) or 10^4 CFU WT *Bp* or *Bp*Δ*ptx* in 15 µl PBS intranasally one time. Three days after inoculation, the animals were sacrificed with CO_2 euthanasia. The lungs were infused with neutral-buffered, 10% formalin fixative solution and immersed in the same fixative. One week following necropsy, tissues were removed from formalin solution, immersed in 70% ethanol, and remained in alcohol until ready for processing. Tissues were subsequently processed, embedded in paraffin, sectioned at approximately 5 μm, and stained with hematoxylin and eosin (HE). A board-certified pathologist (UBM) performed all microscopic evaluations of the HE stained sections.

Microscopic exam consisted of evaluation of the lung for the presence or absence of inflammation. Microscopically, lesion (tissue change or alteration) incidence, severity, and distribution were recorded. If absent (i.e., histologically normal), a score of 0 was assigned. If present, the severity of the lesions was recorded as minimal, mild, moderate, or severe, with severity scores of 1 through 4, respectively, based on an increasing extent and/or complexity of change, unless otherwise specified. Lesion distribution was recorded as none, focal, multifocal, or diffuse, with distribution scores of 0, 1, 2, or 3, respectively. The Group Histopathological Score was calculated by adding individual animal severity + distribution scores.

Cytokine ELISA

Supernatant was taken from lung samples utilized for flow cytometry experiments. Samples were collected and stored at -20° C for cytokine analysis. 100 μl of supernatant from each lung sample was assessed for concentrations of IL-4 and IL-17 utilizing R&D Systems DuoSet ELISA kits following manufacturer's instructions.

Statistics

For experiments determining differences in bacterial loads in the organs of mice, the following statistical analyses were performed using GraphPad PRISM (GraphPad Software, Inc): Two-tailed unpaired Student t-tests, One-way ANOVA, and Two-way ANOVA. For experiments determining differences in immune cell populations, the following statistical analyses were performed using GraphPad PRISM (GraphPad Software, Inc): Two-tailed unpaired Student t-tests, One-way ANOVA, and Two-way ANOVA.

Ethics Statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committees at The University of Georgia at Athens, GA (A2022 04-001-Y1-A0 Bordetella-Host Interactions, A2022 04-025-Y1-A0 Breeding Protocol, and A2022 04-022-Y1-A0 Neonatal Models of Bordetella infection, transmission, and immunity). Mice were consistently monitored for signs of distress over the course of the experiments to be removed from the experiment and euthanized using carbon dioxide inhalation to prevent unnecessary suffering.

RESULTS

Neonatal T cell populations and susceptibility to *B. pertussis* transition in the first 2 weeks of life.

To identify the appropriate aged mouse to assess neonatal susceptibility to *Bp,* C57Bl/6J mouse pups were inoculated with 10^4 CFU of *Bp* at five-, seven-, eleven-, and twenty-five-days-post birth (P5, P7, P11, and P25). Those inoculated at P7, P11, and P25 had roughly 10,000 CFU in their lungs at 3 days post inoculation (dpi) (**Figure 2.1**), demonstrating growth similar to that observed in adult mice (**Figure S2.1**). In contrast, mice inoculated at P5 had approximately 5,000,000 CFU of *Bp* at 3 dpi (**Figure 2.1**), over 500-fold higher numbers than any older mice, indicating that P5 pups are significantly more susceptible to this neonatal pathogen than mice even 2 days older (P7). These results indicate that there is a significant shift in immunological development distinguishing mice inoculated on P5 and P7. This suggests that the increased susceptibility to *Bp* is likely due to some feature(s) particular to the lymphocytes in P5-P8 pups that differs from those in older mice.

A key feature of immunological development is the maturation of the thymus and the T cells which develop therein. Therefore, subsets of T cells were characterized in the lungs of P8, P10, P14, and P28 mice. A comprehensive flow cytometry panel was designed to assess shifts in populations of virtual memory T cells (T_{VM}). Using the gating strategy in **Figure S2.3**, we first identified populations of single positive $CD3^+$ $\alpha\beta$ T cells via expression of $CD3^+NK1.1^-TCR\gamma\delta^-$ and $CD4^+$ or $CD8^+$, then T_{VM} were identified via $CD44^+CD49d^-CXCR3^+Eomes^+$ expression. The panel and gating strategy were validated for specific binding via positive and negative controls (**Figure S2.4**). Shifts in populations of $CD44^{hi}CD49d^-$ to $CD44^{hi}CD49d^+$ antigen experienced T cells were increasingly observed from P8 and P10 to P14 and P28 mice (**Figure 2.2A &2.2B**). The decreased expression of Eomes and CXCR3 in these $CD44^+CD49d^-$ populations followed a similar trend as mouse age progressed; the highest proportions of T_{VM} were observed in P8 mice (**Figure 2.2C and 2.2D**). T_{VM} comprised a significantly larger proportion of single-positive T cells (15%) in the lungs of P8 mice, while only comprising 1-4% of T cells in P10, P14, and P28 mice (**Figure 2.2E**). This trend was also reflected in the total T_{VM} numbers in the lungs of P8 mice, which had ~75x greater numbers than P10, P14, or P28 mice (**Figure 2.2F**).

Since there is no objective cutoff distinguishing “neonatal” mice from later stages of development, we here use the dramatically different sensitivity to *Bp* and predominance of T_{VM}, as the basis for distinguishing P5-P8 mice, herein referred to as “neonatal”, from older mice referred to as “juvenile” (P10 to P21) or “adolescent” (P22 to P30) (**Figure 2.2**). Utilizing this framework, these data demonstrate that neonatal mice with increased populations of T_{VM} are much more susceptible to *Bp*.

Pertussis toxin disrupts neonatal control of *B. pertussis*

To better understand the extraordinary susceptibility of P5 mice to *Bp*, we inoculated them as above and followed the progression over time in the respiratory tract as well as the spleen, which is colonized in severe infections. At 2 hrs post inoculation, most of the initial inoculum was deposited in the lungs, confirming successful inoculation. Within 3 days, *Bp* grew in the lungs over 100-fold to levels exceeding 1,000,000 CFU, indicating a failure of these neonates to control infection (**Figure 2.3A**). *Bp* grew similarly unrestrained in the nasal cavities, from ~100 CFU at 2 hrs post inoculation to over 100,000 CFU by 3 dpi (**Figure 2.3B**). The extraordinary susceptibility of P5 mice was further underscored by the sporadic splenic colonization on 1 dpi that grew to nearly 1,000 CFU in all mice by 3 dpi, indicating consistent systemic dissemination (**Figure 2.3C**), reflecting a serious pneumonic infection and failure of systemic immune control. These results demonstrate that when delivered to neonatal mice (P5), a relatively low dose of *Bp* can efficiently and consistently grow rapidly in the nose and lungs and disseminate to the spleen within 3 dpi, characteristic of severe neonatal disease.

Multiple studies have identified pertussis toxin (PTx) as contributing to severe disease in juvenile (P14) and adult mice [24,31,34], but its effects have not been examined in neonatal (P5-P8) mice, as defined here. To assess the role of PTx in the exceptional virulence of *Bp* in our neonatal model, we compared the wild-type parental strain to an isogenic mutant with an in-frame deletion of the coding region of *ptx* (*Bp*Δ*ptx*)[34]. Inoculation with WT *Bp* and *Bp*Δ*ptx* demonstrated similar recovery from the lungs at 2 hrs post-inoculation, confirming equivalent delivery of both strains to the lungs (**Figure 2.3A**). Within 3 days, *Bp*Δ*ptx* was nearly cleared from the lungs of neonatal mice, with only approximately 100 CFU remaining, while WT *Bp* grew nearly 1000x the original inoculation dose in this same period (**Figure 2.3A**). Despite consistent colonization of the spleen by WT *Bp,* there was no detected systemic dissemination to the spleen by *Bp*Δ*ptx* (**Figure 2.3C**). Complementation of the *Bp*Δ*ptx* mutant via co-inoculation with purified PTx resulted in partial recovery of the phenotype in the lungs (**Figure S2.5**). This indicates that a single bolus delivery of PTx does not precisely mimic the continual secretion from the site of *Bp* microcolonies required for its effects. Together, these results indicate that the neonatal immune system can efficiently and rapidly control lung infection with the *Bp*Δ*ptx* strain, but WT *Bp* substantially disrupts this ability via secretion of PTx.

Conventional infection models inoculate mice with supernaturally high doses of *Bp* (5×10^5 CFU), potentially overcoming the most relevant host immune responses. Though C57Bl/6 adult mice are approximately 10x the size of P5 pups, our much lower inoculation dose of 10^4 CFU is 50x less than that delivered to adults. To examine the potential effects of high inocula in our neonatal mouse model, we used doses equivalent to those used in the conventional adult assays. Increasing the dose of *Bp*Δ*ptx* delivered to P5 pups 10- and 100-fold, to 10^5 and 10^6 CFU, respectively, resulted in the death of most animals (**Figure S2.6**). These results demonstrate that the neonatal immune system can be effective against moderate numbers resembling natural infection but can be overwhelmed by unnaturally large inocula. They also demonstrate that extremely high doses can disrupt the ability to study and understand the natural function of the host immune system.

<u>PTx disrupts rapid immune cell recruitment</u>

PTx has been demonstrated to delay immune cell recruitment to the site of infection by approximately one week in adolescent and adult mice[24,31]. To quantify the effects of PTx on populations of immune cells recruited to neonatal lungs, P5 pups inoculated with WT *Bp* or *Bp*Δ*ptx* were assessed via flow cytometry at 2 hrs, 1 day, and 3 days post inoculation. At 2 hrs post inoculation, pups inoculated with *Bp*Δ*ptx* had significantly higher neutrophil counts in the lungs than pups inoculated with WT *Bp* (**Figure 2.4A**), indicating that PTx interferes with rapid neutrophil recruitment. By 1 dpi, *Bp*Δ*ptx* was present in much lower numbers than WT *Bp* but had recruited significantly higher (3x more) numbers of $CD3^+$ T cells into the lungs (**Figure 2.4B**). Additionally, numbers of T_{VM} in the lungs remained consistent across infection groups and pups deficient in IL-15, which is required for T_{VM} development, demonstrated similar control of *Bp*Δ*ptx* to C57Bl/6 pups **(Figure S2.7 & S2.8)**. By 3 dpi, the mutant was nearly cleared from the lungs and the neutrophil and T cell numbers had already decreased, having substantially resolved the infection. In contrast, WT *Bp* had grown over 100-fold in number, but did not result in substantial increase in the populations of T cells in the lungs (**Figure 2.4B**). Instead, WT *Bp* resulted in significantly higher numbers of macrophages and neutrophils in lungs at 3 dpi, but these levels were not sufficient to clear or control the infection, as the bacterial load was 100-fold higher than the original inoculum and had already disseminated to the spleen (**Figures 2.4A & 2.4C**).

Histopathology of the lungs of infected mice was assessed as a measure of disease severity. To assess resulting inflammatory lesions in the lungs, P5 mice inoculated as above with WT *Bp, Bp*Δ*ptx,* or PBS were histologically assessed at 3 dpi. H&E staining of lung sections of infected or naïve pups revealed that infection with *Bp*Δ*ptx* did not result in observable inflammatory lesions and was indistinguishable from the PBS control pups (**Figures 2.5A, 2.5C, 2.5D, & Table S2.1**). In contrast, WT *Bp* induced substantial inflammation, as evidenced by the presence of inflammatory lesions in 100% of mice assessed (**Figures 2.5B & 2.5D & Table S2.1**).

<u>Pertussis toxin disrupts early cytokine production</u>

Neonatal immune responses are largely anti-inflammatory, which minimizes collateral tissue damage that might result from uncontrolled inflammatory responses to the multitude of mostly harmless bacteria encountered at birth. We therefore assessed concentrations of representative anti-inflammatory (IL-4) and pro-inflammatory (IL-17) cytokines in neonates inoculated with either WT *Bp* or *Bp*Δ*ptx*. At 1 dpi, pups inoculated at P5 with *Bp*Δ*ptx* had significantly higher concentrations of both IL-4 and IL-17 isolated from lung supernatant than naïve and WT *Bp*-inoculated pups, despite lower numbers of bacteria (**Figures 2.6A & 2.6B**). By 3 dpi, when the *Bp*Δ*ptx* infection was nearly controlled, the concentrations of these cytokines returned to levels similar to the naïve control (**Figures 2.6A & 2.6B**). Surprisingly, pups inoculated with WT *Bp* did not have increased IL-4 or IL-17 in lung supernatants at 1 or 3 dpi, despite very high numbers of bacteria, significant immune cell recruitment, and formation of inflammatory lesions. These results suggest that PTx suppresses the production of IL-4 and IL-17 in neonatal mice, thereby allowing the pathogen to grow to high numbers.

DISCUSSION

The high mortality of neonates to particular infectious diseases has long been attributed to an inability to generate effective immune responses; however, this outlook fails to put into context the unique challenges faced by neonates and the immunological mechanisms employed to meet them[39]. Immediately after birth, the naïve neonatal immune system is suddenly and continually thereafter exposed to innumerable microbes, both pathogenic and commensal. A critical requirement of the newborn's immune system is that it avoids dangerously strong inflammatory responses to the many mostly harmless microorganisms. For this reason, the neonatal immune response must be skewed towards anti-inflammatory cells and cytokines[7,40–43]. However, neonates must also rapidly respond to bacterial infections of critical and sensitive organs like the lungs and possess a set of immune cells which can rapidly respond to danger signals. These include myeloid-derived suppressor cells, $CD71^+$ erythroid cells, and T_{VM}, the latter of which respond rapidly to cytokine signals and a broad range of antigens in a memory-like fashion[11,15,36,44–47]. We enumerated population changes of T_{VM}, which are numerous in the neonatal periphery before being replaced by adult T cells, demonstrating a shift in these cell types in post-natal development concurrent with dramatic changes in sensitivity to *Bp*.

Here, we present a novel model focusing on the extraordinary failure of the neonatal immune response to contain a common bacterial pathogen that adults more effectively resist. In this model we distinguish neonatal pups (P5-P8) from a range of older mice that have reduced numbers of T_{VM} in their lungs and behave similarly to older mice when challenged with *Bp*. In stark contrast, mice just two days younger, P5-8, have larger populations of T_{VM} and are highly susceptible to *Bp.* P14 mice were also observed to have a larger population of $CD49d^{+}$ T cells in the lungs, possibly indicating a substantial shift from neonatal to juvenile mice[48]. This model presents a novel opportunity to more accurately assess the neonatal immune response in the relative absence of conventional adult T cells that rapidly emerge in juvenile (P10-P21) mice.

In addition to T_{VM}, neonates are enriched with $CD71^{+}$ erythroid suppressor cells, which have been found to compromise the neonatal response to *Bp* via suppression of the innate immune response[7]. Despite this and other immunosuppressive cells, we demonstrate that P5-P8 neonatal mice very efficiently controlled and nearly eliminated a pertussis toxin-deficient mutant (*Bp*Δ*ptx*) within 3 dpi, indicating that the neonatal immune response can be very effective against bacteria introduced into the lungs, but is highly sensitive to the effects of PTx. The rapid control of *Bp*Δ*ptx* was associated with early accumulation of neutrophils and T cells to the lungs, and early increases in IL-4 and IL-17 concentrations in lung supernatant. This model demonstrates a more profound effect of PTx on these neonatal mice that lack conventional adult T cells compared to juvenile and adult mice[24,49].

PTx is an extensively studied toxin with known effects on several aspects of the adult response to *Bp;* however, its principal role in pathogenesis remains to be determined. Though juvenile mice showed a somewhat larger effect, PTx has a modest effect in adolescent and adult mice compared to the >10,000-fold effect we observe here[24,27,50–54]. The largest effect of PTx previously observed was a ~1,000-fold effect on the rapid antibody-mediated bacterial clearance of *Bp*[31]. In these experiments, transferred antibodies very rapidly cleared *Bp*Δ*ptx* but not WT *Bp* from the lungs of adult mice, indicating that PTx blocks rapid antibody-mediated clearance[31]. That work further implicated neutrophils in the rapid antibody-mediated clearance, suggesting PTx works by blocking their recruitment to the site of *Bp* infection. Here we observed much faster control of *Bp*Δ*ptx* in naïve neonatal mice without the aid of transferred antibodies and observed rapid accumulation of neutrophils and T cells. The naïve neonatal immune system was so efficient in controlling *Bp*Δ*ptx* that there was negligible pathology in the lungs. In stark contrast, WT *Bp* grew rapidly in the lungs and produced substantial inflammatory lesions. Rather than a direct role in inducing pathology, it seems more likely that PTx blocks early immune responses critical to controlling bacterial infection, thus contributing to increased pathology. Altogether, PTx appears to block neonate-specific immune functions that could otherwise very efficiently eliminate *Bp*, allowing it to grow to large numbers and induce greater pathology.

The failure of current *Bp* vaccines to prevent transmission and nasal colonization among infants demonstrates limitations of the previous models used to develop these vaccines and emphasizes the necessity of a better understanding of host interactions, especially in highly sensitive newborns[22,23,55,56]. PTx appears to disrupt critical early responses unique to neonates, potentially explaining the specific sensitivity of neonates to *Bp*. Similar mechanisms may also be used by other pathogens to thwart the neonatal immune system and cause serious disease in this susceptible population[2,3]. Understanding the key functions that are blocked by PTx may reveal novel aspects of neonatal immunity that can guide efforts to protect this vulnerable population. This novel infection model may be useful to better understand how the neonatal immune system can be more effective than the adult in eliminating *Bp*Δ*ptx* and how PTx disrupts it to make newborns so highly susceptible to *Bp*.

FIGURES

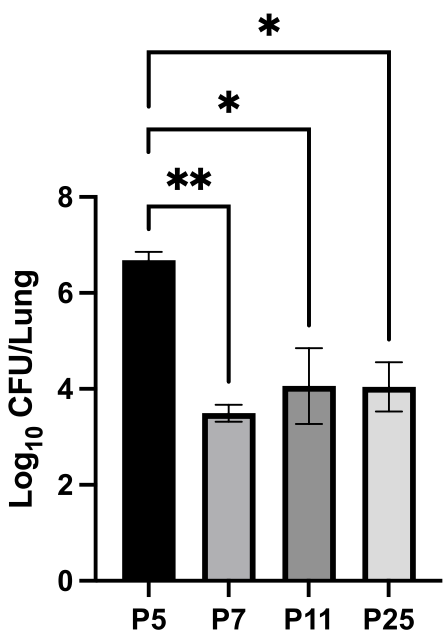

Figure 2.1. P5 pups are significantly more susceptible to WT *Bp* than P7, P11, and P25 mice. C57Bl/6J mice were intranasally inoculated with 10^4 CFU in 15 μl (P5, P7, and P11) or 50 μl (P25). CFU in the lungs at 3 dpi are shown as $\log_{10}$ mean and standard error, n=4. Statistical significance was calculated via One-way ANOVA. *p<0.0332, **p<0.01.

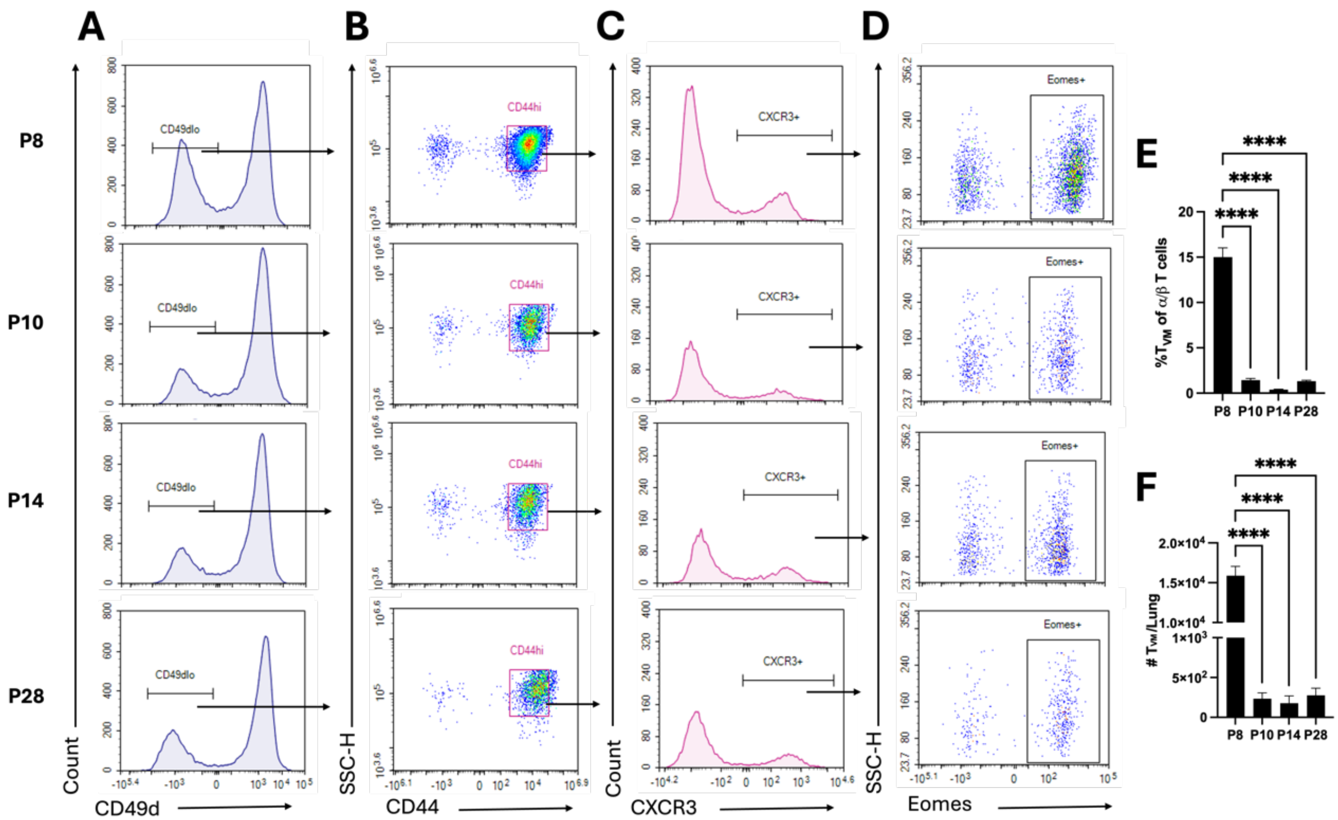

Figure 2.2. P10, P14, and P28 mice have significantly less T_{VM} than P8 mice. $CD49d^{lo}$ T cell populations from the lungs via gating strategy in Supplementary Figure 3. Representative histogram of CD49d expression of $CD3^{+}$ T cells (**A**). Representative density plot of CD44 expression of $CD49d^{lo}CD3^{+}$ T cells (**B**). Representative histogram of CXCR3 expression of $CD44^{+}CD49d^{lo}CD3^{+}$ T cells (**C**). Representative density plot of Eomes expression of $CXCR3^{+}CD44^{+}CD49d^{lo}CD3^{+}$ T cells (**D**). Samples from P8, P10, P14, and P28 C57Bl/6 pups, top to bottom (**A-D**). Proportions of T_{VM} of CD4/CD8 single positive α/β T cells in the lungs from naïve P8, P10, P14, and P28 mice (**E**). Numbers of T_{VM} in the lungs from naïve P10, P14, and P28 mice (**F**). (n=4 per group). Error bars show standard error of the mean. Statistical significance was calculated via One-way ANOVA. ns p> 0.032 ****p<0.0001.

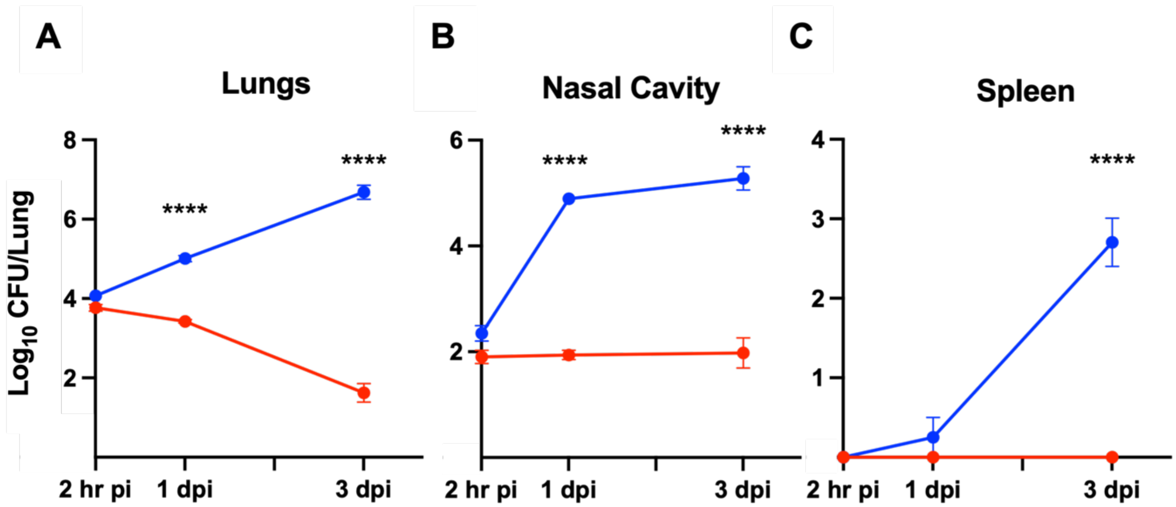

Figure 2.3. Pertussis toxin is required for rapid growth of *Bp* in P5 neonatal mice. Log_{10} CFU of WT *Bp* (blue) and *Bp*Δ*ptx* (red) recovered from the lungs (**A**) nasal cavity **(B)** and spleen **(C)** at 2 hours, 1, and 3 days post inoculation from neonatal C57Bl/6J mice inoculated at P5 (n=4 per strain per timepoint). Statistical significance was calculated via Two-way ANOVA. Error bars show the standard error of the mean. ns p> 0.0332, ****p<0.0001.

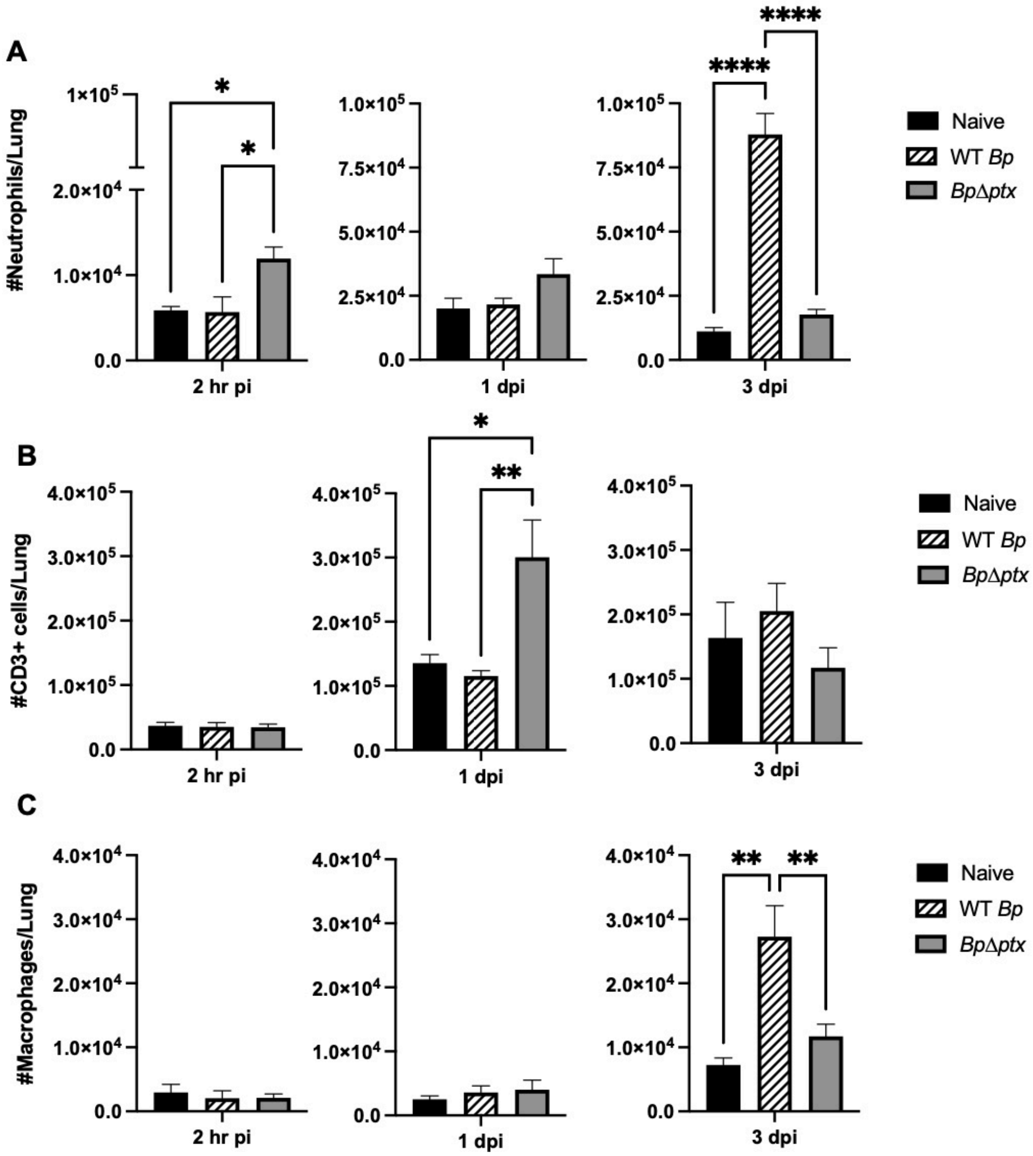

Figure 2.4. Pertussis toxin causes delayed neutrophil and T cell accumulation in the lungs of neonatal mice. Total neutrophils ($CD45^+Ly6G^+CD11b^+$) in lungs of P5 C57Bl/6J mice inoculated with WT *Bp*, *Bp*Δ*ptx*, or uninfected at 2 hours, 1 day, and 3 days post inoculation (**A**). Total T cells ($CD3^+$) in lungs of P5 C57Bl/6J mice inoculated with WT *Bp*, *Bp*Δ*ptx*, or uninfected at 2 hours, 1 day, and 3 days post inoculation (**B**). Total macrophages ($CD45^+Ly6G^-$Siglec-F^-MHCII^+) in lungs of P5 C57Bl/6J mice inoculated with WT *Bp*, *Bp*Δ*ptx*, or uninfected at 2 hours, 1 day, and 3 days

post inoculation (**C**). Statistical analysis was calculated via Two-way ANOVA. Error bars show standard error of the mean (n=5). *p<0.0332, **p> 0.01, and ****p< 0.0001.

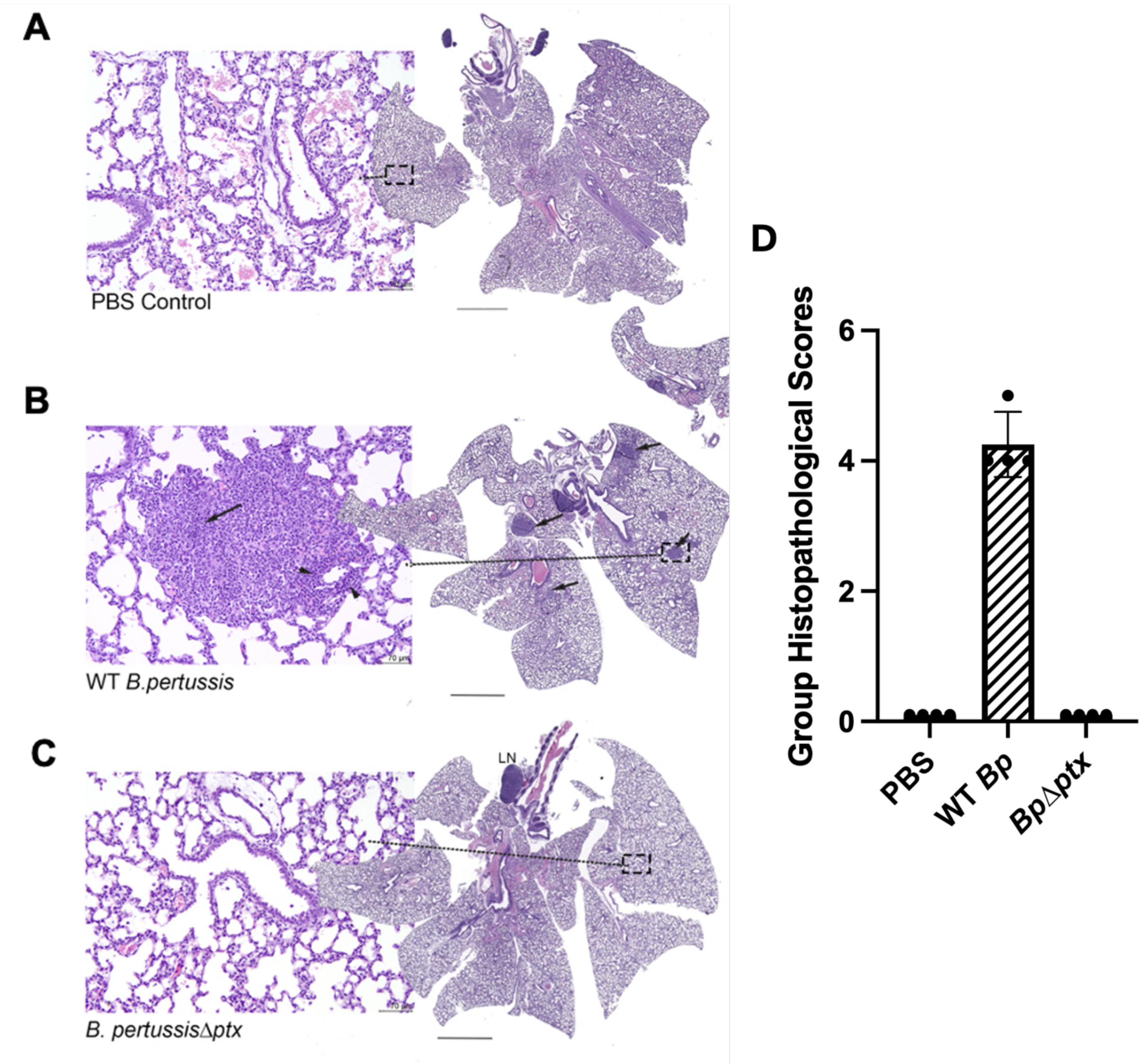

Figure 2.5. Pertussis toxin affects inflammation in the lungs of neonatal C57Bl/6J mice. Representative images from neonatal lung mice exposed to PBS, WT *Bp*, or *Bp*Δ*ptx* at 3 dpi. PBS treated control (**A**). The center image is a whole-slide image from the lung. HE stain. Scale bar = 1.2 μm. The left image represents higher magnification (dashed box) of the lung. There are no

significant tissue alterations. HE stain. Scale bar = 70 μm. *Middle panel*: WT *B. pertussis* (**B**). The center image is a whole-slide image from the lung, with multiple hypercellular foci (arrows). HE stain. Scale bar = 1.2 μm. The left image represents higher magnification (dashed box) of the lung, with large number of neutrophilic and macrophage infiltrates obscuring the alveoli (arrow), and mild number of lymphocytes expanding the perivascular tissues (arrow heads). HE stain. Scale bar = 70 μm. *B. pertussis*Δ*ptx* (**C**). The center image is a whole-slide image from the lung, with a distinct tracheobronchial lymph node (LN). HE stain. Scale bar = 1.2 μm. The left image represents higher magnification (dashed box) of the lung. There are no significant tissue alterations and resemble the lung tissues from PBS control mice. HE stain. Scale bar = 70 μm. (**D**) *Group Histopathological Scores*. In the current figure, Group Histopathological Scores, which were calculated by adding individual animal severity + distribution scores, were highest for the WT *B. pertussis* treated group at 3 dpi. The scores for the *B. pertussis*Δ*ptx* and PBS treated control groups were similar (Supplementary Table 1).

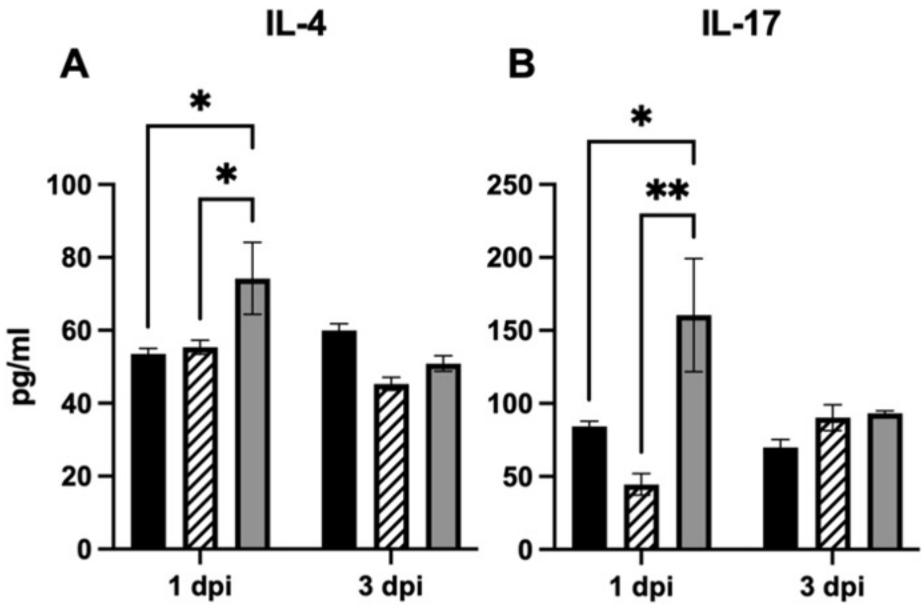

Figure 2.6. Pertussis toxin disrupts IL-4 and IL-17 production. Lung supernatant of C57Bl/6J pups inoculated with WT *Bp* (striped), *Bp*Δ*ptx* (gray), or uninfected (black) and assessed at 1 dpi and 3 dpi. IL-4 pg/ml (**A**) and IL-17 pg/ml (**B**). Statistical significance was calculated via Two-way ANOVA, n=5. Error bars represent standard error of the mean. *p<0.032, **p<0.05.

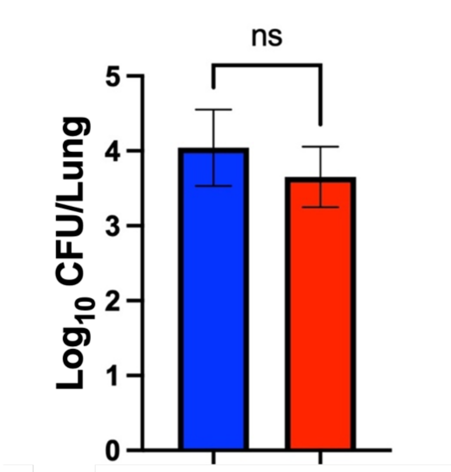

Figure S2.1. Pertussis toxin has a modest effect on adult C57Bl/6J mice at 3 dpi. Mice were intranasally inoculated with 10^4 CFU of WT *Bp* (blue) or *Bp*Δ*ptx* (red) in 50 µl PBS (lungs shown). Error bars show standard error of the mean, n=4. Statistical significance was calculated via student T test. ns>0.0332.

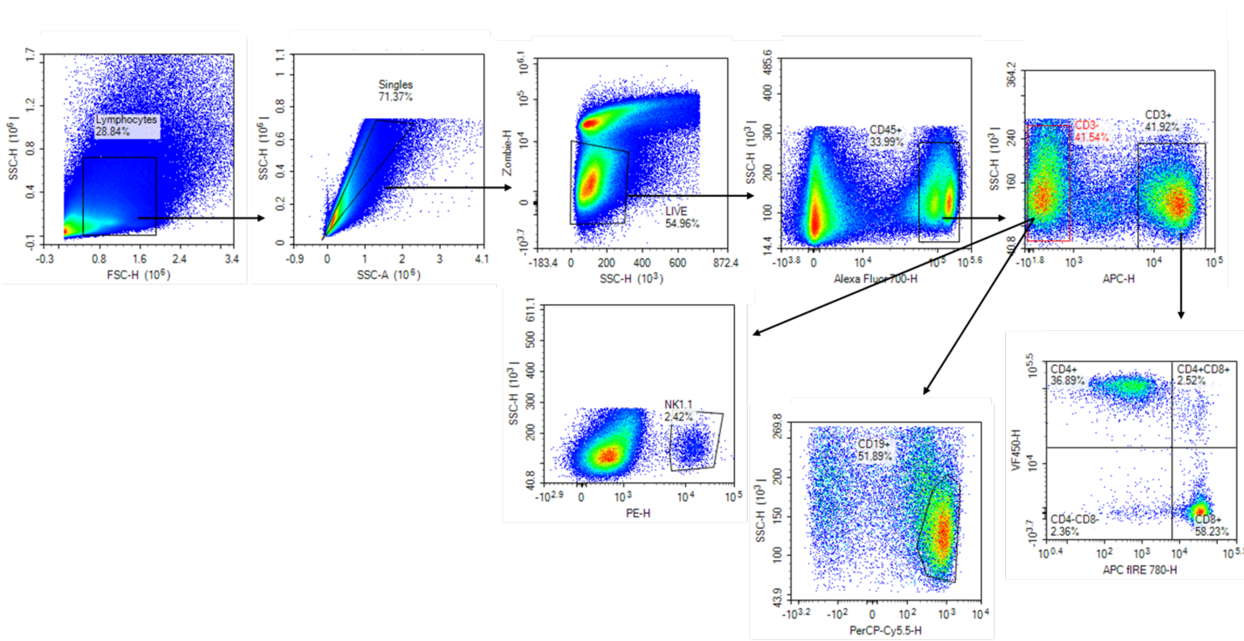

Figure S2.2. Gating strategy for lymphoid panel from lung of naïve P8 C57Bl/6J mouse.

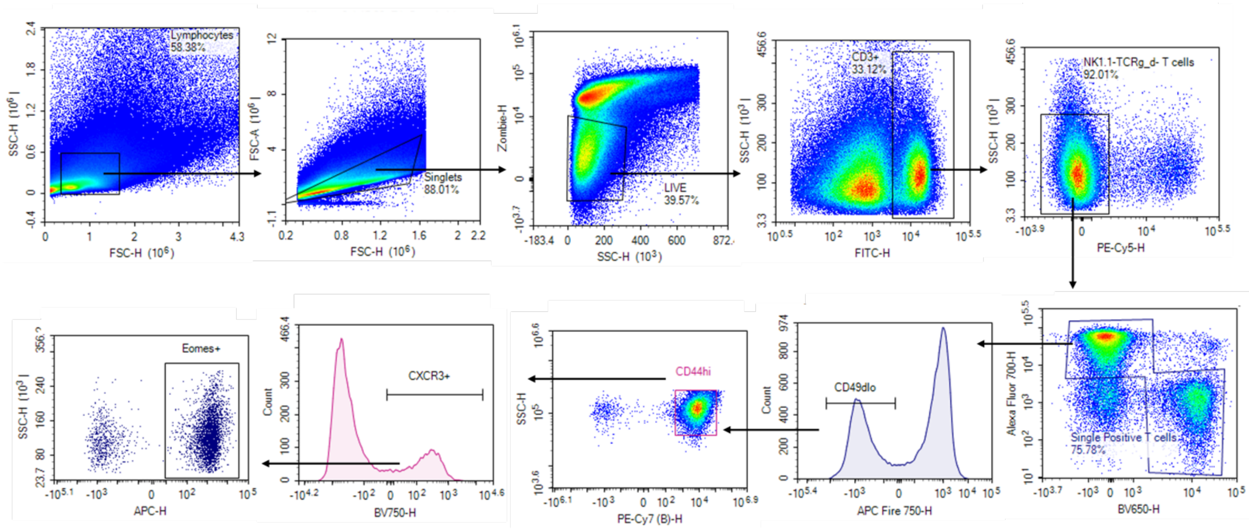

Figure S2.3. Gating strategy for the isolation of virtual memory T cells (T_{VM}) from naïve P8 C57Bl/6J pup lungs.

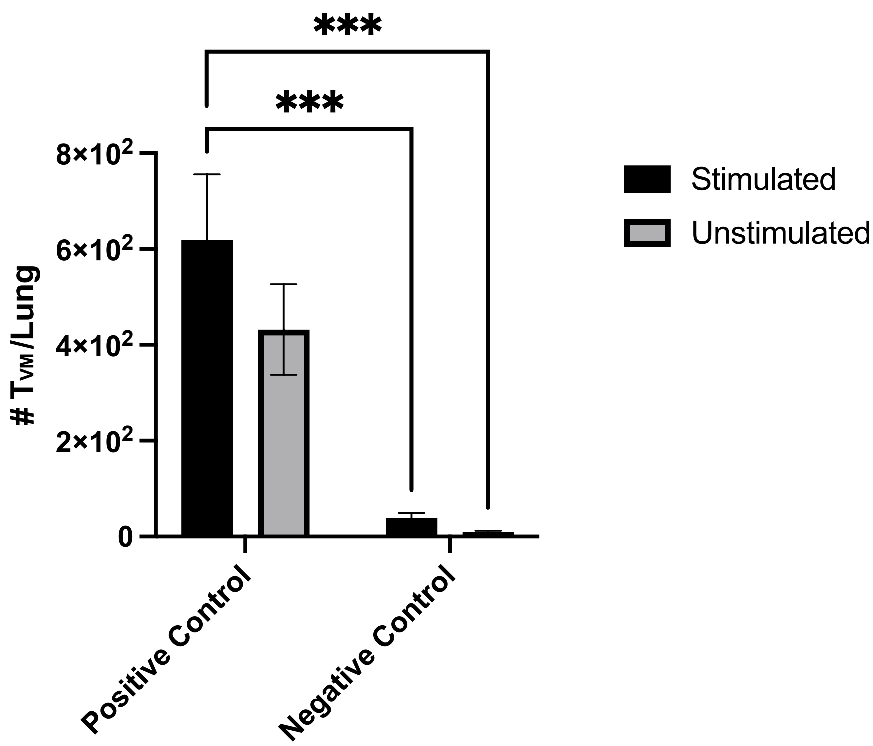

Figure S2.4. Positive and negative controls for T_{VM} panel. Positive control was assessed via TVM from P8 C57Bl/6J pup lungs stimulated with PMA/ionomycin or unstimulated. Negative control was assessed via TVM from P8 B6.129P2-*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom}/J (T cell$^{-/-}$) pup lungs stimulated with PMA or unstimulated. Error bars show standard error of the mean, n=7-8. Statistical significance was calculated via Two-way ANOVA. ns p> 0.032 ***p<0.001.

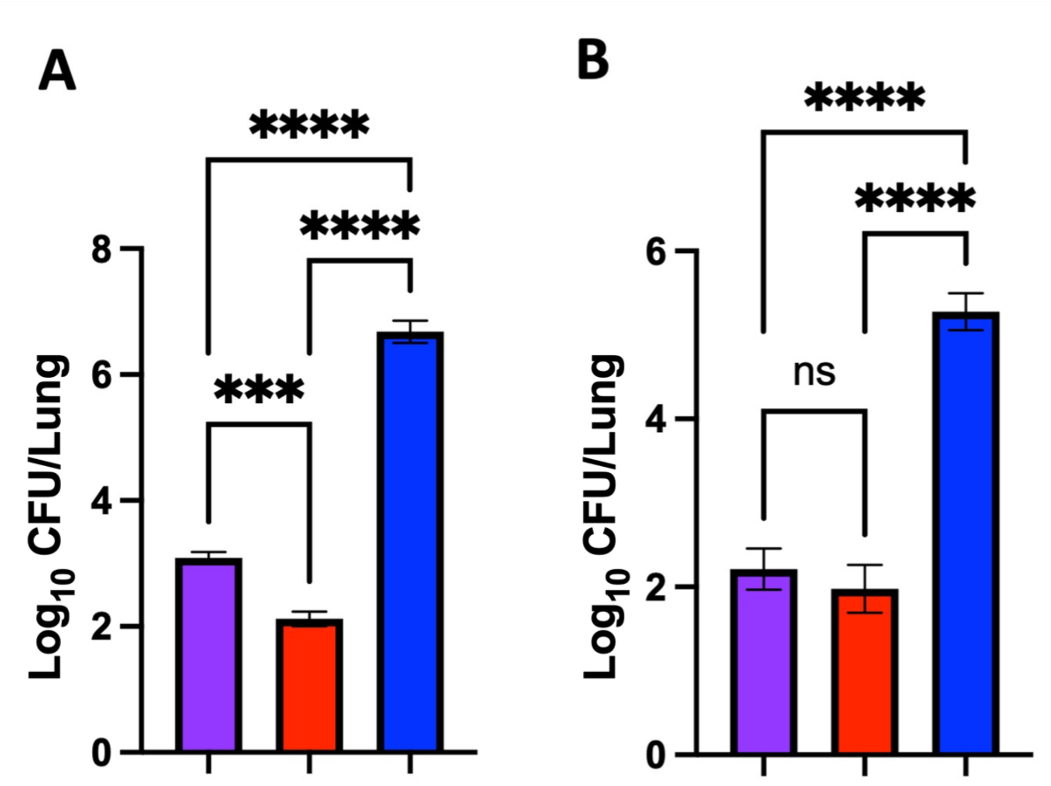

Figure S2.5. Purified pertussis toxin partially rescues ability of *Bp*Δ*ptx* to cause disease in P5 neonatal mice. Log_{10} of CFU recovered from the lungs (A) and nasal cavity (B) from neonatal P5 C57Bl/6J mice at 3 days post inoculation with WT *Bp* (blue), *Bp*Δ*ptx* (red), or *Bp*Δ*ptx* supplemented with purified PTx (*Bp*Δ*ptx* + PTx) (purple) (n=4 per strain). Statistical analysis was calculated via Two-way ANOVA. Error bars show standard error of the mean. ns p> 0.0332, ***p< 0.0002, ****p< 0.0001.

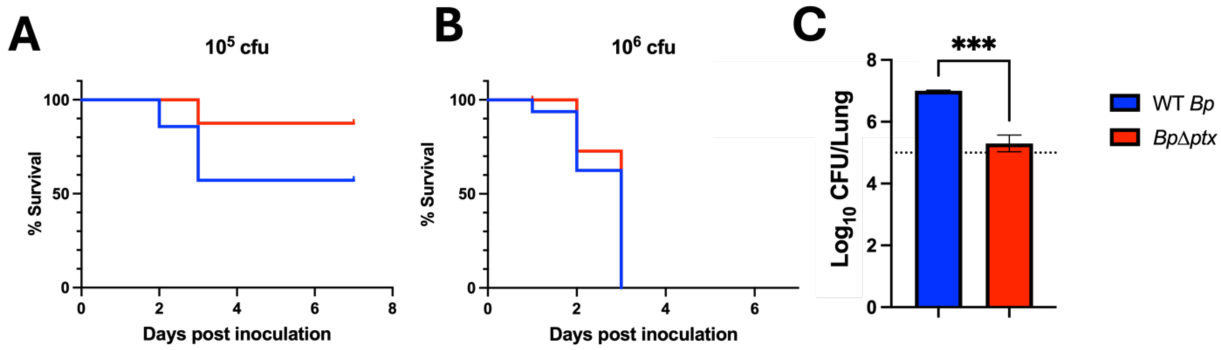

Figure S2.6. Effects of pertussis toxin on neonatal (P5-P8) mice are dose-dependent. Survival of mice inoculated at P5 with 10^5 CFU of WT *Bp* or *Bp*Δ*ptx* in 15 µl over 7 days (A). Survival of mice inoculated at P5 with 10^6 CFU of WT *Bp* or *Bp*Δ*ptx* in 15 µl over 4 days (B). Bacterial recovery from the lungs of mice inoculated with 10^5 CFU of WT *Bp* or *Bp*Δ*ptx* at 3 dpi (C) (dotted line indicates inoculation dose). Statistical analysis was calculated via student's T test. ***$p < 0.0002$.

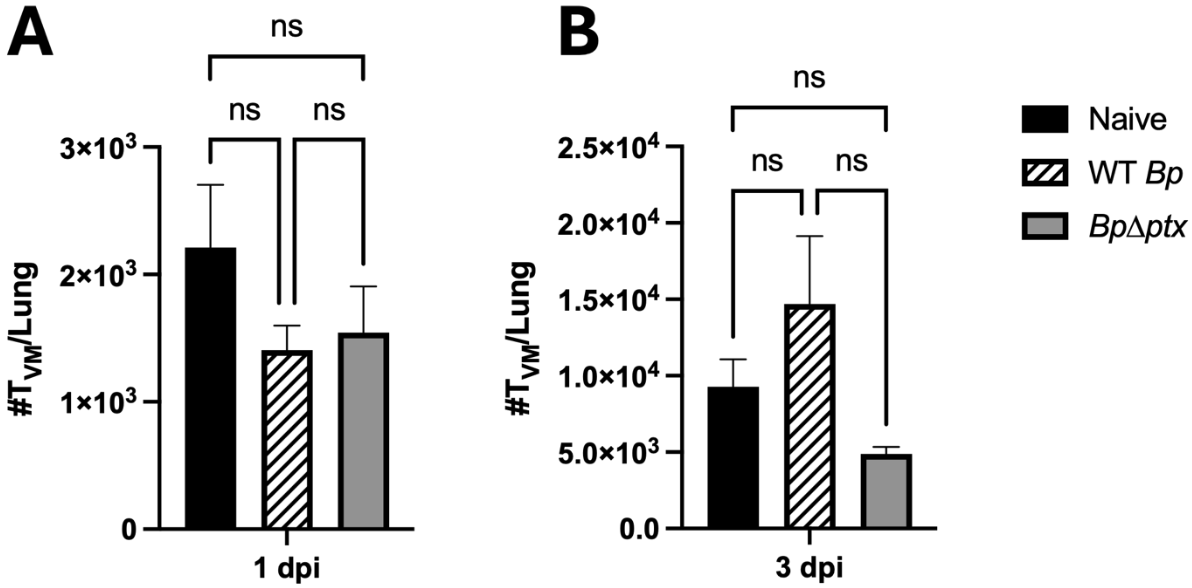

Figure S2.7. T_{VM} populations in neonatal lungs are not affected by *B. pertussis*. Total number of T_{VM} in the lungs of pups inoculated with WT *Bp*, *Bp*Δ*ptx*, or uninfected at 1 (A) and 3 (B) days post inoculation. T_{VM} are identified as $CD3^{+}NK1.1^{-}TCR\gamma/\delta^{-}CD49d^{lo}CD44^{hi}CXCR3^{+}Eomes^{+}$. Statistical analysis was calculated via One-way ANOVA. (n=4 per strain). Error bars show standard error of the mean. ns p> 0.0332.

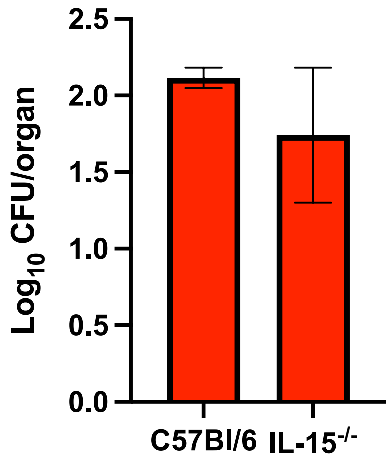

Figure S2.8. IL-15 is not required for neonatal control of *Bp*Δ*ptx*. Log10 CFU of *Bp*Δ*ptx* from lungs of C57Bl/6 and B6(Cg)-*Il15*$^{tm1.2Nsl}$/J (IL-15$^{-/-}$) pups inoculated at P5 and collected at 3 dpi (P8). Statistical analysis was calculated via unpaired student's T-test. (n=4 per strain). Error bars show standard error of the mean. ns p> 0.0332.

Table S2.1. Comparative group histopathological scores for the lungs of P5 C57Bl/6J mice that were infected with WT *Bp*, *Bp*∆*ptx*, or PBS. Assessed 3 dpi (n=4) (Sev= severity, Dis= distribution).

Infection Strain	Pulmonary Lesions			
	Inflammation			
	Sev	Dist	Individual animal Sum Sev + Dist	Group Sum Sev + Dist
PBS control	0	0	0	**0**
	0	0	0	
	0	0	0	
	0	0	0	
B. pertussis	2	2	4	**17**
	3	2	5	
	2	2	4	
	2	2	4	
B. pertussis∆*ptx*	0	0	0	**0**
	0	0	0	
	0	0	0	
	0	0	0	

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CHAPTER 3

NEONATAL NEUTROPHIL-MEDIATED CONTROL OF *BORDETELLA PERTUSSIS* IS DISRUPTED BY PERTUSSIS TOXIN

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ABSTRACT

The increased susceptibility of infants and young children to some diseases has often been explained as the neonatal immune system (NIS) being incomplete and/or underdeveloped. However, our recent work demonstrated that neonatal mice could clear a *Bordetella pertussis* (*Bp*) strain lacking PTx (*Bp*Δ*ptx*) much more efficiently than adult mice, indicating that the NIS can be extremely effective, but this ability is highly sensitive to being blocked by pertussis toxin (PTx). Here, we investigated immunological mechanisms by which neonates efficiently and rapidly clear *Bp*Δ*ptx* to better understand how the NIS functions and how PTx disrupts it. Depleting neutrophils, or blocking their recruitment, inhibited pups' ability to rapidly clear *Bp*Δ*ptx*, revealing a critical role for neutrophils. Pups deficient in complement ($C3^{-/-}$) failed to recruit neutrophils and did not efficiently clear *Bp*Δ*ptx* but recovered these abilities upon treatment with C3a. Neutrophil depletion in $C3^{-/-}$ pups lead to further failure to control *Bp*Δ*ptx*, suggesting that neutrophils and complement have independent roles in rapid clearance of *Bp*Δ*ptx*. Depleting or disrupting neutrophils and complement had negligible effect on the rapid growth of wild type *Bp*, indicating that PTx blocks these otherwise highly effective aspects of the NIS.

INTRODUCTION

Bordetella pertussis (*Bp*) is the causative agent of the respiratory disease "whooping cough". Despite decades of research and wide vaccine coverage, estimates of *Bp* disease exceeds 20 million worldwide each year[1]. Importantly, infants under 2 months old account for 29.3% of all pertussis cases, with approximately 60% of infected infants requiring hospitalization[2]. This extraordinary susceptibility of infants to *Bp* is conventionally considered to be due to the neonatal immune system (NIS) being underdeveloped, incomplete, or ineffective relative to the adult immune system (AIS)[3]. However, we and others have proposed the alternative view that the NIS performs very different functions than the AIS; rather than simply resisting invading pathogens, the NIS must also mediate the acquisition of a healthy microbiota beginning immediately after birth. The subsequent generation of a stable and healthy holobiome is achieved via tightly controlled inflammatory responses unique to the NIS[4,5]. Our recent observation that pertussis toxin (PTx) mediates the extreme virulence of *Bp* provides an opportunity to study both the immunological mechanisms by which the NIS can be highly effective in clearing respiratory infections and how PTx specifically blocks those mechanisms[6].

PTx is an AB_5 toxin uniquely produced by *Bp.* A secreted exotoxin, it has been shown to affect numerous intracellular signaling systems in various cell types by ADP-ribosylating heterotrimeric G proteins, leading to cAMP accumulation[7]. Consequences vary by cell type; for example, PTx has been shown to profoundly affect neutrophils, including decreased L-selectin expression, resulting in delayed recruitment[8,9]. While there are typically low numbers of neutrophils in healthy tissues, they can be rapidly recruited from the periphery in response to infection via a variety of chemoattractant signals[3,6,10–12]. Chemokines such as CXCL1 and products released by complement activation, such as C3a, can attract neutrophils via receptors that signal via PTx-sensitive GPCR pathways[13–16]. There are many studies describing the cellular effects of PTx, but identifying those most relevant to its role in infection has been difficult. Deleting PTx has a relatively modest effect on infection in the conventional assays that deliver large numbers of *Bp* into the lungs of naïve adult mice[17–19]. In this model of severe pneumonic infection in adult mice, PTx has been shown to have much greater impact when antibodies are present, blocking the otherwise rapid antibody-mediated control of *Bp*, explaining its ability to infect hosts despite the presence of anti-*Bp* antibodies and the difficulty in defining antibodies as a "correlate of protection"[8].

Although PTx has long been proposed to be involved in the hyper-virulence of *Bp* in neonates, only recently have newer models been developed to carefully study this. Scanlon *et al*. demonstrated that PTx has greater effects in young (7-14 day old) mice than the more limited effects previously observed in naïve adult mice[20]. But by 14 days old, mice have many aspects of immunity that more closely resemble adults than neonates[3,21]. We therefore examined various timepoints post birth and demonstrated that very young (5-8 day old) mice have a more neonatal-like immune cell repertoire and are much more sensitive to the effects of PTx[6]. The magnitude of the effect of PTx in these 5-day-old (P5) neonatal mice provides a powerful experimental system in which to study immunological mechanisms and how they function in the unique environment of the NIS.

Here, we examine the protective mechanisms by which the NIS can rapidly clear *Bp* infection from neonatal lungs and how these mechanisms may be substantially disrupted by PTx. Depletion of neutrophils via administration of anti-Ly6G antibodies had little effect on response to WT *Bp,* but significantly disrupted the rapid control of *Bp*Δ*ptx*. Anti-CXCL1 treatment also disrupted the rapid recruitment of neutrophils and control of *Bp*Δ*ptx* infection. Additionally, $C3^{-/-}$ pups were not only defective in recruiting neutrophil populations but also unable to control *Bp*Δ*ptx*, demonstrating a key role for complement. Both defects in $C3^{-/-}$ pups were largely compensated by delivery of the chemoattractant C3a. Further, the phenotype of $C3^{-/-}$ pups was exacerbated by depletion of neutrophils, suggesting that complement and neutrophils can work cooperatively or synergistically to control respiratory infection. Wild type *Bp*, expressing PTx, grew very efficiently in all mice, and was not affected by any of the immune disruptions, indicating that in the presence of PTx, neither complement nor neutrophils have any measurable effect. Together, these data reveal that the NIS has highly efficient and rapid mechanisms for efficient control of neonatal respiratory infection that are disrupted by PTx. These results also support the theory that pathogens that are particularly virulent in infants produce factors that specifically target and block NIS functions.

MATERIALS AND METHODS

Bacterial Strains and Growth

Bordetella pertussis strains Tohama 1 (gentamycin-resistant derivative) (WT *Bp*) and BPH101, an isogenic pertussis toxin-deficient derivative (*Bp*Δ*ptx*), have been previously described[20,22–24]. Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% defibrinated sheep blood (Hemostat Laboratories) and either 20 µg/ml streptomycin (Sigma-Aldrich) for *Bp*Δ*ptx* (Sigma-Aldrich) or 20 µg/ml gentamicin (Sigma-Aldrich) for WT *Bp*. Liquid cultures were grown overnight in Stainer-Scholte broth at 37° C to mid-log phase then maintained in 20% glycerol stocks at -80° C for use as inoculum.

Mouse Experiments

Six- to eight-week old female and male C57Bl/6J (00664), B6.129S4-C3^{tm1Crr}/J (C3$^{-/-}$) (029661), and C57Bl/6-Tcrβ/δ$^{-/-}$ (T cell$^{-/-}$) (002122) mice were procured from the Jackson Laboratory (Bar Harbour, ME) and bred in the Harvill mouse colony (University of Georgia, GA). B6.Cg-Padi4$^{tm1.1kmow}$/J (PAD4$^{-/-}$) (030315) mice and PAD4$^{+/+}$ control mice were generously provided by Dr. Balazs Rada at UGA and bred in the Harvill mouse colony (University of Georgia, GA). All mice were maintained in specific pathogen-free facilities, and all experiments were conducted following institutional guidelines. Pups were utilized at five-days-old (P5) and administered the indicated treatments. Pups were lightly sedated with 5% isoflurane (Pivetal) and intranasally inoculated 15 µl of PBS (control) or with 10^4 CFU of WT *Bp,* or *Bp*Δ*ptx*. Pups receiving injections were lightly sedated with 5% isoflurane (Pivetal) and injected at the scruff with 30 μl of PBS, anti-Ly6G depleting antibodies (80 μg) (BE0075-1-5MG, BioXcell), anti-CXCL1 depleting antibodies (0.5 μg) (MAB453R, R&D Systems), or anti-NK1.1 depleting antibodies (0.3 μg) (828623M2, BioXcell) 2 hours prior to inoculation with indicated bacteria. Additional injections were delivered at 1 and 2 dpi to ensure continuous depletion (total of 3 injections by 3 dpi). C3a (6.4 ng) (8085-C3-025, R&D Systems) was delivered to $C3^{-/-}$ pups intranasally concurrently with bacterial inoculation (10^4 CFU/15 μl). At the indicated timepoints, mice were euthanized via CO_2 inhalation and decapitation. Organs were excised and homogenized in 1 ml PBS, serially diluted, and plated on BG agar to quantify bacterial numbers. Colonies were counted following incubation for five days at 37° C.

Flow Cytometry

Lungs were processed and stained as previously described[25]. Viable cells were identified with Zombie Aqua (Biolegend). 0.35 μl of each extracellular antibody was added to each sample. Antibodies/buffers to identify T and B cell populations included 1x DPBS (14190-144, ThermoFischer), Zombie Aqua Live/Dead (0.35 μl of 1:100 dilution) (77143, Biolegend), anti-CD45 AF700 (clone: 30-F11, Biolegend), anti-CD3 APC (clone:17A2, Biolegend), anti-CD4 VF450 (clone:RM4-5, Tonbo Biosciences), anti-CD8 APC Fire 750 (clone:53-6.7, Biolegend), and anti-CD19 PerCP/Cy5.5 (clone: 1D3/CD19, Biolegend). To identify neutrophils ($CD45^+Ly6G^+CD11b^+$) and additional myeloid populations, the following antibodies/buffers were utilized: Brilliant Stain Buffer (50 μl) (566349, BD Biosciences), 1x DPBS (14190-144, ThermoFischer), Zombie Aqua Live/Dead (77143, Biolegend), anti-CD45 AF 700 (clone: 30-F11, Biolegend), anti-MHCII VF450 (clone: M5/114.15.2, Tonbo Biosciences), anti-CD11b PE (clone: M1/70, Tonbo Biosciences), anti-CD11c BV605 (clone: N418, Biolegend), anti-CD64 PerCP-Cy5.5 (clone: X54-5/7.1, Biolegend), anti-Ly6G FITC (clone: RB6-8C5, Tonbo Biosciences), and anti-Siglec F BV650 (clone: E50-2440, BD Biosciences). After staining, cells were fixed with 100 μl of 1:3 dilution of Stabilizing Fixative (338036, BD Biosciences). Data acquisition was performed using the Quanteon (Agilent) and analyzed with NovoExpress (Agilent) following the gating strategies in **Figure S3.1**.

Cytokine Multiplex

To determine concentrations of pro-inflammatory cytokines in lungs of challenged pups, pups were treated and challenged as previously described. Pups were euthanized, lungs excised, placed in 500 μl 1X PBS, and homogenized. Tubes were spun at 2000 g for 5 minutes and supernatant collected and stored at -20° C for 1-6 months until analyzed. The LEGENDplex Mouse Inflammation Panel (13-plex) (740446, BioLegend) was used as directed by the manufacturer and assessed using the Quanteon (Agilent) and analyzed with NovoExpress (Agilent).

pHrodo Phagocytosis Assay

To determine if PTx has substantial effects on phagocytic abilities of neonatal neutrophils, WT *Bp* and *Bp*Δ*ptx* were incubated with pHrodo green (P35373, ThermoFischer) for 45 minutes at 37° C. Lungs were excised from P8 C57Bl/6 pups and processed as described above. Lung cells were then incubated with stained bacteria for 45 minutes. Half of the samples were incubated on ice as control samples to inhibit phagocytosis. The remaining samples were incubated at 37° C. All samples were stained for neutrophils (Ly6G-FITC) and fixed as above. Samples were assessed using the Quanteon (Agilent) and analysis was performed with NovoExpress (Agilent).

Statistics

The following statistical analyses were performed using GraphPad PRISM (GraphPad Software, Inc): Two-tailed unpaired Student t-tests, One-way ANOVA, and Two-way ANOVA as indicated.

Ethics Statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were approved by the Institutional Animal Care and Use Committees at The University of Georgia at Athens, GA (A2022 04-001-Y1-A0 Bordetella-Host Interactions, A2022 04-025-Y1-A0 Breeding Protocol, and A2022 04-022-Y1-A0 Neonatal Models of Bordetella infection, transmission, and immunity). Mice were consistently monitored for signs of distress over the course of the experiments and euthanized using carbon dioxide inhalation to prevent unnecessary suffering.

RESULTS

Pertussis toxin disrupts neonatal neutrophil responses vital to control of *B. pertussis*

Our previous work observed a correlation between the highly efficient control of *Bp*Δ*ptx* with significantly increased populations of neutrophils in neonatal lungs as early as 2 hrs post inoculation (hr pi)[6]. In order to assess the contributions of neutrophils to the highly efficient control of *Bp*Δ*ptx* in neonatal lungs, P5 pups were depleted of neutrophils by delivering 80 μg/30 μl of anti-Ly6G antibodies to the scruff 2 hours prior to challenge with WT *Bp* or *Bp*Δ*ptx* (**Figure 3.1A**). Treatment with anti-Ly6G successfully depleted ~98% of neutrophils, while treatment with a control antibody had no effect on neutrophil populations (**Figures 3.1B-D, S3.2, & S3.3**). Pups depleted of neutrophils had significantly higher numbers of *Bp*Δ*ptx* CFU in the lungs than PBS-treated pups at 1 and 3 days post inoculation (dpi) (**Figure 3.1E**), indicating that neutrophils are required for the efficient control of this respiratory infection. Depleting neutrophils had no effect on CFU of WT *Bp* in the lungs, indicating that neutrophils do not contribute to control of respiratory infection when PTx is present (**Figure 3.1E**). These results suggest that PTx blocks the critical contribution of neutrophils to rapid control of respiratory infection in neonates.

In addition to depleting neutrophils, treatment with anti-Ly6G also reduced T cell numbers in neonates challenged with *Bp*Δ*ptx* (**Figure S3.4**), likely because neutrophils can mediate the recruitment of T cells[26]. To determine whether the recruited T cells contribute to the rapid clearance of *Bp*Δ*ptx*, T cell$^{-/-}$ (TCR$\beta^{-/-}\delta^{-/-}$) pups were challenged with *Bp*Δ*ptx.* T cell$^{-/-}$ pups were found to control *Bp*Δ*ptx* similarly to C57Bl/6 pups, indicating that T cells are not required for the NIS to rapidly control *Bp*Δ*ptx.* Together these data indicate that neutrophils mediate T cell accumulation but rapidly clear *Bp*Δ*ptx* independent of T cell contribution (**Figure S3.5**). This is a striking finding, since T cells are required in control of both WT *Bp* and *Bp*Δ*ptx* in adult mice, revealing substantial differences between NIS and AIS[27,28].

<u>Rapid neutrophil recruitment is critical to neonatal inflammatory responses</u>

In order to elucidate the very rapid control of *Bp*Δ*ptx* in neonatal mice, we investigated the inflammatory cytokines present in lung supernatant of challenged mice. To this end, we used a pro-inflammatory cytokine multiplex kit to assess how depletion of neutrophils affected cytokine production in the lungs. Compared to pups treated with PBS, pups treated with anti-Ly6G had significantly lower amounts of GM-CSF, IL-23, IL-7, and TSLP at 2 hr pi (**Figure 3.2A**). At 1 and 3 dpi, anti-Ly6G treated pups, bearing larger numbers of *Bp*Δ*ptx*, had significantly increased amounts of IL-7, IL-11, IL-27, IL-33, IFN-β, and TSLP (**Figure 3.2B-C**). This suggests that neutrophils likely have a role in the generation of an early pro-inflammatory responses. When neutrophils are depleted, bacteria grow to greater numbers and a slower cytokine response is observed, generated either by the residual neutrophils or by some other cell type.

<u>CXCL1 mediates recruitment of neutrophils to neonatal lungs but is disrupted by PTx.</u>

To understand how neutrophils rapidly clear *Bp*Δ*ptx*, and how PTx might block this, we considered the possibility that neutrophils capture, kill and/or eliminate *Bp*Δ*ptx* via neutrophil extracellular traps (NETs). The contribution of NETs to control of *Bp*Δ*ptx* was assessed via inoculation of PAD4$^{-/-}$ pups, which lack a key enzyme required for NETosis. *Bp*Δ*ptx* was recovered at similar numbers in the lungs of immunocompetent C57Bl/6 and PAD4$^{-/-}$ pups, indicating that NETosis is not a major mechanism of control of *Bp*Δ*ptx* (**Figure S3.6**). The effects of PTx on neutrophil phagocytosis of bacteria was also assessed via a pHrodo green florescence assay. Neonatal neutrophils demonstrated similar ability to phagocytose WT *Bp* and *Bp*Δ*ptx,* suggesting that PTx does not directly affect neutrophil effector functions in this way (**Figure S3.7**). Amongst various other effects, PTx has been shown to disrupt neutrophil recruitment *in vitro* and *in vivo*. However, this has not been specifically assessed in neonates, nor have specific pathways involved in the disruption been identified. CXCL1 is a powerful chemoattractant which binds CXCR2 on neutrophils, a GPCR which initiates signals for the movement of neutrophils to the site of infection. In order to assess if this mechanism is important for neonatal neutrophil recruitment, and if it is a PTx-sensitive pathway, pups were depleted of CXCL1 and then challenged with WT *Bp* or *Bp*Δ*ptx*. Treatment with anti-CXCL1 successfully reduced numbers of neutrophils in the lungs of pups (**Figure 3.3A-C**). Decreasing lung neutrophil numbers in this way had no effect on the increased numbers of WT *Bp* but prevented the rapid control of *Bp*Δ*ptx* (**Figure 3.3D**). Thus, these results suggest that a primary mechanism by which PTx-disrupts control of *Bp* in neonates is blocking neutrophil recruitment, mediated via the CXCL1/CXCR2 pathway. While this pathway is required for efficient control of *Bp*Δ*ptx*, it is blocked by PTx to prevent rapid clearance of *Bp* in neonates.

<u>Complement-mediated recruitment of neutrophils is disrupted by PTx</u>

The complement system is an important innate immune function that can stimulate other aspects of the immune system[29–32]. Previous research has suggested there is little role for complement in control of *Bp*[33–35]. However, we considered the possibility that complement may have a protective role in neonatal infections, but this protective pathway is partly or wholly blocked by PTx. We therefore examined whether rapid complement-mediated clearance of *Bp*Δ*ptx* from neonatal lungs is disrupted in pups lacking the critical complement component 3 ($C3^{-/-}$). $C3^{-/-}$ pups had higher numbers of *Bp*Δ*ptx* in their lungs, indicating they are defective, relative to C57Bl/6 pups, in their ability to rapidly control *Bp*Δ*ptx,* revealing a critical role for complement in the efficient function of NIS in the lungs (**Figure 3.4B**). $C3^{-/-}$ pups were similar to immunocompetent C57Bl/6 pups in failing to control WT *Bp* (**Figure 3.4A**), indicating that PTx abrogates the effects of complement. Together these results suggest that complement can contribute to very efficient control of respiratory infection in neonatal lungs, but this effect is disrupted by PTx. Flow cytometric analysis demonstrated a profound failure of $C3^{-/-}$ pups to recruit neutrophils to the lungs at various timepoints (**Figure 3.4C-D**), suggesting complement has effects involving the recruitment or activation of neutrophils. Importantly, $C3^{-/-}$ pups challenged with WT *Bp* had no neutrophil recruitment to the lungs relative to C57Bl/6 pups, emphasizing our previous observations that in the presence of PTx, neutrophils are poorly recruited and have little to no contribution to controlling infection (**Figure 3.4C**). These data also suggest that the effects of C3 and the complement system might be mediated by neutrophil recruitment. To test this hypothesis, we delivered purified C3a, a major mediator of neutrophil recruitment from the complement cascade, to $C3^{-/-}$ pups. C3a was delivered along with the *Bp*Δ*ptx* inoculum to ensure immune cell recruitment to the sight of bacterial inoculation in the lungs. Co-inoculation of *Bp*Δ*ptx* with C3a resulted in significantly more neutrophils in the lungs at 1 and 3 dpi, as well as significantly reduced CFU in the lungs (**Figure 3.4E-F**). Thus, the complement system contributes to neonatal control of lung infection by recruiting neutrophils via the chemoattractant C3a, but this recruitment is disrupted by PTx.

Neutrophils and complement have additive effects in the rapid control of *Bp*Δ*ptx*

Our results above (**Figure 3.4)** provide strong evidence that a major role of the neonatal complement system is to recruit neutrophils, but $C3^{-/-}$ pups were not completely defective in clearing *Bp*Δ*ptx,* suggesting there are additional pathways involved. Occam's razor supports considering a simple likely pathway by which complement attracts and activates neutrophils to mediate opsonophagocytic killing of bacteria. Alternatively, it is also possible that complement and neutrophils could have independent effects. To distinguish the effects of neutrophils in the presence and absence of complement, C57Bl/6 pups and $C3^{-/-}$ pups were depleted of neutrophils prior to challenge. Depleting neutrophils substantially blocked the rapid (within one day) ~99% reduction in *Bp*Δ*ptx* numbers in both C57Bl/6 and $C3^{-/-}$ pups (**Figure 3.5A**), indicating that neutrophils have important effects independent of complement. $C3^{-/-}$ pups had higher CFU of *Bp*Δ*ptx* regardless of neutrophil depletion, indicating that complement has effects that are not mediated by neutrophils. Importantly, $C3^{-/-}$ pups depleted of neutrophils had similar CFU of *Bp*Δ*ptx* as C57Bl/6 had WT *Bp*, indicating that in the absence of neutrophils and complement, PTx has no measurable effect. This also strongly suggests that a key role of PTx is to block the rapid effects of complement and neutrophils observed by 1 dpi. By 3 dpi, a different pattern emerged; while depleting neutrophils substantially blocked the further reduction of *Bp*Δ*ptx* in C57Bl/6 mice, depletion of neutrophils in $C3^{-/-}$ pups had no significant effect (**Figure 3.5B**). The observation that by 3 dpi $C3^{-/-}$ pups depleted of neutrophils had lower numbers of *Bp*Δ*ptx* than WT *Bp* indicates that PTx blocks some remnant immune function independent of complement and neutrophils, the effects of which become more prominent between 1 and 3 dpi (**Figure 3.5B**). Together these results indicate that the remarkably efficient NIS clearance requires both complement and neutrophils in the first day, and that these immune mechanisms are blocked by PTx. However, by

3 dpi, other mechanisms come into play that can compensate for the lack of complement and neutrophils and can be highly effective but are also blocked by PTx.

DISCUSSION

Here, we add to the growing evidence the NIS is not simply an incompletely developed AIS, but a competent, highly evolved system which can very rapidly and efficiently eliminate bacteria from the respiratory tract; neonates eliminate *Bp*Δ*ptx* from lungs much more efficiently than adult mice, which take weeks to clear this same strain[8,17–19]. This highly efficient anti-microbial defense of neonates allowed us to probe the mechanisms involved, and to demonstrate that they required rapid neutrophil recruitment and for the first time uncover the involvement of both complement and specific chemokines. The observation that this rapid immune clearance is severely blocked by the effects of pertussis toxin (PTx) further supports the importance of this toxin to the extreme virulence of *Bp* in newborns. Together, these data demonstrate that neutrophils and complement activation have multiple critical roles that appear to be particular to neonatal immunity, and that the underlying mechanisms can be probed in this experimental system.

We have previously demonstrated that PTx delays neutrophil recruitment to the lungs by ~7 days in adults and 3 days in neonates, allowing *Bp* to rapidly grow in numbers early in the infection process[6,8]. Here, we identify for the first time the roles of two separate neutrophil recruitment pathways (CXCL1 and C3a) that contribute to the highly efficient neonatal immune clearance, both of which are known to signal via PTx-sensitive GPCRs[12,13]. In fact, the absence of C3 resulted in nearly complete lack of neutrophil recruitment in pups challenged with WT *Bp*. C3-dependent neutrophil recruitment has not been previously observed in neonates and may enable novel approaches to enhance neutrophil recruitment to clear infections in infants.

The importance of multiple roles of neutrophils in the neonatal response is supported by the observation that when neutrophils are depleted, there is substantial dysregulation of pro-inflammatory cytokines. Importantly, none of the inflammatory cytokines assessed here are known to be solely or directly produced by neutrophils, suggesting that neutrophils may instead serve as important regulators in the cascade of these cytokines rather than being the direct source of production[36–48]. Similar observations have been made in adult mouse models, with neutrophil depletion resulting in less inflammation systemically[49]. Additionally, we did not observe a role for NETosis in control of *BpΔptx,* therefore differences in inflammatory cytokines are likely not a result of increased NET production. Importantly, our observations challenge the current hypothesis that neonates are generally unable to generate inflammatory responses or efficiently control infection in the lungs.

In this experimental model focused on the NIS, we observe more profound effects of PTx on both immune cell recruitment and bacterial burden than previously observed in the AIS[6,8]. Since the bacterial burden in the lungs differs dramatically in the presence or absence of PTx, there are complexities in interpreting the cytokine responses. Since similar trends were observed in both challenged and unchallenged pups treated with anti-Ly6G, we can distinguish neutrophil-mediated effects from those confounded by differing bacterial burden. It will be important going forward to clearly distinguish the direct effects of PTx from effects that are indirectly mediated by diverging bacterial burdens. Previous work in adults has demonstrated that increasing the inoculation dose of *BpΔptx* to match the high burden of WT *Bp* in the lungs at a later timepoint does not result in similar immune responses[18]. An analogous approach in this experimental model should allow further analysis of the efficient neonatal immune response stimulated by *BpΔptx* that is disrupted by PTx.

The theory that the NIS is distinct and unique from the AIS is supported by evidence that neonates are in fact not highly susceptible to all pathogens. For example, infections with *Streptococcus pneumoniae* are highly uncommon in infants, recently found to be due to early neonatal neutrophils having enhanced CD11b-dependent opsonophagocytosis[50]. Neonatal neutrophils are also known to have prolonged activity and decreased inhibitory receptors relative to adult neutrophils[51,52]. While there is evidence that neonatal neutrophils do not travel as far in response to chemokine stimulation as do adult neutrophils[53], there is even more evidence that they have efficient complement-mediated killing ability and complement receptor expression[29,30,54–56].

A major obstruction in efforts to unravel interactions between immune components has been the lack of appropriate models. Even in studies utilizing juvenile (P7-P14) mouse models, slight changes in age or treatment resulted in substantially different phenotypes[6,20]. However, our individual observations on the effects of PTx on immune cell activities and contributions of neonatal immune cells to efficient bacterial clearance are consistent with those previously reported[6,29,31,53,57,58]. The interactions of various immune cells are likely to be as complex and highly dynamic in neonates as in adults. It is important to note that in the absence of PTx, neutrophils were only required in moderate numbers for efficient control of infection and that they also appear to have a role in the recruitment of additional immune cells. These complex dynamics are important to understand as they are likely contributing to the extraordinary susceptibility of neonates to WT *Bp*[8].

Few neonatal models have been able to characterize immune components which are required for rapid and efficient control of pathogens. Here, we describe the requirement of neutrophils and complement-mediated mechanisms for control of *Bp* when these functions are not disrupted by PTx. With this work, we outline the very effective neonatal immune response which occurs upon inoculation with *Bp*Δ*ptx*, leading to rapid control of the bacterial infection with minimal inflammation. By assessing the signals and responses initiated by *Bp* but disrupted by PTx, we may also uncover similar mechanisms by other pathogens and gain greater understanding of the effective neonatal immune response.

Our work demonstrates that the NIS can be highly efficient, clearing *Bp*Δ*ptx* from the lungs even faster than the AIS. However, the presence of PTx disrupts this neutrophil- and complement-mediated control. By using *Bp*Δ*ptx,* we can better understand how the NIS can rapidly mount an effective immune response. Additionally, we can use this knowledge and experimental system to better understand how PTx or other pathogen-derived factors enable extreme virulence in newborns. This information also can inform how we define correlates of protection in human infants to identify courses of treatment which are unique to the NIS. This will enable the development of targeted treatments to combat pathogens which cause particularly severe disease in newborns.

FIGURES

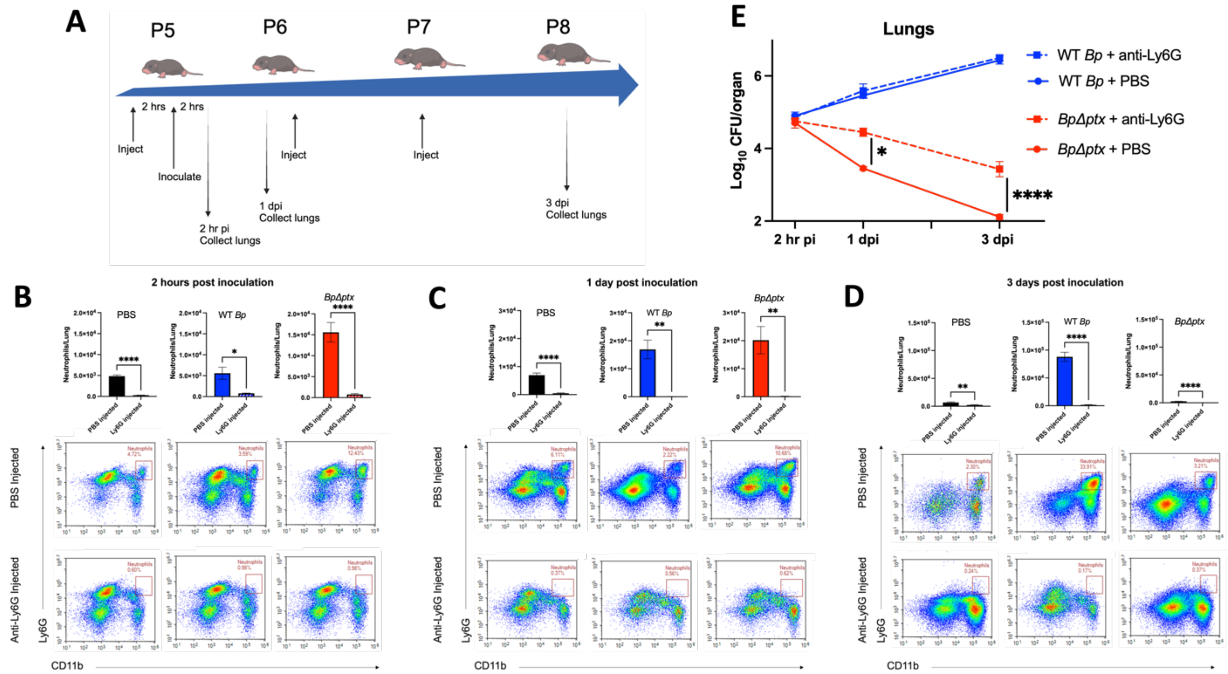

Figure 3.1. Neutrophils are required for rapid control of *Bp*Δ*ptx* in neonatal lungs. Schematic of anti-Ly6G treatment and bacterial inoculation (**A**). Neutrophils per lungs and representative density plots of neutrophils ($CD45^{+}Ly6G^{+}CD11b^{+}$) from lungs of pups treated with PBS or anti-Ly6G at 2 hrs (**B**), 1 dpi (**C**), and 3 dpi (**D**). PBS challenged (black), WT *Bp* challenged (blue), *Bp*Δ*ptx* challenged (red). PBS injected (solid) and anti-Ly6G injected (stripe). Statistical significance was calculated via unpaired Student's T test. *$p<0.0332$, **$p<0.0021$, ****$p<0.0001$. Experiments were completed in triplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint). CFU recovered from lungs at 2 hrs, 1 dpi, and 3 dpi (**E**). WT *Bp* challenged (blue line), *Bp*Δ*ptx* challenged (red line). PBS injected (solid line) and anti-Ly6G injected (dashed line). Experiments were completed in triplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint). Statistical significance was calculated via Two-way ANOVA. *$p<0.0332$, ****$p<0.0001$.

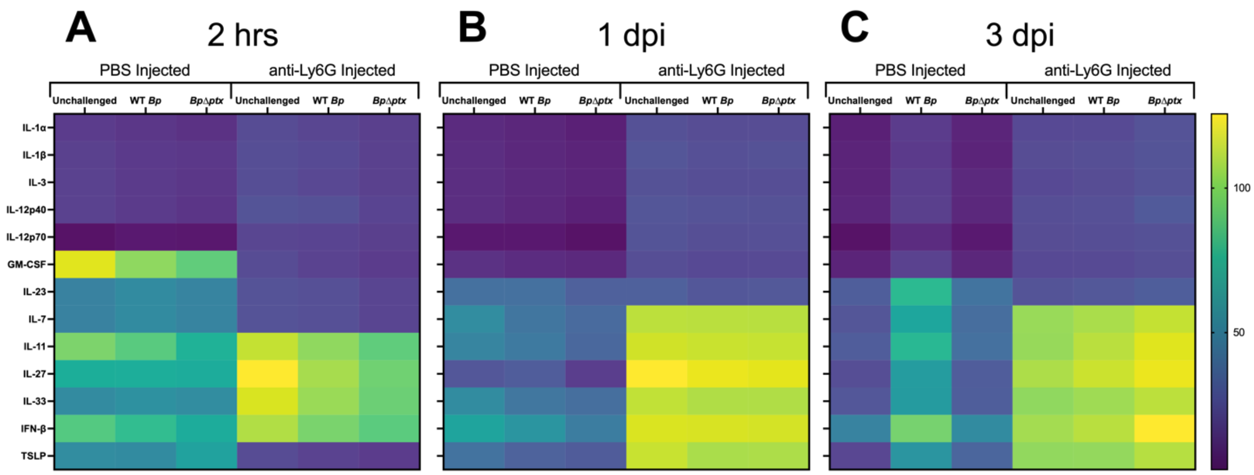

Figure 3.2. Neutrophils are critical to regulation of neonatal inflammatory cytokines. Analysis of inflammatory cytokines from lung supernatants at 2 hrs (**A**), 1 dpi (**B**), and 3 dpi (**C**). (n=3-5 pups per treatment/timepoint).

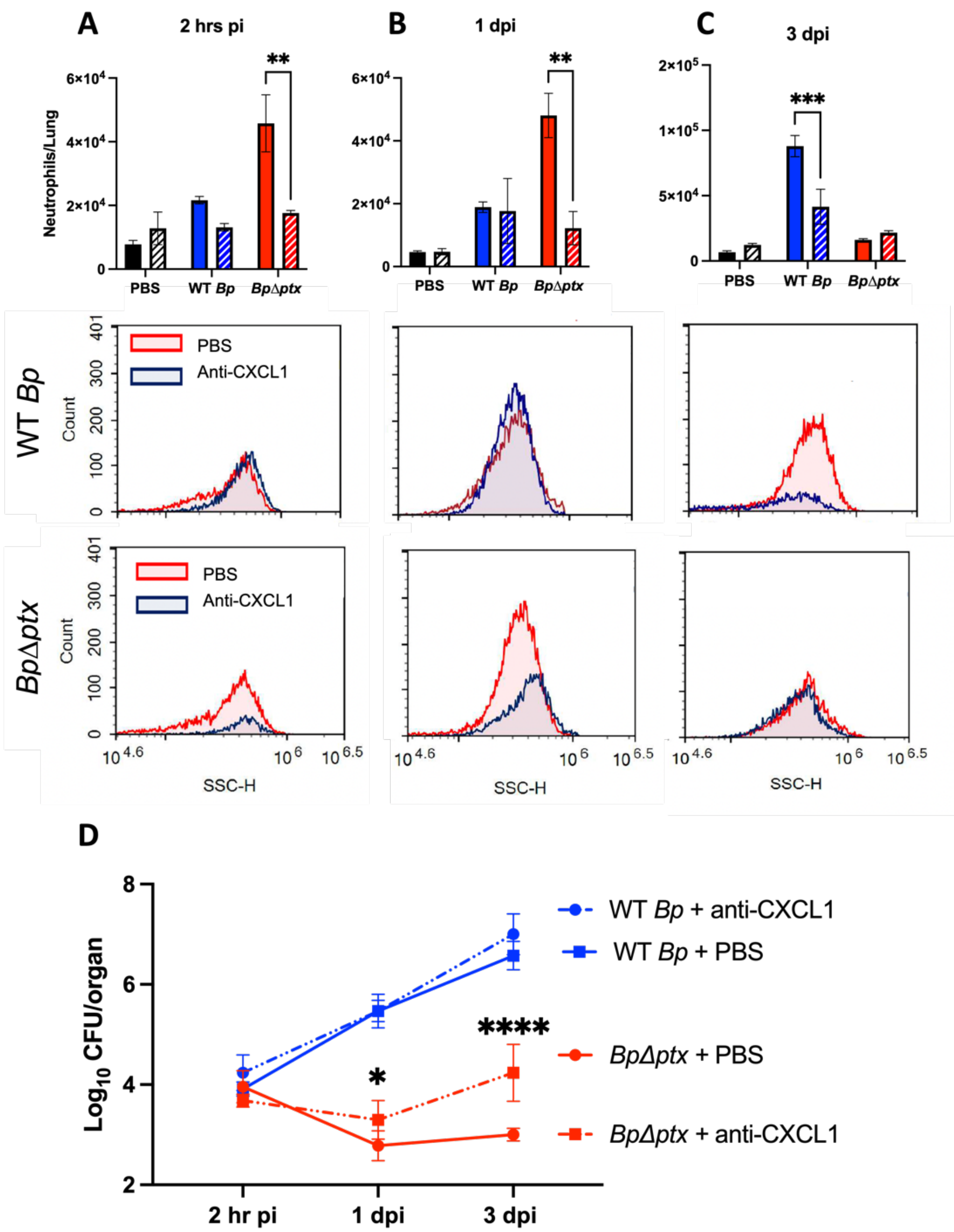

Figure 3.3. CXCL1 mediates neonatal neutrophil recruitment to the lungs. Neutrophils ($CD45^+Ly6G^+CD11b^+$) per lung at 2 hrs (**A**), 1 dpi (**B**), and 3 dpi (**C**). Top panel displays total neutrophil numbers in all groups. Middle panel is representative histogram of pups challenged with WT *Bp* and treated with PBS (red) or anti-CXCL1 (blue) at each timepoint. Bottom panel is representative histogram of pups challenged with *Bp*Δ*ptx* and treated with PBS (red) or anti-CXCL1 (blue) at each timepoint. CFU recovered from lungs at 2 hrs, 1 dpi, and 3 dpi (**D**). WT *Bp*

challenged (blue line), *Bp*Δ*ptx* challenged (red line). PBS injected (solid line) and anti-CXCL1 injected (dashed line). Experiments were completed in triplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint). Statistical significance was calculated via one-way ANOVA. *$p<0.0332$, **$p<0.0021$, ***$p<0.0002$, ****$p<0.0001$.

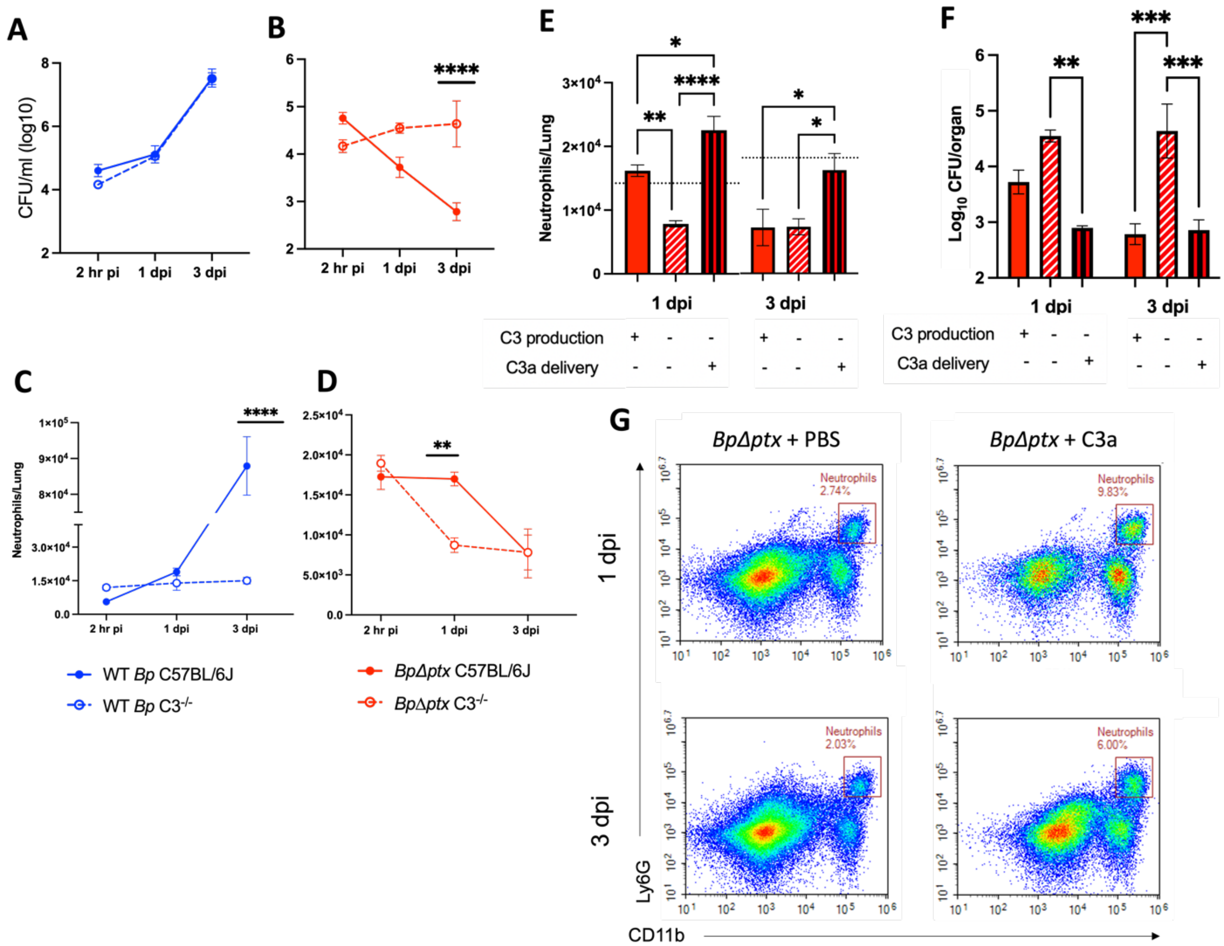

Figure 3.4. C3 contributes to neutrophil recruitment to neonatal lungs. CFU per lung from C57Bl/6 (solid line) or C3$^{-/-}$ pups (dashed line) challenged with WT *Bp* (blue) (**A**) or *Bp*Δ*ptx* (red) (**B**) at 2 hrs, 1 dpi, and 3 dpi. Neutrophils recovered per lung from C57Bl/6 (solid line) or C3$^{-/-}$ pups (dashed line) challenged with WT *Bp* (blue) (**C**) or *Bp*Δ*ptx* (red) (**D**) at 2 hrs, 1 dpi, or 3 dpi. Neutrophils from lungs of C57Bl/6 (solid red bar) or C3$^{-/-}$ pups challenged with *Bp*Δ*ptx* (red and white striped bar) or *Bp*Δ*ptx* with 6.4 ng (red and black striped bar) C3a at 1 and 3 dpi. Average neutrophils per lung of unchallenged C3$^{-/-}$ pups at indicated timepoint (**E**). Dashed line represents average neutrophils per lung in C57Bl/6 pup at indicated timepoint. CFU per lung from C57Bl/6 (solid red bar) or C3$^{-/-}$ pups challenged with *Bp*Δ*ptx* (red and white striped bar) or *Bp*Δ*ptx* with 6.4 ng C3a (red and black striped bar) at 1 and 3 dpi (**F**). Representative density plots of neutrophils

($CD45^+Ly6G^+CD11b^+$ from $C3^{-/-}$ pups challenged with *Bp*Δ*ptx* or *Bp*Δ*ptx* + C3a at 1 and 3 dpi (**G**). Experiments were completed in triplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint). Statistical significance was calculated via Two-way ANOVA. *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001.

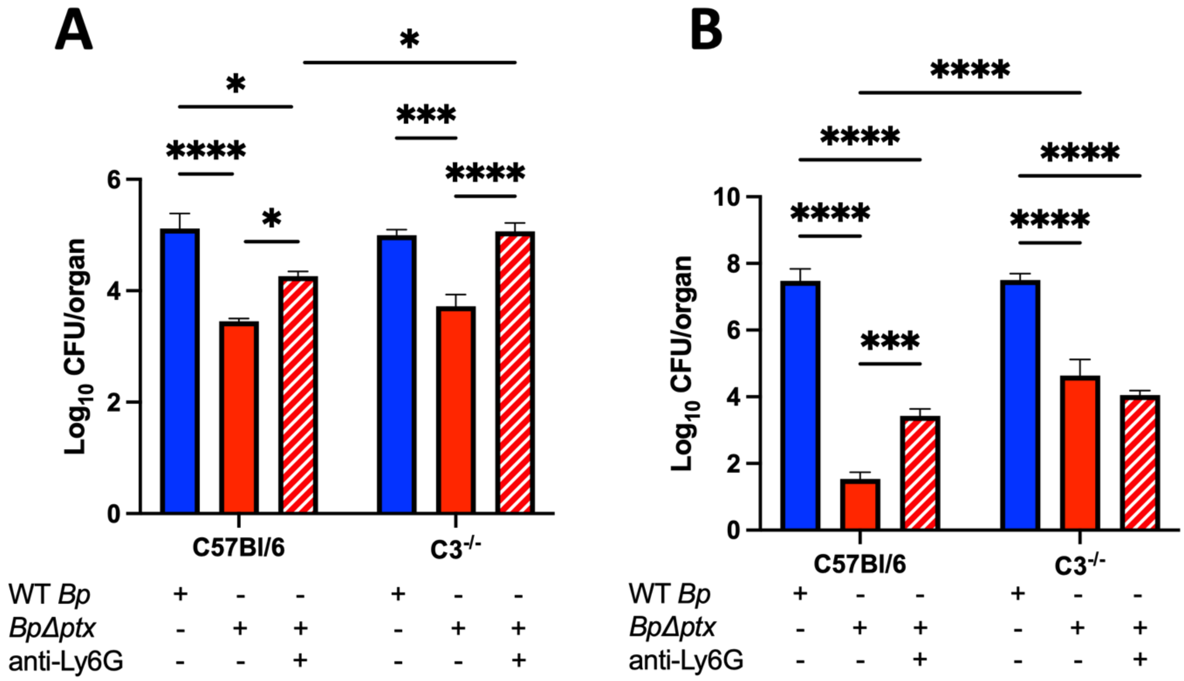

Figure 3.5. Neonatal neutrophils and complement have individual contributes to control of *B. pertussis* at early timepoints but are disrupted by PTx. CFU per lung from C57Bl/6 or $C3^{-/-}$ pups treated with PBS (solid bar) or anti-Ly6G (striped bar) and challenged with WT *Bp* (blue) or *Bp*Δ*ptx* (red) at 1 dpi (**A**) and 3 dpi (**B**). Experiments were completed in triplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint). Statistical significance was calculated via Two-way ANOVA. ns $p>0.0332$, *$p<0.0332$, ***$p<0.0002$, ****$p<0.0001$.

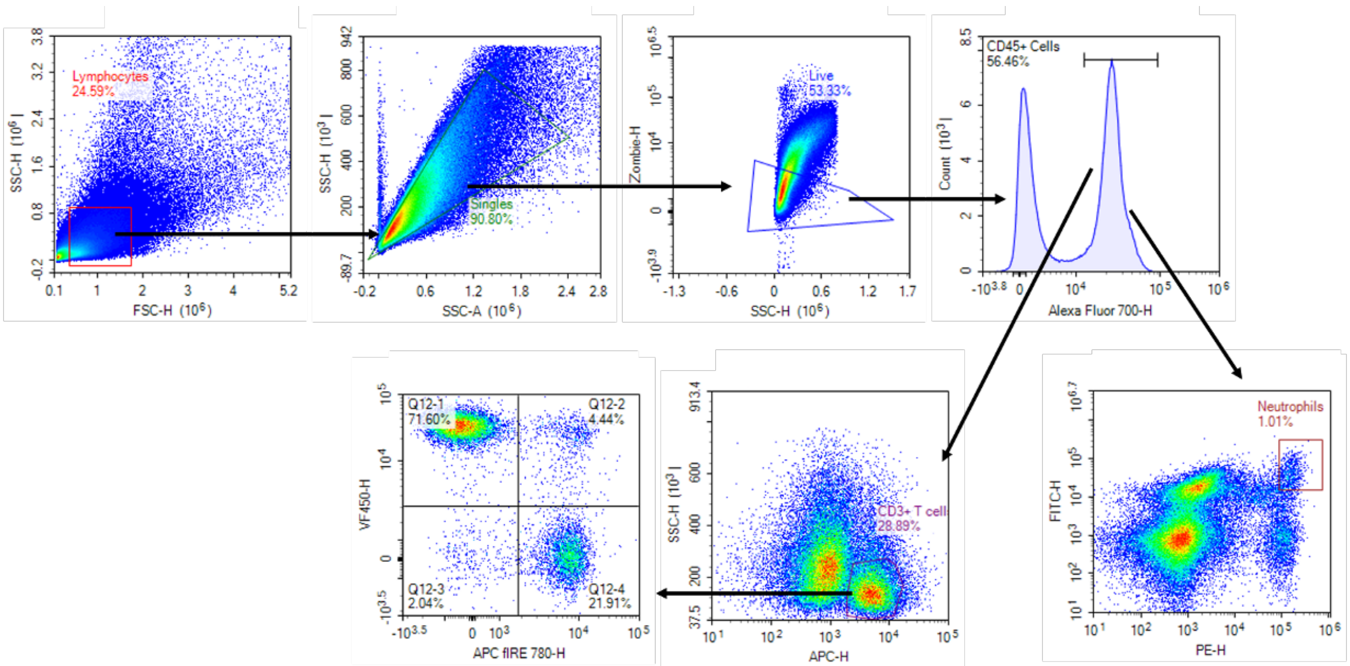

Figure S3.1. Gating strategy to isolate T cells ($CD45^+CD3^+$) and neutrophils ($CD45^+Ly6G^+CD11b^+$) from naïve P8 C57Bl/6 lungs.

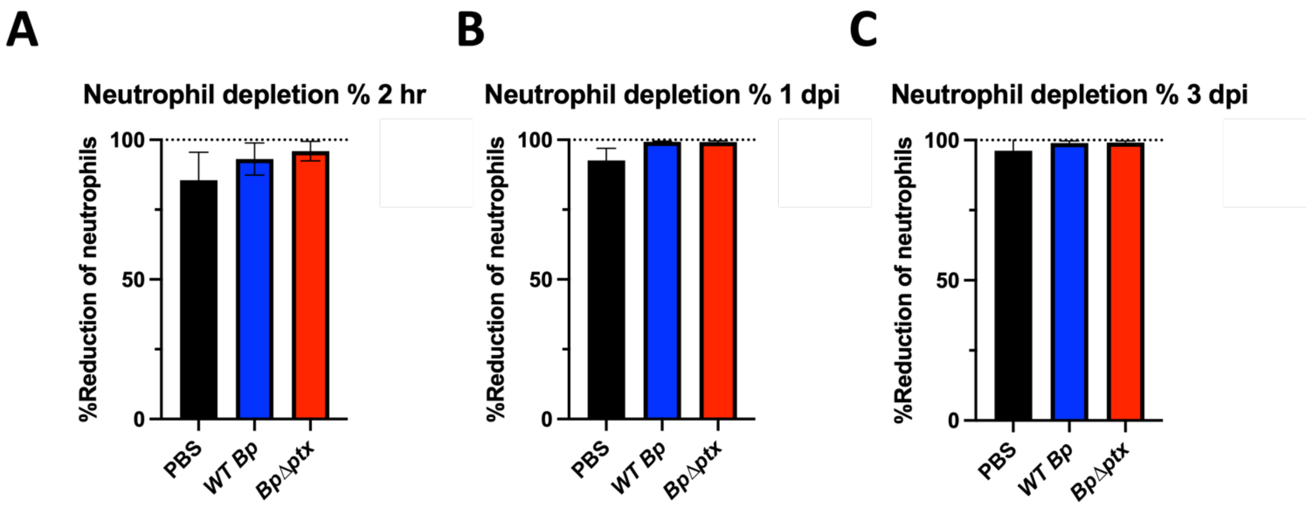

Figure S3.2. Effect of anti-Ly6G treatment on neutrophil populations in lungs of pups. Pups were inoculated with PBS (black), WT *Bp* (blue), or *Bp*Δ*ptx* (red) at 2 hrs (**A**), 1 dpi (**B**), and 3 dpi (**C**). Percent reduction of neutrophils displayed as percent compared to PBS-treated pups (dotted line). Experiments were completed in triplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint).

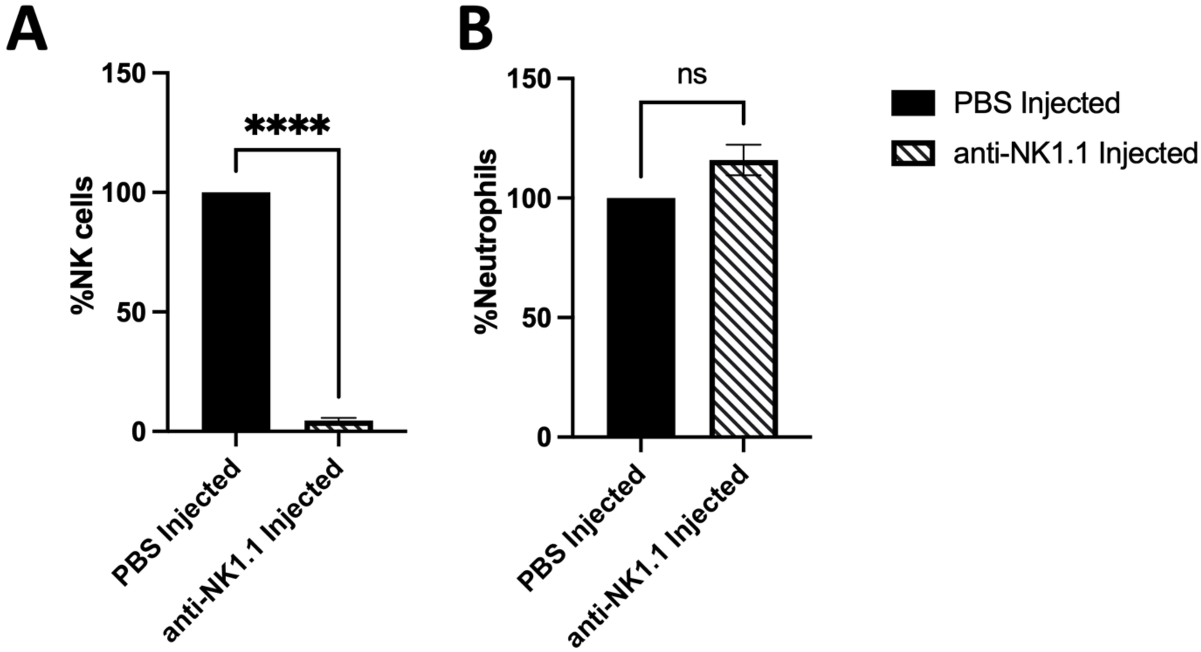

Figure S3.3. Effect of anti-NK1.1 treatment on NK cell and neutrophil populations. NK cell (**A**) and neutrophil (**B**) populations in the lungs. PBS injected (solid bar), anti-NK1.1 injected (striped bar). Experiments were completed in duplicate. Error bars show standard error of the mean (n=3-4 pups per treatment/timepoint). Statistical significance was calculated via unpaired Student's T test. ns p>0.0332, ****p<0.0001.

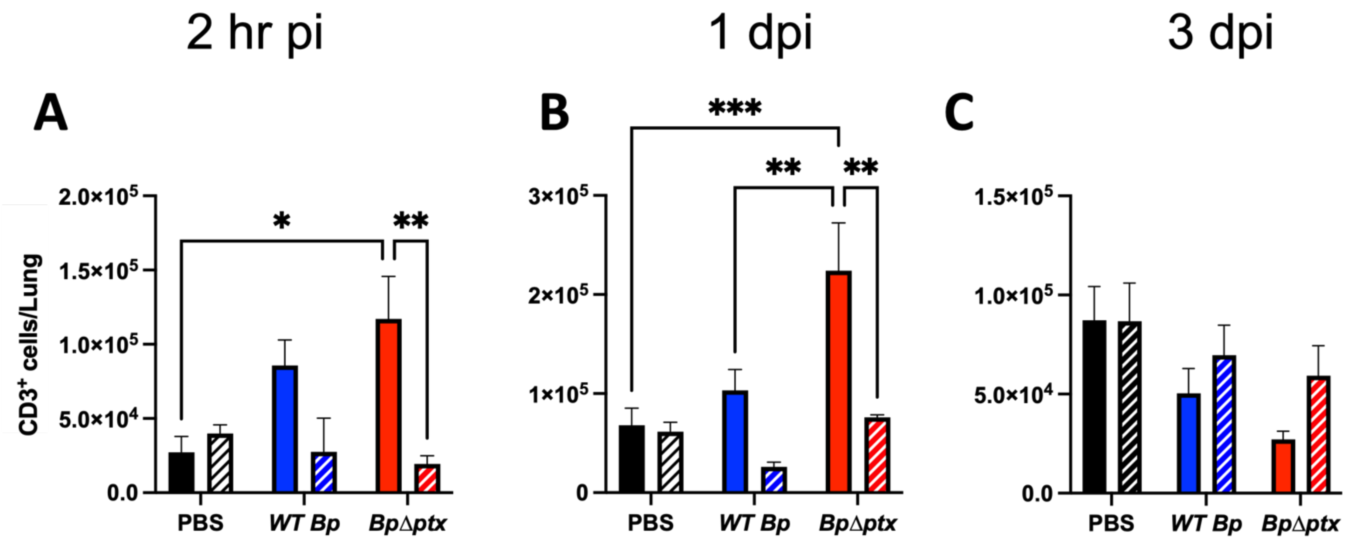

Figure S3.4. Effect of anti-Ly6G treatment on T cell populations. Data shows lungs of pups challenged with PBS (black) WT *Bp,* (blue) or *Bp*Δ*ptx* (red) at 2 hrs pi (**A**), 1 dpi (**B**), and 3 dpi (**C**). Solid bars represent pups treated with PBS and striped bars represent pups treated with anti-Ly6G. Experiments were completed in triplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint). Statistical significance was calculated via Two-way ANOVA. ns p>0.0332, *p<0.0332, ***p<0.0002.

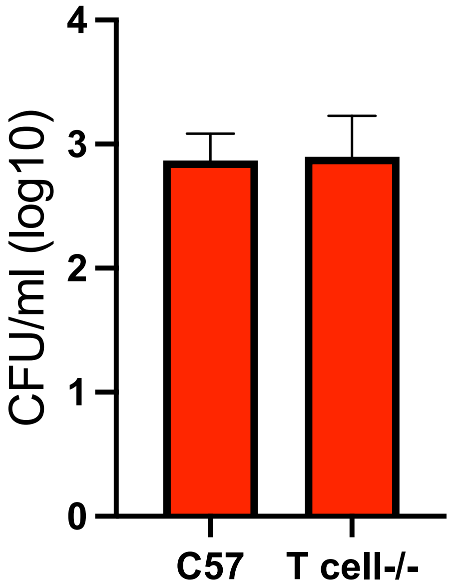

Figure S3.5. T cells are not required for control of *Bp*Δ*ptx*. C57Bl/6 and T cell$^{-/-}$ P5 pups inoculated with *Bp*Δ*ptx* and assessed in lungs at 3 dpi. Experiments were completed in duplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint). Statistical significance was calculated via unpaired Student's T test.

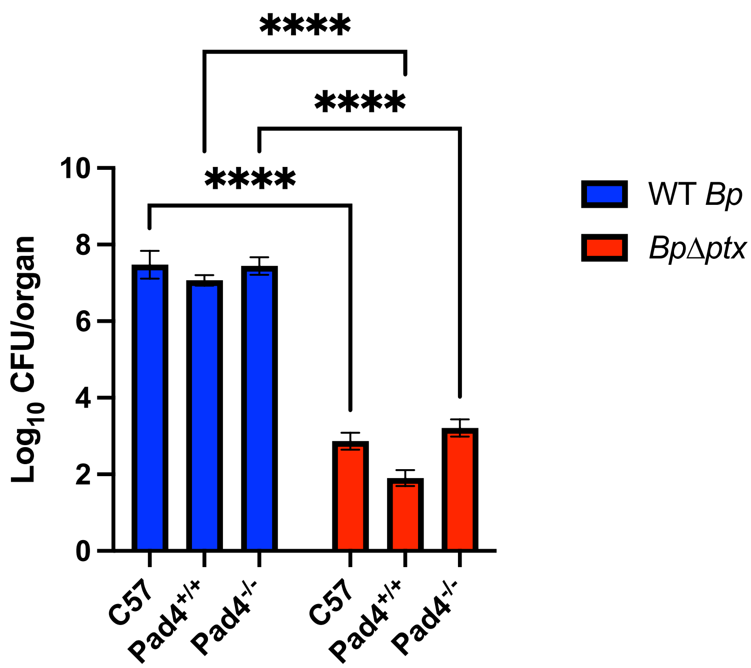

Figure S3.6. NETosis does not contribute to control of *Bp*Δ*ptx*. Inoculation of C57Bl/6, Pad4$^{+/+}$, and Pad4$^{-/-}$ P5 pups with WT *Bp* or *Bp*Δ*ptx* and collected lungs at 3 dpi. Experiments were completed in triplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment/timepoint). Statistical significance was calculated via Two-way ANOVA. ****$p<0.0001$.

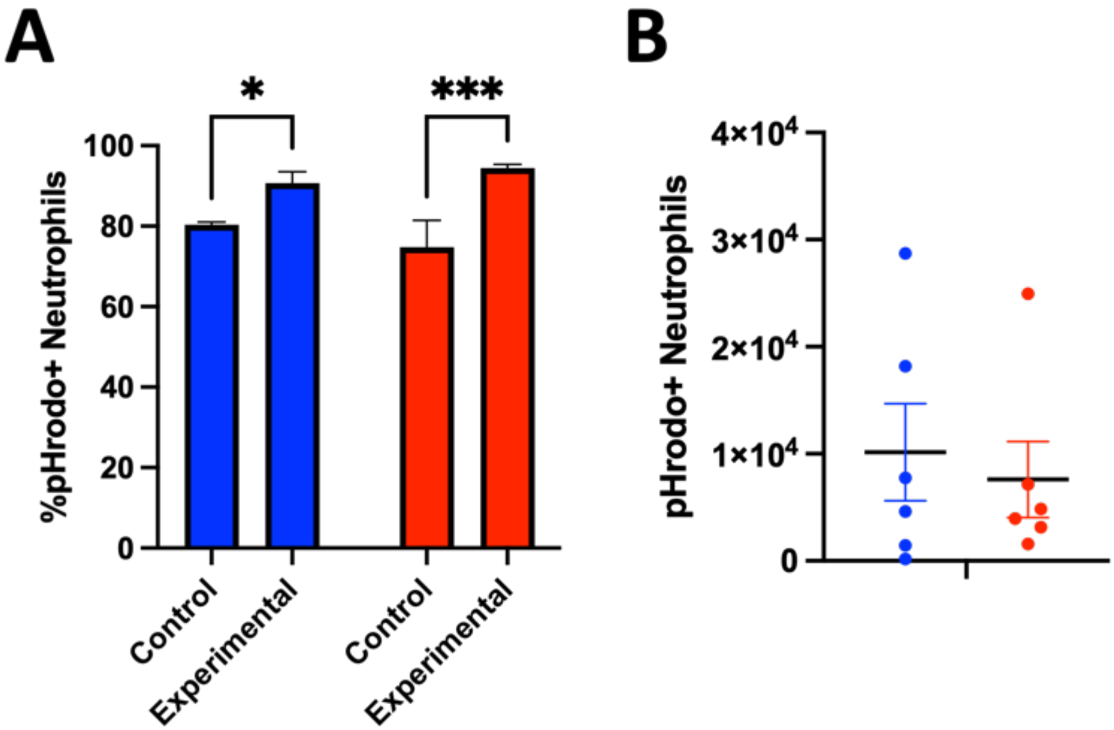

Figure S3.7. PTx does not affect ability of neonatal neutrophil to phagocytose bacteria. Incubation of lung cells with pHrodo green stained WT *Bp* (blue) and *Bp*Δ*ptx* (red) does not show significant effects of PTx on neutrophil phagocytosis. Percent pHrodo+ signals of total neutrophils per sample. Control samples incubated on ice and experimental group incubated at 37° C (**A**). Total events shown represent events from experimental group incubated at 37° C subtracted from control group incubated on ice (**B**). Experiments were completed in duplicate and pooled. Error bars show standard error of the mean (n=5-6 pups per treatment). Statistical significance was calculated via unpaired Student's T test.

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CHAPTER 4

COMPLEMENTARY MODELS OF THYMIC DISRUPTION REVEAL NOVEL NEONATAL-LIKE T CELL RESPONSES TO *BORDETELLA PERTUSSIS*

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ABSTRACT

The immune system of neonates is vastly different from that of adults and often associated with increased susceptibility to some pathogens. One of the most important aspects of this susceptibility is the transition from neonatal immune responses, expressed immediately post-birth, to perinatal immune responses, expressed several weeks after birth. This process is primarily driven by thymus development and the T cells generated; however, the contribution of T cells generated during this process to control of pathogens is poorly understood. Unfortunately, attempts to understand the roles of T cells developed at various stages are often confounded by the rapid development of the thymus in mice. Here, we utilize two complementary models of disrupted thymic development to assess the effects of thymic development on responses to a neonatal pathogen, *Bordetella pertussis* (*Bp*). Utilizing a genetic model of disrupted thymic development, we show that post-birth thymus development is critical for controlling *Bp.* With a complementary surgical model, mice thymectomized at P0 and P7 had substantially different T cell responses to *Bp*, with the later demonstrating an early CD8 response associated with more rapid control. These two models agree that T cells generated at distinct stages of development demonstrate different contributions to control of *Bp,* which could explain the increased susceptibility of young infants to *Bp.* Further work to understand the functions of T cells generated at each developmental phase, and their roles in pathogen defense can greatly improve our understanding of the neonatal immune system and contribute to development of new vaccines or therapeutics against neonatal pathogens.

INTRODUCTION

Bordetella pertussis (*Bp*) is the causative agent of the respiratory illness "whooping cough". Once one of the leading causes of death in children, vaccines against *Bp* were first introduced in the 1910's to prevent severe disease[1,2]. Today, while numerous vaccine formulations are utilized worldwide, recent estimates suggest that there remain approximately 24 million cases of whooping cough per year[3–5]. Importantly, children under six months of age suffer the most severe complications, with 40% of *Bp*-infected infants being hospitalized and at risk of long term complications, including pneumonia, brain damage, and asthma, among others[3]. Unfortunately, the neonatal immune system (NIS) is poorly understood and as such, the mechanisms which render this specific group susceptible to severe *Bp* disease remain unknown.

A primary obstacle to unraveling the interaction between the NIS and *Bp* is the rapid maturation of the thymus in mice. This primary lymphoid organ is responsible for the production of T cells and has a complex maturation process that affects T cell development, a process still not fully understood. T cells develop differently based on the specifics of their thymic environment, which changes with staged thymic maturation, as do the T cells available in the periphery also vary by age[6]. The presence of these subtypes likely correlates to the unique requirements of the host at the neonatal, perinatal, and adult stages, and thus how they respond to pathogens[6–8]. We have recently published a novel murine model of infection with *Bp* which utilizes early timepoints and a short timecourse to assess the neonatal response to *Bp* in the absence of adult T cells. We observed increased susceptibility of these neonatal mice to *Bp* which was mediated by pertussis toxin. However, with this "natural" pup model, we have observed changes in T cell populations and in sensitivity to *Bp* by P8, both suggesting a marked shift toward a more perinatal response, so we can only assess the first 3 days of *Bp* infection (P5 to P8) as primarily representing a neonatal immune response[9]. The very short duration of such neonatal pup infection experiments prevents the study of more prolonged *Bp* infections in neonates, which typically last several weeks in distinct stages.

Here, we utilize mouse models which are complementary to our novel pup model to assess extended neonatal-like responses to *Bp*. These complementary models include surgical removal of the thymus at day of birth (P0) or 7-days postnatal (P7) and N-terminal deletion of the FoxN1 gene[10,11]. These models allows us to investigate the responses generated by mice with both specifically timed disruption of thymic-dependent T cell production (thymectomy) and general thymic development disruption (FoxN1$^{\Delta/\Delta}$)[10–12]. Both models were utilized here to assess the neonatal responses to prolonged *Bp* infections which could not be observed with the abbreviated timeline featured in our neonatal pup model. Here, we observe substantial numbers of a neonatal T cell subset in the lungs of all thymically disrupted mice, similar to populations in P5-P8 pups[9]. FoxN1$^{\Delta/\Delta}$ and P0 thymectomized (thyx) mice were significantly more susceptible to *Bp* than their respective immunocompetent controls which successfully controlled *Bp.* However, P7 thyx mice controlled bacterial burden in the lungs and nasal cavity similarly to sham controls, suggesting that T cells generated in the first 7 days of life are capable of controlling infection similarly to T cells generated thereafter. This ability to control *Bp* in P7 thyx mice was associated with increased CD8$^+$ T cells in the lungs at ~7 dpi. The failure to control *Bp* in P0 thyx and FoxN1$^{\Delta/\Delta}$ mice was associated with partial or complete failure to generate a CD4$^+$ T cell response, respectively. These results suggest that T cells generated at and before P7 can mediate control and are skewed toward CD8$^+$ subsets, a unique response not previously observed in neonatal or adult mouse models. We discuss the utility of these models to study how the immune responses to pathogens develop in early life, rapidly in mice and more gradually in humans.

MATERIALS AND METHODS

Bacterial Strains and Growth

The *B. pertussis* strain Tohama 1 (gentamycin resistant derivative) (WT *B. pertussis*) has been previously described[13,14]. Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% defibrinated sheep blood (Hemostat Laboratories) and 20 μg/ml gentamycin (Sigma-Aldrich). Liquid cultures were grown overnight in Stainer-Scholte broth at 37° C to mid-log phase then maintained in 20% glycerol stocks at -80° C for use as inoculum.

<u>Mouse Experiments</u>

Eight-to ten-week old female and male FoxN1$^{+/\Delta}$, FoxN1$^{\Delta/\Delta}$, and C57Bl/6 (00664) mice were maintained in the Manley mouse colony (University of Georgia, GA). C57Bl/6 mice underwent thymectomy or sham surgery at day of birth or seven-days post birth according to appropriate animal use protocols approved by UGA IACUC. All mice were maintained in specific pathogen-free facilities, and all experiments were conducted following institutional guidelines and IACUC approved protocols. Mice were lightly sedated with 5% isoflurane (Pivetal) and inoculated (5 X 10^5 CFU suspended in 50 μl PBS) by pipetting the inoculum as droplets into their external nares to be inhaled. At the indicated timepoints mice were euthanized via CO_2 inhalation. Organs were excised and processed for flow cytometry. An aliquot of lung suspension was serially diluted and plated on BG agar to quantify bacterial numbers. Colonies were counted following incubation for five days at 37° C.

<u>Flow Cytometry</u>

Lungs were processed and stained as previously described[15]. Viable cells were identified with Zombie Aqua (Biolegend). 0.35 μl of each extracellular antibody or 1 μl of each intracellular antibody was added to each sample. Antibodies to identify T and B cell populations included anti-CD45 AF700 (clone: 30-F11, Biolegend), anti-CD3 APC (clone:17A2, Biolegend), anti-CD4 VF450 (clone:RM4-5, Tondo Biosciences), anti-CD8 APC Fire 750 (clone:53-6.7, Biolegend), and anti-CD19 PerCP/Cy5.5 (clone:1D3/CD19, Biolegend). Antibodies to identify virtual memory T cells (T_{VM}) included anti-CD8 BV650 (clone: 53-6.7, BD Biosciences), anti-CD4 AF700 (clone:RM4-55, Biolegend), anti-CD44 PE/Cy7 (clone: IM7, Biolegend), anti-CD49d APC Fire 750 (clone:R1-2, Biolegend), anti-CD3 FITC (clone: 17A2, Biolegend), anti-TCRg/d PE/Cy5 (clone: GL-3, Invitrogen), anti-NK1.1 PE (clone: PK136, Biolegend), anti-Eomes APC (clone: Dan11mag, Invitrogen), and anti-CXCR3 BV750 (clone: CXCR3-173, BD Biosciences). Intracellular staining for Eomes was performed as previously described[16]. Stimulation of cells from $FoxN1^{+/\Delta}$ and $FoxN1^{\Delta/\Delta}$ was performed for four hours as previously described[17,18]. Antibodies to identify $CD4^{+}$ IFN-γ producing T cells included: anti-CD45 AF700 (clone: 30-F11, Biolegend), anti-CD3 APC (clone: 17A2, Biolegend), anti-CD4 VF450 (clone: RM4-5, Tondo Biosciences), anti-CD8 APC Fire 750 (clone: 53-6.7, Biolegend), and anti- IFN-γ BV785 (clone: XMG1.2, Biolegend). The acquisition of the data was performed using the Quanteon (Agilent) and analysis was performed with NovoExpress (Agilent) following the gating strategies in **Figures S4.1, S4.3, & S4.4**.

Statistics

For experiments determining differences in bacterial loads in the organs of mice, the following statistical analyses were performed using GraphPad PRISM (GraphPad Software, Inc): Two-tailed unpaired Student t-tests, One-way ANOVA, and Two-way ANOVA. For experiments determining differences in immune cell populations, the following statistical analyses were performed using GraphPad PRISM (GraphPad Software, Inc): Two-tailed unpaired Student t-tests, One-way ANOVA, and Two-way ANOVA.

Ethics Statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committees at The University of Georgia at Athens, GA (A2022 04-001-Y1-A0 Bordetella-Host Interactions, A2022 04-025-Y1-A0 Breeding Protocol, and A2022 04-022-Y1-A0 Neonatal Models of Bordetella infection, transmission, and immunity). Mice were consistently monitored for signs of distress over the course of the experiments to be removed from the experiment and euthanized using carbon dioxide inhalation to prevent unnecessary suffering.

RESULTS

Mouse models of neonatal-like immunity display unique T cell populations

To assess contributions of T cells generated at various stages of development in mice, thymectomy was performed at day of birth (P0 thyx) or 1-week-post birth (P7 thyx). The previously described FoxN1$^{\Delta/\Delta}$ genetic model was also utilized to assess the contributions of T cells generated prior to birth to infection[10–12]. Thus, mice utilized here lack either thymus-matured T cells (FoxN1$^{\Delta/\Delta}$), T cells generated after the first day of birth (P0 thyx), or T cells generated after the first week after birth (P7 thyx). To validate the mouse models, overall CD3^{+} T cell populations in the lungs of mice were assessed and compared to appropriate surgical or genetic controls. Overall, all mice with disrupted thymic development have significantly decreased CD3^{+} T cell numbers and proportions in naïve lungs compared to control but have similar numbers of neutrophils and B cells (**Figure 4.1. S4.1, & S4.2**).

Our previously published work demonstrated that a marker of the neonatal immune system in the lungs is the presence of large populations of fetal liver-derived virtual memory T cells (T_{VM}) [9,19,20]. To determine if similar populations are observed in mice with a neonatal-like immune system, we assessed populations of T_{VM} in our genetic and surgical models (**Figure S4.3**). Indeed, mice with disrupted thymic development had significantly more T_{VM} in the lungs than control mice (**Figure 4.2**). This suggests that mice with disrupted thymic development have similar unique populations of T cells as neonatal pups, providing strong evidence that these complementary models of neonatal-like immunity can be utilized to assess longer-term neonatal responses.

<u>Mice with disrupted thymic development are highly susceptible to *Bordetella pertussis*</u>

We have previously demonstrated that neonatal (P5) mice are highly susceptible to *Bp* in a short term (3 day) infection model[9]. The complementary models describe above provide an opportunity to study neonatal immunity during extended time frames more reflective of human infections, and thereby to define immune and bacterial pathogenic mechanisms mediating susceptibility to prolonged infections. To define the sensitivity of thymectomized and FoxN1$^{\Delta/\Delta}$ mice, adult mice were inoculated with 5×10^5 CFU and bacterial burden was assessed in lungs and nasal cavity at 7, 14, and 28 days post inoculation (dpi). Control mice (P0 sham, P7 sham, and FoxN1$^{+/\Delta}$) reduced *Bp* burden by ~99% in the lungs and nasal cavity by 28 dpi (**Figure 4.3A-F**). P0 thyx mice behaved similar to controls until 14 dpi but had significantly higher bacterial burden than control mice at 28 dpi (**Figure 4.3A-B**). FoxN1$^{\Delta/\Delta}$ mice demonstrated significant failure to control *Bp* in the lungs across the entire timecourse, with no reduction in CFU by 28 dpi (**Figure 4.3E**). While FoxN1$^{\Delta/\Delta}$ had minimal control of *Bp* in the nasal cavities up to 14 dpi, the burden of *Bp* at 28 dpi was significantly higher than control mice (**Figure 4.3F**). Surprisingly, P7 thyx mice did not demonstrate any significant differences in CFU compared to controls, demonstrating similar ability to control *Bp* as immunocompetent mice (**Figure 4.3C-D**). Therefore with these three models of disrupted thymic development, we observe three distinct responses to *Bp*, suggesting that different immune responses are generated by each mouse model.

Thymic development significantly affects T cell responses to *Bordetella pertussis*

In order to assess differential immune cell responses to *Bp,,* T cell kinetics in the lungs of mice were assessed. T cells in FoxN1$^{\Delta/\Delta}$ mice failed to expand in numbers and proportion compared to controls (**Figure 4.4C**). However, P0 and P7 thyx mice demonstrated sufficient expansion of T cells after infection and had similar kinetics during infection as control mice. Despite failure to control *Bp* in the lungs, P0 thyx mice had similar total T cell populations at 7 and 14 dpi; P7 thyx mice, which controlled *Bp* similarly to control mice, had significantly reduced T cell numbers in the lungs (**Figure 4.4A-B**). Additionally, no significant differences in neutrophils were observed, suggesting that innate immune responses to *Bp* are similar irrespective of thymic development (**Figure S4.4**). FoxN1$^{\Delta/\Delta}$ mice had no significant increases in CD4 or CD8 populations but had significantly reduced CD4^{+} T cells compared to control mice (**Figure 4.5C**). P0 thyx mice had similar trends of reduced CD4^{+} T cells compared to control mice, though not as completely as FoxN1$^{\Delta/\Delta}$ mice (**Figure 4.5A&C**). P7 thyx mice also had less CD4^{+} T cells but had significantly more CD8^{+} T cells than control mice at 7 dpi (**Figure 4.5B**). This suggests that in the perinatal environment, CD8^{+} T cells may be associated with control of *Bp,* rather than CD4^{+} T cells which are important for control in the adult environment.

CD4^{+} T cells from FoxN1$^{\Delta/\Delta}$ mice fail to produce IFN-γ upon stimulation with *Bordetella pertussis*

In order to determine if the inability of FoxN1$^{\Delta/\Delta}$ mice to control *Bp* is solely due to lack of expansion of T cells, the functional abilities of T cells were assessed. CD4^{+} T cells from FoxN1$^{\Delta/\Delta}$ control mice demonstrated similar ability to produce IFN-γ under positive and negative control conditions (**Figure 4.6 & S4.5**). However, CD4^{+} T cells from FoxN1$^{\Delta/\Delta}$ mice were unable to produce IFN-γ upon stimulation with *Bp*. This suggests that while T cells from FoxN1$^{\Delta/\Delta}$ mice have the functional capabilities to produce IFN-γ, evidenced by efficient production after stimulation with PMA/ionomycin, they are not stimulated by whole *Bp*. Thus, in addition to failing to expand in numbers, T cells from FoxN1$^{\Delta/\Delta}$ mice fail to respond similarly to control mice, likely contribution to a failure to control *Bp*.

DISCUSSION

The ability to accurately assess the NIS and response to infection via animal models has long eluded researchers. We have previously developed a short term neonatal infection model to assess early responses to *Bp* in neonates[9], and identified significant differences from the early immune response in adults. Despite its numerous strengths, this short term model is limited in its ability to study the response of the NIS during longer term infections. Here, we utilized complementary models of neonatal-like immunity to assess immunity to long-term infection without the confounding factors of the developing immune system. Using a surgical model, removing the thymus at two different stages of development, and a previously described genetic model with a life-long neonate-like thymus, we are able to observe distinct responses to *Bp* associated with neonatal thymic development stage[10–12]. We also describe a novel association between the generation of a CD8-skewed response and control of *Bp* in mice thymectomized at P7. This new information provides significant insight into the unique NIS and its effective response to *Bp.*

Neonates demonstrate increased susceptibility to pathogens such as *Bp,* however the mechanisms which mediate this susceptibility are poorly understood. To determine if increased susceptibility to *Bp* can be observed using complementary models of neonatal-like immunity, FoxN1$^{\Delta/\Delta}$, P0 thyx, and P7 thyx mice were inoculated with *Bp*. The model which most closely resembles the pup response to *Bp* was the FoxN1$^{\Delta/\Delta}$ mice, followed by P0 thyx mice. This was associated with decreasing development/cellular output of the thymus, a link which has previously had limited evidence. In fact, T cells which are matured in the thymus and then exit to the periphery vary greatly by the stage of thymic development. For example, neonatal T cells which exit the thymus from P1-7 have increased expression of PD-1 and CD31, resulting in limited CD8 functions and increasing signal threshold required for activation[21,22]. $CD4^+$ T cells generated by P7 also have low acquisition of CD69 and CD103 in response to RSV inoculation, resulting in limited generation of resident memory T cells[23]. Neonatal $CD8^+$ T cells also, however, have decreased expression of genes related to exhaustion, suggesting that they persist for longer periods of time in the naïve state than T cells generated in the adult thymus[24]. These characteristics can also be affected by signals from dendritic cells and other APCs, which also shift during murine development[25–27]. However, it was observed that incubating neonatal or adult APCs with adult T cells resulted in an adult-like TH1 phenotype, while interactions between adult or neonatal APCs and neonatal T cells generated a neonatal-like TH2 phenotype[25]. This suggests that responses generated are determined by the developmental origin of the T cells, not the APCs. Therefore, our complementary models not only benefit from persistent presence of T cells generated at distinct stages, but also from the lack of additional, confounding factors which shift during development.

One of the most important processes for responding to infections is T cell proliferation. We observed that T cells generated by day of birth (P0 thyx), or those produced by the end of the first week of life (P7 thyx), can proliferate in response to *Bp,* similar to T cells in control mice. T cells which first leave the thymus are highly proliferative[28–30], but require different signals than T cells from the adult thymus, namely self-peptide/self MHC interaction in the periphery[28]. This proliferative ability is not observed in T cells from FoxN1$^{\Delta/\Delta}$ mice, likely due to the immature state of the thymus in these mice, which are unable to generate T cells which can successfully respond to these signals[11,28]. Despite overall increases in T cells numbers in the lungs, P0 thyx mice failed to demonstrate sufficient control of *Bp* by 28 dpi. Further assessment of these T cell populations demonstrated that control mice developed a CD4-skewed response which the P0 thyx mice lacked. While a CD4-skewed response is required in adult mice to control *Bp,* P7 thyx mice demonstrated a strong $CD8^+$ T cell response, while still controlling *Bp* similar to sham controls. Though $CD8^+$ T cells are typically associated with control of viral infections, neonatal $CD8^+$ T cells have a greater ability to develop into effector cells[31–34]. It has also been demonstrated that fetal-derived $CD8^+$ T cells adopt different roles than adult-derived $CD8^+$ T cells during infection, specifically into short-lived effector cells and long-lived memory cells, respectively[35]. Additionally, neonatal $CD4^+$ T cells have differential methylation patterns, which results in repressed host defense pathways[36]. Overall, these complementary models demonstrate three distinct responses which model the T cells available at each developmental stage and agree with previous neonatal models without the confounding factors of additional developing immune components.

Thymectomy is a common model utilized to assess neonatal-like immunity, though few studies have specifically utilized this model to assess the roles of T cells at various stages of development in response to infection. Various groups have assessed the populations and characteristics of T cells which are prevalent in the periphery after thymectomy, including NKT cells, Tregs, and T cell expansion kinetics[29,37–40]. Genetic models have also been utilized, with the most common being the nude mouse model, which completely lack the FoxN1 transcription factor ($FoxN1^{null}$), making them athymic and have abnormal fur and whisker development[41]. These mice differ from the $FoxN1^{\Delta/\Delta}$ mice utilized here in that $FoxN1^{\Delta/\Delta}$ mice have normal growth of fur and whickers, and thymic growth up to the double negative 3 (DN3) stage, characterized by T cells which lack expression of CD4, CD8, and CD25, but express CD44[10–12,41,42]. Utilizing the $FoxN1^{\Delta/\Delta}$ mice allows us to observe T cells generated in a thymus which ceases development at the DN3 stage, and thus assess the role of earliest versions of T cells in infection[10,11]. T cells generated in the early thymus are known to have a different TCR repertoire than those generated later[10,43–45]. They also have differential AE integrin and CCL25 responses, which can have an effect on responses to infection[33]. Importantly, work from previous thymectomy models demonstrate that $CD8^+$ T cells are more rapidly induced in the neonatal thymus, which could explain why they expand rapidly in P7 thyx mice, likely contributing to control[32–34]. Previous assessment of $FoxN1^{\Delta/\Delta}$ mice suggests that T cells can be hyperresponsive or hyporesponsive to antigen[12]. Our results here suggest that T cells in these mice may be hyporesponsive to *Bp* antigen, evidenced by a severe lack of T cell proliferation and IFN-γ production. The use of three complementary models which express three distinct stages of thymic maturation has allowed us to assess the role of T cells generated at various stages of development in response to *Bp*. We also describe a novel observation of increased $CD8^+$ T cells in the lungs of P7 thyx mice which is associated with control of *Bp.* This work suggests that T cells generated at distinct stages have novel roles or functions which have yet to be described and further work could aid in the discovery of novel therapeutics for infants.

Infants are significantly more susceptible to particular pathogens and our ability to assess the interactions between the NIS and these pathogens is severely limited[46–53]. Our results suggest that there are distinct responses that occur based on development stage, with the response generated by P7 thyx mice being distinct from, yet similarly effective to, that of control adult mice with a fully developed thymus. Specifically, these mice generate a dramatic $CD8^+$ T cell response after inoculation with *Bp.* This unique response may explain why treatments and vaccines which aim to generate a more adult-like response in infants fail to provide sufficient responses. This also suggests that therapeutics which aim to expand the $CD8^+$ response in neonates against bacterial infections may enhance their ability to control infection, a hypothesis which could be tested with the P0 thyx and FoxN1$^{\Delta/\Delta}$ models.

Overall, the utilization of several, complementary models of neonatal-like immunity offer a unique opportunity to assess and test the neonatal immune responses which may be very effective. While these models are still susceptible to limitations, they can allow for new observations to be made and for applicable comparisons of developmental stages which have not been succinctly made prior. This work, in combination with our short term pup model, has contributed new evidence which supports the theory that neonates have a distinct immune response to the neonatal pathogen *Bp.* This suggests that the neonatal immune responses should be considered separate from the adult, and thus vaccines and treatments should be specifically developed for the unique NIS.

FIGURES

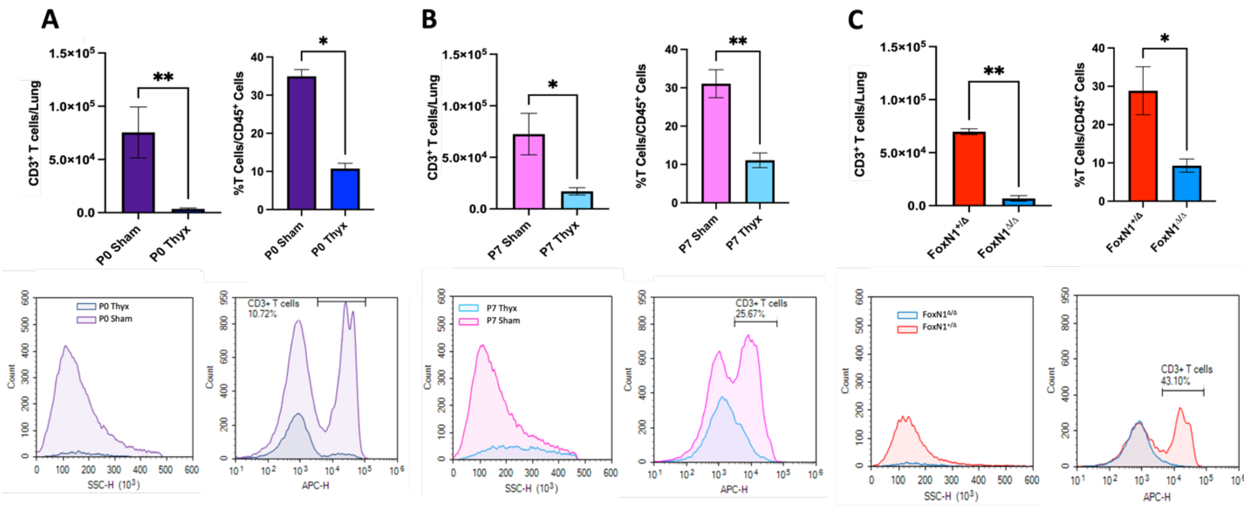

Figure 4.1. **Mice with disrupted thymic development have decreased T cell lung populations**. T cells isolated from the lungs of naïve P0 Sham/Thyx mice (**A**), P7 Sham/Thyx mice (**B**), FoxN1$^{+/\Delta}$/FoxN1$^{\Delta/\Delta}$ mice (**C**). Top left panel represents total CD45^{+}CD3^{+} T cells per lung. Top right panel represents percent T cells of CD45^{+} cells in the lung. Bottom left panel displays representative histogram of total CD3^{+} cells per lung. Bottom right panel displays representative comparative histogram of CD3^{+} expression among CD45^{+} cells per lung. Statistical significance was calculated via unpaired Student's T test. *p<0.0332, **p<0.0021. Experiments were completed in triplicate. Error bars show standard error of the mean (n=5 mice).

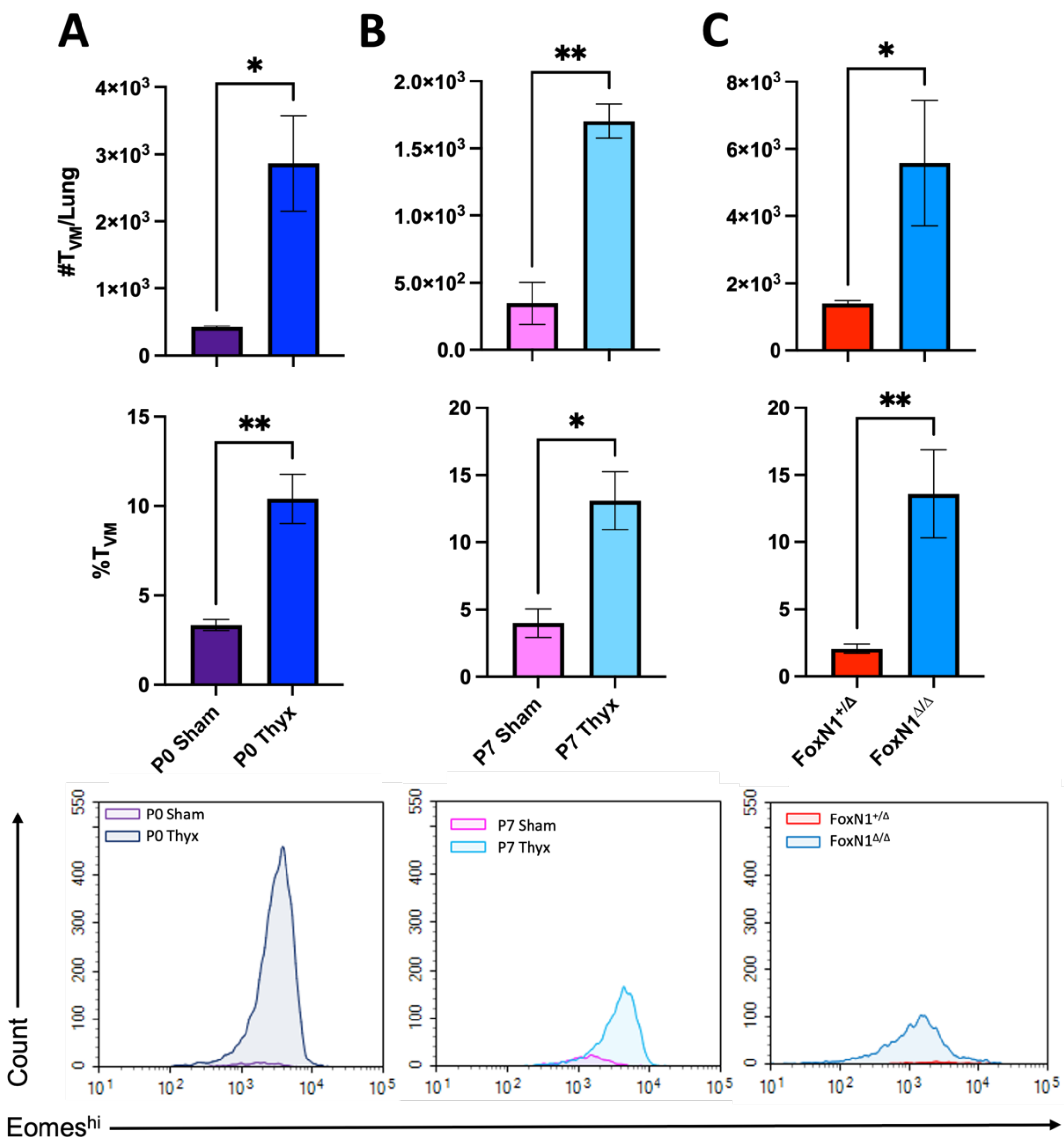

Figure 4.2. Mice with disrupted thymic development have increased populations of virtual memory T cells. T_{VM} isolated from the lungs of naïve P0 Sham/Thyx mice (**A**), P7 sham/thyx mice (**B**), FoxN1$^{+/\Delta}$/FoxN1$^{\Delta/\Delta}$ mice (**C**). Top panel represents total number of T_{VM} per lung. Middle panel represents percent T_{VM} of total $CD3^{+}$ cells from lungs. Bottom panel displays representative comparative histogram of T_{VM} at terminal gate ($CD3^{+}CD49d^{lo}CD44^{hi}CXCR3^{+}Eomes^{+}$). Statistical

significance was calculated via unpaired Student's T test. *p<0.0332, **p<0.0021. Experiments were completed in triplicate. Error bars show standard error of the mean (n=5 mice).

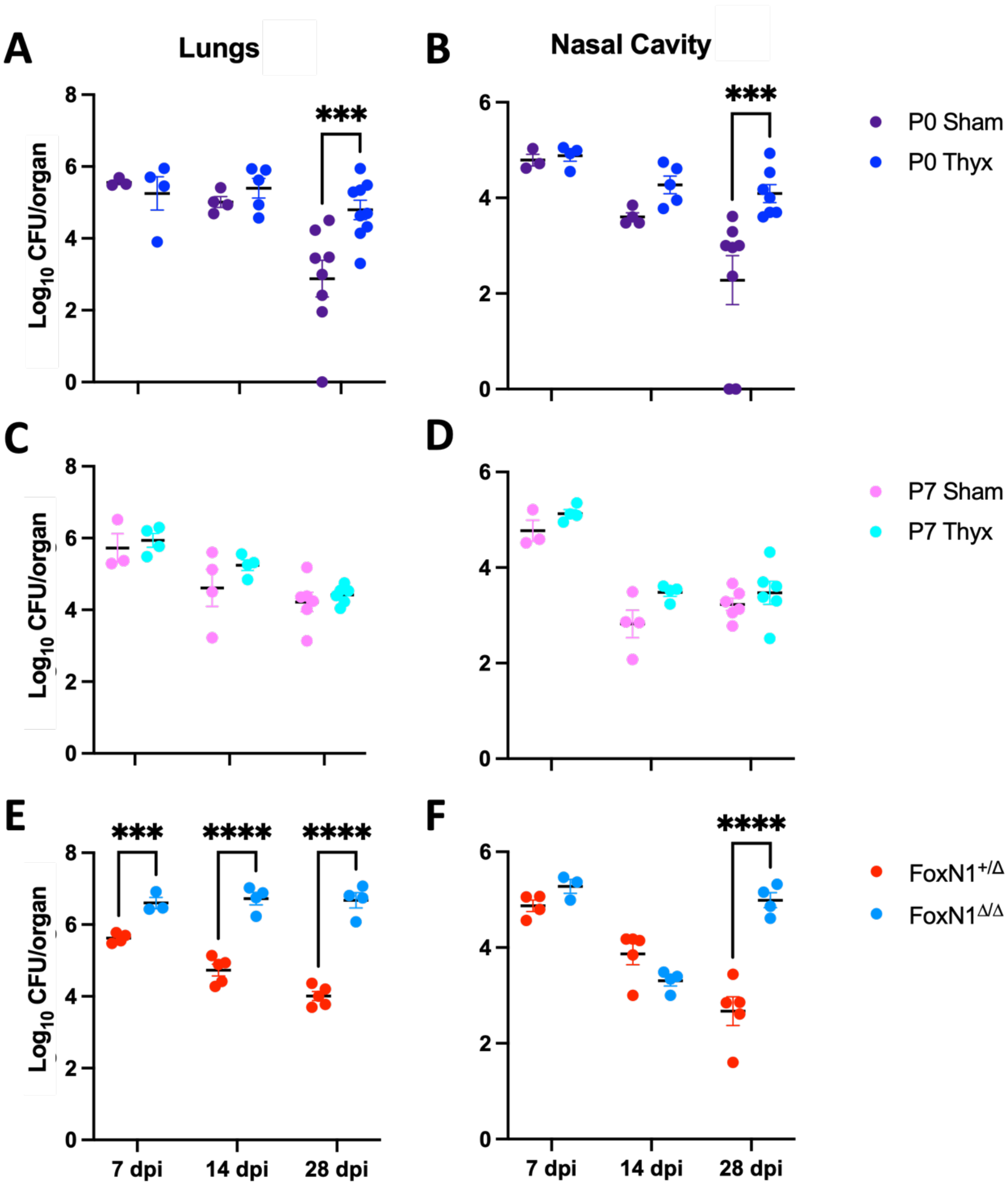

Figure 4.3. Mice with early disrupted thymic development are susceptible to *Bp*. CFU of *Bp* recovered from lungs (**A, C**, and **E**) and nasal cavities (**B, D,** and **F**) from P0 sham/thyx mice (**A-B**), P7 sham/thyx mice (**C-D**), FoxN1$^{+/\Delta}$/FoxN1$^{\Delta/\Delta}$ mice (**E-F**). Experiments were completed in

triplicate. Statistical significance was calculated via Two-way ANOVA. ***p<0.0002, ****p<0.0001. Error bars show standard error of the mean (n=5 mice per treatment/timepoint).

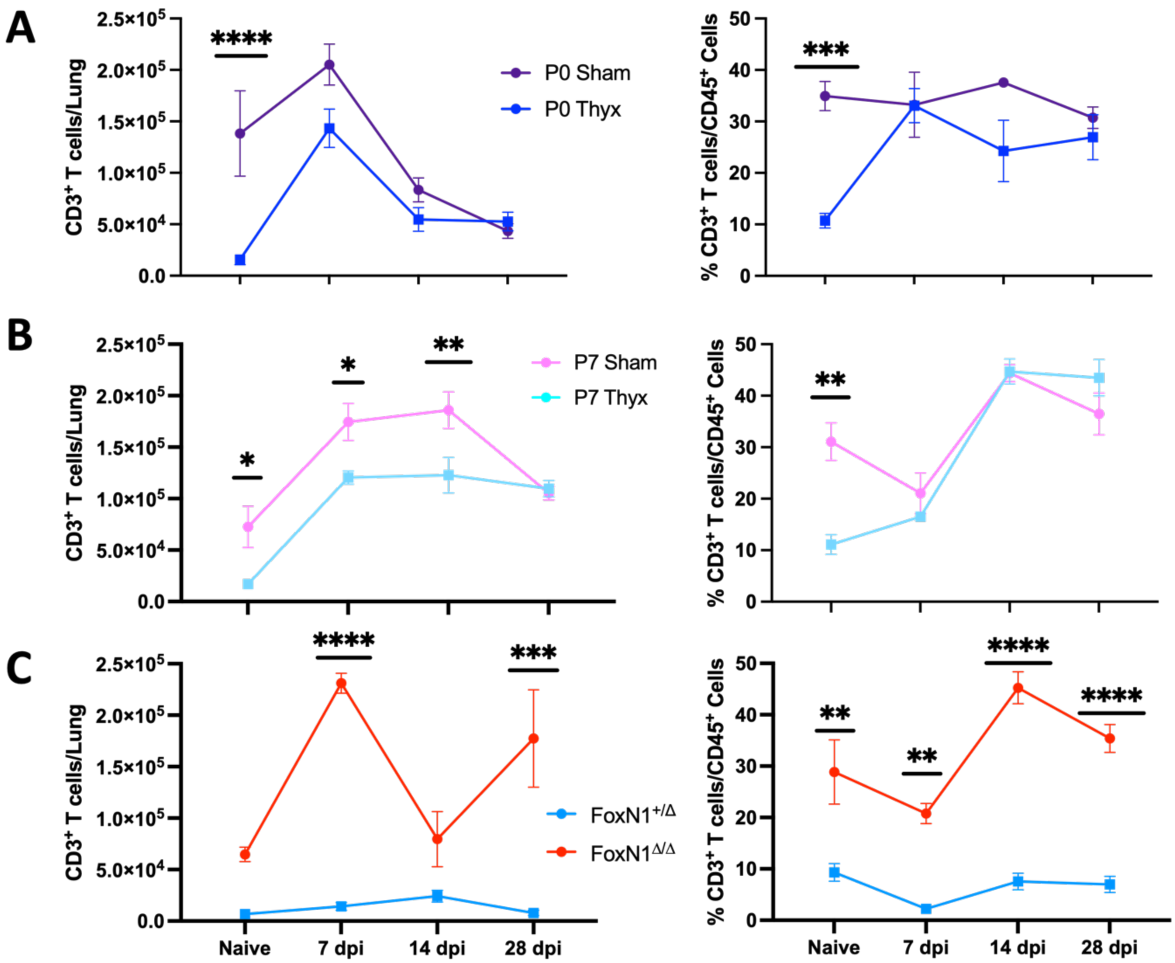

Figure 4.4. Mice with disrupted thymic development have unique T cell kinetics. T cells isolated from the lungs of P0 sham/thyx mice (**A**), P7 sham/thyx mice (**B**), FoxN1$^{+/\Delta}$/FoxN1$^{\Delta/\Delta}$ mice (**C**) at various timepoints. Left panel represents total T cells in lungs of mice. Right panel represents percent T cells of CD45^{+} cells in lungs. Experiments were completed in triplicate. Statistical significance was calculated via Two-way ANOVA. *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001. Error bars show standard error of the mean (n=5 mice per treatment/timepoint).

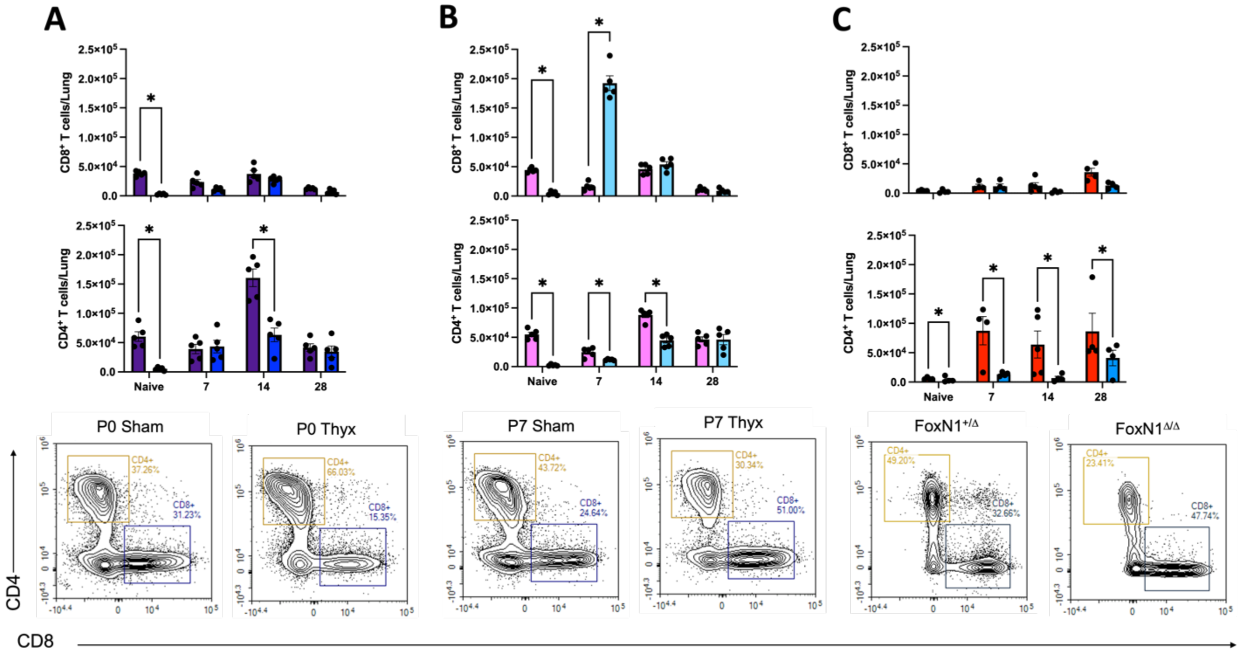

Figure 4.5. Mice with disrupted thymic development have varied CD4⁺ and CD8⁺ responses. CD8 and CD4 T cells in lungs of P0 Sham/Thyx mice (**A**), P7 Sham/Thyx mice (**B**), FoxN1$^{+/\Delta}$/FoxN1$^{\Delta/\Delta}$ mice (**C**). Top panel represents total CD8$^+$ T cells in lungs. Middle panel presents total CD4$^+$ T cells in lungs. Bottom panel is representative contour plot of CD4 and CD8 expression of CD3$^+$ T cells in the lungs at 7 dpi. Statistical significance was calculated via Two-way ANOVA. *p<0.0332. Error bars show standard error of the mean (n=5 mice per treatment/timepoint).

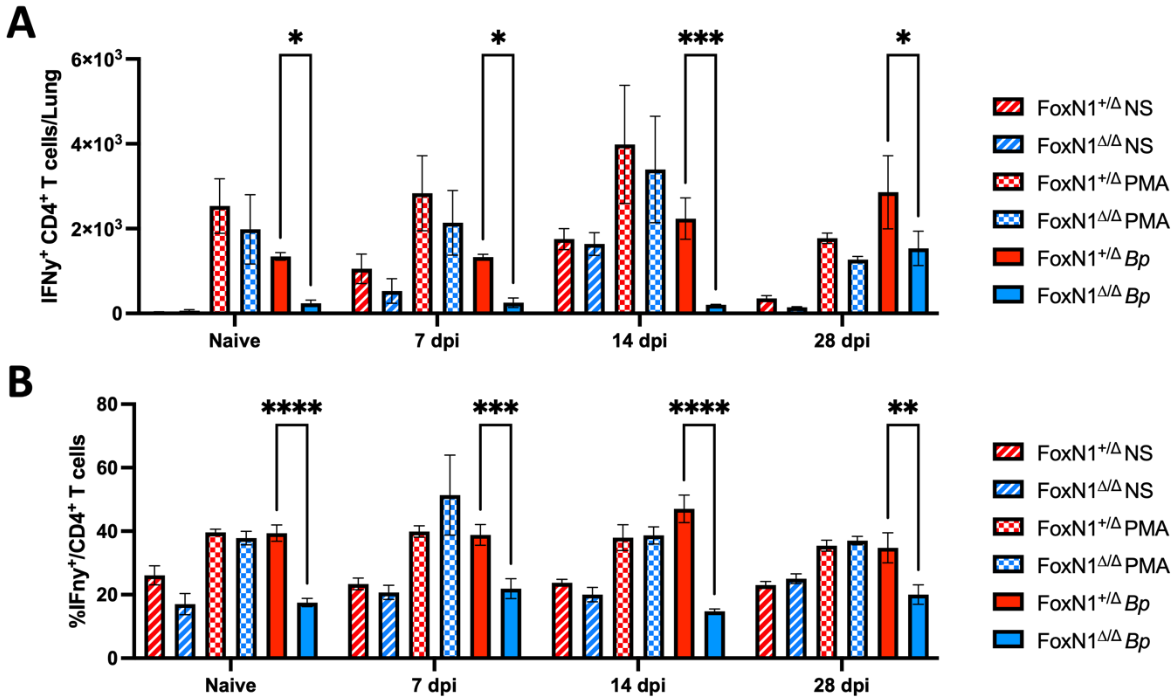

Figure 4.6. CD4$^+$ T cells from FoxN1$^{\Delta/\Delta}$ mice fail to produce IFN-γ after stimulation with *Bp*. IFN-γ producing CD4+ T cells from lungs of FoxN1+/Δ and FoxN1Δ/Δ mice inoculated with *Bp*. Total numbers (**A**) and proportions (**B**). NS= Non stimulation, PMA=PMA/ionomycin stimulation, *Bp*= *Bp* stimulation. Statistical significance between treatment groups was calculated via One-way ANOVA. *p<0.0332. **p<0.0021, ***p<0.0002, ****p<0.0001. Error bars show standard error of the mean (n=4-5 mice per treatment/timepoint).

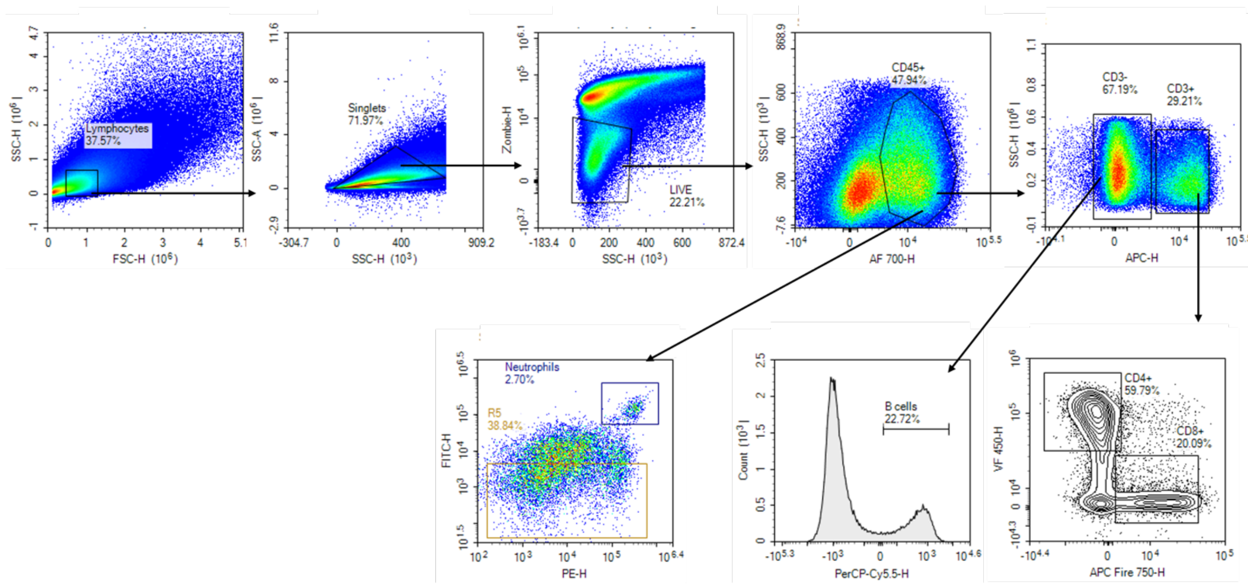

Figure S4.1. Gating strategy for T cells, B cells, and neutrophils from lung of naïve FoxN1$^{+/\Delta}$ mouse.

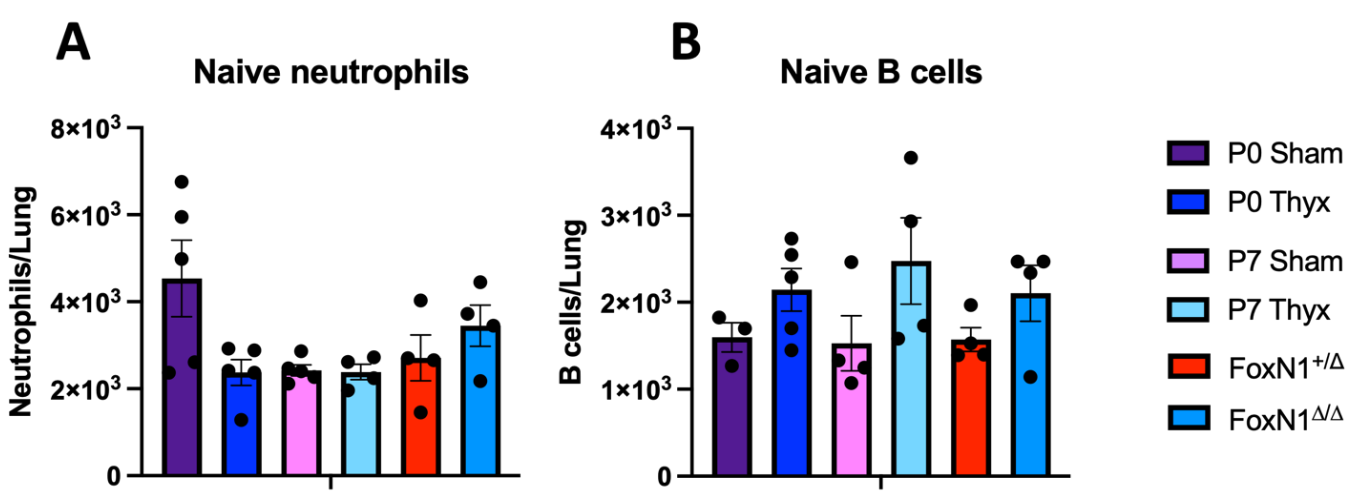

Figure S4.2. Neutrophils ($CD45^+Ly6G^+CD11b^+$) and B cells ($CD45^+CD3^-CD19^+$) from lungs of naïve mice. Error bars show standard error of the mean (n=4-5 mice).

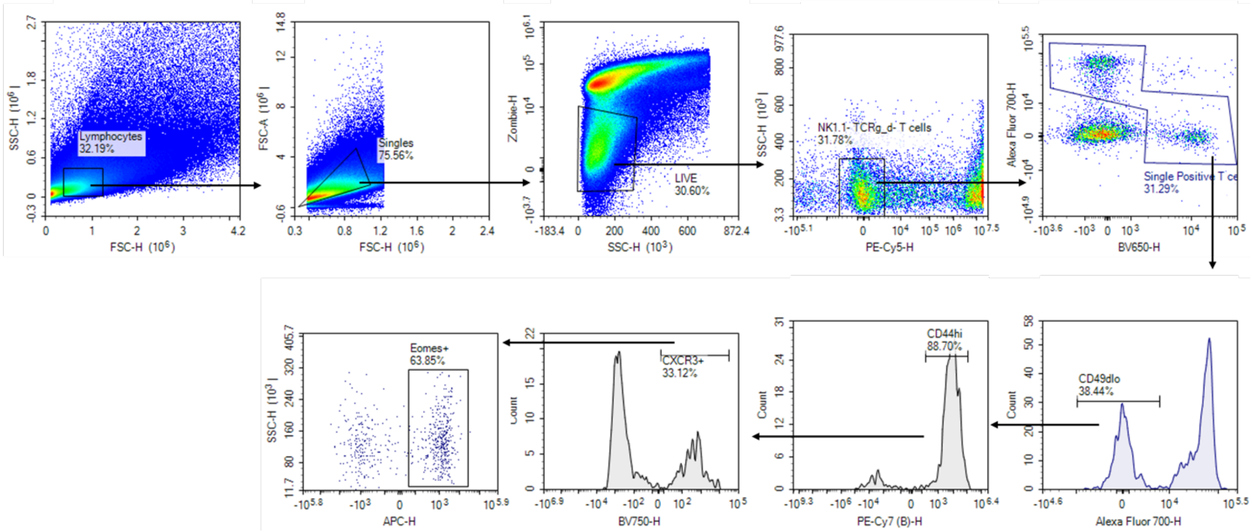

Figure S4.3. Gating strategy for virtual memory T cells (T_{VM}) from naïve FoxN1$^{+/\Delta}$ mouse.

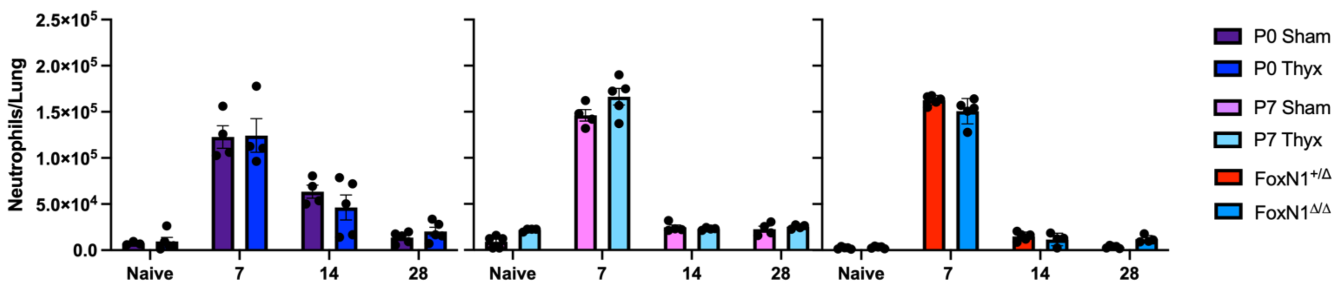

Figure S4.4. Neutrophils from lungs of naïve and *Bp*-inoculated mice. Neutrophils identified as CD45$^+$Ly6G$^+$CD11b$^+$. n=4-5 mice per timepoint. Error bars represent standard error of the mean.

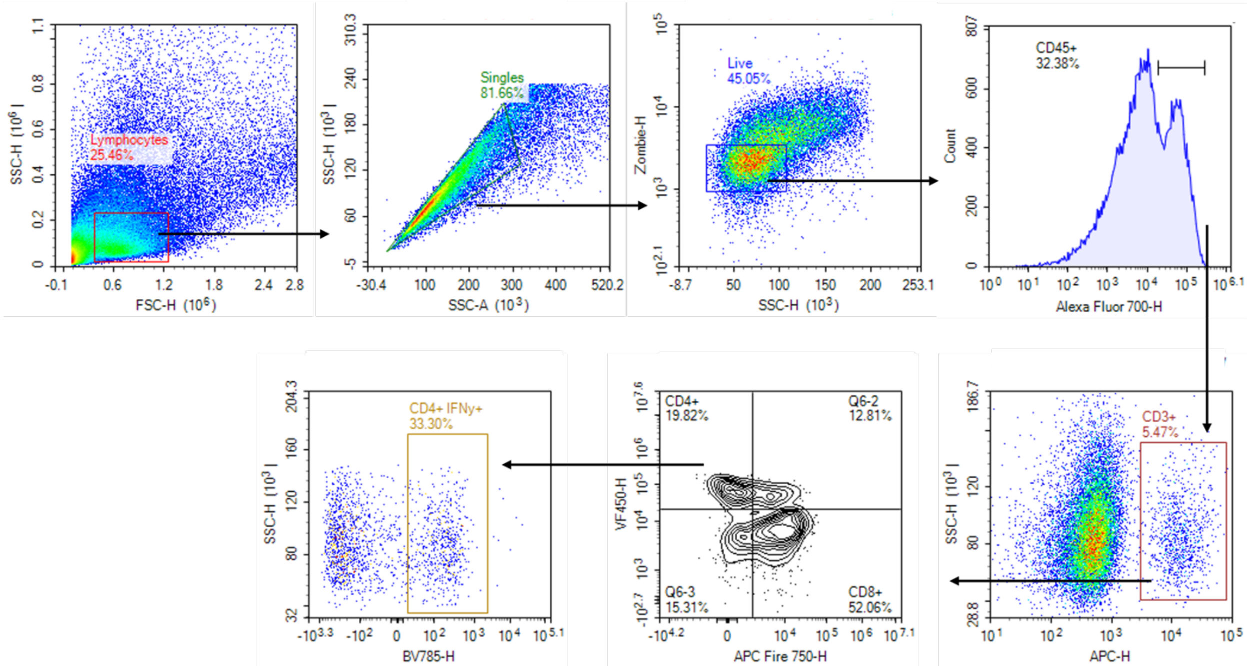

Figure S4.5. Gating strategy for IFN-γ producing CD4^{+} T cells. Representative images from Naïve FoxN1$^{+/\Delta}$ mice stimulated with PMA/ionomycin.

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CHAPTER 5

CONCLUSIONS

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OVERVIEW

In the work described here, I demonstrate the importance of specific assessment of the neonatal immune system and establish several neonatal mouse models that are likely to be highly useful in future studies. Using these models, I have demonstrated that neonatal immune responses to *Bp* can be highly efficient but are severely disrupted by PTx. This disruption is primarily mediated by interrupting neutrophil recruitment via CXCL1 and C3a pathways. Complementary surgical and genetic models of neonatal-like immunity via incomplete or disrupted thymic development were also utilized to assess neonatal-like responses to *Bp.* Importantly, T cells developed in the first week of life were sufficient to control *Bp,* since mice thymectomized at P7 were similar to control mice, a response which was associated with increased $CD8^+$ T cell responses. Together, this work has revealed several novel aspects of the NIS and its response to respiratory infections, which can guide the development of enhanced treatments of newborns.

ASSESSMENT OF THE NEONATAL IMMUNE SYSTEM VIA MURINE MODELS

The characterization of the NIS and responses to infection has long been a goal which is confounded by a number of factors. Specifically, "neonatal" murine infection models often span a large range which extends well into adulthood (Chapter 1). My work here clearly demonstrates that utilization of younger mice up to 8-days-post birth (P8) allows for the assessment of the NIS in the relative absence of adult immune cells or functions (Chapter 2). Based on this new information, additional experiments can be completed to gain a greater understanding of the NIS without confounding factors associated with the AIS. This could have important implications on our knowledge of the neonatal immune responses and current therapeutics.

This novel pup inoculation model was utilized in combination with monoclonal depleting antibodies and congenic mutant mice to demonstrate that neonatal neutrophils and complement system are required for control of *Bp*Δ*ptx* (Chapter 3). In order to gain additional information about the NIS, the contribution of additional immune cells to the neonatal response should also be assessed with this model. These include the Mac II and III macrophage subsets, which have roles in immune regulation and suppression of inflammatory responses. The role of all macrophages could be further probed by first inoculating CSF1-deficient pups with WT *Bp* or *Bp*Δ*ptx* and comparing the bacterial recovery to C57Bl/6 controls. If the CSF1$^{-/-}$ pups have increase bacterial recovery compared to C57Bl/6 pups, then macrophages are likely to be required for control of *Bp.* In order to assess the specific contribution of Mac III macrophages to infection, alveolar macrophages could be depleted in C57Bl/6 pups via clodronate liposomes followed by inoculation with WT *Bp* or *Bp*Δ*ptx.* If these mice show similar bacterial recovery as C57Bl/6 controls, then alveolar macrophages are likely not required for control of *Bp*. Stimulating the cells from C57Bl/6 pups with *Bp* or PMA/ionomycin to determine the cytokines produced by macrophages upon stimulation would also be important. The contribution of DCs to neonatal responses should also be assessed due to their roles in regulation of both innate and adaptive immune responses. To this end, subsets of DCs in the lungs of C57Bl/6 pups inoculated with WT *Bp* or *Bp*Δ*ptx* should be examined. Particular focus would be on cDC1 and mreg DCs as these are specifically replete in P1-P7 pups. DCs from pup lungs could also be isolated and antigen presentation assays performed to determine the ability of the various subsets to present antigen. This work would be particularly important for understanding how neonates generate responses to vaccination.

While the P5-P8 pup model is well suited for assessing innate immune functions, the genetic and surgical models described in Chapter 5 more directly manipulate T cells and are better suited to specifically assess the important roles of neonatal T cells in various responses to infections. With these models, I was able to observe the neonatal immune response to a longer *Bp* infection that was not feasible with the pup model (3 dpi). Thus, additional can completed with these models to examine long term infections or therapeutics in the NIS. Specifically, these mice can be utilized to assess how the NIS responds to acellular pertussis vaccination without the confounding factors involved with the natural pup model, including their rapid maturation which limits the ability to assess vaccination responses. Based on their responses during *Bp* infection, it would be hypothesized that both FoxN1$^{\Delta/\Delta}$ and P0 thyx mice would generate a suboptimal response to the vaccine, which is characterized by expansion of the CD4^{+} T cell compartment and IFN-γ production. Thus, it would be anticipated that vaccination of these mice would not result in protection against *Bp* infection and that P7 thyx mice would generate a protective response, however such a response generated would vary from controls. Specifically, both CD4^{+} and CD8^{+} T cells would be expanded, which may or may not be able to make IFN-γ. This would likely be a distinct response from controls, likely including a TH2-skewed response, but that it would still be protective, resulting in significantly reduced CFU in the lungs. This would greatly inform how we define efficacy of neonatal vaccines and neonatal responses.

While complementary neonatal-like models represent an opportunity to assess and validate independently the contribution of neonatal T cells to responses to infection, they are limited in that they do not express additional neonatal-like features of the innate immune system, such as neutrophils. One possible approach to improve these neonatal-like models would be to perform a bone marrow transplant with irradiation shielding of the abdominal cavity. With this method, FoxN1$^{\Delta/\Delta}$ or P0/P7 thyx mice could retain the immune cells in the lungs but the hemopoietic cells in bone marrow would be eliminated and then replaced by HSCs from neonatal mice. Thus, innate cells such as neutrophils and macrophages would replenish the populations in the lungs which naturally apoptose. In this way, we would be able to examine the interactions between neonatal T cells and innate cells in longer infections and in response to vaccination.

Beyond the understanding of the primary neonatal immune responses to pathogens, these neonatal models can also be used to assess the effects of maternal vaccination on neonatal responses. Due to the more recent shifts in recommendations for pregnant mothers to get vaccinated during pregnancy to establish immunity in the infants prior to birth, animal models will be required to understand the consequences of such vaccination schemes. These neonatal mouse models can be utilized in combination with maternal vaccination to assess such effects on both protection and development of the resulting offspring.

In addition to the development of novel murine models, additional innovative ways to analyze neonatal immune mechanisms need to be developed. In most research comparing neonatal and adult responses, the tests and timepoints used are primarily based on previous work utilizing adults. In my work described here, the timepoints utilized to examine neonatal immune responses to *Bp* are significantly different from those which would be utilized for adults; thus, the neonatal immune response was clearly assessed. In this way, current and new methods must be optimized for the appropriate timepoints and assays which will effectively demonstrate the capabilities of the NIS. In this way, we can truly understand how the NIS functions and where neonatal immune responses are truly effective and where they are not.

CONTRIBUTION OF NEUTROPHIL RECRUITMENT AND ACTIVITIES TO NEONATAL RESPONSES TO *BORDETELLA PERTUSSIS*

The efficient functions and contributions of neutrophils to disease responses has been well described in adult mouse models. However previous work has suggested that neonatal neutrophils lack the same abilities as adult neutrophils. Here, I demonstrate that neonatal neutrophils can very efficiently be recruited to the neonatal lungs after inoculation with *Bp*Δ*ptx.* Neutrophils were shown to be required for control of *Bp*Δ*ptx,* as pups depleted of neutrophils via anti-Ly6G antibodies failed to control *Bp*Δ*ptx* (Chapter 3). This deviation from previous reports could be due to a number of factors, including the age of pups at the time of inoculation or analysis. Specifically, we see a modest increase in neutrophils at 2 hrs post inoculation, a timepoint which is not often assessed in adult models. This work not only shows the importance of neonatal neutrophils to combating bacterial infections, but also underscores the speed at which neonatal neutrophil function, and thus the timepoints at which neonatal immune responses should be assessed.

In addition to the ability of neonatal neutrophils to get to the site of infection, the effector functions of neutrophils at the site of infection must also be considered. My work assessed the contribution of NETosis to neonatal control of *Bp* via *Pad4*$^{-/-}$ pups and demonstrated that NETosis is not a required function for control of *Bp*Δ*ptx*. In an in vitro assessment of phagocytosis of *Bp* by neonatal neutrophils via a pHrodo assay PTx did not modulate the ability of neonatal neutrophils to phagocytose bacteria. Neonatal neutrophils appear to be employing another mechanism which significantly reduces bacteria in the lungs without causing damaging inflammation. It is possible that neonatal neutrophils could be killing the bacteria via degranulation, but the molecules that are released do not cause significant inflammation. This could be evaluated by measuring levels of granules in lung supernatants of pups or blocking neutrophil degranulation. This will be important to assess since at this early timepoint of 2 hrs, there are similar bacterial numbers in pups inoculated with WT *Bp* and *Bp*Δ*ptx,* a factor which could perhaps explain why the significant populations of neutrophils in the lungs at 3 dpi have little effect on CFU of WT *Bp.*

In addition to the requirement for neutrophils for control of *Bp*Δ*ptx,* CXCL1 was identified as an important recruitment mechanism for neutrophils via treatment of pups with anti-CXCL1 antibodies, which lead to significantly reduced neutrophils and increased *Bp*Δ*ptx* in the lungs. Importantly, this is also a primary recruitment axis for neutrophils in adults, suggesting that neonatal and adult neutrophils are recruited via similar mechanisms, though they appear to occur at different rates. In order to further characterize the mechanisms by which neonatal neutrophils are recruited and how this is disrupted by PTx, additional experiments should determine if PTx has a role in disrupting production of CXCL1 by neonatal epithelial cells, or if PTx functions by disrupting neonatal neutrophils' ability to respond to CXCL1. To test this, recombinant CXCL1 would be delivered to C57Bl/6 pups inoculated with WT *Bp*. If these pups have increased neutrophil recruitment and decreased bacterial burden, then PTx likely does not affect the ability of neonatal neutrophils to respond to CXCL1 via CXCR2. However, if there is no change based on CXCL1 treatment, then PTx likely disrupts neutrophil recruitment via CXCR2 activity. This information would be important for identifying pathways that can be targeted by therapeutics to improve neonatal neutrophil migration and activities.

Similar to observations of neonatal neutrophils, the complement system in neonates has largely been described as irrelevant to pathogen defense, due to low levels of complement proteins in human neonatal cord blood. As such, there has been very little work completed on the role of complement in neonatal immune responses to pathogens. However, the very rapid control of *Bp*Δ*ptx* suggested that innate factors which require few initiating signals are contributing. Indeed, $C3^{-/-}$ pups were unable to control *Bp*Δ*ptx* similarly to C57Bl/6 pups, which was associated with decreased neutrophils recruitment. Delivery of recombinant C3a to $C3^{-/-}$ pups recovered neutrophil recruitment to the lungs and resulted in significant reduction of *Bp*Δ*ptx.* This represents a possible therapeutic mechanism to recover the necessary neutrophil recruitment for control of *Bp.* To test this, the optimum dose of C3a for chemotaxis of neutrophils to adult lungs would be identified and C3a would be co-inoculated with WT *Bp* to determine if this results in similar reduction of CFU at 3 dpi. It could also be assessed if treatment with C3a after inoculation with WT *Bp* would be effective, even in the presence of increasing amounts of PTx. In addition to mediating improved neutrophil recruitment, delivery of C3a could also improve macrophage recruitment, as neonatal neutrophils traffic via binding of CR3 to C3bi, which would be in higher amounts after administration of C3a. The work completed here on neonatal neutrophils and complement is summarized in **Figure 5.1** and represents the proposed model of immune responses initiated upon inoculation with *Bp* and *Bp*Δ*ptx.* Continued assessment of the contribution of neonatal neutrophils to controlling bacterial infection will greatly increase our understanding of the NIS and likely lead to the development of novel therapeutics.

ROLE OF T CELLS IN NEONATAL RESPONSES TO *BORDETELLA PERTUSSIS*

T cells are one of the most important immune cells in infection due to their role in regulating immune responses. T cells in the periphery vary based on the needs of the host, with neonatal T cells having vastly different functions than adult T cells. However, it is difficult to assess the roles of T cells in infection in neonatal pup models due to the rapid maturation of the thymus. In fact, the work described in Chapter 2 did not identify a requirement for T cells in the neonatal response; control of *Bp*Δ*ptx* was similar in the lungs of T $cell^{-/-}$ and C57Bl/6 pups. However, as conventional antigen-specific T cells typically take time to expand in numbers and contribute more to adaptive responses that are prominent later in infection, the pup model is likely not the ideal model to assess the contribution of conventional T cells. Indeed, the complementary models of neonatal-like immunity which have disrupted thymic development demonstrate T cells are required to control/clear infection (Chapter 4). Specifically, the thymectomy experiments suggest that T cells generated on day of birth or prior are not sufficient to control *Bp* as efficiently as control mice, but T cells generated within the first week of life are sufficient to control *Bp* similarly to control mice. This ability to control *Bp* was associated with a novel observation of expanded $CD8^+$ T cells, an unusual observation due to the well-known role of $CD4^+$ T cells in control of bacterial infections. This suggests that the activities and roles of $CD4^+$ and $CD8^+$ T cells may vary based on the age of mouse at the time of their development.

To determine if $CD8^+$ T cells are required to control *Bp* in P7 thyx mice, anti-CD8 depleting antibodies can be administered to adult P7 thyx mice at 6-8 weeks old, with control P7 thyx mice receiving an isotype control antibody. These mice would then be subsequently inoculated with *Bp,* and bacterial recovery assessed at various timepoints. If anti-CD8 treated mice had similar control of *Bp* as the isotype-treated P7 thyx mice, then it could be concluded that the increase in $CD8^+$ T cells is likely due to some other factor associated with thymectomy at P7 and that $CD8^+$ T cells are not required for control of *Bp*. However, if anti-CD8 treated P7 thyx mice have significantly more CFU in the lungs than isotype-control mice, then it could be concluded that $CD8^+$ T cells are required for control of *Bp* in P7 thyx mice, specifically $CD8^+$ T cells made in the first 7 days of life which then persist in the periphery after thymectomy. This information would be extremely valuable, as there are currently few documented reports of $CD8^+$ T cells being required for control of *Bp* in neonates.

In addition to determining if the $CD8^+$ T cell population observed in P7 thyx mice are required for control of *Bp,* the specific subset of these T cells should also be assessed. This would be particularly important as there are a number of $CD8^+$ T cell subsets which are uniquely expressed in the neonatal period (eg. T_{VM}, $\alpha\beta^+/\gamma\delta^+$). A tamoxifen time stamping experiment should also be performed to determine if the T cells expanding in response to infection emigrated from the thymus at a particular stage of thymic development. Importantly, several neonatal T cell subsets are characterized by their production of IFN-γ, thus the ability of $CD8^+$ T cells from P7 thyx mice to produce IFN-γ could also be examined.

An important mechanism in protection against *Bp* in adults is IFN-γ production by $CD4^+$ T cells. To determine if the failure of $FoxN1^{\Delta/\Delta}$ mice to control *Bp* was primarily due to failure to expand $CD3^+$ T cell populations in the lungs, the specific ability of $CD4^+$ T cells from these mice to produce IFN-γ was assessed. Importantly, $CD4^+$ T cells from $FoxN1^{\Delta/\Delta}$ mice had similar levels of IFN-γ production after PMA/ionomycin stimulation but failed to produce IFN-γ after stimulation with *Bp* compared to $FoxN1^{+/\Delta}$ mice. This suggests that T cells from $FoxN1^{\Delta/\Delta}$ mice have the ability to produce IFN-γ, but this is blocked by *Bp*. Identifying the mechanism by which this disruption occurs will be extremely important, as it could inform the specific susceptibility of neonates to *Bp.* To determine if disruption occurs via some bacterial-derived factors, cells from $FoxN1^{\Delta/\Delta}$ mice would be stimulated with mutants of *Bp* which lack various virulence factors to determine if increased IFN-γ production is detected. However, as is discussed in Chapter 1, neonatal cells have a feedback loop which generally represses IFN-γ production. Thus, it is possible that using PMA/ionomycin for positive controls is artificial as it overcomes this repression and a different mechanism for positive control should be used. Specifically, a positive control which preferentially activates neonatal cells could be identified via assessing molecules which particularly stimulate TH2 responses. Additionally, it would be useful to understand how different adjuvants could improve production of IFN-γ or other cytokines. These experiments should also be completed with the P0 and P7 thyx mice to determine if this inability to make IFN-γ after stimulation with *Bp* is consistent in T cells made at birth and after, or if this is unique to T cells generated prior to birth.

SUMMARY AND IMPLICATIONS

Overall, the work completed here demonstrates that neonatal immune responses are not simply an ineffective version of adults, but distinct and effective. These models can be utilized in a number of ways to assess responses and mechanisms by which neonates differ from adults. This work also shows that neonatal mice can effectively control *Bp*, but this ability is disrupted by PTx. Understanding the mechanisms which mediate both the control of *Bp*Δ*ptx* and the disruption by PTx will inform the development and optimization of treatments and vaccines for infants against *Bp* and how other pathogens may disrupt the NIS in similar mechanisms to PTx.

FIGURES

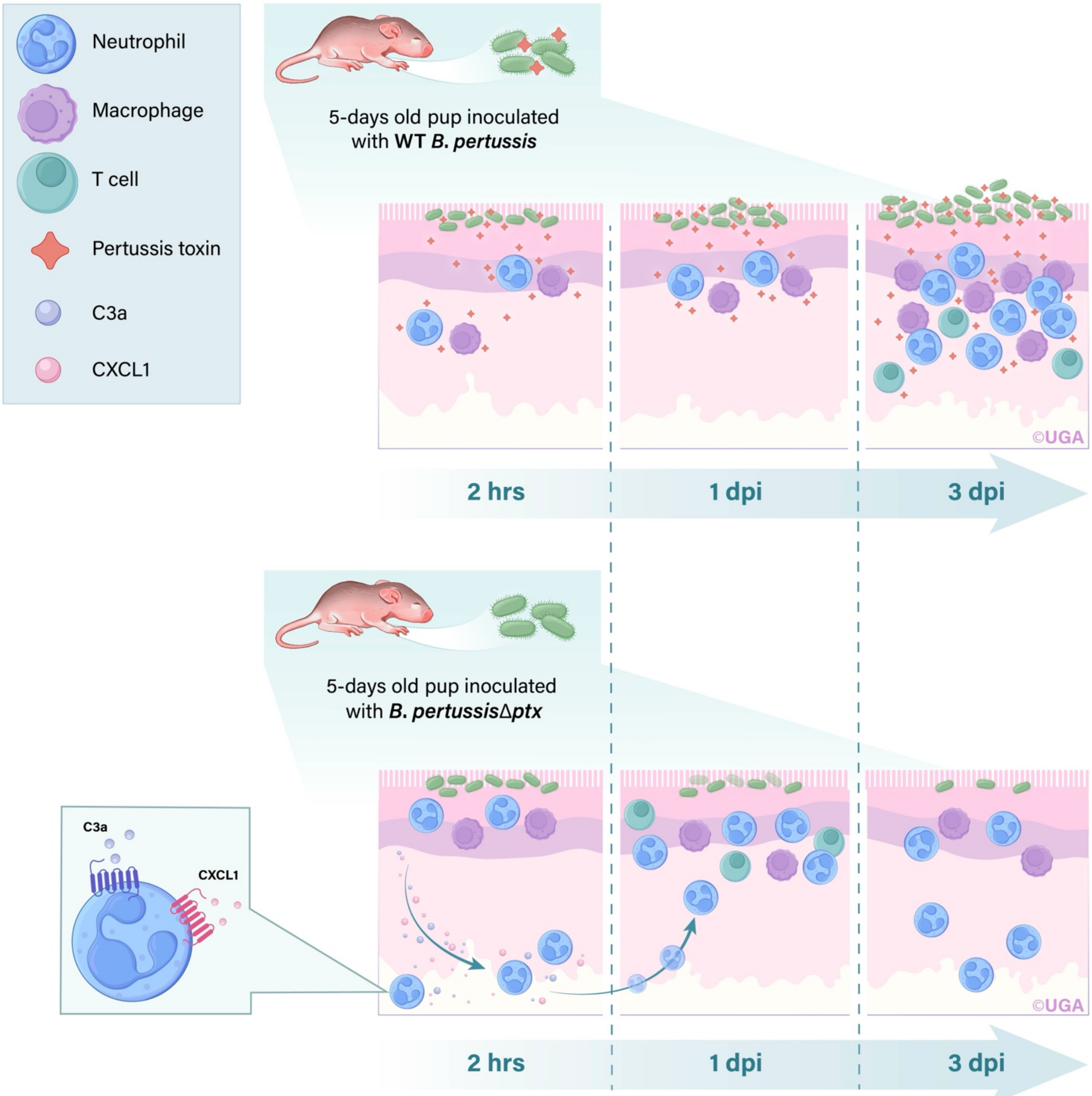

Figure 5.1. Summary figure of mechanisms of disruption of neonatal immune responses by PTx during infection with WT *B. pertussis*. Top section displays response upon inoculation of neonatal lungs with WT *Bp* and bottom section displays response upon inoculation of neonatal lungs with *B. pertussis*Δ*ptx.* Arrows represent recruitment of cells. Lightly shaded bacterial cells represent ghost cells (dying).