# The Neonatal Immune System

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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 response is not generated. 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, which is better studied and understood than is the neonatal one, efficiently generates pro-inflammatory responses which mediate the efficient control of most pathogens. The neonatal immune system, however, is 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 response, a seemingly impossible feat. Therefore, during this transition from near-sterile-fetal to microbe-rich neonatal environment, the immune system is evolved to respond to novel antigens primarily with anti-inflammatory TH2 responses to prevent unnecessary inflammation which can severely harm the infant.

neonatal immune system | neutrophils | lymphocytes | immune cells | pathogens

## 1. 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 [1][2]. 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 [3][4]. 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 [5]. This includes populations of unique cells and subsets of immune cells that specifically make up the neonatal immune system. 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 *Bordetella pertussis*, 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.

# 2. Innate Immune System

## 2.1. 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 [6]. Neutrophils are derived from the granulocyte-macrophage progenitor (GMP) and proliferative neutrophil precursor (preNeu), which expand under microbial stress before migrating to the periphery [6]. Their short half-life requires that they be consistently replenished in the periphery [6]. 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 [7]. 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 [8]. Multiple studies suggests 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 h 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 [9]. 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 neonatal immune system 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 [10]. Neonatal mice (P5) inoculated with a mutant of *Bordetella pertussis* lacking the pertussis toxin demonstrated neutrophil accumulation to the lungs as early as 2 h after inoculation, also demonstrating that neonatal neutrophils can respond very rapidly to the lungs. A subsequent increase in T cells in the lungs suggests that neonatal neutrophils can also efficiently recruit T cells. These responses, however, were not observed in P5 pups inoculated with wildtype *B. pertussis*, suggesting that pertussis toxin disrupts neutrophil accumulation in the lungs [11].

The immunosuppressive effects of pathogens on neonatal neutrophils, however, can be curtailed via pre-treatment with drugs that stimulate the neonatal immune system. Pretreating neonatal mice (P5–P7) with alum 24 h before the induction of polymicrobial sepsis improved phagocytosis by peritoneal neutrophils resulting in decreased pathogen numbers by 24 h post-challenge (P6–P8). Alum pretreatment also increased numbers of NET-positive neutrophils and the expression of co-stimulatory molecules (CD80 and CD86) [12]. 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 for definitive results. 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 [13]. 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 [14][15][16]. 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 [17]. The production of ROS by neutrophils has also been implicated in acute lung injury of neonatal mice [18][19]. 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, an opportunity some pathogens may take advantage of.

## 2.2. 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 are also a member of the Group 1 innate lymphoid cells (see below). They function 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 and are the primary NK cell population by 8 weeks [20][21]. Mice lacking FcRn have low levels of immature NK cells, indicating that FcRn plays a role in their development. These NK cells also 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 [22]. However, there is also evidence that neonatal NK cells can function to control pulmonary infections. P2 pups inoculated with *Chlamydia muridarum* had a significant accumulation of NK cells in the lungs by 3 dpi (P5) accompanied by decreased bacteria in the lung [23]. Additionally, the ablation of erythroid suppressor cells in P6 pups inoculated with *B. pertussis* resulted in increased NK cell accumulation in the lungs and increased protection against *B. pertussis* by 4 dpi (P10) [24]. This evidence suggests that there is a protective role of neonatal NK cells in controlling infections in the neonatal lung.

In addition to producing granules to mediate microbe killing, NK cells are also 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) [25]. 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 [17]. Conversely, IFN-γ can also suppress production of antibodies [26][27].

## 2.3. Dendritic Cells

Dendritic cells are antigen-presenting cells which can induce T cell activation via phagocytosis of pathogens and the presentation of antigens. 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*). cDC1 was 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. Interestingly, the *mreg*-expressing DCs were not detected prior to birth but were present in high numbers at P7 before decreasing at P21. This suggests a unique migratory DC subset in neonatal murine lungs [27]. 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 [28]. 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 [29]. 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) [28][29]. $CD11b^+$ 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 [29]. 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 [28][29][30]. Neonatal lungs had more cDC1 subsets; however, these proportions shift during development, with adults having more cDC2s in the lungs [29][31][32]. 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 [30][31]. This was accompanied by increased production of IL-10, further repressing activation of $CD8^+$ T cell responses [30].

One of the major issues with the neonatal immune system 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 [33]. 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 [33]. The shift in the ability to produce IL-12 and generate a TH1 response occurs at P6 in mice when the DC populations increase and subsets shift away from the neonatal pattern and toward the adult-like one, causing a shift in the response to be primarily TH1 [34].

## 2.4. Macrophages

Macrophages are immune cells that phagocytose pathogens and are derived from the same progenitor as neutrophils are [35]. Single-cell sequencing identified unique clusters of macrophages that shift from fetal development to post-birth [27]. 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 [27]. 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 at P39 [36][37]. Another cluster of alveolar macrophages (Mac III) was also observed at P1 and P7. These clusters of macrophages (Mac I, II, and III) expressed 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 [27]. This work displays the dramatic shifts in macrophage populations and phenotypes that occur during neonatal lung development. Additionally, one of the most important subsets in the neonatal lung are alveolar macrophages due to their anti-inflammatory tendency to prevent damaging inflammation. Fetal monocytes develop into alveolar macrophages by P7 and persist for approximately 3 months [38]. Differentiation of monocytes into alveolar macrophages was also aided by lung basophils [39]. The persistence and maintenance of alveolar macrophages requires GM-CSF and neonatal neutrophil-derived 12-hete [38][40]. An additional subset of macrophages which exist in the lungs are M2 macrophages. These are in lungs at highest amount from P14–P21 and have roles in tissue remodeling and immunosuppression [41].

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 [42]. 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 [43]. The reduction of IL-10 allowed for the increased activation of neonatal macrophages [44]. 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 [12]. The response of neonatal alveolar macrophages in pups inoculated with RSV was also improved by the addition of IFN-γ [45]. Thereby, neonatal macrophages can play important roles in protecting neonates from pulmonary infections; however, their function can be greatly improved via TH1 skewing.

## 2.5. Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are immune cells with 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 ILC2s can be recruited to the lungs, Group 2 ILCs (ILC2s) are naturally resident in the lungs [46][47]. 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 [48]. 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 [49]. Lung ILC2s were found starting at P4 and peaked at P14 then decreased as the lungs matured [50]. ILC development is also dependent on the transcription factor RORα. In P12 lungs, three populations of ILCs were found. One was 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 subsets [51]. 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 [52].

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 [53]. 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 [36][52]. 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. This response also led to RSV immunopathogenesis and was inhibited by IL-33 depletion [36][54]. 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 Group 2 ILCs populations making IL-33 were expanded. This response was attenuated by IL-25-neutralizing antibodies, implicating IL-25 as an additional mediator of ILC activity [55].

## 2.6. 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 [56][57]. While they suppress α/β T cells, they also promote the development of Tregs in an IL-10-dependent manner [58]. 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 [57]. While MDSCs support and protect the neonatal immune system such that it reduces inflammation, they can also then cause reduced responses to pathogens.

# 3. Adaptive Immune System

## 3.1. 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 types 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 until P21 [27]. γ/δ T cells mediate responses to influenza and generate TH2 responses in the lung [59]. 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 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 [29][30]. Additionally, $CD62L^+$ T cells which can migrate to sites of infection are in low numbers in neonatal lungs [60].

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 that allow for rapid responses. Additionally, they can be activated independently of antigens, instead rapidly expanding after cytokine signaling [61][62]. These are observed in highest amount at P8 and are greatly reduced by P10, suggesting they are a significant component of the neonatal immune system [63]. Neonatal mice also have populations of regulatory T cells (Tregs) which are primarily anti-inflammatory and produce TH2 cytokines and responses. Neonates also have substantial populations of Tregs, which have been observed to regulate iBALT [64]. This is in contrast to Tregs in adult lungs, which have been observed to interfere with BALT development [65]. Importantly, P2 neonatal T cells were more likely to develop into Tregs than adult T cells were, but this ability diminished by P14 [66]. Additionally, infection of P2 neonatal mice with influenza required Tregs for clearance at 6–10 dpi (P8–P12) [67]. Tregs were also required for responses to LPS by P2–P5 mice by 3 dpi (P5–P8), though this shifted after the neonatal stage (P12–

P20) [68]. Additionally, beginning at P14, Tregs require PG-L1 for development, indicating a shift in immune system maturation [69].

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 [57]. Despite this, it was observed that P5 mice inoculated with a mutant of *B. pertussis* had an influx of neutrophils into the lungs, followed by T cells at 1 dpi (P6) [63]. However, it has also been observed that T cells are defective in migrating to neonatal alveolar spaces in lungs [10]. 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 [57][70].

## 3.2. 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 like they do in adults [13]. In addition to having lower numbers of B cells than those in adult mice, the composition of the B cell subsets in neonates differs greatly [71]. 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 neonatal immune system, including dysregulated neutrophil migration, T cell activation, and macrophage activation [9][12][30][33][72]. This can limit the neonatal immune response to pathogens. In fact, pups inoculated with RSV demonstrated IFN-I production by AM, but this process was then repressed by IL-10 from Bregs [72].

## 3.3. 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 [73][74]. Neonatal mice (P3) had significantly more expansion of CECs than adult mice had and this resulted in the increased suppression of T cell activation [72]. P6 neonatal mice were replete with $CD71^+$ CECs and highly susceptible to *B. pertussis*, resulting in increased mortality by 8 dpi (P14) [73]. The depletion of CECs in neonates resulted in decreased susceptibility to *B. pertussis* 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 [24][73][74][75]. P6 mice were also found to be susceptible to *L. monocytogenes* and *E. coli* infections in the lungs. 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 had. This suggests a relationship between the relative abundance of CECs in neonatal/juvenile mice and the ability to control bacterial infections [75].

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