HHS Public Access

Author manuscript

J Leukoc Biol. Author manuscript; available in PMC 2024 May 30.

Published in final edited form as:

J Leukoc Biol. 2024 May 29; 115(6): 1143–1153. doi:10.1093/jleuko/qiae020.

Age-dependent NK cell and interferon-gamma deficits contribute to severe pertussis in infant mice

Ashley E. Mitchell,
Karen M. Scanlon,
Emily M. Flowers,
Cassandra M. Jordan,
Ellis J. Tibbs,
Alicia Bukowski,
Danisha Gallop,
Nicholas H. Carbonetti*

Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201

Abstract

Many respiratory infections are selectively injurious to infants, yet the etiology of age-associated susceptibility is unknown. One such bacterial pathogen is *Bordetella pertussis.* In adult mice, innate interferon-gamma (IFN-γ) is produced by natural killer (NK) cells and restricts infection to the respiratory tract. In contrast, infant pertussis resembles disease in NK cell- and IFN-γ -deficient adult mice that suffer disseminated lethal infection. We hypothesized that infants exhibit age-associated deficits in NK cell frequency, maturation, and responsiveness to *B. pertussis*, associated with low IFN-γ levels. To delineate mechanisms behind age-dependent susceptibility, we compared infant and adult mouse models of infection. Infection in infant mice resulted in impaired upregulation of IFN-γ and substantial bacterial dissemination. *B. pertussis*-infected infant mice displayed fewer pulmonary NK cells than adult mice. Furthermore, the NK cells in the infant mouse lungs had an immature phenotype, and the infant lung showed no upregulation of the IFN-γ-inducing cytokine IL-12p70. Adoptive transfer of adult NK cells into infants, or treatment with exogenous IFN-γ, significantly reduced bacterial dissemination. These data indicate that the lack of NK cell-produced IFN-γ significantly contributes to infant fulminant pertussis and could be the basis for other pathogen-induced, age-dependent respiratory diseases.

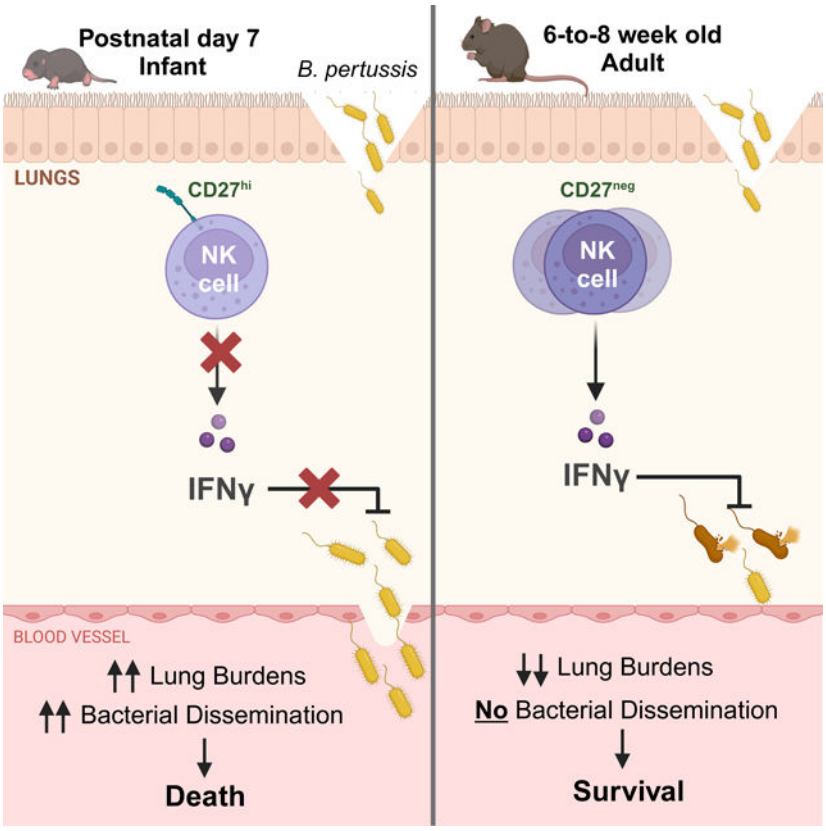
Summary sentence:

Age-dependent deficits in Natural Killer cell functional maturation and IFN-γ production contribute to severe *Bordetella pertussis* infection in infant mice

Graphical Abstract

*Corresponding Author (ncarbonetti@som.umaryland.edu).

The authors declare no competing financial interests.



Keywords

age dependent; Interferon-gamma; NK cells; pertussis (whooping cough); respiratory infection; innate immunity; mucosal immunity

INTRODUCTION

Infants are particularly susceptible to infectious diseases, which may result in severe outcomes specific to this age group [1]. The susceptibility of infants to infection is partly attributed to the lack of components of canonical, adult immune responses [1, 2]. Specific differences in the infant immune system result in less Th1-polarizing cytokine responses and associated susceptibility to other infections [1, 3]. The role of Th1 innate immune responses is understudied in bacterial infections of early life. Here we define critical deficits of immunity that permit systemic disease in an age-dependent mouse model of respiratory bacterial infection.

A bacterial infectious disease of particular concern in infants is pertussis (whooping cough), caused by acute respiratory infection with *Bordetella pertussis*. Typical pertussis disease in adults and children is self-resolving, despite paroxysmal coughing. In infants, however, pertussis can progress to severe pulmonary and systemic complications, often requiring hospital intensive care. 80% of pertussis deaths occur in infants under 3 months of age [4]. Despite the mortality observed in infants, no consistently efficacious therapeutics are

available, and the acellular pertussis vaccine has limited protection in infants before the third dose. The specific immune system features contributing to this age-dependent risk are unknown, hampering the field's ability to move toward a rational vaccine or therapeutic design.

In mouse models of pertussis, adults limit infection to the airways and clear the bacteria within a few weeks [5]. In contrast, infant mice suffer from disseminating lethal infection with critical features of human infant disease, including leukocytosis, bacterial dissemination, and pulmonary hypertension [6–8]. Notably, adult mice lacking the IFNγ receptor (IFNγR) fail to restrict *B. pertussis* infection to the respiratory tract and suffer lethal disseminating infection [9], mirroring pertussis disease in infant mice. Furthermore, adult mice depleted of natural killer (NK) cells, innate lymphoid cells which are significant early producers of IFN-γ during infection [10], also succumb to lethal disseminating *B. pertussis* infection [11]. These findings indicate that NK cells and innate IFN-γ are critical features of the immune response that limits this bacterial infection.

Since infant mice suffer a similar lethal, disseminating infection to that observed in IFNγR-deficient or NK cell-depleted adults, we hypothesized that a deficiency in NK cell function results in reduced IFN-γ levels and heightened susceptibility to pertussis in infants. We studied age-dependent host responses to *B. pertussis* infection in mice. Our findings strongly indicate that NK cells are key coordinators of innate responses that, when immature in infants, fail to restrict bacterial dissemination, resulting in systemic and lethal infection and disease.

MATERIALS AND METHODS

Animals

Mice were housed and bred at the animal facilities at The University of Maryland School of Medicine. C57BL/6 mice were purchased from Charles River or Jackson Labs for in-house breeding. GREAT (B6.129S4-Ifngtm3.1Lky/J) RRID: IMSR_JAX:017581 [12] and CD45.1 (B6.SJL-PtprcaPepcb/BoyJ) RRID: IMSR_JAX:002014 mice were purchased from Jackson Labs. All studies were performed on preweaning animals aged postnatal day 7 (P7) to P21 or adult mice 6–8 weeks. Experimental pups were obtained by timed mating in-house. All animal procedures and experimental treatments were performed under protocols approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine. All mouse strains were maintained in the University of Maryland School of Medicine animal facilities in accordance with institutional guidelines.

Bacterial strains

The strain of *B. pertussis* used was a streptomycin-resistant derivative of Tohama I [Carbonetti et al, 2003 I&I 71:6358]. *B. pertussis* was grown on Bordet–Gengou agar supplemented with 10% defibrinated sheep's blood (Lampire Biological Laboratories) and 200 μg/ml streptomycin at 37°C for 48 h.

Bacterial infections

Bacteria were suspended in PBS at OD600 = 1. The aerosol inoculum was administered via a nebulizer system (Pari Vios) for 20 min as previously described [10], which resulted in doses that ranged from 5×10^5 cfu/mouse to 2×10^6 cfu/mouse depending on mouse age at the time of inoculation. Control mice were sham inoculated by aerosolized PBS. On the indicated days post-inoculation (dpi), lungs and other organs were removed to analyze bacterial burden, transcript levels, protein levels, or flow cytometry. Leukocytosis was quantified as previously described [7]. For survival studies, mice were observed over 21 dpi when the experiment was terminated. Generally, groups of adult mice consisted of 4–6 animals of both sexes and groups of infant mice were litters of 5–9 animals of both sexes.

RNA processing and qRT-PCR

RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed using a reverse transcription system (Promega) per the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with Maxima SYBR green/ROX qPCR master mix (Thermo Scientific) in an Applied Biosystems 7500 Fast real-time PCR system. Transcript levels of cytokine genes were normalized to the hypoxanthine phosphoribosyl transferase (HPRT) housekeeping gene and compared with those from PBS-inoculated age-matched mice to determine fold changes (calculated by $2^{-\Delta\Delta CT}$). Primer sequences were: hprt forward 5′-GCTGACCTGCTGGATTACATTAA-3' and hprt reverse GATCATTACAGTAGCTCTTCAGTCTG-3′; Ifng forward 5′-GGAGGAACTGGCAAAAGGATG-3', and Ifng reverse 5′-GACGCTTATGTTGTTGCTGATGG-3'; IL10 forward 5'-ATTTGAATTCCCTGGGTGAGAAG-3', IL10 reverse5'-CACAGGGGAGAAATCGATGACA-3'; IL12a forward 5'-GCATGTGTCAATCACGCTACCTCC-3', IL12a reverse 5'-GCAGGTTTCGGGACTGGCTAAGAC-3'; IL12b forward 5'-GACATCATCAAACCAGACCCGCCCAAGA-3', IL12b reverse 5'-CGAACAAAGAACTTGAGGGAGAAGTAGG-3'; and IL17a forward 5'-CTGTGTCTCTGATGCTGTTGC-3' and IL17a reverse 5'-GAACGGTTGAGGTAGTCTGAGG-3'; IL17f forward 5'-CATGGTCAAGTCTTTGCTACTGTTG-3', and IL17f reverse 5'-CAACTCTCACAGTGTTATCCTCCAG-3'.

ELISA

After harvest, tissues were flash-frozen in an isopropanol/dry ice bath. The tissue was homogenized in 1–2 ml PBS with protease inhibitor (Roche) to prevent protein degradation. ELISA was performed on dilutions of the tissue samples as per the manufacturer's protocol for IFNγ, IL-18 (Invitrogen), or IL-17, IL-10, IL-12p70, IL-18, IL15, using the MILLIPLEX MAP Mouse TH17 Magnetic Bead Panel on the Luminex Multianalyte System by the University of Maryland School of Medicine Cytokine Core Laboratory.

Interferon-gamma treatment

P7 littermates were weighed to determine starting weights before inoculation with *B. pertussis* at 0 dpi. At random, half of the litter received recombinant murine IFNγ (rIFNγ) (Thermo Fisher) at 60 ng/g body weight i.n. or under isoflurane anesthesia, and the other half received vehicle (PBS) on 1 through 5 dpi. The total volume administered to infant mice was 10–20 μl.

Processing tissue into single-cell suspensions

Spleens were gently dissociated between two frosted glass slides; cells were then suspended in cell preparation buffer (1x PBS, 2 mM EDTA, and 0.5% BSA w/v) and filtered through a 70 μm cell strainer twice to remove debris and generate a single cell suspension. Red blood cells were lysed by adding 2 ml ACK Lysing Buffer (Quality Biologicals, inc.) for up to 1 min at room temperature, followed by washing in cell preparation buffer. Prior to harvest, lungs were perfused by injection through the right ventricle of either 10 ml PBS for mice aged ≤P21 or 20 ml PBS for adult mice to remove circulating leukocytes. The superior lobe was dissected and homogenized for CFU enumeration. The remaining tissue was used to generate single-cell suspensions by incubation of minced lung tissue in digestion buffer containing 2 mg/ml Collagenase Type X1 (Sigma-Aldrich), 20 mg/ml DNase I (Roche), 5 mg/ml Liberase TM (Sigma-Aldrich) and 100 mg/ml Hyaluronidase type IS (Sigma-Aldrich) for 45 mins at 37°C, grinding through a 100 μm cell strainer, separation of leukocytes on a 37% Percoll density gradient (Sigma-Aldrich) and ACK lysis to remove remaining red blood cells. Single-cell suspensions of both spleen and lung were recovered in 2 ml PBS with 2 mM EDTA and counted by trypan blue staining.

Flow cytometry and FACS analysis

2×10^6 cells were stained with either Live Dead Fixable Aqua (ThermoFisher) or Zombie NIR (Biolegend) per manufacturers' instructions in 1x PBS containing 2 mM EDTA. Cells were washed twice in FACS buffer (1x PBS, 0.5% w/v BSA, 2 mM EDTA) and resuspended in Fc Block (Biolegend) for 15 mins on ice. Cells were then stained with antibodies at 4°C for 15 min. All fluorochrome-conjugated monoclonal antibodies were purchased either from BD Pharmingen (La Jolla, CA, USA), Biolegend (San Diego, CA, USA), or Invitrogen (Waltham, MA, USA). The following fluorescent conjugated antibodies were used: PE-CD19 (clone 6D5), APC-eF780-CD3e (clone 145–2C11) or AF700-CD3e (clone 500A2), FITC- or Pacific Blue-CD4 (clone GK1.5), AF700-CD45 (clone 30-F11), APC-CD8a (clone 53–6.7), PerCP-Cy5.5-NK1.1 (clone PK136), BV60-CD27 (clone LG.3A1) and Pe-Cy7-CD69 (clone H1.2f3). After staining, cells were fixed with 2% PFA for 10 mins at room temperature. After fixation, cells were washed and resuspended in 350 uL of FACS buffer and acquired on either the LSR II (BD) or Aurora Spectral Flow Cytometer (Cytek). Analysis was performed using FlowJo 10.9.0 (BD). Fluorescence minus one (FMO) for each antibody was used to determine gating. Gating strategies to identify NK, NKT-like and T cells are shown in Fig. S2A and B. In the GREAT mouse experiments, lung single-cell suspensions of age-matched *B. pertussis*-infected WT C57BL/6 mice were used as the FMO control for YFP-IFN-γ expression and autofluorescence extraction.

NK cell adoptive transfer

Spleen cell suspensions from uninfected adult mice were prepared as described above at 1×10^8 cells/ml for direct transfer or to purify NK cells. NK cells were purified by negative magnetic selection (StemCell Technologies) per manufacturer instructions. To confirm NK cell purity, 250,000 cells from either unfractionated splenocytes or NK cell enrichment were stained with viability dye 7-AAD (Biolegend), PE-CD45.2 (clone A2,), FITC-, PerCP-cy5.5-, or PE-NK-1.1 (clone PK 136), APC-eF780-CD3e (clone 145–2C11) and PE-Cy7-CD69 and purity was confirmed by flow cytometry. P6 CD45.1 recipient mice were injected i.p. with 50 μl whole splenocytes (1.25×10^6 cells), purified NK cells (1.25×10^6 cells), or PBS as control one day prior to bacterial inoculation (−1 dpi). At 7 dpi, tissues were harvested for assessment of bacterial loads and flow cytometry analysis to detect transferred cells in recipient mice.

Statistical analysis

Data were analyzed, and graphs were made using GraphPad Prism 9.0 statistics software. Each plot represents mean values with standard deviations. The student's *t*-test was used for determining the significance between two means and one-way ANOVA with Dunnett's/Sidak's/Tukey's multiple comparisons tests to determine the significance between multiple groups.

RESULTS AND DISCUSSION

Resistance to *Bordetella pertussis* develops with age.

We previously characterized a postnatal day 7 (P7) infant mouse model of *B. pertussis* infection [7]. We hypothesized that, as in humans, resistance to severe and lethal *B. pertussis* infection would increase during postnatal development. To determine the age-associated resistance to pertussis disease, we inoculated mice of increasing postnatal age (P7, P10, P14, or P21) or 6-to-8-week-old adult mice with *B. pertussis*. We assessed bacterial burdens and monitored survival (Fig. 1A). Aerosol inoculation of all age groups resulted in productive infection, and bacterial lung burdens at 24 h post-inoculation were not significantly different between P7, P10, and P14 mice (Fig. S1A). P21 mice burdens were comparable to adult mice (Fig. S1A). At the peak of infection (7 dpi), bacterial lung loads were progressively lower with increasing age (Fig. 1B). Mirroring the primary site of infection, bacterial dissemination into the blood, liver, and spleen declined with increasing age and was absent in adult mice (Figs. 1B, S1B). Pertussis-induced lethality was also age-associated. Consistent with our previous studies [7], P7 infant mice succumbed to infection from 9 dpi and reached 100% mortality by 16 dpi. In stark contrast, most P10, P14, and P21 mice survived the infection (Fig. 1C). In infected P7 infant mice, lung and liver bacterial loads increased over time and were at the highest level at 9 dpi (last time point assessed) (Fig. S1D). In contrast, lung bacterial loads in infected adult mice peak at 4–7 dpi and decline thereafter [13].

As mentioned above, pertussis disease severity in humans also stratifies by age, and clinical complications are most significant in young infants. For example, leukocytosis develops soon after the onset of pertussis disease in infants, and higher levels of leukocytosis

correlate with fatal outcomes [8]. Accordingly, we assessed leukocytosis and monitored weight change to measure disease severity in mice to gain insight into age-dependent disease outcomes during acute infection. Leukocytosis was observed in mice inoculated at P7 or P10 but not in older mice (Fig. S1C). Disease severity paralleled lethality, as infected P7 mice failed to match the weight gain of uninfected control mice from 3 dpi, with a significant decline at 10 dpi. In contrast, infected older mice continued to gain weight similarly to control mice (Fig. S1E).

In summary, our data indicate that age at inoculation impacts pertussis-associated disease severity during the acute phase of infection. We hypothesized that the age-related susceptibility to pertussis and insufficient infection control is attributable to defects in the infant immune response in this mouse model.

B. pertussis-infected infant mice display impaired IFN-γ responses, and intranasal rIFN-γ treatment enhances bacterial clearance and reduces disease.

Clearance of *B. pertussis* from the respiratory tract of adult mice is driven by the production of IFN-γ and IL-17 from innate and adaptive immune cells [14–16]. A likely contributing factor to the susceptibility of infants to severe pertussis is the different and developing immune system at this age. In both mouse and human studies, it is well established that young infants have an inherent bias toward an immunosuppressive milieu, which is characterized by low production of pro-inflammatory cytokines, such as IFN-γ and IL-17, and high levels of IL-10 [2, 17–19]. We compared lung production of IFN-γ and IL-17 in P7 infant and adult mice at 7 dpi. Consistent with previous reports [20], adult mice significantly upregulated lung IFN-γ (Fig. 2A, B) and IL-17 (Fig. 2C, and S1F, G) at the mRNA and protein levels during infection. In contrast, these cytokines were not significantly upregulated in P7 infant mice during infection (Fig. 2A-C and S1F, G). Interestingly, P7 infant mice, but not adult mice, displayed upregulation of IL-10 (Fig. 2D, S1H). The imbalanced early cytokine response in infant mice may explain the lack of a protective immune response initiated by IFN-γ and other activating cytokines.

Studies in adult mice have established the protective role of IFN-γ during *B. pertussis* infection. Adult mice lacking the *Ifng* gene (GKO), the IFN-γ cell surface receptor (IFNγR−/− mice), or NK cells (significant IFN-γ producers during infection) develop a prolonged, disseminating, and lethal infection [9, 11, 21], recapitulating the age-dependent disease phenotype observed in our infant mice. Since IFN-γ responses were not observed during infection in infants, we hypothesized that supplementation with IFN-γ during infection would ameliorate severe disease symptoms in infants. To test this, P7 infant mice were inoculated with *B. pertussis*. Intranasally administered recombinant IFN-γ (rIFN-γ) daily from 1–5 dpi (Fig. 2E). At 7 dpi, bacterial lung burden and systemic dissemination were significantly reduced (6-fold in lungs and 10- and 17-fold in the spleen and liver, respectively) in rIFN-γ-treated mice compared to vehicle control (Fig. 2F). Furthermore, using weight change as an indicator of severe disease, treatment of infant mice with rIFN-γ reversed early disease severity, with rIFN-γ-treated infants gaining weight at nearly the same rate as uninfected infants (Fig. 2G). Intraperitoneal rIFN-γ treatment of infected infant mice did not significantly reduce lung burdens or systemic dissemination (Fig. S1I),

indicating that local effects of IFN-γ at the primary infection site are necessary to control the infection. Together, these results demonstrate the importance of localized IFN-γ within the lung to confine bacteria to the respiratory tract and prevent dissemination. Notably, the protective effect of rIFN-γ has been demonstrated in age-dependent viral infection [22], and we have demonstrated this effect in an age-dependent bacterial infection. These similarities highlight the broader relevance of susceptibility to respiratory infections, which likely share etiology due to age-dependent deficits in IFN-y-driven immune responses.

Infant mice NK cells do not significantly expand during *B. pertussis* infection

In adult mice, NK and NKT-like cells are recruited early during lung infection, providing innate control of *B. pertussis* [11]. Therefore, we hypothesized that age-dependent differences in innate cellular responses to *B. pertussis* infection limit IFN-γ production in infant mice. To evaluate this, we first quantified cellular responses at the primary site of infection from sham-inoculated or *B. pertussis*-infected infant or adult mice at 4 and 7 dpi. At 4 dpi, there were no significant increases in lung NK, NKT-like or T cells induced by infection in adult mice, and only NKT-like cells were significantly increased by infection in infant mice (Fig. S2C), although lung NK cell numbers were significantly higher in infected adults than infected infants. At 7 dpi, the numbers of NK and NKT-like cells in uninfected infant lungs and spleens were not significantly different from those populations in adult mice (Fig. 3A, B, D, E). Critically, unlike adult mice which displayed significant increases in NK and NKT-like cell numbers upon infection (Fig. 3A, B), infection in infant mice resulted in no significant increase in these cell populations (Fig. 3A, B). These data suggest that defects in leukocyte trafficking or proliferation at the site of primary infection may be responsible for the lack of bacterial control during infant *B. pertussis* infection in this model. Pertussis toxin [23, 24], a virulence factor secreted by *B. pertussis*, is an inhibitor of chemokine signaling and can impact immune cell trafficking during infection. Therefore, we considered whether there were systemic differences in the cellular response to *B. pertussis,* with the possibility that NK cells were present in the peripheral tissues but unable to enter lung tissue. We quantified cellular responses in the spleen during sham-inoculated or *B. pertussis*-infected infant or adult mice to test these. No significant increase was observed in the number of NK, NKT-like, or T cells in the spleen at 7 dpi in either infant or adult mice (Fig. 3D-F), although these cells were generally higher in adult mice than in infant mice. While immune cell trafficking may be impacted by pertussis toxin [23, 24], we observed no significant changes in NK cells numbers in the infant's spleen during infection, which suggests an intrinsic defect in the infant NK cell response, not attributable to trafficking defects. As a result, subsequent evaluation of IFN-γ production in the infant mice focused on the primary site of infection.

Infant NK cells are immature and unable to produce IFN-γ during *B. pertussis* infection.

Our results demonstrate that *B. pertussis*-infected infant mice have lower NK and NKT-like cell numbers and lower lung IFN-γ production than infected adult mice. We utilized IFN-γ-YFP reporter (GREAT) mice [25] to identify the primary cellular sources of IFN-γ in the lungs of *B. pertussis*-infected infant and adult mice at 7 dpi (Fig. 4A). We compared the number of IFN-γ-producing NK, NKT-like, CD4 and CD8 T cells among total leukocytes. There were no differences in the number of IFN-γ-producing NKT-like, CD4, and CD8

T cells between *B. pertussis*-infected infant and adult mice (Fig. 4B, C and Fig. S2D). However, the number of IFN-γ-producing NK cells was significantly lower in infected infant mice than in adult mice, which were the predominant producers of IFN-γ (Fig. 4B, C and Fig. S2D).

Using flow cytometry, we compared lung NK cell phenotypes to investigate the mechanisms underlying the lower IFN-γ production in infants. We sought to characterize intrinsic or extrinsic factors that impact infant NK cell responses. CD69 expression on total NK cells was used to evaluate activation during infection. There were no significant differences in CD69 expression between infant and adult mice (Fig. S2E). Despite the difference in IFN-γ production between infant and adult mouse NK cells, similar levels of CD69 expression on total NK cells suggest that infant and adult NK cells have similar abilities to sense environmental cues, such as cytokines.

The maturation status of NK cells can also influence their capacity to respond in peripheral tissues during infection [26–32]. During postnatal development, NK cells undergo continuous maturation, generating different subsets in peripheral organs, such as the lung [33, 34]. $CD27^{hi}$ NK cells, representing an immature subset, have reduced ability to produce IFN-γ, while $CD27^{lo}$ mature NK cells exhibit the highest effector functionality [35]. Once mice reach adulthood, NK cell maturation is complete; thus, most NK cells are $CD27^{lo}$ and fully competent. *B. pertussis*-infected infant lung NK cells expressed high levels of CD27, indicating that they remain immature in the context of infection, while adult NK cells were $CD27^{lo}$ and mature (Fig. 4D, E). Therefore, an increased proportion of immature NK cells within the total NK cell numbers may contribute to increased susceptibility to pertussis in infants, further supported by a minute proportion (6.26 ± 1.7) of infant NK cells able to produce IFN-γ (Fig. S2D).

A possible explanation for the relative lack of NK cell IFN-γ responses in infants is a deficiency in cytokines that activate NK cells. IL-12 (dimeric IL-12p70) and IL-18 are the predominant NK cell-activating cytokines for IFN-γ production, especially when present in combination, and can stimulate human NK cells to produce IFN-γ in response to *B. pertussis* in vitro [36–38]. In infants, the capacity to produce IL-12 during bacterial infections is not fully developed because infants have antigen-presenting cells which produce lower cytokine levels [39–42]. Indeed, IL-12 response from infant or cord blood cells after B. *pertussis* vaccination or in response to *B. pertussis*-specific vaccine antigens are markedly reduced compared to adult PBMCs [43, 44]. We found that IL-12p70 production was upregulated in *B. pertussis*-infected adult mouse lungs but not in infected P7 infant mice (Fig 4F). *Il-12a* gene expression increased in the lungs of infected mice in an age-dependent manner (Fig. S2F), and *Il-12b* gene expression was upregulated in the lungs of infected adults but not P7 infant mice (Fig. S2G). Interestingly, IL-18 production was consistent between naïve and infected adult mice, while infected infants upregulated IL-18 to match adult levels (Fig. S2H). IL-15 also acts as a general activation signal for NK cells during bacterial infection [45], and was not significantly upregulated in either group of mice (Fig. S2I). Lung IFN-γ gene expression and protein levels, which were reduced in infant mice inoculated at P7 (Fig. 2), were significantly upregulated by infection in infant mice inoculated at P10 and older (Fig. S2J), corresponding to the stepwise, age-dependent

increase in *Il-12a* gene expression and age-associated protection. Therefore, we propose that deficient IL-12p70 production is a significant contributor to the lack of IFN-γ production by NK cells in infected infants.

In summary, we identified that infant NK cells are deficient in IFN-γ production in response to *B. pertussis* infection, and this correlated with the immaturity of this cell type within the lung. This deficit in innate IFN-γ is related to multiple features that antagonize the infant innate Th1 response, including lower IL-12p35 expression and IL-12p70 production, higher IL-10 production, and a previously identified type I and type III IFN deficit [46]. Infant mice have multiple deficits in activation pathways which contribute to functional innate Th1 immunity against *B. pertussis*. Consequently, overcoming the infant blockade against Th1 polarization through vaccination or during natural infection would likely require a multifaceted approach and is discussed in greater detail below.

Adoptive transfer of functional adult NK cells reduces bacterial dissemination and pertussis disease in infant mice.

Thus far, our results suggest that infant susceptibility observed during acute *B. pertussis* infection may be due to diminished IFN-γ production by NK cells. Therefore, we performed a series of adoptive transfer experiments using adult immune cells to investigate whether functional defects in infant NK could be compensated for by a mature population (Fig. 5A). One day before bacterial inoculation, infant mice were injected with either 1.25×10^6 highly enriched adult spleen NK cells (Fig. S3A, B), an equivalent number of unfractionated adult splenocytes, or vehicle control (PBS). At 7 dpi, flow cytometry to detect the transferred cell populations in the lungs and systemic organs of the recipient mice revealed that donor adult NK cells were found primarily at the site of infection in recipient mice (Fig. 5B, C and Fig. S3C, D). We assessed the bacterial burdens in the lungs, blood, spleen, and liver. There were no significant differences in lung burdens at 7 dpi between any experimental groups (Fig. 5D). Interestingly, the infants that received enriched adult NK cells had significantly reduced bacterial dissemination in the blood compared to controls (Fig. 5E). Seven out of 10 mice in the enriched NK cell transfer group had blood CFU counts below the detection limit. In addition, this reduction in infant blood CFU numbers mirrored the low levels of blood CFU observed in infected adult mice. We also observed a reduction in spleen and liver CFU numbers in infant mice that received enriched adult NK cells suggesting reduced bacterial dissemination. However, these differences did not reach statistical significance (Fig. S3E). The reason for this difference between dissemination levels in the blood versus the organs is unclear. Blood CFU provides a snapshot of the number of bacteria in circulation at this time point, perhaps reflecting initial dissemination from the lungs into the systemic circulation. The liver and spleen CFU may result from accumulation of bacteria from blood draining over multiple days and additional bacterial proliferation in these organs. Additional IFN-γ-dependent cell mechanisms may contribute to clearance from these organs. The selective recruitment of enriched adult NK cells into the lungs, but not the spleen or the liver, after adoptive transfer into infant mice suggests that NK cells are important in preventing initial dissemination into the circulation.

These findings demonstrate that the adoptive transfer of functionally competent NK cells can reduce bacterial dissemination in infant mice. Moreover, it indicates that functional defects in infant lung NK cell responses permit bacterial dissemination, a feature of severe disease observed in infant mice but absent in adults during *B. pertussis* infection. Homing of adult NK cells to the infant lung indicates that NK cells participate via an unknown mechanism confining bacteria to the primary site of infection. In contrast, infant NK cells cannot provide early, innate infection control, likely due to deficient IFN-γ production.

Overall, our study revealed that during acute *B. pertussis* infection, mice display progressive age-dependent protection from dissemination and lethality, closely mirroring the progressive immunity seen in human disease. We also observed minimal expansion of infant lung NK cell numbers and high levels of CD27 on the NK cells that were present, which suggests an immature state with limited effector functions. Most notably, we associated high CD27 expression with an inability to produce IFN-γ. Consistent with our data, an analysis of human peripheral blood cells over the lifespan showed that NK cell frequencies and maturation were the lowest after birth [47]. A study of adult human volunteers challenged with *B. pertussis* demonstrated that expansion of blood NK cells correlates with protection against *B. pertussis* nasal colonization [48] *B. pertussis*-infected adult mice showed similar results with a significant expansion of mature NK cells to the respiratory tract. Ultimately, we identified that mature NK cells, through IFN-γ production, limit bacteria in the respiratory tract. In contrast, the immature, infant NK cells permit dissemination to the blood and peripheral organs. These findings highlight the important role of NK cells in early protection against *B. pertussis* infection and demonstrate the relevance of our models to human infection.

Our data indicate that T cells may not significantly contribute to preventing early extrapulmonary dissemination of *B. pertussis* infection. *B. pertussis* respiratory infection of adult T cell-deficient mice results in neither bacterial dissemination nor lethality [49], whereas infected adult NK cell-depleted mice succumb to disseminating infection [50]. IFN-γ-secreting Th1 responses are essential for clearance of *B. pertussis* from the lungs, preventing systemic disease and providing lasting immunity [51, 52], but CD4 T cell-dependent IFN-γ production occurs late in infection (after 3–4 weeks) in adult mice [53]. Although, we observe no significant differences in early CD4 T cell IFN-γ production between *B. pertussis*-infected infant and adult mice, CFU kinetics in infant mice at late time points suggest that age-dependent differences in CD4 T cell responses may significantly impact the disease. In a pioneering study, in vitro stimulation of infected infant PBMCs with *B. pertussis*-specific antigens demonstrated that human infants have the capacity to produce Th1 responses to *B. pertussis* [54]. Our study identified potential deficits in infant immune polarization that may affect the capacity to mount an effective Th1 response in vivo and contribute to age-dependent severity. Further research is needed to determine the impact of CD4 Th1 responses during late infection.

The lack of innate NK cell IFN-γ may contribute to age-related susceptibility to other respiratory diseases in addition to pertussis, such as respiratory syncytial virus (RSV) or influenza virus. RSV is a common pathogen that can cause severe respiratory illness in infants and young children [55]. Previous studies have shown that NK cells play an

important role in the immune response to RSV infection and that IFN-γ produced by NK cells can limit viral replication and promote the clearance of infected cells [56]. We believe the data here could inform the development of new treatments or vaccines that target NK cells and stimulate the production of IFN-γ to enhance immune responses to respiratory pathogens and provide a valuable pre-clinical model of early life to a pulmonary bacterial infection.

A coordinated immune response involving multiple cell types and cytokines is likely necessary for controlling any bacterial infection, including pertussis. Consistent with that idea, type I and III IFN responses to *B. pertussis* infection in infant mice are also deficient [46]. The deficiency in these IFN responses may contribute to the inability to raise a productive IFN-γ response in infants, in parallel with the limitation in IL-12p35 expression identified here. Effective vaccination against pertussis would ideally result in Th1-polarized CD4 T cells. Unfortunately, achieving that means overcoming a blockade against Th1 polarization in infants with multiple deficient or inhibited activation pathways. However, babies born to mothers vaccinated with the acellular pertussis vaccine during pregnancy had elevated IL-12 responses in cord blood but reduced NK and monocyte numbers [57]. In concept, this tells us that in human infants, there is some capacity to upregulate IL-12 without identified deleterious effects. Many groups are exploring how adding adjuvants to the pertussis vaccine formulation may bolster innate immunity and increase vaccine responses. Adjuvants targeting pattern recognition receptors TLR7/8 [58, 59], Dectin-1 [60], or STING [61] can promote IL-12 and IFN-γ production and that infant cells can be induced to elicit a Th1 response. Our results show that inducing IFN-γ from NK cells may produce an innate protective response against respiratory infection in infants. The combination of IL-12 and IFN-γ has the potential to go beyond early innate control and induce CD4 Th1 responses during vaccination. Therefore, our findings are important for both therapeutic and vaccination approaches to the control of respiratory infectious diseases.

Little is known about the impact of age on innate lymphoid cell maturation and subsequent effector functions in the lungs. This study used an age-dependent mouse model to determine whether functional defects in infant NK cell responses contribute to severe disease. We identified that infant mice with a profound defect in NK cell functional maturity develop impaired responses to *B. pertussis*. Furthermore, our data suggest that the capacity to produce IL-12/IFN-γ increases throughout postnatal development and highlight how age-related differences in NK cell responses could impact protection during natural infection and vaccination in early life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Da'Kuawn Johnson, Ciaran Skerry, David Rickert, Jeremy Ardanuy, and Rainer Butler for help with some experiments, and Tonya Webb, Nevil Singh, Rossana Trotta, Eileen Barry, Martin Flajnik, and Stephania Cormier for helpful advice and feedback on the manuscript. We acknowledge the assistance of the University of Maryland School of Medicine Cytokine Core Lab and the University of Maryland Greenebaum Comprehensive Cancer Center Flow Cytometry Shared Services. This work was supported by NIH grants AI141372, AI156042 (to

NHC) and a University of Maryland, Baltimore Institute for Clinical and Translational Research grant. AEM was partly supported by NIH grant T32 AI095190.

REFERENCES

1. Kollmann TR, Kampmann B, Mazmanian SK, Marchant A, Levy O. (2017) Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny. Immunity 46, 350–363. [PubMed: 28329702]
2. Dowling DJ and Levy O. (2014) Ontogeny of early life immunity. Trends Immunol 35, 299–310. [PubMed: 24880460]
3. Levy O. (2005) Innate immunity of the human newborn: distinct cytokine responses to LPS and other Toll-like receptor agonists. J Endotoxin Res 11, 113–6. [PubMed: 15949138]
4. Cherry JD, Wendorf K, Bregman B, Lehman D, Nieves D, Bradley JS, Mason WH, Sande-Lopez L, Lopez M, Federman M, Chen T, Blumberg D, Johnston S, Schwenk HT, Weintrub P, Quinn KK, Winter K, Harriman K. (2018) An Observational Study of Severe Pertussis in 100 Infants ≤120 Days of Age. Pediatr Infect Dis J 37, 202–205. [PubMed: 28737623]
5. Carbonetti NH, Artamonova GV, Mays RM, Worthington ZEV (2003) Pertussis Toxin Plays an Early Role in Respiratory Tract Colonization by *Bordetella pertussis*. Infection and Immunity 71, 6358–6366. [PubMed: 14573656]
6. Scanlon KM, Chen L, Carbonetti NH (2022) Pertussis Toxin Promotes Pulmonary Hypertension in an Infant Mouse Model of Bordetella pertussis Infection. J Infect Dis 225, 172–176. [PubMed: 34145457]
7. Scanlon KM, Snyder YG, Skerry C, Carbonetti NH (2017) Fatal Pertussis in the Neonatal Mouse Model Is Associated with Pertussis Toxin-Mediated Pathology beyond the Airways. Infect Immun 85.
8. Winter K, Zipprich J, Harriman K, Murray EL, Gornbein J, Hammer SJ, Yeganeh N, Adachi K, Cherry JD (2015) Risk Factors Associated With Infant Deaths From Pertussis: A Case-Control Study. Clin Infect Dis 61, 1099–106. [PubMed: 26082502]
9. Mahon BP, Sheahan BJ, Griffin F, Murphy G, Mills KH (1997) Atypical disease after Bordetella pertussis respiratory infection of mice with targeted disruptions of interferon-γ receptor or immunoglobulin μ chain genes. The Journal of experimental medicine 186, 1843–1851. [PubMed: 9382883]
10. Spits H, Bernink JH, Lanier L. (2016) NK cells and type 1 innate lymphoid cells: partners in host defense. Nature Immunology 17, 758–764. [PubMed: 27328005]
11. Byrne P, McGuirk P, Todryk S, Mills KHG (2004) Depletion of NK cells results in disseminating lethal infection with Bordetella pertussis associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. Eur J Immunol 34, 2579–88. [PubMed: 15307190]
12. Reinhardt RL, Liang H-E, Locksley RM (2009) Cytokine-secreting follicular T cells shape the antibody repertoire. Nature Immunology 10, 385–393. [PubMed: 19252490]
13. Carbonetti NH, Artamonova GV, Andreasen C, Bushar N. (2005) Pertussis toxin and adenylate cyclase toxin provide a one-two punch for establishment of Bordetella pertussis infection of the respiratory tract. Infect Immun 73, 2698–703. [PubMed: 15845471]
14. Misiak A, Wilk MM, Raverdeau M, Mills KH (2017) IL-17-Producing Innate and Pathogen-Specific Tissue Resident Memory gammadelta T Cells Expand in the Lungs of Bordetella pertussis-Infected Mice. J Immunol 198, 363–374. [PubMed: 27864475]
15. Mosley YC, Lu F, HogenEsch H. (2017) Differences in innate IFNgamma and IL-17 responses to Bordetella pertussis between BALB/c and C57BL/6 mice: role of gammadeltaT cells, NK cells, and dendritic cells. Immunol Res 65, 1139–1149. [PubMed: 29052125]
16. Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, Lavelle EC, McLoughlin RM, Mills KH (2013) Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. PLoS Pathog 9, e1003264.
17. Belderbos ME, van Bleek GM, Levy O, Blanken MO, Houben ML, Schuijff L, Kimpen JL, Bont L. (2009) Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal

blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clin. Immunol. 133, 228–37. [PubMed: 19648060]

18. Langrish CL, Buddle JC, Thrasher AJ, Goldblatt D. (2002) Neonatal dendritic cells are intrinsically biased against Th-1 immune responses. Clin. Exp. Immunol. 128, 118–123. [PubMed: 11982599]
19. Yu JC, Khodadadi H, Malik A, Davidson B, Salles E, Bhatia J, Hale VL, Baban B. (2018) Innate Immunity of Neonates and Infants. Front Immunol 9, 1759. [PubMed: 30105028]
20. Andreasen C, Powell DA, Carbonetti NH (2009) Pertussis Toxin Stimulates IL-17 Production in Response to Bordetella pertussis Infection in Mice. PLoS ONE 4, e7079. [PubMed: 19759900]
21. Barbic J, Leef MF, Burns DL, Shahin RD (1997) Role of gamma interferon in natural clearance of Bordetella pertussis infection. Infect Immun 65, 4904–8. [PubMed: 9393774]
22. Empey KM, Orend JG, Peebles RS Jr., Egaña L, Norris KA, Oury TD, Kolls JK (2012) Stimulation of Immature Lung Macrophages with Intranasal Interferon Gamma in a Novel Neonatal Mouse Model of Respiratory Syncytial Virus Infection. PLOS ONE 7, e40499.
23. Andreasen C. and Carbonetti NH (2008) Pertussis toxin inhibits early chemokine production to delay neutrophil recruitment in response to Bordetella pertussis respiratory tract infection in mice. Infect Immun 76, 5139–48. [PubMed: 18765723]
24. Kirimanjeswara GS, Agosto LM, Kennett MJ, Bjornstad ON, Harvill ET (2005) Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of Bordetella pertussis. J Clin Invest 115, 3594–601. [PubMed: 16294220]
25. Reinhardt RL, Liang H-E, Bao K, Price AE, Mohrs M, Kelly BL, Locksley RM (2015) A novel model for IFN-γ-mediated autoinflammatory syndromes. Journal of Immunology (Baltimore, Md.: 1950) 194, 2358–2368. [PubMed: 25637019]
26. Beli E, Clinthorne JF, Duriancik DM, Hwang I, Kim S, Gardner EM (2011) Natural killer cell function is altered during the primary response of aged mice to influenza infection. Mech Ageing Dev 132, 503–10. [PubMed: 21893080]
27. Hayakawa Y. and Smyth MJ (2006) CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. J. Immunol. 176.
28. Kim S, Iizuka K, Kang H-SP, Dokun A, French AR, Greco S, Yokoyama WM (2002) In vivo developmental stages in murine natural killer cell maturation. Nature Immunology 3, 523–528. [PubMed: 12006976]
29. Michel T, Poli A, Domingues O, Mauffray M, Thérésine M, Brons NHC, Hentges F, Zimmer J. (2012) Mouse Lung and Spleen Natural Killer Cells Have Phenotypic and Functional Differences, in Part Influenced by Macrophages. PLoS ONE 7, e51230.
30. Swann JB, Hayakawa Y, Zerafa N, Sheehan KC, Scott B, Schreiber RD, Hertzog P, Smyth MJ (2007) Type I IFN contributes to NK cell homeostasis, activation, and antitumor function. J Immunol 178, 7540–9. [PubMed: 17548588]
31. Takeda K, Oshima H, Hayakawa Y, Akiba H, Atsuta M, Kobata T, Kobayashi K, Ito M, Yagita H, Okumura K. (2000) CD27-mediated activation of murine NK cells. J Immunol 164, 1741–5. [PubMed: 10657619]
32. Watt SV, Andrews DM, Takeda K, Smyth MJ, Hayakawa Y. (2008) IFN-gamma-dependent recruitment of mature CD27(high) NK cells to lymph nodes primed by dendritic cells. J Immunol 181, 5323–30. [PubMed: 18832688]
33. Andrews DM and Smyth MJ (2010) A potential role for RAG-1 in NK cell development revealed by analysis of NK cells during ontogeny. Immunol Cell Biol 88, 107–16. [PubMed: 19949422]
34. Samson SI (2003) GATA-3 promotes maturation, IFN-gamma production, and liver-specific homing of NK cells. Immunity 19, 701–11. [PubMed: 14614857]
35. Vahlne G, Becker S, Brodin P, Johansson MH (2008) IFN-γ Production and Degranulation are Differentially Regulated in Response to Stimulation in Murine Natural Killer Cells. Scandinavian Journal of Immunology 67, 1–11. [PubMed: 18028287]
36. den Hartog G, Schijf MA, Berbers GA, van der Klis FR, Buisman A-M (2022) Bordetella pertussis induces interferon gamma production by natural killer cells, resulting in chemoattraction by respiratory epithelial cells. The Journal of infectious diseases 225, 1248–1260. [PubMed: 32219323]

37. Kroes MM, Mariman R, Hijdra D, Hamstra HJ, van Boxtel K, van Putten JPM, de Wit J, Pinelli E. (2019) Activation of Human NK Cells by Bordetella pertussis Requires Inflammasome Activation in Macrophages. Front Immunol 10, 2030. [PubMed: 31507615]
38. Kroes MM, Miranda-Bedate A, Jacobi RHJ, van Woudenbergh E, den Hartog G, van Putten JPM, de Wit J, Pinelli E. (2022) Bordetella pertussis-infected innate immune cells drive the anti-pertussis response of human airway epithelium. Scientific Reports 12, 3622. [PubMed: 35256671]
39. Arulanandam BP, Mittler JN, Lee WT, O'Toole M, Metzger DW (2000) Neonatal administration of IL-12 enhances the protective efficacy of antiviral vaccines. J Immunol 164, 3698–704. [PubMed: 10725728]
40. Chen CJ, Hou JW, Chiang BL (2014) The difference in immune response and IL-12p35 methylation between newborns and adults. J Biomed Sci 21, 76. [PubMed: 25139335]
41. Goriely S, Vincart B, Stordeur P, Vekemans J, Willems F, Goldman M, De Wit D. (2001) Deficient IL-12(p35) gene expression by dendritic cells derived from neonatal monocytes. J Immunol 166, 2141–6. [PubMed: 11160266]
42. Kovarik J, Martinez X, Pihlgren M, Bozzotti P, Tao MH, Kipps TJ, Wild TF, Lambert PH, Siegrist CA (2000) Limitations of in vivo IL-12 supplementation strategies to induce Th1 early life responses to model viral and bacterial vaccine antigens. Virology 268, 122–31. [PubMed: 10683334]
43. Dirix V, Verscheure V, Goetghebuer T, Hainaut M, Debrie AS, Locht C, Mascart F. (2009) Monocyte-derived interleukin-10 depresses the Bordetella pertussis-specific gamma interferon response in vaccinated infants. Clin Vaccine Immunol 16, 1816–21. [PubMed: 19846681]
44. Tonon S, Goriely S, Aksoy E, Pradier O, Del Giudice G, Trannoy E, Willems F, Goldman M, De Wit D. (2002) Bordetella pertussis toxin induces the release of inflammatory cytokines and dendritic cell activation in whole blood: Impaired responses in human newborns. European Journal of Immunology 32, 3118–3125. [PubMed: 12385032]
45. Wang J, Li F, Zheng M, Sun R, Wei H, Tian Z. (2012) Lung natural killer cells in mice: phenotype and response to respiratory infection. Immunology 137, 37–47. [PubMed: 22612500]
46. Ardanuy J, Scanlon K, Skerry C, Fuchs SY, Carbonetti NH (2020) Age-Dependent Effects of Type I and Type III IFNs in the Pathogenesis of *Bordetella pertussis* Infection and Disease. The Journal of Immunology 204, 2192–2202. [PubMed: 32152071]
47. Jalali S, Harpur CM, Piers AT, Auladell M, Perriman L, Li S, An K, Anderson J, Berzins SP, Licciardi PV, Ashhurst TM, Konstantinov IE, Pellicci DG (2022) A high-dimensional cytometry atlas of peripheral blood over the human life span. Immunology & Cell Biology 100, 805–821. [PubMed: 36218032]
48. Diks AM, De Graaf H, Teodosio C, Groenland RJ, De Mooij B, Ibrahim M, Hill AR, Read RC, Van Dongen JJM, Berkowska MA (2023) Distinct early cellular kinetics in participants protected against colonization upon Bordetella pertussis challenge. Journal of Clinical Investigation 133.
49. Harvill ET, Cotter PA, Miller JF (1999) Pregenomic comparative analysis between bordetella bronchiseptica RB50 and Bordetella pertussis tohama I in murine models of respiratory tract infection. Infect Immun 67, 6109–18. [PubMed: 10531274]
50. Mahon BP, Sheahan BJ, Griffin F, Murphy G, Mills KH (1997) Atypical disease after Bordetella pertussis respiratory infection of mice with targeted disruptions of interferon-gamma receptor or immunoglobulin mu chain genes. J Exp Med 186, 1843–51. [PubMed: 9382883]
51. Blanchard-Rohner G. (2022) Novel approaches to reactivate pertussis immunity. Expert Rev Vaccines 21, 1787–1797. [PubMed: 36400443]
52. Schmitt P, Borkner L, Jazayeri SD, McCarthy KN, Mills KH (2023) Nasal vaccines for pertussis. Curr Opin Immunol 84, 102355.
53. McGuirk P, Mahon BP, Griffin F, Mills KH (1998) Compartmentalization of T cell responses following respiratory infection with Bordetella pertussis: hyporesponsiveness of lung T cells is associated with modulated expression of the co-stimulatory molecule CD28. Eur J Immunol 28, 153–63. [PubMed: 9485195]
54. Mascart F, Verscheure V, Malfroot A, Hainaut M, Pierard D, Temerman S, Peltier A, Debrie AS, Levy J, Del Giudice G, Locht C. (2003) Bordetella pertussis infection in 2-month-old infants promotes type 1 T cell responses. J Immunol 170, 1504–9. [PubMed: 12538714]

55. Griffiths C, Drews SJ, Marchant DJ (2017) Respiratory Syncytial Virus: Infection, Detection, and New Options for Prevention and Treatment. Clin Microbiol Rev 30, 277–319.

56. Bhat R, Farrag MA, Almajhdi FN (2020) Double-edged role of natural killer cells during RSV infection. Int Rev Immunol 39, 233–244. [PubMed: 32469615]

57. Rice TF, Diavatopoulos DA, Guo Y, Donaldson B, Bouqueau M, Bosanquet A, Barnett S, Holder B, Kampmann B. (2021) Modification of innate immune responses to *Bordetella pertussis* in babies from pertussis vaccinated pregnancies. eBioMedicine 72.

58. Dowling DJ, Barman S, Smith AJ, Borriello F, Chaney D, Brightman SE, Melhem G, Brook B, Menon M, Soni D, Schuller S, Siram K, Nanishi E, Bazin HG, Burkhart DJ, Levy O, Evans JT (2022) Development of a TLR7/8 agonist adjuvant formulation to overcome early life hyporesponsiveness to DTaP vaccination. Sci Rep 12, 16860. [PubMed: 36258023]

59. Makris S. and Johansson C. (2021) R848 or influenza virus can induce potent innate immune responses in the lungs of neonatal mice. Mucosal Immunol 14, 267–276. [PubMed: 32576926]

60. Lemoine S, Jaron B, Tabka S, Ettreiki C, Deriaud E, Zhivaki D, Le Ray C, Launay O, Majlessi L, Tissieres P, Leclerc C, Lo-Man R. (2015) Dectin-1 activation unlocks IL12A expression and reveals the TH1 potency of neonatal dendritic cells. J Allergy Clin Immunol 136, 1355–68.e1–15. [PubMed: 25865351]

61. Lirussi D, Weissmann SF, Ebensen T, Nitsche-Gloy U, Franz HBG, Guzman CA (2021) Cyclic Di-Adenosine Monophosphate: A Promising Adjuvant Candidate for the Development of Neonatal Vaccines. Pharmaceutics 13.

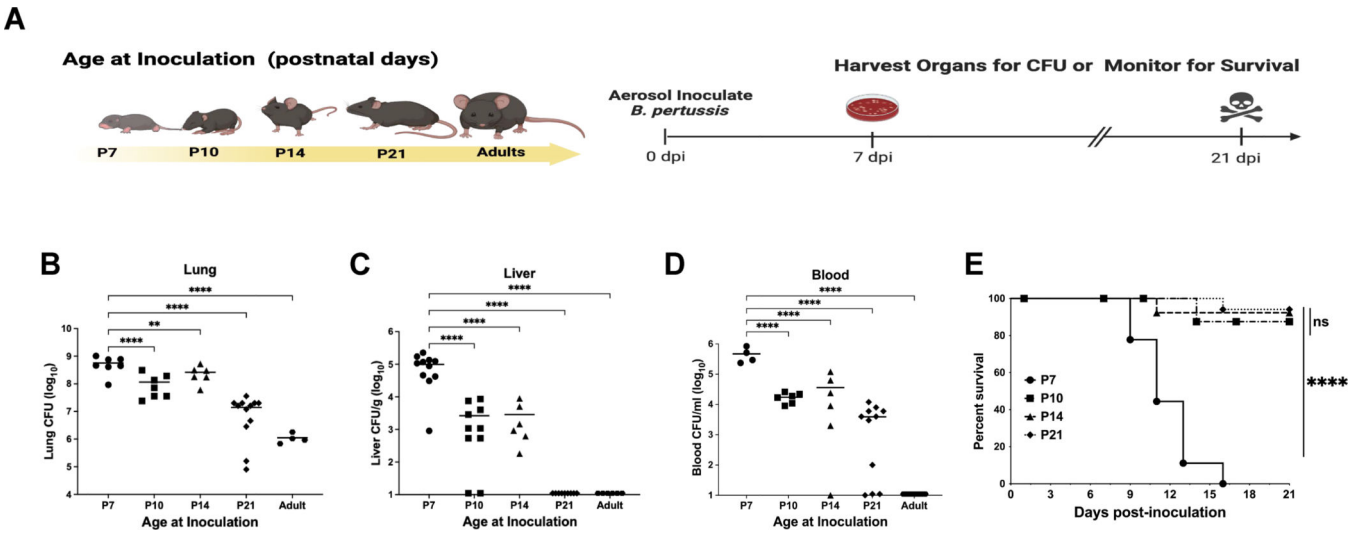


Figure 1:

Resistance to *Bordetella pertussis* develops with age. (A) Schematic outline of the experimental design. C57BL/6 litters at indicated postnatal (P) ages or 6-to-8-week-old (adult) mice were inoculated with *B. pertussis*, and at 7 days post-inoculation (dpi), some mice were euthanized, and organs were harvested. Other mice were monitored for survival until 21 dpi. (B-D) Colony forming units (CFU) were quantified from (B) lungs, (C) liver, and (D) blood at 7 dpi in mice inoculated at the indicated age. 4–12 mice/group. Statistical significance was analyzed using Ordinary One-way ANOVA with Dunnett's multiple comparisons tests. *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.
(E) Survival of mice inoculated with *B. pertussis* at the indicated ages up to 21 dpi—survival curves of represented groups from a single replicate of experiments performed at least two times. Survival curve significance was determined by Log-rank (Mantel-Cox) test. ****p≤0.0001.

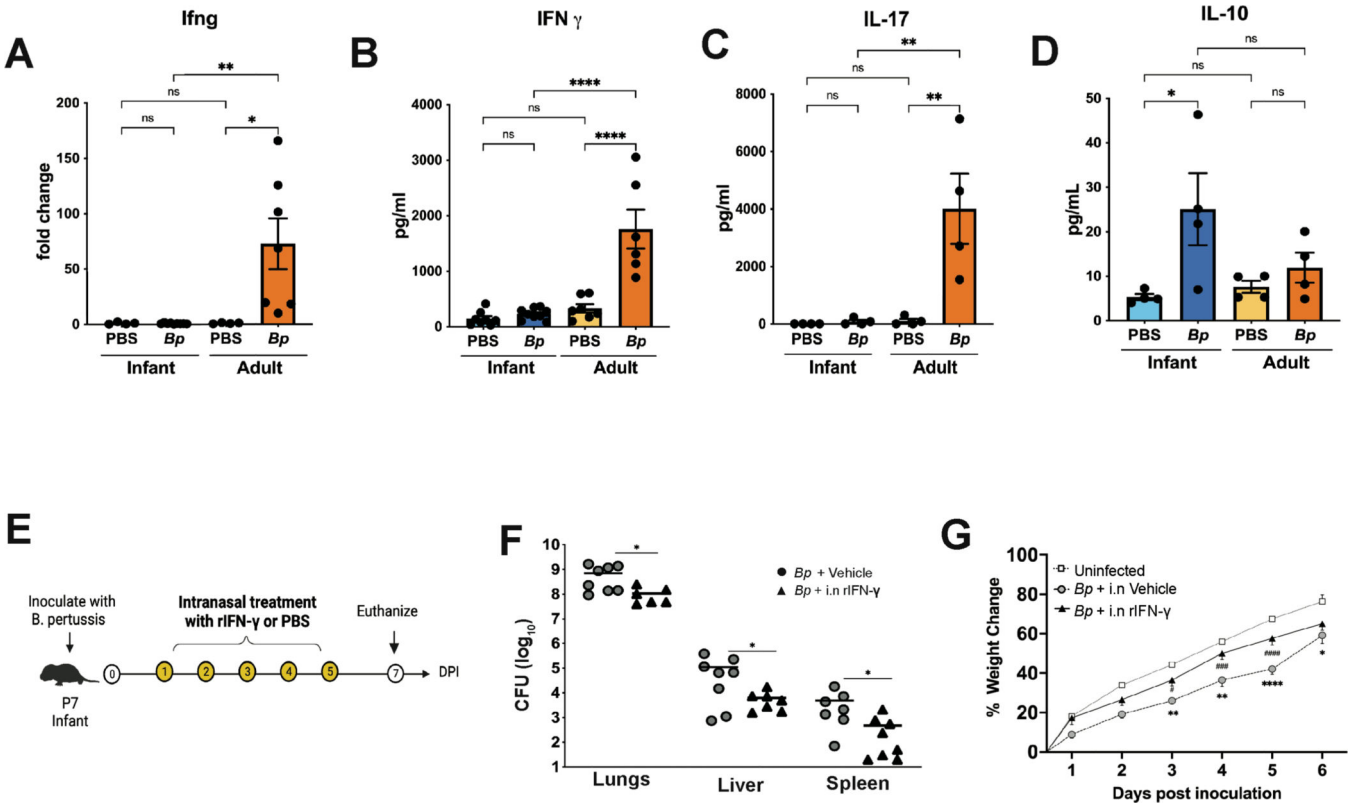


Figure 2:

IFN-γ is not produced, and intranasal treatment with recombinant murine interferon-gamma (rIFN-γ) improves bacterial clearance and ameliorates disease in *B. pertussis*-infected infant mice. (A-D) P7 infant and adult mice were inoculated with *B. pertussis* (Bp) or sham-inoculated with PBS. At 7 dpi, mice were euthanized, and lungs were harvested and processed for qRT-PCR or ELISA. (A) *Ifng* transcript upregulation (fold change versus sham-inoculated mice), (B) IFN-γ protein levels, (C) IL-17A protein levels, and (D) IL-10 protein levels were quantified from lung homogenates from the indicated experimental groups. (E) Schematic of the experiment. P7 infant mice were aerosol inoculated with *B. pertussis* (0 dpi), and from 1–5 dpi, mice received 60 ng/g rIFN-γ or PBS (vehicle) control via intranasal administration. At 7 dpi, mice were euthanized, lungs and systemic organs were harvested, and *B. pertussis* CFU counts were determined. (F) Bacterial burden in the lungs, liver, and spleen of *B. pertussis* infected treated and control mice (n=9 per group) at 7 dpi. Individual mice are represented as a single data point pooled from two individual experiments and presented as mean ± SEM. (G) Percent body weight changes relative to initial body weight at inoculation (0 dpi) up to 6 dpi in uninfected mice or *B. pertussis*-infected mice treated intranasally with rIFN-γ or PBS (vehicle) control. Statistical significance was analyzed using Ordinary One-way ANOVA with Šídák's multiple comparisons test. p >0.05 ns (not significant), *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.

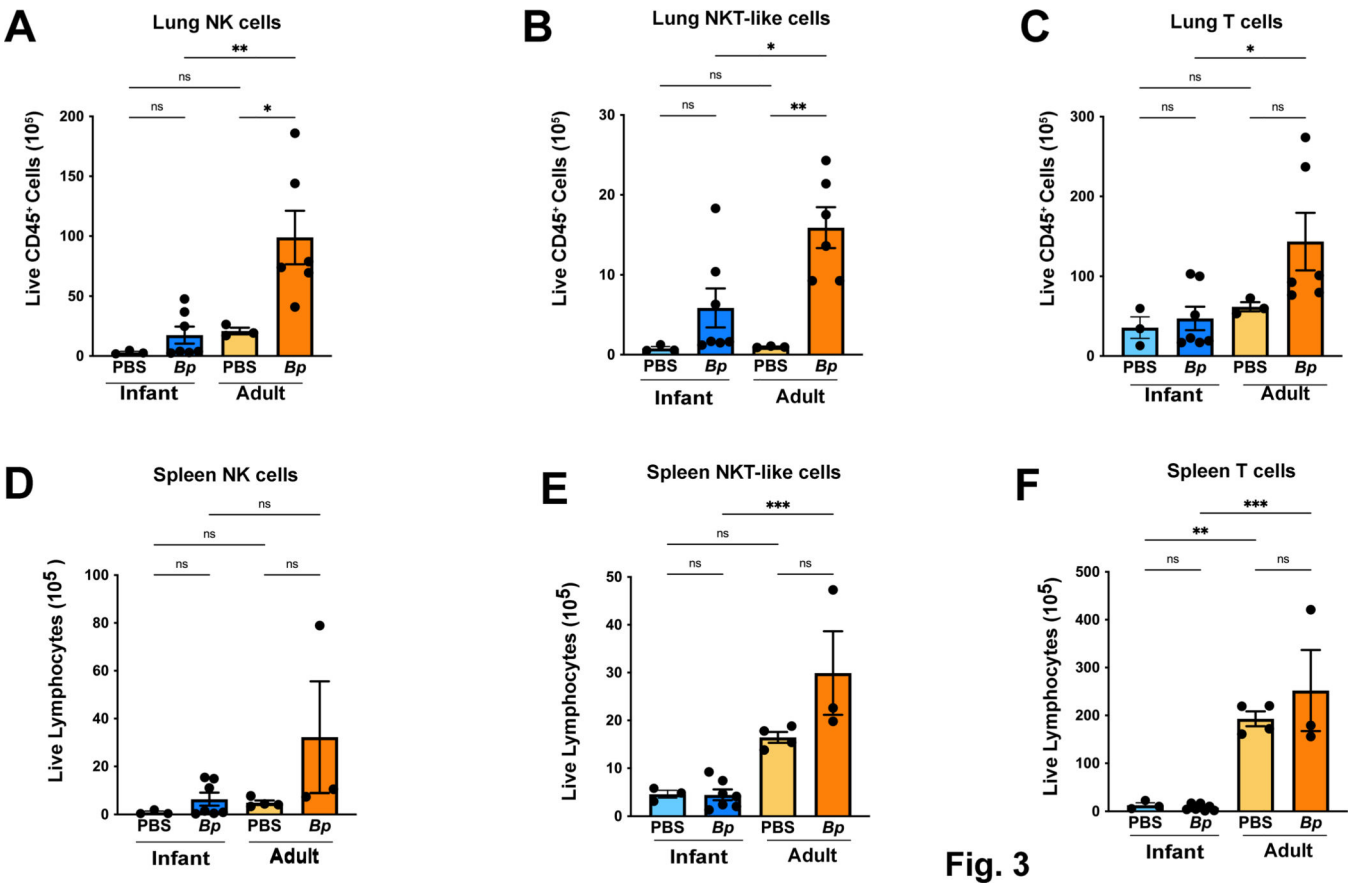


Fig. 3

Figure 3:

Infant mice NK cells do not significantly expand during *B. pertussis* infection. P7 infant and adult mice were inoculated with *B. pertussis* or PBS sham inoculum. At 7 dpi, lungs and spleens were harvested for flow cytometry. Top, the numbers of (A) NK cells, (B) NKT-like cells, or (C) T cells are based on the total number of Live CD45 cells in the lungs. Bottom, the numbers of (D) NK cells, (E) NKT-like cells, or (F) T cells are based on the total number of live lymphocytes in the spleen. Symbols represent individual mice (n = 3–7 mice/group), and bars indicate mean ± SEM. Statistical significance was analyzed using Ordinary One-way ANOVA with Tukey's multiple comparisons test. *p≤0.05, ***p≤0.001, ****p≤0.0001.

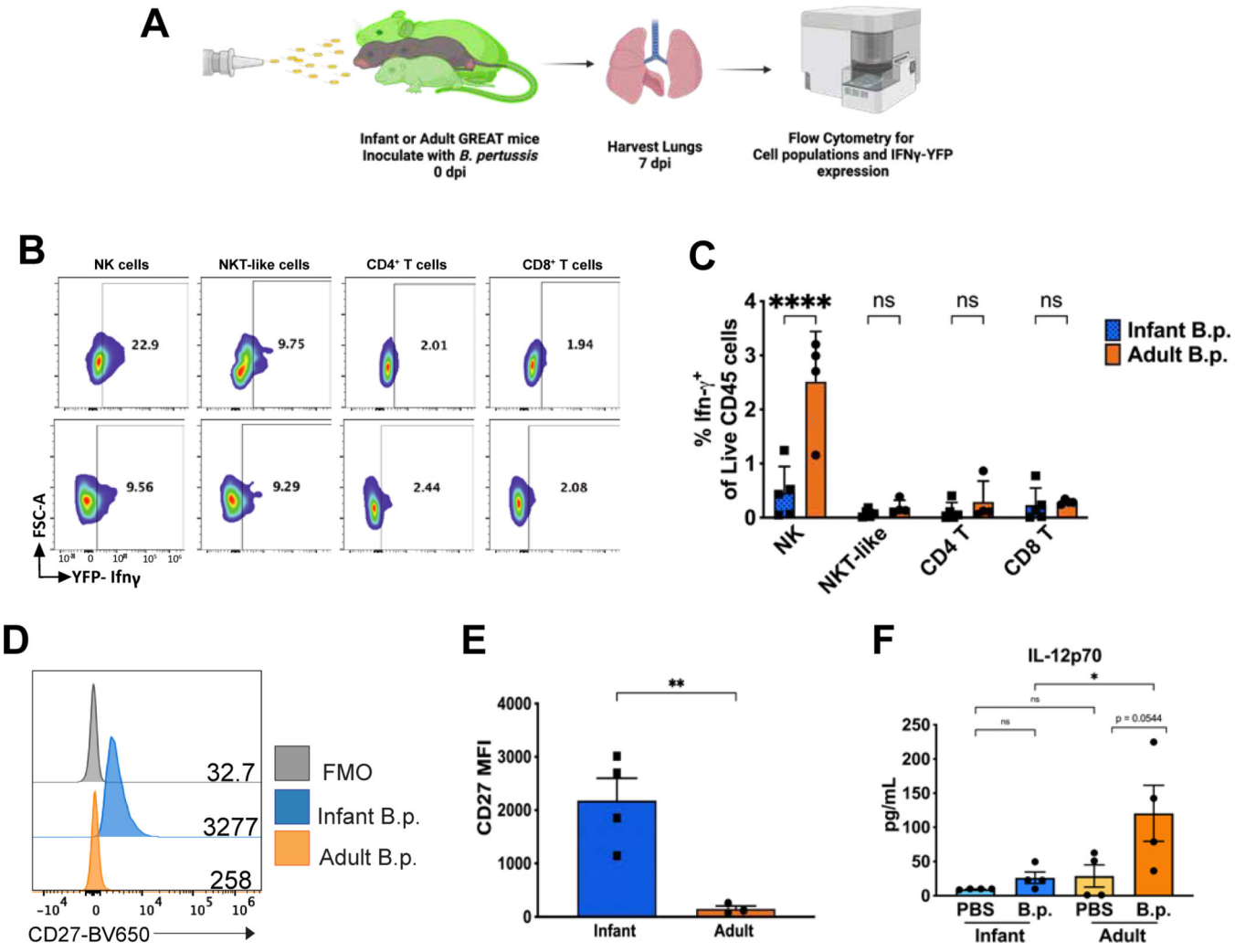


Figure 4:

Infant mice NK cells produce less IFN-γ than adult NK cells during *B. pertussis* infection and have an immature phenotype. (A) Schematic outline of the experimental design. P7 infant and adult GREAT (IFN-γ-YFP reporter) mice were inoculated with *B. pertussis* or PBS, and lungs were harvested for flow cytometry and other assays at 7 dpi. (B) Flow cytometry analysis showing the percentage of IFN-γ-expressing cells among live $CD45^+$ NK, NKT-like, $CD4^+$, and $CD8^+$ T cells in adult mice (upper panels) and infant mice (lower panels). Plots are from single mice and are representative of 4–5 mice per group. (C) Graphed data corresponding to results in (B) from all mice (n = 4–5 mice per group). (D) Histogram showing expression of CD27 on lung NK cells from *B. pertussis*-infected infant and adult mice. (E) Mean fluorescence intensity of CD27 expression on lung NK cells from *B. pertussis*-infected infant and adult mice. (F) Production of lung IL-12p70 in *B. pertussis*-infected and PBS sham-inoculated infant and adult mice. (C, E, and F) Symbols represent individual mice (n = 4–5 mice/group), and bars indicate mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001, Ordinary One-way ANOVA with Tukey's multiple comparisons test.

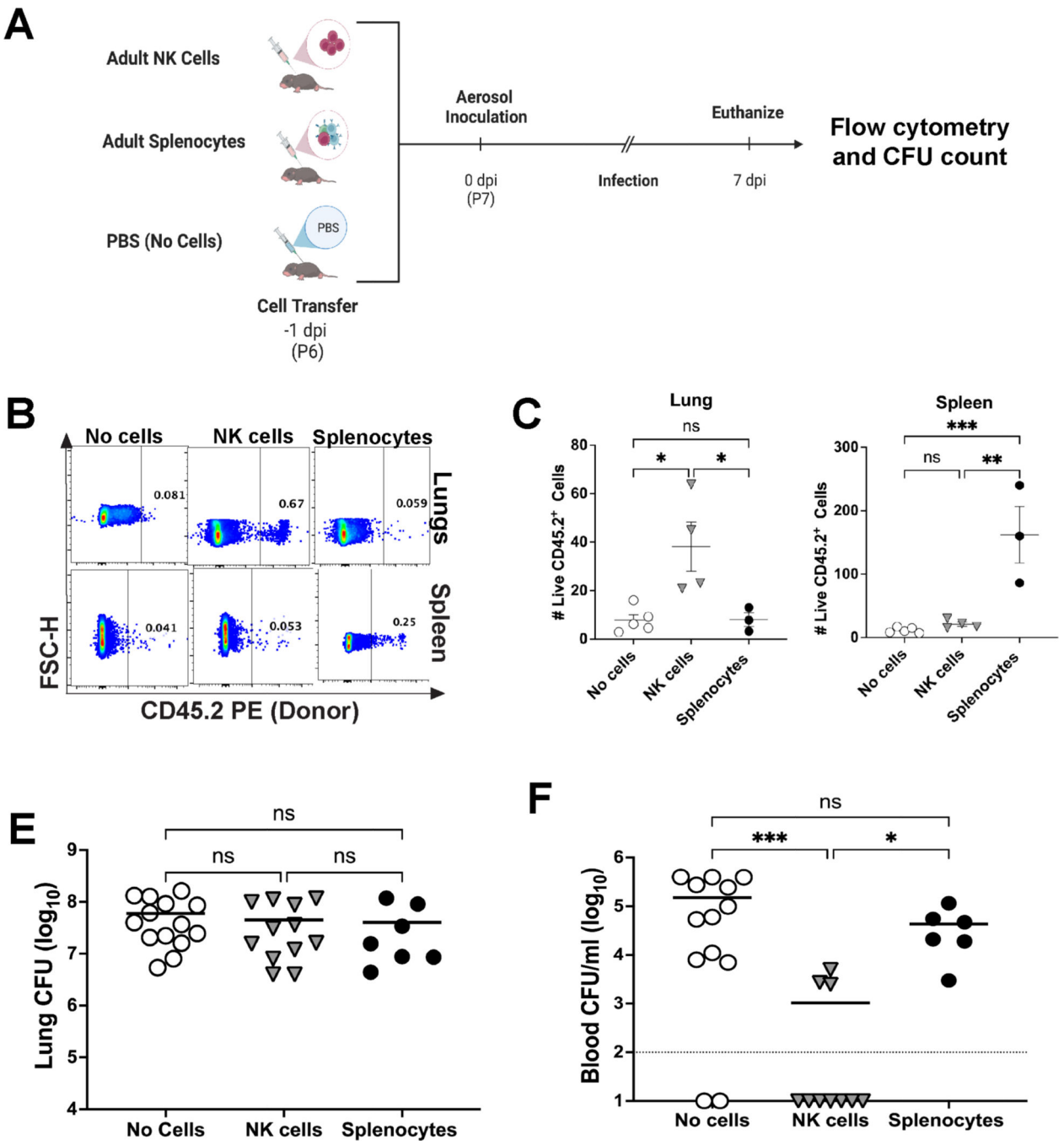


Figure 5:

Adoptive transfer of NK cells from adult mice to infant mice reduces bacterial dissemination during *B. pertussis* infection. (A) Schematic outline of the experimental design. Purified splenic NK cells or whole splenocytes from adult CD45.2 mice (or PBS control) were adoptively transferred to infant CD45.1 mice at P6. At P7, mice were inoculated with *B. pertussis* and euthanized at 7 dpi, and tissue was harvested for flow cytometry and bacterial CFU assessment. (B) Flow cytometry analysis of the percentage of donor CD45.2$^+$ cells in the lungs or spleen of infant mice receiving no cells, NK cells, or whole splenocytes

from adult mice. (C) Number of donor CD45.2$^+$ cells in the lungs or spleen of infant mice receiving no cells, NK cells, or whole splenocytes from adult mice (n = 3–5 per group). (D, E) Bacterial burden in the lungs (D) and blood (E) of *B. pertussis*-infected infant mice receiving no cells, NK cells, or whole splenocytes from adult mice at 7 dpi (n = 7–14 per group). (C, D, and E) Symbols represent individual mice, and bars indicate mean ± SEM. *p≤0.05, **p≤0.01, ***p≤0.001, Ordinary One-way ANOVA with Tukey's multiple comparisons test.