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Intestinal Commensal Bacteria Mediate Lung Mucosal Immunity and Promote Resistance of Newborn Mice to Infection

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Abstract

Immature mucosal defenses contribute to increased susceptibility of newborn infants to pathogens. Sparse knowledge of age-dependent changes in mucosal immunity has hampered improvements in neonatal morbidity due to infections. Here, we report that exposure of neonatal mice to commensal bacteria immediately after birth is required for a robust host defense against bacterial pneumonia, the leading cause of death in newborn infants. This crucial window was characterized by an abrupt influx of interleukin (IL)-22 producing group 3 innate lymphoid cells ($IL22^+ILC3$) into the lungs of newborn mice. This influx was dependent on sensing of commensal bacteria by intestinal mucosal dendritic cells. Disruption of postnatal commensal colonization or selective depletion of dendritic cells interrupted the migratory program of lung IL-22^+ILC3 and made the newborn mice more susceptible to pneumonia, which was reversed by transfer of commensal bacteria after birth. Thus, the resistance of newborn mice to pneumonia relied on commensal bacteria-directed ILC3-influx into the lungs, which mediated IL-22-dependent host resistance to pneumonia during this developmental window. These data establish that postnatal colonization by intestinal commensal bacteria is pivotal in the development of lung defenses in mice.

Graphical abstract

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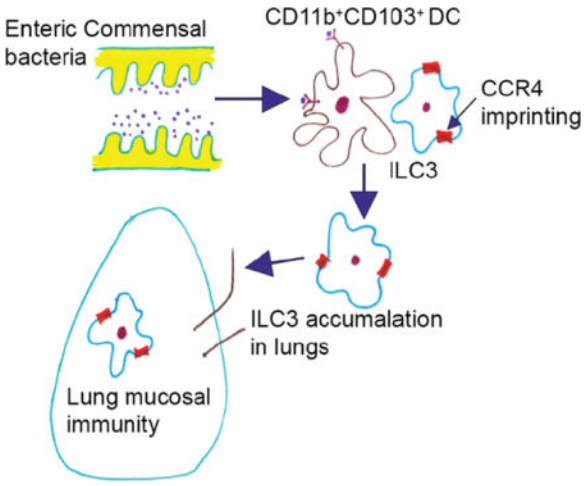

Introduction

Development of the immune system requires a sequential series of timed and coordinated events that begin early in fetal life and continue through the early postnatal period (1). Disruption of immune development during the early neonatal period results in abnormal postnatal immune responses that are more dramatic and persistent than those after disruption during adult life, highlighting the importance of the neonatal period as a critical developmental window (2). While several host genetic and environmental factors modulate the development of the immune system during fetal and early postnatal life (3), few are as important as the continued interaction with commensal bacteria, which is not only the most intimate environmental exposure (4, 5), but also represents a challenge to the developing immune system (6, 7).

Commensal colonization, which begins at birth, progresses through a choreographed succession of bacterial species and evolves rapidly during the first month of life (8). These evolving microbial signals are hypothesized to play a critical role in the functional programming of immune cells. Modern childbirth practices like caesarean deliveries (9) and increased use of antibiotics in early life (10) not only alter the pattern of intestinal commensal colonization in the newborn, but are also associated with increased risk of sepsis and pneumonia (10–14), suggesting that intestinal commensal bacteria can promote the resistance of newborn infants to pneumonia. The interaction between host and the intestinal commensal bacteria extends beyond the local enteric environment and influences immune homeostasis at peripheral sites, exemplified by intestinal complications during respiratory disease and vice versa (15, 16). Nevertheless, the mechanistic basis of cross talk between the intestinal commensal bacteria and innate lung defense, the so-called gut-lung axis, remains poorly defined (17) and the developmental pathways underlying the association between commensal colonization in the early postnatal period and development of lung immunity in newborns remain unexplored.

Here, we show that interactions between host and the intestinal commensal bacteria shape the repertoires of immune cells in the newborn mouse lung and importantly directs the

postnatal ontogeny of IL-22 producing type 3 innate lymphoid cells (ILC3), a group of sentinel cells that maintain homeostasis at mucosal barrier sites. This postnatal influx of IL-22^{+}ILC3 promotes the resistance of neonatal mice to pneumonia. This crosstalk is mediated by mucosal dendritic cells (DC), which capture signals from intestinal commensal bacteria. Disruption of commensal bacteria interrupted the migratory program of ILC3, impairing their ability to traffic to the lungs and rendering the newborn mice more susceptible to pneumonia, which was reversed by exogenous IL-22 or through adoptive transfer of ILC3. Reconstitution of intestinal commensal bacteria restored the expression of CCR4 on the ILC3, restored the ability of ILC3 to migrate into the lungs and promoted IL-22 dependent resistance to pneumonia in newborn mice.

Results

Postnatal colonization by commensal bacteria promotes resistance to pneumonia in newborn mice

Prior epidemiological studies show that human infants whose mothers received frequent antibiotics before birth, or who were delivered by Caesarean section, not only had altered intestinal commensal bacteria (18, 19), but also had increased risk of developing pneumonia (20, 21). This led us to hypothesize that early life exposure to commensal bacteria promotes resistance to pneumonia in newborns. To test this hypothesis, we exposed pregnant mouse dams to a combination of ampicillin, gentamicin, and vancomycin, three commonly used antibiotics in pregnant women and human newborns (22)(Fig. 1A), beginning 5 days before delivery. Antibiotics were discontinued immediately after birth and newborn mice were challenged intratracheally with *Streptococcus pneumoniae* (*S. pneumoniae*) serotype 19 A, a leading cause of pneumonia in human newborns (23).

This early life antibiotic exposure reduced not only the total number of commensal bacteria (Fig. 1B) but also disrupted the succession of bacterial species in the intestine of newborn mice (Fig. S1A,B, Table S1A). Six hours post infection, we observed an increased bacterial load in the lungs and the bronchial lavage (BAL) fluid (Fig. S1C) and increased susceptibility in newborn mice whose dams were exposed to antibiotics (ABX-exposed) as compared to age-matched mice whose dams were not exposed to antibiotics (ABX-free) (Fig. 1C). Germ-free (GF) mice, which lack commensal bacteria, similarly were more susceptible to challenge with *S. pneumoniae* as compared to the age-matched conventionally raised (CNV) mice (Fig. 1D). We paralleled these observations using *Escherichia coli* K1 or *Candida albicans*, other common causes of pneumonia in newborns (Fig. S1D,E) (24). Since disruption of commensal bacteria in infancy is associated with increased susceptibility to inflammatory disorders like allergen-induced airway hyperreactivity (25) and colitis (26) in later life, we ascertained whether disruption of postnatal commensal colonization led to durable changes in host resistance to infection. We found that increased susceptibility to pneumonia after early life ABX-exposure persisted beyond the neonatal period, until at least four weeks of age (Fig. 1E). This persistence in susceptibility contrasted with the transient susceptibility to infection that occurs in ABX-exposed adult mice (27, 28), highlighting the critical nature of commensal exposure in early life.

We reversed the commensal disruption in ABX-exposed newborns by transferring intestinal contents from a newborn mouse in the early postnatal period as done previously (Fig. 1A) (29). Reconstitution of intestinal commensal bacteria restored resistance to pneumonia in ABX-exposed and GF newborn mice (Fig. 1C,D). This protection against *S. penumoniae* persisted beyond the neonatal period. ABX-exposed mice that received intestinal contents in the early postnatal period likewise showed increased resistance to infection at least for as long as four weeks after birth compared to their littermates that did not receive intestinal bacterial reconstitution (Fig. 1E,F).

Whether lung-resident commensal bacteria educate the mucosal immune system, like the intestinal commensal bacteria, remains a source of controversy (30, 31) and the effect of early life antibiotics on lung commensal colonization in human newborns remains unexplored. We found no difference in the composition of lung commensal bacteria in ABX-free and ABX-exposed mice (Fig. S1F,G, Table S1B), perhaps related to our experimental strategy of limiting ABX-exposure to the pregnant dams and not the newborn mice. Lack of differences in lung-resident commensal bacteria in the ABX-exposed newborn mice as compared to ABX-free newborns suggest that intestinal commensal bacteria rather than lung commensals mediate the resistance to pneumonia, although this possibility cannot be completely excluded.

Postnatal colonization by commensal bacteria promotes interleukin (IL)-22 dependent mucosal defenses in newborn mice

We hypothesized that disruption of commensal colonization mediated changes in the expression of genes related to various aspects of innate lung defense. We carried out RNA sequencing analysis of lung mucosal RNA isolated from newborn mice on day 0-4. Unsupervised analysis revealed consistent transcriptional changes in ABX-exposed newborns as compared to ABX-free murine newborns (Fig. S1H, Table S2). Differentially expressed genes included interleukin (IL)-22, a cytokine critical in lung epithelial repair (Fig. S1H) (32, 33) and host defense against pathogens (32, 34, 35). We found decreased concentrations of IL-22 in the BAL fluid of ABX-exposed or GF newborn mice as compared to ABX-free newborn mice (Fig. 1F). We confirmed these observations in human newborns, finding reduced concentrations of IL-22 in the BAL fluid from human newborns exposed to prolonged duration of ABX (Fig. 1G, Table 1).

Reconstitution with intestinal contents from age-matched neonatal mice restored IL-22 levels in BAL fluid of ABX-exposed or GF newborn mice (Fig. 1G). Similarly, treatment with recombinant IL-22 intratracheally restored host resistance to pneumonia in ABX-exposed newborn mice (Fig. 1H). To interrogate the importance of IL-22 in newborn's resistance to pneumonia, we blocked IL-22 signaling with an IL-22 neutralizing antibody (36). Treatment of newborn mice with an anti-IL-22 antibody blocked the restoration of host resistance in ABX-exposed newborn mice after reconstitution of intestinal commensal bacteria (Fig. 1I). IL-22 acts via a transmembrane receptor complex that consists of IL-22R1, a receptor subunit that is shared by related cytokine IL-20 (37). We found no difference in concentrations of IL-20 in BAL fluid of ABX-exposed or GF newborn mice compared to ABX-free newborn mice (Fig. S1I). Blockade of IL-20 signaling by treatment

with a neutralizing antibody directed against IL-20 (38) did not block restoration of host resistance in ABX-exposed newborn mice after reconstitution of intestinal commensal bacteria (Fig. S1J) (36). These findings demonstrate a central and non-redundant role for IL-22 in host defense against pneumonia (32, 33) and importantly, implicate IL-22 as a critical mediator by which commensal bacteria promote resistance to pneumonia in newborn mice . IL-22 bioactivity is negatively regulated by IL-22-binding protein (IL-22BP), a secreted receptor that binds to soluble IL-22 with higher affinity than IL-22R1 and functions as an antagonist (39). We did not evaluate the role of endogenous IL-22BP in our study. Several lung resident immune cells are known to secrete IL-22BP(40), but the role of endogenous IL-22BP in pulmonary host defense remains unclear and may represent an additional regulatory layer in the ontogeny of lung defense in the newborn.

Disruption of commensal bacteria in the early postnatal period leads to durable changes in the repertoire of IL-22 producing immune cells in the lungs of newborn mice, contributing to increased susceptibility to pneumonia

The identity of the IL-22-producing cells in the newborn mouse lung is unknown. We found that neither neutrophils ($CD45^+Ly6G^+$) nor macrophages ($CD45^+F4/80^+$) nor T cells ($CD45^+CD4^+$) were a significant source of IL-22 in the lungs of newborn mice (Fig. 2A). The majority of IL-22 producing cells in the murine newborn lung were lineage negative ($CD45^+CD3^-CD8^-CD11b^-CD19^-MHCII^-F4/80^-CD161^-Ly6G^-$) lymphocytes. We further characterized these lineage-negative lymphocytes based on expression of surface markers CD4, CD117, CD127, NkP46 or CCR6 and transcription factors RORγt, T-Bet or Eomes. More than 90% of IL-22 producing cells were lineage negative ($CD3^-CD8^-CD11b^-CD19^-MHCII^-F4/80^-CD161^-Ly6G^-F4/80^-$) lymphocytes expressing surface markers NKp46, CCR6, CD117 and transcription factor RORγt identifying them as ILC3 lymphoid cells (Fig 2A, S2A).

We tested these observations in human newborns. ILC3 ($CD45^+CD3^-CD8^-CD14^-CD19^-CD69^-$ $ROR\gamma t^+$), but not neutrophils ($CD45^+CD3^-CD8^-CD19^-CD69^+$), NK cells ($CD45^+CD3^-CD8^-CD19^-CD56^+$), $CD4^+$ T cells ($CD45^+CD3^+CD4^+$) or $CD8^+$ T cells ($CD45^+CD8^+$) were a primary source of IL-22 in the lungs of human newborns (Fig. 2B, S2B). These findings illustrate an important difference in the cellular sources of IL-22 in the lung of newborn humans compared to adult humans, as several groups have reported that NK cells (41), Th17 (42) and γδ T cells (43) are the principal sources of IL-22 in adult human lungs.

ILC3 lymphoid cells developmentally depend on RORγT and continuously express this transcription factor (44, 45). Therefore, to interrogate the importance of ILC3 in the resistance of newborn mice to pneumonia, we bred transgenic mice expressing cre recombinase under the control of the RORγt promoter (46) with transgenic mice expressing inducible diphtheria toxin receptor (iDTR) (47) to generate $Ror\gamma t^{DTR}$ mice. Treatment of newborn $Ror\gamma t^{DTR}$ mice with diphtheria toxin (DT) decreased the number of ILC3 in the lungs (Fig. 2C, S2C), reduced IL-22 in BAL fluid (Fig. S2D) and made the DT-treated $Ror\gamma t^{DTR}$ newborn mice more susceptible to pneumonia (Fig. 2D). Adoptive transfer of lung ILC3 restored host resistance to pneumonia in newborn $Ror\gamma t^{DTR}$ mice treated with

DT (Fig. 2D). Together, these data confirm that IL-22$^+$ILC3 are necessary and sufficient in promoting host resistance to pneumonia in newborn mice (48).

We sought to determine if disruption of commensal colonization alters the repertoire of IL-22 producing cells in the newborn mouse lung. We found significantly decreased ($P<0.01$) numbers of IL-22$^+$ILC3 (Fig. 2E–G) but not neutrophils or T cells or NK cells (for all, $P>0.05$) (Fig. S2E) in the lungs of ABX-exposed or GF newborn mice as compared to ABX-free newborn mice. The decrease in the numbers of IL-22$^+$ILC3 persisted beyond the newborn period till at least four weeks of life (Fig. 2E). We confirmed these observations in human newborns and found significantly decreased numbers of lung IL-22$^+$ILC3 in the BAL fluid of human newborns exposed to prolonged duration of antibiotics (Fig. 2H). We then questioned if reversing the commensal disruption would correct the immune alterations in ABX-exposed newborn mice. We found that reconstitution with commensal bacteria restored the numbers of IL-22$^+$ILC3 in the lungs of ABX-exposed or GF newborn mice (Fig. 2F,G), although individual IL-22 expression did not change (Fig. S2F). These data illustrate that disruption of commensal bacteria in early postnatal development alters the repertoire of IL-22-producing cells in the newborn lungs.

Commensal bacteria direct the postnatal trafficking of IL-22$^+$ILC3 in the murine newborn lung

We tested whether reduced numbers of IL-22$^+$ILC3 in the lungs of GF or ABX-exposed newborn mice could be explained by differences in proliferation or apoptosis of IL-22$^+$ILC3. We assessed cell proliferation or apoptosis by quantifying the number of IL22$^+$ILC3 lymphoid cells expressing Ki67 or annexin, respectively. We found that an increased number of ILC3 in the lungs was not due to changes in proliferation or apoptosis (Fig. S2G). We, therefore, hypothesized that a decrease in the absolute numbers of IL-22$^+$ILC3 in ABX-exposed newborn mice was due to a reduced ability of ILC3 from ABX-exposed newborns to traffic preferentially to the lungs. To test this, we used a competitive trafficking assay (49) to determine the advantage of ILC3 isolated from ABX-free newborn mice to traffic to the lungs as compared to ILC3 from ABX-exposed newborn mice. We found that ILC3 from ABX-exposed newborn mice had decreased ability to traffic selectively into the lungs, but not the spleen or small intestine as compared to ILC3 isolated from ABX-free newborn mice (Fig. 2I,J).

We then asked if reversing the commensal disruption would restore the ability of ILC3 to traffic to the lungs. We similarly determined the advantage of ILC3 isolated from ABX-exposed newborn mice that had received transfer of commensal bacteria to traffic to the lungs as compared to ILC3 from ABX-exposed newborn mice that had received no such transfer. Reconstitution of commensal bacteria restored the ability of ILC3 from ABX-exposed newborns to traffic selectively to the lungs (Fig. 2I,J). Tissue-selective ILC3 trafficking has been described for the small intestine and secondary lymphoid tissues (50, 51). Our data unveils a role for intestinal commensal bacteria in selective trafficking of ILC3 into the lungs.

Commensal bacteria modulate expression of CCR4 on ILC3 lymphoid cells and direct their postnatal trafficking into lung

Chemokines control the trafficking and positioning of immune cells and are critical for development and recruitment of immune cells in disease (52). We first identified the repertoire of chemokine receptors on IL22^{+}ILC3 from the lungs or small intestine (SI) of newborn mice. We found that C-C chemokine receptor (CCR) 4 was highly expressed by a majority of IL-22^{+}ILC3 from the newborn murine lung but not from the newborn murine SI (Fig. S3A). We found no difference in expression of CCR6, 7, 9 or C-C chemokine ligand (CCL) 20 nor C-X-C chemokine receptor (CXCR) 3 or 5 on IL-22^{+}ILC3 from the newborn lung or SI (Fig. S3A). Tissue-selective ILC3 trafficking has been described for the intestine (50, 51), but not for the lungs. Like the intestine, the lung has a large mucosal surface, which is in continuous contact with the environment and therefore could potentially benefit from lung-selective ILC3 trafficking.

We hypothesized that exposure to commensal bacteria in early life may modulate the expression of lung-specific homing receptors on IL-22^{+}ILC3 lymphoid cells and thus increase their ability to traffic to the lungs. We examined the numbers and frequencies of CCR4 expressing IL22^{+}ILC3 in the lungs of ABX-exposed or ABX-free newborn mice. We found that the majority of IL22^{+}ILC3 from the lungs of ABX-free newborn mice were CCR4^{high} as compared to the lung ILC3 from ABX-exposed mice or GF mice, which were CCR4^{low} (Fig. 3A). We found no difference in expression of CCR6, 7, 9 CCL20, CXCR3 or CXCR5 on IL22^{+}ILC3 from the lungs of ABX-free mice or ABX-exposed mice (Fig. 3A). CCR4 is critical for homeostatic trafficking of T lymphocytes (53) and Tregs (54) into the lungs. CCL17, one of the ligands for CCR4 is expressed by the lung epithelium (55). We therefore hypothesized that commensal bacteria use a similar mechanism to direct trafficking of ILC3 into the newborn lungs. To test this hypothesis we determined the capability of ILC3 isolated from newborn mice lacking CCR4 (*Ccr4*$^{-/-}$) to traffic to the lungs as compared to ILC3 from age-matched wild-type littermates. We found that ILC3 from newborn *Ccr4*$^{-/-}$ mice had decreased ability to traffic into the lungs as compared to ILC3 isolated from wildtype littermates (Fig. 3B). Furthermore, the newborn *Ccr4*$^{-/-}$mice were more susceptible to pneumonia compared to wildtype littermates (Fig. 3C). We then asked if an adoptive transfer of wildtype ILC3, which express CCR4 and therefore traffic into the lungs, could improve host resistance in newborn *Ccr4*$^{-/-}$mice. We found that adoptive transfer of ILC3 from wildtype newborn mice to age-matched *Ccr4*$^{-/-}$mice restored host resistance to pneumonia (Fig. 3C). These data illuminate a role for CCR4 in trafficking of ILC3 into the lungs and promoting newborn's resistance to pneumonia. Nevertheless, the origin of ILC3 that traffic to the lungs is unknown. ILC3 are concentrated within the SI (51), and the spatial proximity of ILC3 with commensal bacteria in the small intestine supports the notion that ILC3 in intestinal mucosa may be directed by the commensal bacteria to traffic to the lung.

CD103^{+}CD11b^{+} DC capture antigen from commensal bacteria and induce expression of CCR4 on ILC3

We sought to identify the mechanisms by which intestinal commensal bacteria induce the expression of CCR4 on ILC3. Murine ILC3 do not express pattern recognition receptors and

therefore are unlikely to directly sense the commensal bacteria (56). Mononuclear phagocytes like DC and macrophages, not only detect a range of microbial signals (57), but can also cross talk with ILC3 in the intestine (58). We found that the majority of mononuclear phagocytes in the intestine of newborn mice were $CD45^+CD11b^+CD103^+F4/80^-$ cells ($CD103^+CD11b^+$ DC) (Fig. 3D), consistent with previous findings (59). Transcription factor ZBTB46 is selectively expressed by $CD103^+$ $CD11b^+$ DCs and their committed progenitors but is not expressed by monocytes, macrophages, or other lymphoid or myeloid lineages (60, 61). We used mice that express diptheria toxin receptor (DTR) under the control of Zbtb46, (*Zbtb46*^{DTR}), which allows for the depletion of $CD103^+$ $CD11b^+$ DC (60) after treatment with DT. Twenty-four hours later, there was a decreased number of intestinal $CD11b^+CD103^+$ DCs (Fig. S3C). Depletion of $CD103^+CD11b^+$ DCs was associated with a decrease in IL-22 in the BAL (Fig. S3D), reduced numbers of ILC3 in the lungs and increased susceptibility to pneumonia of DT-treated *Zbtb46*^{DTR} newborn mice (Fig. 3E,F). Adoptive transfer of $CD103^+$ $CD11b^+$ DC into age-matched newborn *Zbtb46*^{DTR} mice treated with DT ($CD103^+CD11b^+$ DC-depleted newborn mice) restored the number of lung ILC3 and improved the resistance of newborn mice to pneumonia (Fig. 3E,F).

There is increasing evidence that intestinal DCs act as conductors of ILC traffic to the intestine and secondary lymphoid tissue (50). We tested whether $CD103^+CD11b^+$ DCs induced CCR4 expression on $IL\text{-}22^+ILC3$. We co-cultured $CD103^+CD11b^+$ DCs isolated from the small intestine of ABX-free or ABX-exposed newborn mice with ILC3 isolated from the lungs of ABX-free or ABX-exposed newborn mice. $CD103^+CD11b^+$ DCs isolated from the small intestine of ABX-free newborn mice were more efficient than $CD103^+CD11b^+$ DCs isolated from the small intestine of ABX-exposed newborn mice at inducing CCR4 expression on $IL\text{-}22^+ILC3$ (Fig. 3G,H) and restoring the ability of lung ILC3 from ABX-exposed animals to migrate in response to the CCR4 ligand, CCL17 *in vitro* (Fig. 3I). We found no change in expression of CCR6, 7, 9 CCL20, CXCR3 or CXCR5 (Fig. 3G). Conversely, co-culture of $CD11b^+CD103^-$ DCs isolated from small intestine of either ABX-free or ABX-exposed newborn mice did not change the CCR4 expression on $IL\text{-}22^+ILC3$ (Fig. S3E), suggesting $CD11b^+CD103^-$ DCs were not as efficient as $CD11b^+CD103^+$ DCs at modulating CCR4 expression on $IL\text{-}22^+ILC3$. These observations are consistent with other reports (62–64), which suggest that distinct DC subsets respond differentially to similar environmental cues.

Finally, to test the hypothesis that $CD103^+CD11b^+$ DCs capture antigen from commensal bacteria and direct the trafficking of ILC3 into the lungs, we treated newborn *Zbtb46*^{DTR} mice with DT before reconstitution with commensal bacteria. Depletion of $CD103^+CD11b^+$ DCs abrogated the increase in ILC3 number in ABX-exposed newborn *Zbtb46*^{DTR} mice after reconstitution with intestinal commensal bacteria (Fig. 3J). Treatment of *Zbtb46*^{DTR} newborn mice with DT depleted the $CD103^+CD11b^+$ DCs in extra-intestinal sites including the lungs. $CD103^+CD11b^+$ DCs are exceedingly rare in the newborn murine lungs (65). Therefore intestinal $CD103^+CD11b^+$ DCs, but not lung resident $CD103^+CD11b^+$ DCs are likely to play a major role in selective trafficking of ILC3 to the lung. Since previous reports (66, 67) implicated IL-1β in crosstalk between intestinal mononuclear phagocytes and small intestine ILC3, we measured IL-1β in the supernatant from co-cultures of DCs and ILC3.

We found no difference in the IL-1β levels in co-culture supernatants of $CD103^+$ $CD11b^+$ DCs isolated from the small intestine of ABX-free or ABX-exposed newborn mice and co-cultured with ILC3, (Fig. S3F), suggesting that IL-1β may not be involved in cross-talk between DCs and ILC3.

Discussion

Distinct immune responses specifically adapted for fetal and early postnatal life render newborns more vulnerable to infection (1). Lack of understanding of immune development in early life contributes to our inability to reduce neonatal morbidity due to pneumonia, which unfortunately kills more newborns than any other cause. While a series of coordinated events control the development of a newborn's immune system (3), few are as important as the interaction with successive waves of commensal bacteria, which colonize the newborn's intestine immediately after birth (4, 5). Disruption of commensal colonization in the critical window of the early postnatal period has enduring consequences for the developing immune system, exemplified by increased risk of inflammatory disorders like asthma and increased risk of respiratory infections beyond infancy in those infants exposed to prolonged antibiotic treatment or delivered by caesarean section (10–14). To develop therapeutic interventions to decrease morbidity in the newborn period and beyond, we need to better understand the role of commensal colonization in the development of the newborn immune system.

Prior studies investigating commensal bacteria-driven immune maturation have prioritized the use of GF mice (68). It is now accepted that the immune system while not immature in early life, differs fundamentally from immune responses in adults (1). Importantly, the newborn's intestine is colonized by successive waves of diverse commensal bacteria, and newborn's intestinal microbiota is fundamentally distinct from that of adult mice (29). Thus, commensal reconstitution studies in adult GF mice do not fully capture the complex interaction between the developing host and evolving microbiota. We exposed pregnant mouse dams to a combination of ABX, used commonly to treat mother and infants. This not only disrupted the sequential colonization of the newborn's intestine by different commensal bacteria but also made the newborn mice more susceptible to pneumonia, replicating the key observations from epidemiological studies (10–14). Perhaps more importantly, the susceptibility to pneumonia in ABX-exposed newborn mice persisted beyond the newborn period, in contrast to previous reports (28) (27), showing that ABX exposure in adult mice induced only transient susceptibility to infection. Reversing the commensal disruption restored the resistance to pneumonia in the ABX-exposed newborn mice and, importantly, this protection persisted beyond the newborn period. These results coupled with other recent reports (25, 26) highlight the importance of therapeutic interventions addressing commensal dybiosis in early life that have lasting consequences.

T cells (32) and NK cells (41) are primary mediators of innate mucosal defense against respiratory pathogens in adults. We found that at birth, the mouse lung is populated by a few $IL\text{-}22^+$ T cells or NK cells, but is home to a significant number of $IL\text{-}22^+$ ILC3 innate lymphoid cells. While $IL22^+$ILC3 maintain tissue homeostasis in the small intestine, the role of $IL22^+$ILC3 in lung mucosal defense remains a source of controversy (69). ILC3 were dispensable for protection against Influenza A pneumonia (70). Reports ascribing an

important role for ILC3 in lung immune homeostasis have used adult mice deficient in recombination activating gene **(***Rag) 2* or interleukin 2 receptor, gamma (*Il2rg)* (48, 71), which are profoundly immunodeficient, or treated with antiCD90.2 antibodies (35) resulting in nonspecific depletion of several immune cell types thus confounding interpretation of results from these animals. We, therefore, generated *Rorγt*^{iDTR} mice, a genetic tool to selectively deplete ILC3. By first showing that ILC3 depletion in early life rendered the newborn mice more susceptible to pneumonia, we were able to demonstrate that IL-22^{+} ILC3 were necessary to promote the resistance of newborn mice to pneumonia. Second, by restoring the resistance to pneumonia in ILC3 depleted-newborn mice after adoptive transfer of ILC3, we established that ILC3 were sufficient to promote newborn's resistance to pneumonia. One limitation of *Rorγt*^{iDTR} mice is potential depletion of the RORγt^{+} T cells after DT treatment. However, both human newborns (72) as well as murine newborns (73) are relatively lymphopenic compared to adults. Absolute numbers of IL22^{+} T cells were low in the newborn mice (Fig. 2B), suggesting that depletion of RORγt^{+} T cells in our *Rorγt*^{iDTR} mice may have had only a marginal effect on the newborn's resistance to infections.

Having shown that ILC3 played a critical role in promoting the resistance of newborn mice to pneumonia, we investigated the role of commensal bacteria in the postnatal development of lung IL22^{+}ILC3. Several groups have demonstrated that colonization with commensal bacteria increased the number of IL22^{+}ILC3 in the small intestine of adult GF mice (74, 75). Similarly, an increase in the number of IL-22^{+}ILC3 in the postnatal period has been described for the small intestine (76, 77) but not for the lung, raising the possibility that this phenomenon is restricted to the newborn's intestine. Our data challenge this assumption and illuminate a role for intestinal commensal bacteria in the postnatal accumulation of IL-22^{+}ILC3 in the lungs. We noted an abrupt increase in the number of IL-22^{+}ILC3 in the lungs immediately after birth and observed an age-dependent increase in the number of lung IL22^{+}ILC3, peaking at two weeks of age. This postnatal increase in lung IL22^{+}ILC3 was absent in GF or ABX-exposed newborn mice and was reversed by transfer of commensal bacteria in the early postnatal period. Previous studies have used transcriptional activation and cytokine production to delineate a role for commensal bacteria in the ontogeny of ILC3 (75, 76). But tissue selective-trafficking as a mechanism through which commensal bacteria direct the ontogeny of ILC3 in postnatal lungs has not been investigated. ILC3 are thought to establish residency in the developing intestine by tissue-specific migration (50, 51). We show that exposure to commensal bacteria in early life directs the lung-specific IL-22^{+} ILC3 trafficking and thus contributes to the postnatal accumulation of IL22^{+}ILC3 in the newborn lung. This study did not address the potential role of commensal bacteria in long-term maintenance of lung IL-22^{+}ILC3, which needs to be clarified in the context of recent reports that ILC homeostasis at peripheral sites is dependent primarily on self-renewal (78).

Small intestine-specific trafficking of ILC3 depends on expression of chemokine receptors CCR9 and α4β7 by ILC3 (50). In contrast, we found that lung-specific trafficking of IL-22^{+}ILC3 was mediated by CCR4, a homing receptor also used by other immune cells (79) for homeostatic trafficking into the lung. CCR4 is activated by chemokine CCL17 or CCL20. CCL17 is expressed by the lung epithelium (55). There is evidence that intestinal

DCs act as conductors of ILC3 traffic to the small intestine and secondary lymphoid tissue but not to the lungs (50, 51). We, therefore, evaluated the role of intestinal DCs in instructing IL-22$^+$ILC3 to traffic to the lung and found that intestinal CD103$^+$CD11b$^+$ DCs increased expression of the lung homing receptor CCR4 by IL-22$^+$ILC3. The ability to up-regulate CCR4 expression was dependent on the exposure of intestinal CD103$^+$CD11b$^+$ DCs to commensal bacteria as CD103$^+$CD11b$^+$ DCs from ABX-exposed newborn mice failed to increase the expression of CCR4 on IL22$^+$ILC3, suggesting that the ability to induce CCR4 expression and thus direct the tissue-specific migration of IL22$^+$ILC3 is a cell extrinsic property. Many factors such as components of bacterial cell walls, bacterial metabolites, and intestinal epithelial cell-derived cytokines are known to condition the functional properties of CD103$^+$CD11b$^+$ DCs (80). Identification of this signal remains an area of active investigation. The role of lung CD103$^+$CD11b$^+$ DCs in lung-specific trafficking of IL-22$^+$ILC3 remains unclear. The rarity of CD103$^+$CD11b$^+$ DCs in the newborn murine lung (65) precluded us from evaluating whether these cells could induce IL22$^+$ILC3 to traffic to the lungs as efficiently as intestinal CD103$^+$CD11b$^+$ DCs, a potential limitation of our study.

In conclusion, our data demonstrate the importance of commensal exposure in a defined developmental window during the newborn period in the development of pulmonary mucosal immunity in mice. We illuminate a critical role for intestinal commensal bacteria in lung-selective trafficking of IL22$^+$ILC3. This was mediated by intestinal CD103$^+$CD11b$^+$ DCs, which induced expression of the lung homing signal CCR4 on the IL22$^+$ILC3. Lung-selective trafficking contributed to the postnatal accumulation of IL22$^+$ILC3, promoting the newborn's resistance to pneumonia. These data also potentially explain the association between caesarean delivery or widespread use of antibiotics and an increased risk of infections in newborn infants. Finally, similar mechanisms could influence the development of other pulmonary inflammatory disorders like asthma, which is also associated with caesarean delivery and antibiotic use in early life (81) and lead to new therapeutic agents to mitigate the risk associated with early-life antibiotic exposure in children.

Materials and Methods

Study design

Institutional Animal Care and Use Committee at Cincinnati Children's Hospital Medical Center (CCHMC) approved all the animal studies (IACUC2014-0055), which were carried out in accordance with NIH's *Guide for the Care and Use of Laboratory Animals*. We bred *Rorγt*-Cre mice with *Rosa26*-iDTR mice to generate *Rorγt*^{iDTR} mice. We maintained C57/BL6, *Rosa26*-iDTR mice, *Rorγt*-Cre or *Zbtb46*^{DTR} mice at CCHMC animal facility. We maintained the germ-free (GF) C57/BL6 neonatal mice in plastic isolator cages with autoclaved feed and water at CCHMC Germ-Free Core facility. After birth, neonatal mice from multiple litters were pooled and redistributed to control for the founder effect and to minimize in-cage variations. We used neonatal C57/BL6, *Zbtb46*^{DTR}, *Rorγt*^{DTR} or GF C57/BL6 mice between postnatal ages P1 and P14 and appropriate, age-, sex- and genetic strain-matched controls to account for any variations in data. We treated pregnant female mice (C57/BL6 or Z*btb46*^{DTR}) with sterile drinking water mixed with three different

antibiotics (ampicillin, gentamicin, and vancomycin; all at 1 mg ml^{-1}) starting from embryonic day 15 until the delivery of neonatal mice. We determined group sizes necessary for adequate statistical power analysis using preliminary data sets. There was no randomization designed in the experiments, and we did not exclude any samples. The investigators were not blinded to group allocation during collection and analysis of the data.

Murine neonatal pneumonia studies

We grew *S. pneumoniae* serotype 19A (ATCC 700674) or *E. coli* serotype K1 (82) or *C. albicans* (37°C, 200 rpm) in tryptic soy (TS) broth to log-phase growth. To mimic *S. pneumoniae* or *E. coli* or *C. albicans* pneumonia, we inoculated neonatal mice (postnatal day 4 or 14) with either *S. pneumoniae* serotype 19A (10^5 CFU g^{-1}) or *E. coli* (10^4 CFU g^{-1}) or *C. albicans* (10^5 CFU g^{-1}) respectively via i.t. route. The animals were examined every 6 h for signs of distress and were euthanized 72 h later or earlier if moribund. To assess bacterial burden we homogenized the lung in sterile PBS. We plated serial dilutions of lung homogenates or BAL fluid in TSB agar plates and incubated (37°C, overnight) to count the number of CFU.

We pooled intestinal contents from no ABX-exposed P2 newborn mice. We transferred intestinal contents (200 μg in 50 μl PBS) or vehicle (50 μl PBS) to antibiotic-exposed neonatal P2 mice by a single oral gavage via fine polyethylene tubing as described before (83).

Isolation and characterization of IL-22$^+$ cells in the murine neonatal lung

We pooled and cut the freshly lungs from 3-4 newborn mice and incubated (37 °C, 30 min) the cut tissues with shaking (150 r.p.m.) in digestion buffer (RPMI 1640 with 10% FBS, 15 mM HEPES, 1% penicillin/streptomycin (wt/vol) and 300 U ml^{-1} collagenase VIII) and pressed through a 100-μm nylon strainer to obtain single-cell suspension. The pooled preparation constituted a single data point in our analysis. We then incubated (37 °C, 5 h, 5% CO_2) the cells (1×10^7) in culture medium containing RPMI 1640 with 10% FCS, 1× nonessential amino acids, 10 mM HEPES, 2 mM L-glutamine (all from Invitrogen) and 1% penicillin/streptomycin with 1:1,000 Golgi Stop (554724, BD Biosciences), 10 ng/ml phorbol 12-myristate 13-acteate (PMA) and 500 ng/ml calcium ionophore A23187 (both from Sigma-Aldrich). We washed and incubated (4 °C, 10 min) the cells (1×10^7) with anti-mouse CD16/CD32 and then re-incubated (4 °C, 30 min) with anti-mouse CD3 antibody (145-2C11), anti–mouse CD4 antibody (GK1.5), anti–mouse CD8 antibody (53–5.8), anti–mouse CD11b antibody (M1/70), anti–mouse CD19 antibody (6D5), anti-mouse Ly6G antibody (1A8), anti-mouse F4/80 antibody (BM8), anti-mouse CD117 antibody (2B8), anti–mouse NKp46 antibody (29A1.4), anti-mouse CCR4 antibody (2G12), anti-mouse CCR6 antibody (29-2L17), anti-mouse CCR7 antibody (4B12), anti-mouse CCR9 antibody (9B1), anti-mouse CCL20 antibody (114906), anti-mouse CXCR3 antibody (173), anti-mouse CXCR5 antibody (L138D7), anti-mouse Ki67 antibody (16A8) (all diluted 1:100, Biolegend). For intracellular staining, we washed and fixed (4 °C, 60 min) the surface-stained cells in 1× Cytofix/Cytoperm buffer (BD Biosciences) and permeabilized them (4 °C, overnight) using 1× Permeabilization Buffer (BD Biosciences) according to manufacturer instructions. We stained the cells intracellularly with anti–mouse IL-22

antibody (5164) or anti–mouse RORγt antibody (Q31-378) or anti-mouse T-bet antibody (4B10) or anti-mouse Eomes antibody (WD1928) (all diluted 1:50, Biolegend) and then washed (2×) and resuspended them in flow cytometry buffer. We collected the data with LSRII (BD Biosciences) and analyzed the data with FlowJo (Treestar).

Isolation and characterization of antigen presenting cells in the murine newborn intestine

We pooled and cut the freshly resected terminal ilea from 3-4 neonatal mice into 2- to 5-mm pieces and incubated (37°C,15 min) them in extraction buffer (HBSS, 15 mM HEPES and 1 mM EDTA) to remove the epithelial cells. We then incubated (37°C, 30 min) the cut tissues with shaking (150 rpm) in digestion buffer (RPMI 1640 with 10% FBS, 15 mM HEPES, 1% penicillin/streptomycin (wt/vol), and 300 U ml-1 collagenase VIII) and pressed through a 100-μm nylon strainer to obtain single-cell suspension. The pooled preparation constituted a single data point in our analysis. We then incubated (4 °C, 10 min) the cells (1×10^7) with anti-mouse CD16/CD32 and then re-incubated (4 °C, 30 min) with anti–mouse CD45 antibody (30-F11), anti–mouse CD103 antibody (2E7), anti-mouse CD11b antibody (M1/70), anti-mouse CD11c antibody (N418), anti-mouse MHCII antibody (M5/114.15.2), anti–mouse F4/80 antibody (BM8) and anti-mouse CX3CR1(SA011F11) (all diluted 1:100, Biolegend) and then washed (2×) and resuspended them in flow cytometry buffer. We collected the data with LSRII (BD Biosciences) and analyzed the data with FlowJo (Treestar).

Treatment of neonatal mice with neutralizing antibodies, recombinant IL-22 or diphtheria toxin

We injected neonatal B6 mice with an anti-IL22 antibody (8E11, a gift from Wenjun Ouyang) or anti-IL20 antibody (Clone PA5-47092, Invitrogen) or anti-IgG2A (54447, R&D) (all 5 μg per g body weight) via i.p. route on P1. For specific cell depletion, we treated neonatal *Rorγt*^{iDTR} or *Zbtb46*^{DTR} mice with diphtheria toxin (1.5 ng, R&D) or vehicle via i.p. route on P1. We assessed ablation efficiency by flow cytometry 24 h later. For gain of function studies, we treated ABX-exposed neonatal mice with recombinant IL-22 (10 μg per g body weight) (cat. 414-CS, R&D) or vehicle via i.t. route on P2.

Adoptive transfer of ILC3 or CD103$^+$CD11b$^+$ DCs

For adoptive transfer, we pooled lungs or SI from 8-10 P2 newborn mice. We harvested approximately 1×10^6 Lin$^-$CCR6$^+$ from the pooled specimens by positive and negative selection as done previously by other groups (71). This resulting cell population was > 97% enriched for ILC3 (71). We harvested 1×10^7 intestinal CD103$^+$CD11b$^+$ DC from the pooled SI specimens by positive and negative selection as done previously (84). We adoptively transferred Lin$^-$CCR6$^+$ cells (0.5×10^6 cells/animal) or CD103$^+$CD11b$^+$ DC (0.5×10^6 cells/animal) via i.p. route on P2.

In vivo ILC3 migration assay

We first isolated lung Lin$^-$CCR6$^+$ cells (>97% ILC3) by positive and negative selection as done previously (71). We then incubated (37 °C, 20 min) ILC3 (1×10^7) from P3 GF or ABX-exposed newborns with carboxyfluorescein succinimidyl ester (CFSE) (5 mM). We

incubated ILC3 from control (ABX-free) neonatal mice with chloromethyl-benzoyl-amino-tetramethylrhodamine (CMMTR) (10 mM). We quenched with an equal volume of 10% FBS, diluted 10 × with PBS and resuspended the cells in RPMI1640, supplemented with 2% FBS and 2 mM glutamine. We co-injected 10^6 cells of each population into ABX-exposed neonatal mice via i.p. route. We euthanized host mice 24 h. later and determined the numbers of injected ILC3 migrating into the lungs, spleen and SI by flow cytometry as done previously (50). We calculated the relative homing index as follows. [$CFSE^+$ ILC3 in organ A]/[$CMMTR^+$ ILC3 in organ A] ÷ [$CFSE^+$ ILC3 in injected cells]/[$CMMTR^+$ ILC3 in injected cells] as described before (49). We performed similar experiments after reconstitution of commensal bacteria and with lung Lin^-CCR6^+ cells from *Ccr4*$^{-/-}$ or WT mice.

Cell co-culture and chemotaxis assay

We isolated Lin^-CCR6^+ cells (>97%ILC3) from lungs (71) or $CD103^+CD11b^+CD11c^+$ cells from the intestine of ABX-exposed or ABX-free newborn mice (P4). We then co-cultured lung ILC3 and intestinal $CD103^+CD11b^+$ DC or $CD103^-CD11b^+$ (10^5 cells each) in following combinations (ILC3 from ABX mice + DC from no-ABX mice, ILC3 from ABX-mice + DC from ABX mice or ILC3 from ABX-free mice + DC from ABX-free mice or ILC3 from ABX-free mice + DC from ABX mice, ILC3 from ABX or ABX-free mice alone or DC from ABX and ABX-free mice alone) in round-bottom plates in RPMI1640 supplemented with 2% FBS, 2 mM glutamine, and 50 μM β-mercaptoethanol. We harvested the supernatants and incubated the remaining cells were incubated with Golgi Plug and subsequently analyzed by flow cytometry. For chemotaxis assay, we loaded ILC3 (10^6 cells in 100 μl RPMI 2% FBS) into transwell inserts with pore size of 5 μm (Corning Transwell) and placed in wells containing ±20 nM CCL17 in RPMI1640, supplemented with 2% FBS, 2 mM glutamine, and 50 μM β-mercaptoethanol. We incubated (2 h, 37 °C) the plates and then analyzed the migrated and input cells by flow cytometry. We expressed results as percentages of ILC3 in migrated wells as compared to input wells as described before (50).

Sample collection and analysis of commensal bacteria in the lungs and small intestine of newborn mice

Given the technical challenges in collecting adequate bio specimens for 16s sequencing from newborn mice, we opted to pool bio-specimens from 8-10 newborn mice from 3 separate litters per treatment group. An unexpected benefit of this approach is the control of variations in the commensal bacteria attributed to founder and cage effect (85). We collected the entire left lobe of the lung using one heat treated sterile scissor per animal as described previously by other groups (86). We then cut open the terminal ileum using sterile scissors and removed the intestinal contents using sterile plastic loops as done previously (29). We snap froze (−80°C) the specimens for subsequent analysis (−80°C). We extracted the bacterial DNA from the whole lung or the intestinal contents using QIAamp DNA stool Mini Kit (Qiagen) using a previously described protocol (86) and quantified 16S ribosomal (r) DNA by RT-PCR using degenerate primers as described before (87). To analyze the commensal bacteria, we amplified the V2 region of microbial 16S rRNA by high fidelity PCR with barcoded 8F and 338R universal primers with A and B sequencing adaptors respectively and bifido primers (Roche) and sequenced them with Genome Sequencer GS-

FLX Titanium system (Roche) at University of North Carolina Microbiome core facility (Chapel Hill, NC). The reagents used for DNA extraction, polymerase chain reaction and sequencing reaction are a common source of contamination in microbiome sequencing studies (88). To control for variation in the reagents, we processed all the samples using a common batch of DNA extraction reagents and PCR reagents. We included appropriate controls including a negative control (no template) and positive community control (intestinal contents from age-matched mice) from each batch of harvested tissues when performing 16 rRNA PCR or 16S rRNA sequencing. We decoded and processed the sequences using the QIIME software package (Version 1.7) and custom R package code (89). Analysis of the sequences from negative control indicated presence of several bacterial species suggesting potential contamination from the DNA extraction or sequencing reagents. The dominant bacterial species in the negative control were Rhodocyclae (17%), Rhizobiales (15%) Argobacterium (14%), Micrococcus (10%), Hydrogenophilus (9%), Neiserria (5%), Lysinibacillus (4%), Micrococcus (4%) and Tenericutes (4%). The relative abundance of these contaminants in experimental samples was less than 3% (Supplemental Table 1) and thus unlikely to alter the conclusions. We used phylogenetic diversity (PD) to compute and visualize α diversity and unweighted and weighted Unifrac for β diversity. We tested the observed differences in Unifrac distances between antibiotic treated groups and across different ages for significance using a *t*-test, and we corrected the reported *P* values for multiple comparisons using a Monte Carlo permutation procedure with 10,000 iterations. We deposited all data sets in a publicly available database (Figshare) and can be accessed at https://figshare.com/s/52f4aa2f8035fd505cf1.

Transcriptomic analysis

We sequenced high-quality RNA from the whole lung at CCHMC Sequencing Core Facility with an Illumina HiSeq 2500. We performed data alignment with TopHat, followed by gene quantification (FPKM) using Cufflinks. We carried out differential expression analysis with both FPKM and read count-based methods. We performed pathway and network analyses with Altanalyze as described before (90). We deposited all data sets in a publicly available database (Figshare) and can be accessed at https://figshare.com/s/52f4aa2f8035fd505cf1.

Human newborn studies

The Institutional review board at CCHMC approved all the human studies. The biological samples were initially collected from infants who underwent clinical evaluation of either upper airway obstruction or tracheaobronchomalacia, common anomalies of the airways in infants, after obtaining informed consent from the parents (IRB approval #2013-3309). The bronchial lavage (BAL) fluid samples were centrifuged (4°C, 10 min, 400 g). The resultant supernatant was frozen (−80 °C) and the cells were cryopreserved (−150 °C) in 90% FBS and 10% DMSO. We used the frozen supernatant and cryopreserved cells in our analysis (IRB approval #2015-7983). Since pneumonia (48) and asthma (71) are associated with ILC3 activation in the lungs, we excluded infants with a history of pneumonia (defined as worsening of respiratory status, increase or change in the quality of respiratory secretions, temperature instability with radiographic changes) or wheezing. We then selected infants who received antibiotics (ABX-exposed group) or no antibiotics (No ABX-group) and matched the respective treatment groups for gestational age, age at the time of procedure and

history of mechanical ventilation. Characteristics of the subjects are provided in Table 1. After thawing, we incubated (37°C, 5 h, 5% CO_2) the cells (0.5×10^6) in culture medium containing RPMI 1640 with 10% FCS, 1× nonessential amino acids, 10 mM HEPES, 2 mM L-glutamine (all from Invitrogen) and 1% penicillin/streptomycin with 1:1,000 Golgi Stop (554724, BD Biosciences), 10 ng/ml phorbol 12-myristate 13-acteate (PMA) and 500 ng/ml calcium ionophore A23187. We immunophenotyped the cells in BAL fluid as described before (91). We stained cells with anti-human CD3 antibody (SP34-2), anti-human CD8 antibody (B9.11), anti-human CD14 antibody (RMO52), anti-human CD19 antibody (J3-119), anti-human CD45 antibody (J.33), anti-human CD56 antibody (NK901), anti-human CD69 antibody (TP1.55.3) and anti-human NKp46 antibody (9E2) (all diluted 1:1000, Biolegend). For intracellular staining, we washed and fixed (4 °C, 60 min) the surface-stained cells in 1× Cytofix/Cytoperm buffer (BD Biosciences) and permeabilized them (4 °C, overnight) using 1× Permeabilization Buffer (BD Biosciences) according to manufacturer's instructions. We stained the cells intracellularly with anti-human RORγt antibody (AFKJS-9), anti-human T-bet antibody (4B10) and anti-human IL-22 antibody (BG/IL22) (all diluted 1:50, Biolegend) and then washed (2×) and resuspended them in flow cytometry buffer. We collected the data with LSRII (BD Biosciences) and analyzed the data with FlowJo (Treestar).

ELISA

We measured IL-22 or IL-20 in the murine (69) or human BAL fluid (92) using commercially available kits (eBioscience) as described before. We measured IL-1β in the cell culture supernatant using commercially available kits (eBioscience) as per manufacturer's instructions.

Statistical analyses

Each data point represents a pool of 3-4 newborn mice that were pooled before the isolation of leukocytes from the indicated tissue. All data met the assumptions of the statistical tests used. We compared differences between groups by either unpaired two-tailed Student's *t*-test or ANOVA or Wilcoxon signed-rank test. We used the Kaplan-Meier log-rank test to compare survival between groups. (All in GraphPad Prism 6.0). P-values are indicated as follows: * $p \leq 0.05$ or ** $p \leq 0.01$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One sentence summary

Postnatal colonization by intestinal commensal bacteria directs migration of innate lymphoid cells into the lungs and promotes newborn resistance to pneumonia.

Accessible Summary

Interactions between host and intestinal commensals shape the development of immune cells in intestine. We report that host-commensal interactions extend beyond the local environment and shape the repertoires of immune cells at extra-intestinal sites like the lungs. Exposure to commensals in the developmental window of newborn period directs lung-selective trafficking of innate lymphoid cells (ILC3), a group of sentinel cells that maintain mucosal homeostasis. This was mediated by intestinal DCs, which induced expression of the lung homing signal CCR4 on the ILC3. Lung-selective ILC3 trafficking promoted the newborn's resistance to pneumonia. These data explain the association between widespread use of antibiotics and an increased risk of pneumonia in newborn infants.

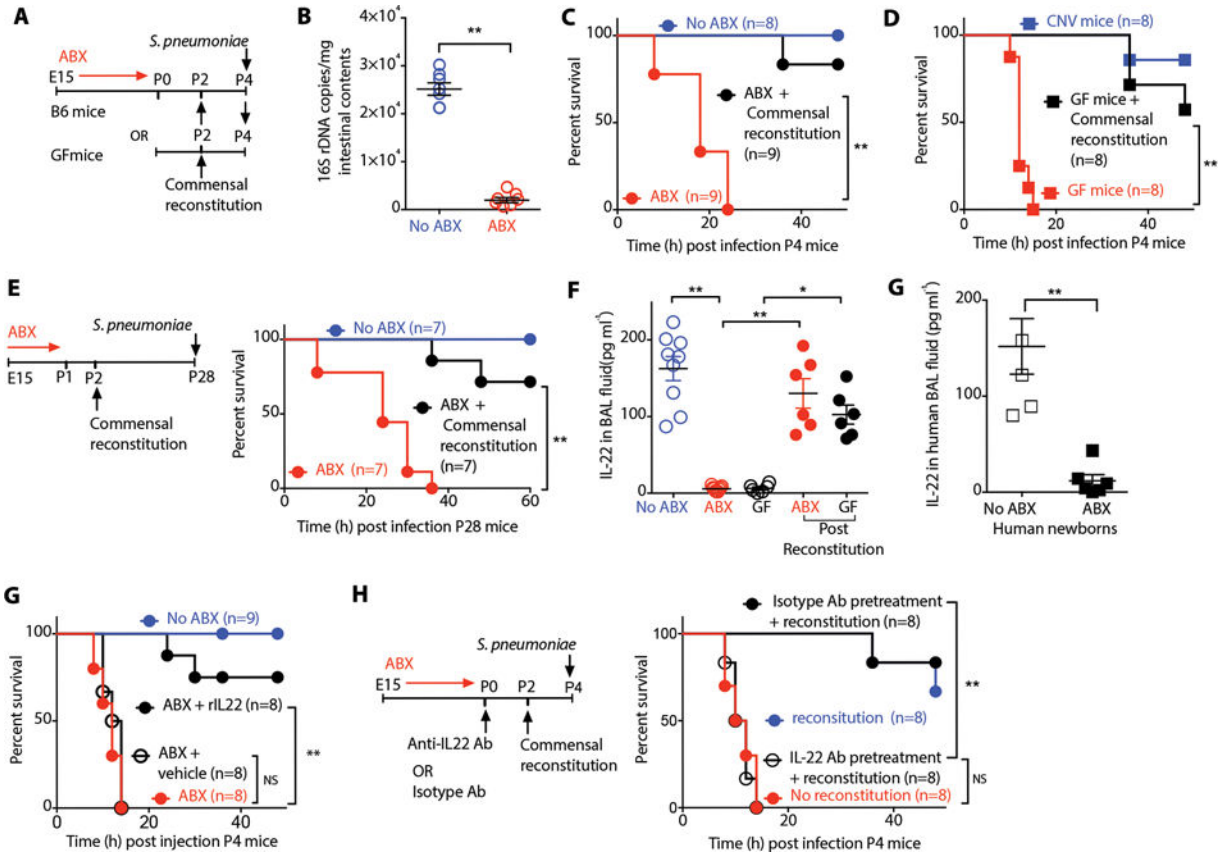

Figure 1. Intestinal commensal bacteria promote resistance to *S. pneumoniae* in newborn mice via IL22

(A) Study design. (B) Intestinal commensal bacteria enumerated in postnatal day 4 (P4) newborn mice exposed to antibiotics (ABX) or no antibiotics (ABX-free) quantified using real-time PCR.. (C) Survival of ABX-free or ABX-exposed P4 mice or ABX-exposed newborn mice reconstituted with intestinal commensal bacteria and infected with *S. pneumonia*. (D) Survival of germ-free (GF) or conventionally housed (CNV) mice or age-matched GF mice reconstituted with intestinal commensal bacteria and infected with *S. pneumoniae*. (E) Survival of ABX-free or ABX-exposed P14 mice or ABX-exposed newborn mice reconstituted with intestinal commensal bacteria and infected with *S. pneumoniae*. (F) The amount of IL-22 in the bronchial lavage (BAL) fluid of P4 ABX-free or ABX-exposed mice or GF newborn mice or ABX-exposed or GF newborn mice reconstituted with intestinal commensal bacteria in early life. None of the newborn mice in this experimental group were inoculated with *S. pneumoniae.* (G) The amount of IL-22 in the BAL fluid of human newborns who were exposed to ABX or no ABX. (H) Survival of P4 ABX-free or ABX-exposed newborn mice treated with IL-22 intratracheally and infected with *S. pneumoniae*. (I) Survival of P4 ABX-free or ABX-exposed newborn mice treated with anti-IL22 antibody or isotype control antibody before reconstitution with intestinal commensal bacteria and infection with *S. pneumoniae*. Data are representative of three independent experiments. Results are shown as the mean ± s.e.m (Student's t-test or ANOVA or Wilcoxon signed-rank test). *P ≤ 0.05; **P ≤ 0.01. Number of individual animals [n] are indicated.

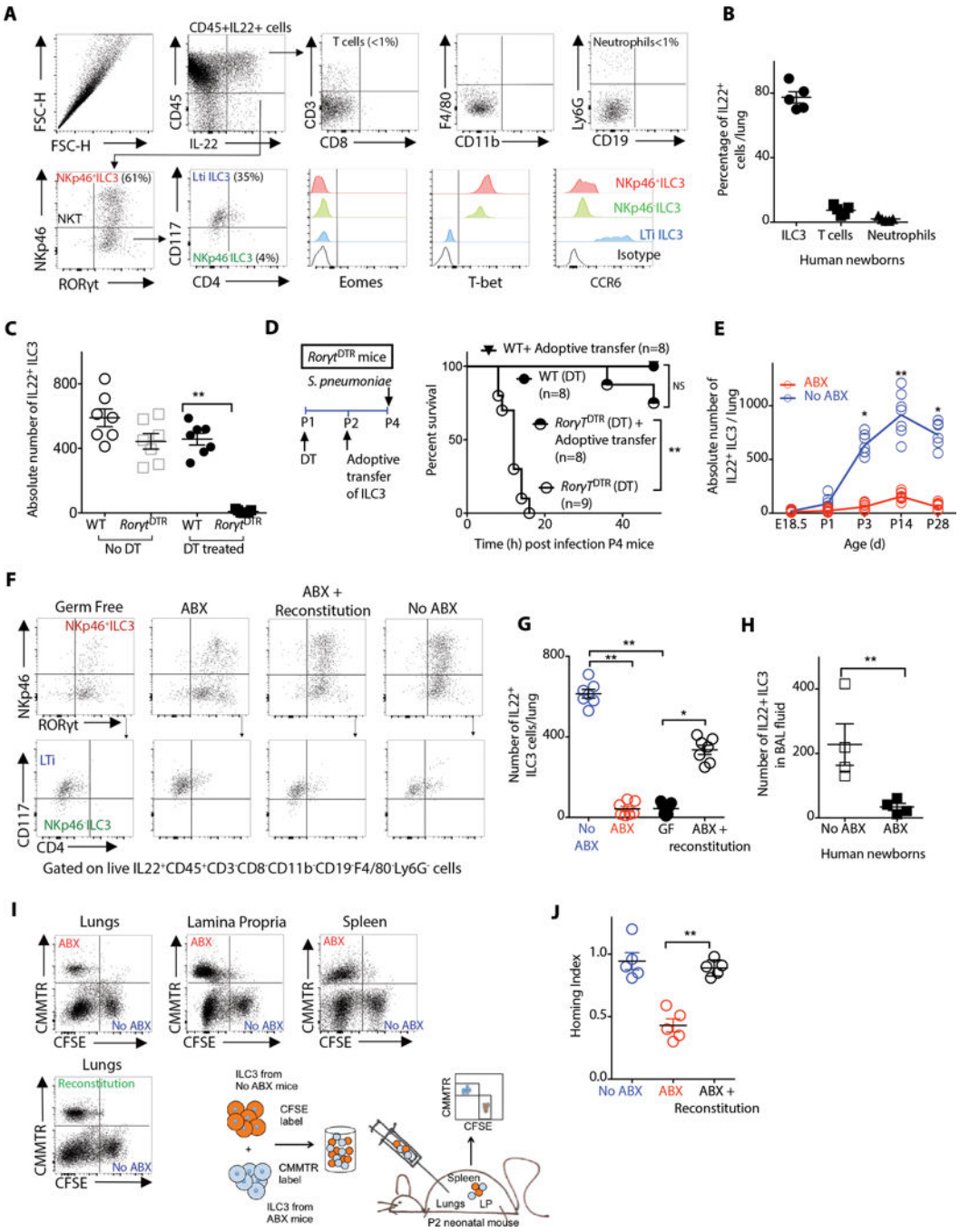

Figure 2. Intestinal commensal bacteria direct postnatal trafficking of IL-22^{+}ILC3 innate lymphoid cells to murine newborn lung

(A) Representative flow cytometry plots of distinct subsets of IL-22^{+} cells in the lungs of postnatal day 4 (P4) newborn mice. Shown are relative frequencies of IL22^{+} T cells (CD45^{+}CD3^{+}) or neutrophils (CD45^{+}Ly6G^{+}) or lineage negative (CD3^{-}CD8^{-}CD11b^{-}CD19^{-}F4/80^{-}CD161^{-}Ly6G^{-}F4/80^{-}) lymphocytes in the lungs of P4 newborn mice. (B) The relative frequencies of distinct subsets of IL-22^{+} cells in the bronchial lavage (BAL) fluid of human newborn infants.. (C) Absolute numbers of IL22^{+}ILC3 in the lungs of P4 wild-type (WT) or *Rorγt^{iDTR}* newborn mice treated with diphtheria toxin (DT) or no DT.

(D) Survival of P4 WT or *Rorγt*^{iDTR} newborn mice treated with DT (ILC3-depleted) that received adoptive transfer of ILC3 and then were infected with *S. pneumoniae*. (E) The absolute number of IL-22^{+}ILC3 in the lungs of ABX-free or ABX-exposed newborn mice at different time points after birth. (F) Representative flow cytometry plots and (G) absolute numbers of IL-22^{+}ILC3 in the lungs of P4 GF or ABX-free or ABX-exposed or ABX-exposed newborn mice reconstituted with intestinal commensal bacteria in early life. (H) The absolute numbers of IL-22^{+}ILC3 in the BAL fluid of human newborns exposed to ABX or no ABX. (I) ILC3 from P4 ABX-free newborn mice were labeled with carboxyflourosceinsuccimidylester (CFSE). ILC3 from age-matched P4 ABX-exposed or ABX-exposed newborn mice reconstituted with commensal bacteria were labeled with chloromethylbenozylaminotetramethylrhodamine (CMMTR). An equal number of CFSE- or CMMTR-labeled ILC3 were adoptively transferred into ABX-exposed newborn mice. Representative flow cytometry plots and absolute numbers of CFSE^{+} or CMMTR^{+} ILC3 in lung, spleen or small intestine were determined 12 h following adoptive transfer. (J) Relative capability of ILC3 from ABX-free or ABX-exposed or ABX-exposed newborn mice reconstituted with intestinal commensal bacteria in early life to traffic to the lungs (Homing index). Data and plots are representative of three independent experiments. Results are shown as the mean ± s.e.m (Student's t-test or ANOVA or Wilcoxon signed-rank test). *P ≤ 0.05; **P ≤ 0.01. Number of individual animals [n] are indicated.

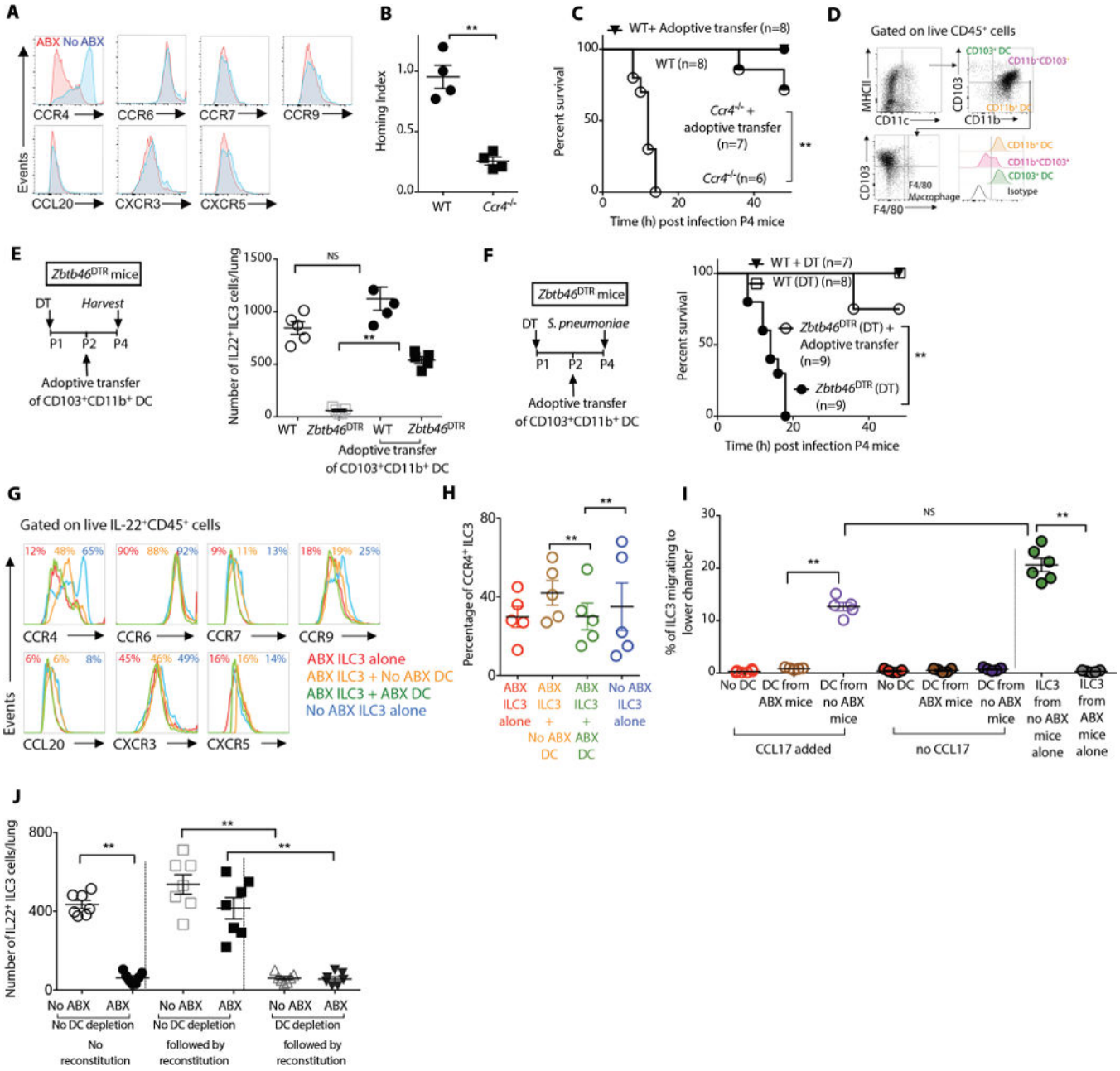

Figure 3. Intestinal dendritic cells mediate cross talk between commensal bacteria and IL22^{+}ILC3 innate lymphoid cells and induce ILC3 to traffic to the murine newborn lung

(A) Representative flow cytometry histograms showing expression of C-C chemokine receptor (CCR) 4, 6, 7 and 9, C-X-C chemokine receptor (CXCR) 3 and 5 and C-C chemokine ligand (CCL) 20 by IL22^{+}ILC3 innate lymphoid cells in the lung of postnatal day 4 (P4) ABX-free or ABX-exposed newborn mice. (B) An equal number of ILC3 from P4 WT or *Ccr4*$^{-/-}$ newborn mice were adoptively transferred into age-matched ABX-exposed newborn mice, and the ability of ILC3 to traffic to the lungs (homing index) was determined. (C) Survival of P4 WT or *Ccr4*$^{-/-}$ newborn mice that received adoptive transfer of WT ILC3 after infection with *S. pneumoniae.* (D) Representative flow cytometry plots

and relative frequencies of distinct subsets of mononuclear phagocytes in the small intestine of P4 newborn mice. (E) The absolute numbers of IL22^{+}ILC3 in the lungs of P4 WT or *Zbtb46*^{DTR} newborn mice treated with DT (CD11b^{+}CD103^{+} DC-depleted) that received adoptive transfer of CD11b^{+}CD103^{+} DCs. (F) Survival of P4 WT or *Zbtb46*^{DTR} newborn mice treated with DT (CD11b^{+}CD103^{+} DC-depleted) that then received adoptive transfer of CD11b^{+}CD103^{+} DCs, after infection with *S. pneumoniae* . (G) ILC3 isolated from lungs of P4 ABX-exposed mice were co-cultured with CD11b^{+}CD103^{+} DCs isolated from age-matched ABX-exposed or ABX-free mice and examined for surface expression of various chemokine receptors. A representative flow cytometry plot is shown and (H) relative frequencies of IL-22^{+}ILC3 cells expressing CCR4. (I) ILC3 isolated from lungs of P4 ABX-exposed mice were co-cultured either alone or with CD11b^{+}CD103^{+} DCs isolated from age-matched ABX-exposed or ABX-free newborn mice. The ability of these ILC3 to migrate *in vitro* in response to a gradient of the chemokine ligand (CCL) 17 is shown. (J) The absolute numbers of IL22^{+}ILC3 in the lungs of P4 *Zbtb46*^{DTR} newborn mice either exposed to ABX or no ABX that were then treated with DT (CD11b^{+}CD103^{+} DC-depleted) or no DT (no DC depletion) before they were reconstituted with commensal bacteria. Data and plots are representative of three independent experiments. Results are shown as the mean ± s.e.m (Student’s t-test or ANOVA or Wilcoxon signed-rank test,). *P ≤ 0.05; **P ≤ 0.01. Number of individual animals [n] are indicated.

Table 1

Demographic characteristics of human newborn infants.

Patient Demographics				Clinical History											
Sex	**Race**	**GA (wks)**	**Age at exam (days)**	**Lobe lavage**	**Asthma**	**Chronic Cough**	**History of Mechanical Ventilation**	**Ventilator Dependent**	**History of Pneumonia**	**Acute Respiratory Failure**	**Interstitial Lung Disease**	**Steroids**	**Antibiotic use**	**Indications for study**	**Study Findings**
M	Caucasian	34	56	RLL	No	No	Yes	Yes	No	No	Yes	Yes	Yes	Surfactant Deficiency	Intestitial lung disease
M	Caucasian	35	54	RLL	No	No	Yes	Yes	No	No	No	Yes	Yes	airway obstruction	tracheal rings
M	Caucasian	36	66	RUL	No	No	No	No	No	No	No	No	No	airway obstruction	normal exam
M	Caucasian	36	37	LLL	No	No	Yes	No	No	No	No	No	No	ALTE	normal exam
F	African American	36	68	RUL	No	No	No	No	No	No	No	No	No	airway obstruction	laryngomalacia
F	Caucasian	37	72	RUL	No	No	Yes	Yes	No	No	No	Yes	No	subglottic stenosis	subglotiic stenosis
M	African American	37	51	RLL	No	No	No	No	No	No	No	No	No	airway obstruction	tracheal compression
M	Caucasian	37	68	RLL	No	No	No	No	No	No	No	No	Yes	airway obstruction	larynomalaia
F	African American	37	48	RUL	No	No	Yes	No	No	No	No	Yes	Yes	Stridor	laryngeal edema
M	African American	37	54	LLL	No	No	No	No	No	No	No	No	Yes	airway obstruction	normal exam
M	Caucasian	38	80	Lingula	No	No	No	No	No	No	No	No	No	tracheomalacia	mild tracheomalacia
M	Other	38	76	RUL	No	No	Yes	No	No	No	No	Yes	No	tracheomalacia	tracheomalacia
F	Caucasian	39	54	RLL	No	No	No	No	No	No	No	Yes	No	airway obstruction	laryngeal hemangioma
M	Caucasian	39	42	RUL	No	No	No	No	No	No	No	No	Yes	tracheomalacia	tracheomalacia
M	Caucasian	40	61	RLL	No	No	No	No	No	No	No	No	Yes	tracheomalacia	mild tracheomalacia