The developing T-cell compartment of the neonatal lung orchestrates an atypical response to respiratory syncytial virus

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Authors contribution

All authors contributed to important elements to the work presented in this paper and reviewed and approved the final version of this manuscript. T.D. designed the various antibody combination staining, validated them in vitro, designed and performed the
animal trials, performed the RSV quantifications in animal tissues and serum micro-neutralization assays, acquired, assembled, analyzed and interpreted the data, prepared the figures and wrote the manuscript. M.B. and B.Z established the RSV quantification and serum micro-neutralization assay protocols and designed and performed the animal trial. B.I.O.E. participated in the execution of the animal trial. K.M. and H.P. participated in the execution of the animal trial and evaluated the lung pathology. A.F. assisted in the animal trial and performed part of the immunohistochemistry. L.B. computed the data from WBC and PMN counts in the circulation with the R software and generated the related figures. C.B. helped with clinical observations, interpretation of the data, and reviewed the manuscript. N.R. participated in the design and execution of the animal trial, organized the partitioning of animals in stables, and monitored the clinical scores. M.P.A. conceived the idea, overviewed, designed, led and performed the animal trial, and edited the manuscript.

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Abstract

Rationale. Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection in infancy, potentially aided by an inappropriate immune response. Sparse information is available for the distal lung, mostly because data arose from non-invasive samplings of peripheral blood and nasal aspirates.

Objectives. To determine the neonatal immune response to RSV in the bronchoalveolar space and better understand why neonates are at greater risk of developing severe disease.

Methods. We used the newborn lamb, a state-of-the-art translational model of human RSV infection, offering ease sampling and full accessibility to lower airways. Using a multiparameter flow cytometry assay, we evaluated the frequency and activation/maturation state of the major subsets of the developing T-cell compartment.

Measurements and Main Results. The T-cell compartment of the healthy developing lung was very distinct to that seen in adults. We observed a high frequency of type 2 CD4+ (Th2) and CD8+ (Tc2) T-cells, both being a large source of IL-4, which declined progressively over time. Remarkably, RSV infection exacerbated this pro-type 2 environment, rather than inducing a type 2 response per se. Neonatal regulatory T-cell (Treg) suppressive functions occurred very early to dampen those Th2 and Tc2 responses, while γδ T-cells dropped and failed to produce IL-17. The disease severity was related to the magnitude of these T-cell responses.
Conclusion. The atypical neonatal immune response to RSV consists of distinct T-cell subsets that tightly cooperate, namely a combined bronchoalveolar influx of Treg, Th2 and Tc2 cells, associated with a depletion of γδ T-cells.

Keywords: Respiratory syncytial virus; neonatal immunity; bronchiolitis; immunopathogenesis; T cells immunity
**Introduction**

The human respiratory syncytial virus (RSV) is a seasonal virus, known as the major cause of lower respiratory tract infection during early childhood (1, 2). Worldwide, RSV leads to around 160,000 deaths each year (3). Although there are risk factors for RSV severity such as prematurity and congenital heart disease, the majority of hospitalized infants are previously healthy (4-7), suggesting that the disease is partially linked to inherent properties of early life immunity. The precise understanding of the immune-driven susceptibility of neonates is essential, considering there is no licensed vaccine and available prophylactic treatments are mostly based on palivizumab, a neutralizing antibody used in high-risk group infants (8).

The first exposure to pathogens occurs during the early postnatal period and is critical for lung colonization by immune cells. There is increasing evidence that the overall neonatal T-cell compartment is distinctive during this temporal window (9). Upon stimulation neonatal T-cells tend to differentiate into regulatory T-cells (Tregs), to facilitate self-tolerance to developing organs (10). Also, the developing lung is characterized by a type 2 immune phenotype, and the pulmonary T-cell responses present a T helper type 2 (Th2) bias (11, 12). Remarkably, neonatal γδ T-cells, a T-cell subset enriched at mucosal barrier sites, have an impaired capacity to respond to stimulation (13-16).

Due to the aforementioned reasons, young infants do not mount a classical pulmonary immune response to pathogens such as RSV, which may contribute to high morbidity and mortality (17, 18). A likely explanation could be a combined contribution of viral load (19) and an inappropriate and/or dysfunctional immune response, through an over-exuberant inflammatory response or a biased T-cell response (9, 20, 21).

Neonatal T-cells may have both protective and harmful effects in responses to RSV
infection. While children with T-cell deficiencies cannot efficiently clear the virus (22, 23), it remains controversial whether the severity of RSV disease is associated with a bias towards type 2 cytokines such as IL-4 (24-26).

Knowledge of the course of RSV disease is still limited due to ethical and technical difficulties associated with the investigation of the immune response in human neonates. The neonatal lamb model of RSV infection recapitulates most of the features of paediatric disease. Indeed, RSV is a natural pathogen of ruminants. Experimentally-infected animals clinically present rhinitis, coughing and respiratory distress. Typical pulmonary lesions are bronchiolitis and interstitial pneumonia associated with inflammatory infiltrates (27-29). This model, immunologically related to human, was exploited to have full access to the small airways and follow the pulmonary immune cell features over the course of RSV disease.

Materials and methods

Virus

RSV-A2 strain was obtained from the American Type Culture Collection (ATCC® VR-1540™). RSV-ON1-H1 strain was isolated from nasopharyngeal aspirates of a child below the age of five years with confirmed RSV infection, hospitalized at Hannover Medical School, Germany. The caregivers gave informed consent for sampling and all steps were conducted in compliance with good clinical and ethical practice and approved by the local ethical committee (permission number 63090/2012) (30). Virus propagation and titre determination used standard methods, described in the online supplement.
Animals

The experiments were approved by the cantonal veterinary authority, and performed in compliance with the Swiss animal protection law under the authorization BE125/17. Animals were housed in the high containment animal facility. Postpartum, the acclimatization lasted 3-6 days before infection. In accordance with the principles of the 3Rs, suckling lambs were held together with their mothers and fed ad libitum. More details about the housing are given in the online supplement.

The transtracheal RSV inoculation was performed under sedation and analgesia by intramuscular injection of a mixture of midazolam (Dormicum®, Roche) 0.2 mg/kg and butorphanol (Morphasol®, Dr. E. Graeub AG) 0.2 mg/kg. The trachea was punctured between the proximal tracheal rings with a 20G needle and a volume of 2 ml of virus (10^8 PFU for RSV-A2; 10^7 PFU for RSV-ON1-H1) or PBS (mock-treated controls) were injected slowly.

Histopathology and Immunohistochemistry

The methods used in the present work were described elsewhere (31).

Cell suspension preparation

Cell isolation used standardized methods, described in the online supplement.

Human RSV neutralization assay

Serum neutralization assay used standardized methods, described in the online supplement.

RNA isolation and quantitative PCR
The primer and probe sequences used to detect RSV have been previously described (32).

**Flow cytometry**

The different ovine immune cell subtypes were identified by flow cytometry (FCM) using an eight-step, seven-color staining protocols. All antibodies used for the procedure, as well as their clones, host, working dilution, and references, are listed in Table E1. Combination stainings analyzed plasmacytoid dendritic cell (pDC), γδ T-cells, Tregs, CD4 and CD8 T-cell subsets. For the acquisitions, $10^6$ events were accumulated for each sample. The experimental schedule is summarized in Table E2. For the counts of cell subtypes, we calculated the ratio (number of events in the gated cell subtype) to (number of all events, excepting cell aggregates and debris). FCM acquisitions were performed on a FACS Canto II (BD Bioscience) using the DIVA software and further analyzed with FlowJo (TreeStar).

**Statistical Analysis**

Statistical analysis was done using GraphPad Prism 8 software. To determine differences between two groups, non-parametric paired Wilcoxon tests or Mann–Whitney U-tests were used as appropriate. Associations were tested using the Spearman rank correlation test. A p<0.05 was considered significant.
Results

Respiratory disease following neonatal RSV infection

Selected time-points were as follow: 3 and 6 days post-infection (p.i.), to evaluate the innate and early adaptive responses; 14 days p.i. to assess the adaptive mechanisms and virus clearance; 42 days p.i. to investigate the impact of RSV infection on the immune cell colonization of the developing lung. As controls, non-infected newborns and adults were used (Figure 1A).

We quantified RSV in BALs (cellular and fluid fractions) and in the lung tissue. Although RSV clearance was slower in the cellular fraction of BALs, it became undetectable in any sample at day 42 p.i., demonstrating an efficient clearance (Figure 1B). Immunohistochemistry of lung tissue sections revealed the presence of RSV-antigens in type I pneumocytes and macrophages (Figure 1C).

Clinically, RSV infected neonates showed signs of rhinitis with clear nasal discharge, occasional coughing and wheezing. Macroscopical lesions were visible at days 3-6 p.i, including failure of pulmonary collapse and focal, dark-red areas of subpleural pulmonary consolidation and atelectasis. Histological lesions, present at days 3-6 p.i consisted of multifocal-coalescing areas of lymphocytic and histiocytic infiltrates within interalveolar septae (interstitial pneumonia) and accumulation of inflammatory cells within alveolar spaces and bronchioles (bronchiolitis) (Figure 1D). Longitudinal measurements of white blood counts and neutrophil counts in the circulation showed no difference between mock and infected neonates (Figure E1).

Neonatal RSV infection activates the initiators of the adaptive immune response

When quantified in the BALs, pDC numbers of mock neonates were similar to those of healthy adults. Upon RSV infection, we observed a trend to pDC recruitment as
early as 3 days p.i., and this was enhanced at day 6 p.i. (p<0.05). However, this pDC recruitment was transitional and tightly regulated, since day 14 p.i corresponded to a return to steady-state levels (Figure 2A,B). One out of six infected neonates failed to display any pDC recruitment; this animal was the only one for which RSV was undetectable in the fluid fraction of BAL (Figure 1B). To verify whether the extent of pDC recruitment was linked to the magnitude of RSV shedding, pDC counts were plotted as a function of RSV copies in the fluid fraction of BALs. A significant association was found (Figure 2C). We next evaluated if the recruited pDCs displayed a mature and activated phenotype. A significant CD86 upregulation was found at days 3-6 p.i. (Figure 2D,E). This upregulation was transitional and back to steady-state levels at day 14 p.i.

**Neonatal RSV infection leads to a transient depletion of γδ T-cells**

γδ T-cell potential impairment in the bronchoalveolar space could contribute to RSV immunopathogenesis (13-15). Their numbers in the BALs of mock neonates were comparable to those of healthy adults. However, RSV infection led to a significant decrease in γδ T-cell frequency at day 14 p.i.. Nevertheless, this γδ T-cell depletion was entirely reversed at day 42 p.i. (Figure 2F,G). To exclude the possibility that the γδ T-cell reduction could be due to migration or relocation, we calculated their counts as a function of the percentage of Live/Dead-positive cells. A negative association was found, suggesting that neonatal γδ T-cells death is linked to the presence of RSV in the bronchoalveolar space (Figure 2H). Finally, BAL γδ T-cells collected from infected neonates failed to produce detectable levels of IL-17.

We next ascertained whether neonatal γδ T-cells are characterized by an immature phenotype. Remarkably, in BALs of healthy adults, half of the γδ T-cell pool was
composed of mature CD25+ cells, whereas the latter were absent in mock neonates at day 3. The proportion of mature γδ T-cells progressively strengthened over time, without reaching the level detected in healthy adults. RSV infection exerts an effect on this maturation of neonatal γδ T-cells. Not only the observed depletion was rapidly followed by a complete replenishment at day 42 p.i., but also the restoration was accompanied by a marked increase in the percentage of CD25-positive cells, compared to mock neonates (Figure 2I,J).

Neonatal RSV infection exacerbates the type 2 cytokine environment

Assessment moved on to adaptive immune response, and more particularly to IL-4 versus IFN-γ production by T-cells (Figure 3A). In BALs, mock neonates had a very low number of CD4+ and CD8+ T-cells compared to healthy adults. RSV infection led to their strong recruitment, albeit with distinct kinetics. An unexpected reduction of CD4+ T-cell recruitment was seen as soon as 14 days p.i. This contradicted the values obtained for CD8+ T-cells, where the significant enhancement following RSV infection was maintained. When we considered both CD4+ and CD8+ fractions at a later time-point, the numbers were comparable to those of mock neonates (Figure 3B).

Surprisingly, mock neonates had high frequencies of Th2 cells, which declined progressively over time. Elevated amounts of spontaneous produced IL-4 were a signature of the developing CD4+ fraction, since it was no longer detectable at day 42, or in healthy adults. Upon infection, this analysis revealed a trend towards an increase in the frequency of those Th2 cells, whereas there was no consistent influence on IFN-γ induction (Figures 3C,D). Consequently, RSV infection in early life exacerbates the overall lung pro-Th2 cytokine environment, rather than inducing a Th2 immune response per se.
High frequencies of IL-4-expressing CD8$^+$ T-cells (Tc2) were also found in mock neonates. Again, a progressive decline of Tc2 cells occurred up to day 42 p.i., comparable to negligible levels measured in healthy adults. RSV disease induced a greater overall Tc2-derived cytokine environment in BALs. This exacerbation was tightly regulated, reduced to a level similar to healthy adults at day 14 p.i. Again, no consistent influence of RSV infection was seen on IFN-γ induction (Figure 3E,F).

Altogether, these results highlight the role of Tc2 cells as an important source of IL-4 production during neonatal RSV infection.

We then tested the activation and differentiation of naïve T lymphocytes into memory subsets. The most striking observation was achieved at day 42 p.i.; the percent contributions of different subsets seen in RSV-infected neonates was highly superimposable to that found in healthy adults. This was not the case for mock neonates who had lower frequencies of activated memory T-cells, particularly for the CD8$^+$ fraction (Figure 3G). Consequently, RSV infection in early life modulates the activation and the maturation of the T-cell pool.

Next, we monitored the presence of RSV neutralizing antibodies (NAb) (Figures 3H, E2). In the serum of healthy adults and mock neonates, no RSV neutralizing antibodies were detected. In contrast, RSV-infected neonates have naturally acquired NAb at day 13-14 p.i., except one animal. Notably, two siblings had RSV NAb preceding infection; this was passively transmitted by their mother whose serum also had a strong RSV-neutralization (Figure E3). We extended the assay to later time points to test the persistence of RSV neutralization. No loss of humoral memory was measured up to 42 days p.i (Figure 3I). Altogether, these results indicate that despite an inappropriate cell-mediated immune response, neonates can clear RSV by mounting an efficient humoral response.
Neonatal RSV infection rapidly induces Treg suppressive functions to dampen the Th2 and Tc2 responses

The shortened kinetics observed for CD4+ T-cells was elaborated by determining whether this was due to an early Treg rather than a classical CD4+ T-cell response (Figure 4A). RSV infection elicited a rapid Treg recruitment in BALs that mirrored the one previously observed for CD4+ T-cells. This Treg dynamic is specific for the airway mucosa, since Treg frequencies remained unchanged in the lung tissue (Figure 4B).

We then noticed a trend for Treg subset overrepresentation following RSV infection (Figure 4C). The proportion of Tregs declined progressively over time, tending towards similar levels to those detected in healthy adults. RSV infection promoted enhancement of TGF-β production by Tregs, however not significant (Figure 4D).

When Treg counts were calculated as a function of non-Treg CD4+ counts, an association was found, suggesting that CD4+ T-cell pool tends to differentiate into Tregs. When Treg counts were calculated as a function of CD8+ counts, an association was still found, confirming that CD8+ T-cells and Tregs colonized the alveolar space in a coordinated manner. By plotting CD8+ count as a function of non-Tregs CD4+ counts, it appeared that T-cell response was mainly driven by the CD8+ fraction (high numbers at days 6 and 14 p.i.) (Figure 4E). In peribronchial LNs following RSV infection, CD8+ counts remained stable over the course of the disease, showing that their accumulation is airway mucosa-specific (Figure 4F). Moreover, unlike the situation described for the BAL compartment, RSV infection did not promote a further increase of Tc2 cells (Figure 4G).
Distinct cellular immune response in neonates compared to adults following RSV infection

We followed the same approach to dissect the cellular immune response in RSV-infected adults. While the virus clearance was comparable between adults and neonates (Figure 5A), respiratory lesions were absent in adults (Figure 5B). The rapid and robust pDC recruitment observed in infected neonates did not occur in infected adults. However, lung-resident pDCs of the latter were prompt to react, displaying an even more mature and activated phenotype. Importantly, this mature pDC response was also tightly regulated (Figure 5C,D).

The strong decrease in γδ T-cell counts observed in infected neonates was absent in adults. Moreover, adult γδ T-cells produced high amounts of IL-17, suggesting that IL-17-mediated immunity is deficient in early life (Figure 5E,F) (15). This IL-17 response was accompanied by an activated phenotype, as indicated by the enhanced proportion of CD25+ cells (Figure 5G). From these results, we could ascertain that neonates lack a mature and functional γδ T-cell pool in the lower airways.

For the CD4+ compartment, no significant recruitment was found (Figure 5H). The percentage of IL-4-producing cells was very low, and RSV infection had no consistent influence on IFN-γ induction (Figure 5I). Another clear difference was the contraction of effector memory pool (CD25+CD45RO+) at day 14 p.i., indicative of a specific immune response (Figure 5J).

The recruitment of CD8+ T-cells was delayed compared to neonates, only detectable at day 14 p.i. (Figure 5K). Again, the percentage of IL-4-producing cells was low (Figure 5L), whereas the precipitous contraction of effector memory pool at day 14 p.i. suggested a RSV-specific CD8+ T-cell response (Figure 5M). From these results, it
became clear that the inaptitude of neonates to mount an effector T-cell response is tentatively substituted by a fast and massive Th2/Tc2 cell recruitment. Finally, we failed to detect any Treg recruitment. However, the rare tissue-resident Tregs were able to produce TGF-β, suggesting that an immune regulation happened (Figure 5N,O).

**The severity of disease relates to the magnitude of bronchoalveolar T-cell responses**

The pulmonary T-cell response was evaluated in neonates infected with RSV-ON1-H1 strain, which induced a milder disease (Figure 6A). We did not observe a γδ T-cell depletion, and the CD8+ T-cell recruitment was delayed. Furthermore, the Treg recruitment was lower compared to RSV A2 infection (Figure 6B). By combining results obtained with both strains, we found a significant association between Treg and pDC counts in BALs (Figure 6C). This indicates that, despite their skew, all distinct subsets of neonatal T-cells cooperate tightly with the magnitude of the cellular immune response against RSV infection.

**Discussion**

To properly assess the immunopathogenesis of RSV, it is essential to select a model that recapitulates the features of human disease. We selected the neonatal lamb, as RSV is a natural pathogen for this species, lung development is similar, and the key features mimic human RSV infection in infants (27, 28). One strength of our experimental approach was the ease sampling and the full accessibility to the lower airways throughout the disease when sampling in infants is mainly restricted to the
upper airways, the latter representing only an approximate surrogate of the distal lung where RSV disease occurs. Moreover, we offer an unprecedented view of the development of pulmonary neonatal immunity in healthy conditions. This process is highly dynamic and tightly regulated, with a short and early stage where colonizing T-cell subsets synergize towards a narrow pro-tolerogenic window, namely a "Treg/Th2/Tc2" environment. This certainly contributes to the maturation of the immune system and may constitute an unsuitable state to resolve RSV infection. The mechanisms underlying the severity of disease are still unclear. Herein, we observed a comparable virus clearance between adults and neonates, reinforcing the assumption of an inappropriate immune response. We observed a rapid and robust recruitment of neonatal pDCs, whose extent correlated with the viral loads of infected neonates. Infected adults did not require this recruitment, since their lung-resident pDCs reacted promptly by maturing to a higher level than that measured in neonates. This partially corroborates recent studies reporting low counts and maturation levels of pDCs in mice and infants (33, 34). This reduced capacity to respond against RSV might be due to a direct disturbance of signaling by RSV itself (35), or an intrinsic deficiency of neonatal pDCs to produce elevated amounts of cytokines (36-38). This neonatal defect would prevent conventional DCs from adequate presentation of viral antigens, contributing to poor specific T-cell responses (39).

Controversial is whether γδ T-cells participate in viral clearance or immunopathology. Herein, the γδ T-cell depletion conflicted with a previous report. Indeed, children with severe RSV bronchiolitis had reduced frequencies of γδ T-cells in peripheral blood, which was explained by the authors as a likely redistribution towards the lungs; unfortunately, they could not evaluate their hypothesis (40). In line with our findings, mice depleted of γδ T-cells before RSV infection display increased viral titers (13). An
alternative hypothesis that would reconcile our study with others, is that γδ T-cell tissue redistribution plays less of a role than its immature state in early life. Peripheral blood γδ T-cells of infants with severe disease failed to produce IFN-γ when restimulated (14), whereas γδ T-cells from neonatal mice had an impaired ability to produce IL-17 (15). Finally, Tc17 and Th17 cells were recently associated with shorter hospital stays and proposed to play a protective role (26). Altogether, our data demonstrate that the protective role of γδ T-cells in RSV infection is largely inefficient in early life. The BALs of neonates displayed a high proportion of Tregs that declined rapidly to levels comparable to healthy adults. As fetal CD4+ T-cells tend to differentiate preferentially into Tregs after stimulation (10), this tolerogenic mechanism is likely to promote self-tolerance to the developing lung. We found that Treg response to RSV infection happened very early in neonates, accompanying CD4+ and CD8+ T-cell expansion rather than shutting them down afterwards. This strong Treg recruitment was absent in adults. We speculate that the neonate immune system faces a dilemma between avoiding potential autoimmune disorders and failing to mount a specific T-cell response to harmful pathogens. Studies conducted in young infants with RSV disease support this statement. A selective depletion of peripheral Tregs was shown, probably due to massive recruitment to the lungs (41). A higher level of TGF-β transcript was measured in neonatal DCs compared to adults (42). In contrast, some reports emphasized the protective role of Tregs, since Treg-depleted young mice had an enhanced RSV disease, with abundant T-cells exhibiting an activated phenotype (43). Another remarkable observation was the strong correlation between pDC and Treg counts. Despite the need for further investigations, it is worth noting that pDC to Treg ratio was linked to the clearance versus persistence of human papillomavirus (44).
Whether the cellular arm of the adaptive pulmonary response is detrimental or beneficial in RSV disease is still debated. Particularly, the contribution of IL-4 to viral clearance versus immunopathogenesis remains controversial (25, 45-47). Recently, a study performed in hospitalized infants showed a possible involvement of Tc2 cells in disease severity. Although convincing, this study was for obvious ethical reasons restricted to nasal aspirates (26). Interestingly, Tc2 cells are increasingly proposed to play an important role in the etiology of asthma (48). Herein, we provide a significant advancement in understanding this yet unclear IL-4 contribution, by showing that type 2 cytokine environment in the lung is a footprint of early life that decreases rapidly with time in healthy conditions. RSV infection circumvents this decline by mediating the massive recruitment of CD4+ and CD8+ T-cells, exacerbating the overall type 2 cytokine environment. We think that the link between IL-4 and RSV disease is the direct consequence of a strong trafficking of endogenous Th2 and Tc2 lymphocytes towards infected pulmonary tissues, rather than an RSV-specific type 2 immune response per se.

The high proportion of pulmonary T-cells producing spontaneously IL-4 was undetectable in the healthy adults. Neither was the RSV-specific immune response of adults oriented towards a Th2 or Tc2 profile. This corroborates a previous report mentioning an endogenously poised cytokine profile toward IL-4 in CD4+ T-cells in cord blood of neonates and infants, establishing a link between IL-4 and development (49). However, we provide new insights by demonstrating that i) CD8+ Tc2 cells are an equal and possibly superior source of IL-4 than CD4+ Th2 cells; ii) this immunological state occurs during a short period in vivo, raising the likelihood that numerous animal trials failed to detect it due to the study design. Remarkably, this transient type 2 cytokine environment was exacerbated upon RSV infection. So far,
similar observations were only made at the systemic level or in nasal aspirates, and authors could only speculate on the relocation to the lower respiratory tract (26, 50).

Herein, not only we show that this happens, but we also unify the link between IL-4 and RSV disease and endogenous IL-4 program in neonates. This finding constitutes an important step towards deciphering why neonates and young infants are particularly at risk.

Conclusion

Our holistic approach provided novel insights into the identification of the host’s contributing factors to RSV immunopathology, strengthening the hypothesis of an inappropriate immune response in the narrow temporal window of early life. This can now be characterized as a combined bronchoalveolar influx of Treg, Th2 and Tc2 cells, associated with a strong depletion of γδ T-cells. These findings constitute a novel basis for further exploration of RSV immunopathogenesis and should be considered in RSV vaccine design, which remains challenging after five decades of effort.
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Figure legends

**Figure 1.** Neonatal RSV infection causes bronchiolitis and interstitial pneumonia. (A) Illustration of the transtracheal RSV infection experimental design. (B) RSV was quantified in the respiratory tract (BAL, cell fraction; Lung; BAL, fluid) by qPCR. (C) RSV detection by immunohistochemistry in macrophages, and type I pneumocytes. (D) Representative histological lung tissue sections from neonates over the course of RSV infection (40X, HE). Lungs of RSV-infected animals (strain A2) show interstitial septae expanded by inflammatory cells (interstitial pneumonia) and accumulation of inflammatory cells within alveoli at days 3 and 6 p.i. No lesions at days 14 and 42 p.i. The lungs of mock neonates allow comparison with normal lung architecture within the first weeks of life. Each symbol represents an individual animal (healthy adults, n=3; mock neonates, n=3; neonates infected with RSV, n=6). Boxplots indicate median value (center line) and interquartile ranges (box edges), with whiskers extending to the lowest and the highest values. Stars indicate significance levels. *, p < 0.05.

**Figure 2.** Neonatal RSV infection activates the initiators of the adaptive immune response and induces a transient depletion of γδ T-cells. (A-E) Neonatal RSV infection induces bronchoalveolar space recruitment of activated pDCs. (A) FCM gating strategy for ovine pDCs. (B) Neonatal RSV infection induces an early and transient bronchoalveolar space colonization by pDCs. (C) Correlation coefficient (r) obtained with pDC counts calculated as a function of RSV copies/10^{12} 18S copies. Significance was reached for BAL fluid but not for BAL, cell fraction. (D-E) A high frequency of recruited pDCs in bronchoalveolar space upregulate CD86 (D,
Figure 3. Neonatal RSV infection exacerbates the type 2 cytokine environment in lung. (A) FCM gating strategy for ovine CD4$^+$ and CD8$^+$ T-cells. (B) RSV infection in neonate accelerates the recruitment of CD4$^+$ and CD8$^+$ T-cells in the bronchoalveolar space. (C-D) A high frequency of recruited CD4$^+$ T-cells in bronchoalveolar space differentiate towards a Th2 cytokine profile (IL-4 production) (C, representative FACS contour plots; D, plot displaying all individuals). (E-F) A high frequency of recruited CD8$^+$ T-cells in bronchoalveolar space differentiate towards a Tc2 cytokine profile (IL-4 production) (E, representative FCM contour plots; F, plot displaying all individuals). (G) RSV infection in neonates modulates the activation (cell surface expression of CD25) and the maturation (cell surface expression of T-cell memory marker CD45RO) of CD4$^+$ and CD8$^+$ T-cell pools in the lung. (H) Neonates infected with RSV can mount an antiviral neutralizing-antibody response. (I) Anti-RSV
Nabs persist over a 42-day long period. Each symbol represents an individual animal (healthy adults, n=3; mock neonates, n=3; neonates infected with RSV, n=6). Boxplots indicate median value (center line) and interquartile ranges (box edges), with whiskers extending to the lowest and the highest values. Stars indicate significance levels. *, p < 0.05; **, p < 0.01.

Figure 4. Neonatal RSV infection rapidly induces Treg suppressive functions to dampen the Th2 and Tc2 responses. (A) FCM gating strategy for ovine CD4⁺ and CD8⁺ T-cells. (B) RSV infection in neonates led to the recruitment of Tregs in the bronchoalveolar space (left panel), but not in the lung (right panel). (C) Progressive decrease with age of Treg subset among the CD4⁺ T-cell fraction (representative FCM contour plots). (D) Frequency of TGF-β-producing Tregs. (E) Correlation coefficient (r) obtained in BALs with Treg counts calculated as a function of non-Treg CD4⁺ counts (left), Treg counts calculated as a function of CD8⁺ counts (middle) and CD8⁺ counts calculated as a function of non-Treg CD4⁺ counts (right). Significance was reached for the three correlations. (F) CD8⁺ T-cell counts remained stable in the peribronchial LNs over the course of neonatal RSV infection. (G) Role of neonatal RSV infection on cytokine production by CD8⁺ T-cells in draining LNs. Upper panel: poor influence on the frequency of IL-4⁺ CD8⁺ T-cells (representative FCM contour plots). Lower panel: slight, but significant enhancement of IL-17⁺ CD8⁺ T-cells (representative FCM contour plots). Each symbol represents an individual animal (healthy adults, n=3; mock neonates, n=3; neonates infected with RSV, n=6). Boxplots indicate median value (center line) and interquartile ranges (box edges), with whiskers extending to the lowest and the highest values. Stars indicate significance levels. *, p < 0.05; **, p < 0.01.
Figure 5. Distinct cellular immune response in neonates compared to adults following RSV infection. (A) RSV was quantified in the respiratory tract (BAL, cell fraction; lung; BAL, fluid) of adults and neonates by qPCR. (B) Representative histological lung tissue sections from RSV-infected neonates and adults at day 6 and 14 p.i. (40X, HE). Adults show no histological lesions, whereas neonates show interstitial pneumonia at day 6 p.i. (C-D) Counts of recruited pDCs and CD86 upregulation in bronchoalveolar space (C, plot displaying all individuals; D, representative FCM contour plots). (E-G) γδ T-cell counts, cytokine production, and maturation in the bronchoalveolar space (E, plot displaying all individuals; F, representative FCM contour plots; G, pie charts integrating all animals). (H-J) Recruitment, activation, and maturation of the CD4+ T-cell pool in the bronchoalveolar space (H, plot displaying all individuals; I, representative FACS contour plots; J, pie charts integrating all animals). (K-M) As in (H-J), but showing CD8+ T-cell pool. (N-O) Recruitment of Tregs and TGF-β production in the bronchoalveolar space (N, plot displaying all individuals; O, representative FACS contour plots). Each symbol represents an individual animal (infected adults, n=3; neonates infected with RSV, n=6). The grey zone corresponds to the interval integrating the values of all corresponding mock controls. Boxplots indicate median value (center line) and interquartile ranges (box edges), with whiskers extending to the lowest and the highest values. Stars indicate significance levels. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 6. The severity of disease relates to the magnitude of bronchoalveolar T-cell responses. (A) Representative histological lung tissue sections from mock neonates and neonates infected with RSV-ON1-H1 and RSV A2 strain (40X, HE). At day 6 p.i. the RSV-ON1-H1 infected animals show mild interstitial pneumonia (upper right corner) with accumulation of inflammatory cells within alveoli. No lesions at day 14 p.i. or mock-infected animals. (B) Comparison of immune cell subset trafficking into bronchoalveolar space after infection with RSV-ON1-H1 or RSV A2 strains. (C) Correlation coefficient (r) obtained in BALs with Treg counts calculated as a function of pDC counts. Each symbol represents an individual animal (infected adults, n=3; neonates infected with RSV, n=6). The grey zone corresponds to the interval integrating the values of all corresponding mock controls. Boxplots indicate median value (center line) and interquartile ranges (box edges), with whiskers extending to the lowest and the highest values. Stars indicate significance levels. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Data availability statement

The data generated and analysed during the current study are available from the corresponding author on reasonable request.
Figure 1

A

Birth

Acclimatization

-6
-3
0
3
6
9
12
15
18
21
24
27
30
33
36
39
42

RSV

*

BALs

Lung

Draining LNs

Clinical status

Blood sampling

Day 3, p.i.

Day 6, p.i.

Day 14, p.i.

Day 42, p.i.

B

BAL, cell fraction

Lung

BAL, fluid

Days p.i.

Days p.i.

Days p.i.

C

Mock

6

RSV

6

D

Mock

3

6

14

42

RSV
Figure 2

A. pDCs

B. pDC counts

C. Days p.i.

D. Adults

E. % of pDCs CD86

F. γδ T cells

G. γδ T-cell counts

H. Days p.i.

I. Adults

J. Adults

RSV copies/10^12 18S copies

r = 0.280

ns

r = 0.655

p = 0.034

r = -0.496

p = 0.014
Figure 3

A. T-cells

B. CD4+ counts

C. CD4+ IFN-γ IL-4

D. % of CD4+ IL-4

E. CD8+ IFN-γ IL-4

F. % of CD8+ IL-4

G. CD4+ and CD8+ subset analysis

H. Serum neutralisation (%)
Figure 5

A. BAL, cell fraction

|          | Adults | Neo. |
|----------|--------|------|
| Days p.i. | 6      | 14   |
| RSV copies×10^{12} copies/μL | p<0.001 | p>0.05 |

B. Lung

|          | Adults | Neo. |
|----------|--------|------|
| Days p.i. | 6      | 14   |
| RSV copies per ml | p<0.001 | p>0.05 |

C. pDCs

|          | Adults | Neo. |
|----------|--------|------|
| Days p.i. | 6      | 14   |
| pDC counts | *     | ***  |

D. BAL, fluid

|          | Adults | Neo. |
|----------|--------|------|
| Days p.i. | 6      | 14   |
| RSV copies | **    | **   |

E. γδ T cells

|          | Adults | Neo. |
|----------|--------|------|
| Days p.i. | 6      | 14   |
| γδ T cell counts | *     | ***  |

F. CD4⁺

|          | Adults | Neo. |
|----------|--------|------|
| Days p.i. | 6      | 14   |
| CD4⁺ counts | **    | **   |

G. Adult Mock

|          | Adults | Neo. |
|----------|--------|------|
| Days p.i. | 6      | 14   |
| Neonate RSV | **    | **   |

H. CD8⁺

|          | Adults | Neo. |
|----------|--------|------|
| Days p.i. | 6      | 14   |
| CD8⁺ counts | *     | ***  |

I. Adults

|          | 6      | 14   |
|----------|--------|------|
| IL-17 | 0      | 0    |

J. Neo.

|          | 6      | 14   |
|----------|--------|------|
| IL-17 | 0.079  | 4    |

K. Adults

|          | 6      | 14   |
|----------|--------|------|
| IFN-γ | 3      | 2    |

L. Neo.

|          | 6      | 14   |
|----------|--------|------|
| IFN-γ | 27     | 7    |

M. Adults

|          | 6      | 14   |
|----------|--------|------|
| CD25⁺CD45RO⁻ | 30    | 60   |

N. Neo.

|          | 6      | 14   |
|----------|--------|------|
| CD25⁺CD45RO⁺ | 40    | 80   |
Figure 6

A

Mock

RSV ON1-H1

RSV A2

6

14

B

pDCs

RSV

RSV A2

RSV

RSV ON1-H1

RSV A2

Counts

10^0

10^2

10^4

10^6

6 14 6 14 6 14

Days p.i.

CD4*

RSV

RSV ON1-H1

RSV A2

Counts

10^0

10^2

10^4

10^6

6 14 6 14 6 14

Days p.i.

CD8*

RSV

RSV ON1-H1

RSV A2

Counts

10^0

10^2

10^4

10^6

6 14 6 14 6 14

Days p.i.

C

γδ T cells

RSV

RSV ON1-H1

RSV A2

Counts

10^0

10^2

10^4

10^6

6 14 6 14

Days p.i.

Tregs

RSV

RSV ON1-H1

RSV A2

Counts

10^0

10^2

10^4

10^6

6 14 6 14

Days p.i.

0.0

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1.0

pDC counts

p = 0.7337

p = 0.0066