SHORT COMMUNICATION

Elevated Th17 Response in Infants Undergoing Respiratory Viral Infection

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IL-17 and T-helper (Th)17 cells contribute to viral airway pathology in human newborns. Because umbilical cord blood T cells fail to differentiate toward the Th17 lineage in the presence of autologous antigen-presenting cells, we asked whether Th17 cells are present in young infants that experience respiratory viral infection. To this end, we analyzed tracheal aspirate samples from infant patients suffering from acute respiratory syncytial virus (RSV) infection and healthy infant controls. Acute RSV infection associates with elevated IL-17 and accumulation of CD161⁺ T cells in acute RSV infected lungs. Correspondingly, local Th17 polarizing cytokines were increased. In peripheral blood, we show that Th17 cells are absent in healthy 1-month-old infants, but are present in acute RSV patients. The triggering of pathogen-associated pattern receptors TLR4 and TLR7 promotes the generation of a Th17-polarizing cytokine environment by 1-month-old infant dendritic cell (DC). We thus conclude that although Th17 cells are absent in healthy newborns, Th17 cells are present in peripheral blood and the airways of infants that experience viral infection, thereby contributing to airway immunopathology. (*Am J Pathol* 2014, 184: 1274–1279; <http://dx.doi.org/10.1016/j.ajpath.2014.01.033>)

Respiratory infections are the most prominent cause of infant mortality worldwide. Primary respiratory syncytial virus (RSV) infection is the most common cause of hospitalization of otherwise healthy infants in developed countries. The clinical course of primary RSV infection is hallmarked by rapid neutrophil infiltration, excessive mucus production, and a delayed CD8 T-cell response.^{1,2} High neonatal susceptibility to respiratory infections has been attributed to a predominant T-helper (Th)2 response due to low production of Th1 cytokines. Although an immature Th1 reactivity is indeed observed during neonatal vaccination to respiratory pathogens,^{3–5} Th2 reactivity cannot fully explain the type of responsiveness observed during primary RSV infection in human neonates.⁶ Recent data in both humans and mice propose the contribution of IL-17–dependent immune activation in RSV airway infection.^{7,8}

Th1 and Th17 differentiation are reciprocally regulated through the requirement of the IL-12p40 subunit for functional IL-12p70 and IL-23, respectively.⁹ In umbilical cord blood the IL-12p70 axis appears suboptimally used, causing a predisposition toward IL-23 production⁹ facilitated by a Th2-polarizing

environment.¹⁰ Despite this preference for IL-23 production, we recently showed a block of Th17 development in co-cultures of cord blood naïve CD4 T cells and autologous antigen-presenting cells.¹¹ However, considering the human immune system rapidly develops during the first month of life, Th17 responses in 1-month-old infants may be distinct from umbilical cord blood. We hypothesized that virally infected neonates would show an increased Th17 response, and therefore we studied T-cell responses in healthy and RSV infected infant samples derived from the airways and the peripheral blood.

Materials and Methods

Participants

Peripheral venous blood samples were obtained from healthy adult volunteers, healthy 1-month-old infants, and

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Figure 1 Th17 cells are present in infants that experience respiratory viral infection. TA and peripheral blood from 48 hours intubated RSV patients ($n = 10$) and healthy infants ($n = 7$) were analyzed by multiplex immunoassay and flow cytometry. **A:** TA IL-17 concentration. **B:** Frequency of CD3⁺CD161⁺ cells in RSV patient TA ($n = 5$) and PBMC ($n = 4$). TA concentrations of IFN- γ (**C**), TNF (**D**), and IL-22 (**E**). Phorbol 12-myristate 13-acetate- and ionomycin-stimulated PBMC from healthy adults ($n = 5$), infants ($n = 7$), and infant RSV patients ($n = 4$) were analyzed by flow cytometry. Intracellular IL-17 in blood CD4⁺ T cells (**F**) and blood CD45RO⁺ CD4 memory T cells (**G**). * $P < 0.05$, ** $P < 0.01$.

infant RSV patients. Tracheal aspirate (TA) was collected during routine cleaning procedures from the ventilation tubes of intubated infant RSV infection patients and uninfected infants who were intubated during minor surgery. Both for TA and peripheral blood samples only healthy infant controls without a history of infection were included. This study was approved by the local medical Ethical Committee of the University Medical Center Utrecht (Utrecht, The Netherlands) and written consent was obtained from the parents of the infant participants.

Blood and TA Cell Isolation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and maintained in RPMI 1640 medium, including 2 mmol/L L-glutamine, 60 mg/mL penicillin, 20 μ g/mL streptomycin (Invitrogen, Carlsbad, CA).

TA cells were harvested by vigorously resuspending aspirates in RPMI 1640 medium, followed by mucus removal via filtering cells twice over sterile 70 μ m cell strainers (BD Biosciences, San Jose, CA).

DC Differentiation and Stimulation

CD14⁺ monocytes were isolated from PBMC using CD14 microbeads (MACS; Miltenyi Biotech, Bergisch, Germany). CD14⁺ monocytes were cultured in presence of 200 ng/mL IL-4/50 ng/mL granulocyte-macrophage colony-stimulating factor for 6 days. DC were stimulated on day 7 of culture with 1 μ g/mL Pam3Cys (Sigma-Aldrich, Zijdrecht, The Netherlands), 1 μ g/mL lipopolysaccharide (LPS; Enzo Life Sciences, Lausen, Switzerland), or 10 μ g/mL Imiquimod (InvivoGen, San Diego, CA). Supernatants and cell lysates in Tripure (Roche, Woerden, The Netherlands) were acquired after 24 hours of stimulation.

Figure 2 RSV infection causes a local Th17-polarizing environment in infant lungs. TA was analyzed by multiplex immunoassay. Concentrations of IL-1β (A), IL-6 (B), and IL-23 (C) in TA from 5 ($n = 10$) and 48 hours ($n = 9$) intubated RSV patients and healthy infants ($n = 7$). $**P < 0.01$, $***P < 0.001$. hr, hours.

Flow Cytometry of TA and PBMC

TA cells were directly surface stained. For intracellular cytokine staining of PBMC, cells were stimulated for 4 hours in culture medium with 5 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), 1 μg/mL ionomycin (Calbiochem, Beeston, UK), and 0.66 μL/mL GolgiStop (BD Biosciences). Cells were resuspended in PBS with 2% fetal bovine serum (fixed and permeabilized when necessary), stained for 30 minutes at 4°C, and measured using FACScanto II (BD Biosciences).

TA and DC Culture Supernatant Cytokine Analysis

TAs that were used for cytokine analysis were snap-frozen and stored at -80°C . Several hours before cytokine measurement, aspirates were thawed and weighed, and diluted in high-performance ELISA (HPE) buffer (Sanquin, Amsterdam, The Netherlands). Aspirates were sonicated twice at 4°C for 30 seconds, mucus spun down, and the supernatant filtered through 0.2 μm spin columns (spin X; Corning Life Sciences, Corning, NY). Cytokines were measured in the filtered supernatant and results were corrected for dilution. TA and DC culture supernatant cytokine concentrations were determined by multiplex immunoassay.

RNA Isolation and Quantitative PCR

RNA isolation from Tripure (Roche) was performed according to the manufacturer's protocol. The resulting RNA pellet was air-dried and resolved in RNase-free water. The cDNA was synthesized using a cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) with random hexamer primers.

Quantitative PCR was performed using the Bio-Rad MyiQV1 and CFX96 systems and iQ SYBR Green ready reaction mix (Bio-Rad, Veenendaal, The Netherlands). Primer sets used were hIL-23p19 (Forward: 5'-TGTTCCCCATATCCAGTGTG-3', Reverse: 5'-TCCTTTGCAAGCAGAACTGA-3') and human GAPDH (Forward: 5'-GTCGGAGTCAACGGATT-3', Reverse: 5'-AAGCTTCCCGTTCTCAG-3').

Statistical Analysis

Data were analyzed using Graphpad Prism (GraphPad Software Inc., La Jolla, CA). The *U*-test was applied for statistical testing between two groups. Comparison of three groups was done using the Kruskal-Wallis test. The *U*-test with Bonferroni correction was used for post hoc testing. A *P* value of at least 0.05 was considered statistically significant. Data are shown as median $\pm$ interquartile range.

Results

Th17 Cells Are Present in Infants that Experience Respiratory Viral Infection

To investigate the contribution of Th17 cells to the inflammatory response to viral airway infection, we analyzed TAs and peripheral blood of infant RSV patients (Supplemental Table S1). Indeed, the concentration of IL-17 was elevated in TAs of infants undergoing RSV infection compared to control aspirates (Figure 1A). Consistently, T cells bearing the Th17 cell surface marker CD161 were increased in RSV-infected TA compared to autologous peripheral blood (Figure 1B), indicating Th17 presence and possibly accumulation in the lung lumen. Th1 cytokines, notably tumor necrosis factor- α and interferon- γ , were poorly induced by RSV infection (Figure 1, C and D). IL-22, a cytokine that is related to IL-17 by the requirement for retinoic acid receptor-related orphan receptor gamma-T for its induction, was unaffected by RSV infection (Figure 1E).

To confirm that RSV infection associates with elevated Th17 cell counts in otherwise healthy 1-month-old infants, we measured IL-17 production in phorbol 12-myristate 13-acetate and ionomycin stimulated peripheral blood CD4 T cells from RSV patients, infant controls, and adult controls using intracellular flow cytometry. In accordance with earlier published results,^{11,12} newborn blood contained fewer Th17 cells than adult blood (Figure 1F). As

the developing newborn immune system contains fewer memory cells, we examined the frequency of Th17 cells within the CD45RO⁺ memory CD4 T cell population. IL-17-producing cells were less abundant in the newborn memory T-cell population (Figure 1G). In RSV patient peripheral blood, however, we observed a significant increase of Th17 cells over age-matched controls, both in the total CD4 T-cell population and within the CD45RO⁺ memory cells (Figure 1, F and G). IFN- γ production was lower in newborn T cells than in adult cells, but was not affected by RSV infection (Supplemental Figure S1). Although Th17 cells are virtually absent in the peripheral blood of 1-month-old infants, these data show they are elevated in RSV patients.

RSV Infection Causes a Local Th17-Polarizing Environment in Infant Lungs

Th17 cell differentiation is driven by pro-inflammatory cytokines. In humans, IL-1 β , IL-6, and IL-23 are known to induce Th17 cells and propagate the activity of this cell type.¹³ As it has been shown that the production of IL-1 β and IL-6 is reduced or even absent in the neonatal immune system,¹¹ we asked whether these cytokines are produced in the viral-infected lungs of RSV patients. We analyzed the TA of patients collected at 5 and 48 hours after hospitalization for these cytokines and compared this to the TA from healthy controls. Neither IL-1 β nor IL-6 can be found in the samples from healthy infants, but are readily observed in patient samples (Figure 2, A and B). IL-23 was found in the TA of healthy infants as well, but was higher in infected patients and appeared to increase over time (Figure 2C). These data suggest that RSV infection induces an environment favorable for Th17 development.

RSV-Mimicking TLR Triggering of DC Induces Th17-Polarizing Cytokine Production

Activated DC are potent producers of polarizing cytokines in the lungs.¹⁴ RSV particles activate DC through engagement of Toll-like receptor (TLR)2, TLR4, and TLR7.¹⁵ We hypothesized that activation of infant DC via TLR signaling can result in a Th17-polarizing cytokine environment. To investigate this, we cultured DC from adult and 1-month-old infant peripheral blood monocytes and stimulated them with the individual TLR ligands Pam3Cys (TLR2), lipopolysaccharide (TLR4), and imiquimod (TLR7). After 24 hours of culture we measured IL-1 β and IL-6 production in the culture supernatants and IL-23p19 mRNA by real-time quantitative PCR, as the supernatant IL-23 protein levels were below detection limit. Both adult and infant DC produced some IL-1 β and IL-6 in the absence of TLR stimulation (Figure 3, A and B). TLR4 activation resulted in an increase in IL-6 production and IL-23 mRNA content in both infant and adult DC, whereas TLR2 stimulation did not induce either of these

Figure 3 RSV-mimicking TLR triggering of DC induces Th17-polarizing cytokine production. Monocyte-derived DC were cultured from healthy adult and healthy infant PBMC and stimulated with Pam3Cys, lipopolysaccharide (LPS), or imiquimod ($n = 3$ to 5). Culture supernatant IL-1 β (A) and IL-6 concentrations (B). C: Relative IL-23p19 mRNA levels. * $P < 0.05$.

cytokines. TLR7 activation triggering induced reverse responses between adult and infant DC: imiquimod increased the amount of IL-23p19 mRNA in infant DC, but not in adult DC (Figure 3C). Thus, TLR4 and TLR7 activation induces

the production of Th17-polarizing cytokines, specifically in infants.

Discussion

Although increased levels of IL-17 in RSV-infected infant plasma¹⁶ and TA was previously demonstrated,^{8,12,17} for the first time to our knowledge, we show the presence of Th17 cells in the airways during infancy. Previously, we showed that cord blood naïve T cells fail to differentiate to Th17 cells in co-culture with autologous antigen-presenting cells, despite the presence of polarizing cytokines.¹¹ Our current data show that 1-month-old infants lack circulating Th17 cells, but we also show that these are infants who experience RSV bronchiolitis. This suggests that the capacity for Th17 development *in vivo* may be acquired quickly after birth. It is likely that immune-related events that occur early during child development potentiate Th17 responses.

In contrast to IL-17, the Th17-related cytokine IL-22 was not elevated in RSV TAs, at least at the time of our measurements. Although both IL-17 and IL-22 are produced by retinoic acid receptor-related orphan receptor gamma-T expressing cells, IL-22 can be repressed by TGF- β in IL-17-producing cells, which allows for uncoupling of these cytokines.¹⁸ Additionally, a multitude of IL-22-expressing cell types that do not produce IL-17 have been identified in mice, and particularly in humans.¹⁹ Therefore, we think it is likely that the IL-17 and IL-22 we measured in TA originate from distinct cellular sources.

To our knowledge, we are the first to compare Th17 responses between healthy 1-month-old infants and RSV patients of similar age. Although our data support a clear Th17 response in RSV-infected newborns, several considerations limit further conclusions. First, although sufficient for statistical analysis, the number of both RSV patients and healthy control subjects was modest. The amount of blood that could be acquired from each participant was further restrictive in several experiments. Second, medical and ethical considerations limited the frequency of sampling for both patients and controls. Finally, practical concerns hampered the selection of controls for analysis of TA. For these experiments we were limited to healthy infant controls that were intubated during minor surgery. As such, we could not match duration of intubation nor obtain fully age-matched controls. In those experiments, the youngest control individuals that we could compare were 224.6 days, on average. However, we found that TAs from RSV-infected infants already contained IL-17 (average age, 47.1 days), in contrast to aspirates from those control infants who were not virally infected. Thus, our data that Th17 cell responses are present in RSV-infected infants are sustained, even compared with somewhat older infants.

The infant-specific induction of IL-23 on TLR7 ligation may contribute to the induction of a Th17-polarizing cytokine environment in newborns. This observation is in

contrast to RSV activation of TLR7 in mice, which limits IL-23 production.²⁰ Our data suggest that RSV stimulation of DC through TLRs could enhance the production of Th17-polarizing cytokines, specifically in infants. Further research is required to provide conclusive insight in the contribution of RSV-mediated TLR activation to immune-polarizing responses in infants.

Together, our data show that RSV infection of infant lungs results in a Th17-polarizing cytokine environment accompanied with Th17 cells, which are otherwise absent in infants. An increased cytokine production on TLR4 and TLR7 triggering in infant DC likely contributes to this effect.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.01.033>.

References

1. Heidema J, Lukens MV, van Maren WW, van Dijk ME, Otten HG, van Vught AJ, van der Werff DB, van Gestel SJ, Semple MG, Smyth RL, Kimpen JL, van Bleek GM: CD8+ T cell responses in bronchoalveolar lavage fluid and peripheral blood mononuclear cells of infants with severe primary respiratory syncytial virus infections. *J Immunol* 2007, 179:8410–8417
2. Lukens MV, van de Pol AC, Coenjaerts FE, Jansen NJ, Kamp VM, Kimpen JL, Rossen JW, Ulfman LH, Tacke CE, Viveen MC, Koenderman L, Wolfs TF, van Bleek GM: A systemic neutrophil response precedes robust CD8(+) T-cell activation during natural respiratory syncytial virus infection in infants. *J Virol* 2010, 84:2374–2383
3. White OJ, Rowe J, Richmond P, Marshall H, McIntyre P, Wood N, Holt PG: Th2-polarisation of cellular immune memory to neonatal pertussis vaccination. *Vaccine* 2010, 28:2648–2652
4. van den Biggelaar AH, Richmond PC, Pomat WS, Phuanukoonnon S, Nadal-Sims MA, Devitt CJ, Siba PM, Lehmann D, Holt PG: Neonatal pneumococcal conjugate vaccine immunization primes T cells for preferential Th2 cytokine expression: a randomized controlled trial in Papua New Guinea. *Vaccine* 2009, 27:1340–1347
5. De Swart RL, Kuiken T, Timmerman HH, van Amerongen G, Van Den Hoogen BG, Vos HW, Neijens HJ, Andeweg AC, Osterhaus AD: Immunization of macaques with formalin-inactivated respiratory syncytial virus (RSV) induces interleukin-13-associated hypersensitivity to subsequent RSV infection. *J Virol* 2002, 76:11561–11569
6. McNamara PS, Smyth RL: The pathogenesis of respiratory syncytial virus disease in childhood. *Br Med Bull* 2002, 61:13–28
7. Mukherjee S, Lindell DM, Berlin AA, Morris SB, Shanley TP, Hershenson MB, Lukacs NW: IL-17-induced pulmonary pathogenesis during respiratory viral infection and exacerbation of allergic disease. *Am J Pathol* 2011, 179:248–258
8. Stoppelenburg AJ, Salimi V, Hennis M, Plantinga M, Huis in 't Veld R, Walk J, Meerding J, Coenjaerts F, Bont L, Boes M: Local IL-17A potentiates early neutrophil recruitment to the respiratory tract during severe RSV infection. *PLoS One* 2013, 8:e78461
9. Gorieli S, Van Lint C, Dadkhah R, Libin M, De Wit D, Demonte D, Willems F, Goldman M: A defect in nucleosome remodeling prevents IL-12(p35) gene transcription in neonatal dendritic cells. *J Exp Med* 2004, 199:1011–1016
10. Tanaka J, Watanabe N, Kido M, Saga K, Akamatsu T, Nishio A, Chiba T: Human TSLP and TLR3 ligands promote differentiation of

- Th17 cells with a central memory phenotype under Th2-polarizing conditions. *Clin Exp Allergy* 2009, 39:89–100
11. de Roock S, Stoppelenburg AJ, Scholman R, Hoeks SB, Meerding J, Prakken BJ, Boes M: Defective TH17 development in human neonatal T cells involves reduced RORC2 mRNA content. *J Allergy Clin Immunol* 2013, 132:754–756.e3
 12. Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, Rodolico G, Querci V, Abbate G, Angeli R, Berrino L, Fambrini M, Caproni M, Tonelli F, Lazzeri E, Parronchi P, Liotta F, Maggi E, Romagnani S, Annunziato F: Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J Exp Med* 2008, 205:1903–1916
 13. Sallusto F, Zielinski CE, Lanzavecchia A: Human Th17 subsets. *Eur J Immunol* 2012, 42:2215–2220
 14. Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL: Regulation of immunological homeostasis in the respiratory tract. *Nat Rev Immunol* 2008, 8:142–152
 15. Klein Klouwenberg P, Tan L, Werkman W, van Bleek GM, Coenjaerts F: The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. *Crit Rev Immunol* 2009, 29:531–550
 16. Larranaga CL, Ampuero SL, Luchsinger VF, Carrion FA, Aguilar NV, Morales PR, Palomino MA, Tapia LF, Avendano LF: Impaired immune response in severe human lower tract respiratory infection by respiratory syncytial virus. *Pediatr Infect Dis J* 2009, 28:867–873
 17. Faber TE, Groen H, Welfing M, Jansen KJ, Bont LJ: Specific increase in local IL-17 production during recovery from primary RSV bronchiolitis. *J Med Virol* 2012, 84:1084–1088
 18. Rutz S, Noubade R, Eidenschenk C, Ota N, Zeng W, Zheng Y, Hackney J, Ding J, Singh H, Ouyang W: Transcription factor c-Maf mediates the TGF-beta-dependent suppression of IL-22 production in T(H)17 cells. *Nat Immunol* 2011, 12:1238–1245
 19. Rutz S, Eidenschenk C, Ouyang W: IL-22, not simply a Th17 cytokine. *Immunol Rev* 2013, 252:116–132
 20. Lukacs NW, Smit JJ, Mukherjee S, Morris SB, Nunez G, Lindell DM: Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. *J Immunol* 2010, 185: 2231–2239