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Augmented Th17-Type Immune Responses in Preterm Neonates Exposed to Histologic Chorioamnionitis:

Chorioamnionitis and Th17-type immunity

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Abstract

Background—Histologic chorioamnionitis (HCA) is a placental inflammatory disorder that frequently precedes preterm delivery. HCA increases risk for long-standing inflammatory injury and may influence immune programming, particularly in preterm (PT) neonates. We hypothesized that HCA exposure is associated with an increased circulating frequency of pro-inflammatory, Th17-type responses.

Methods—Placental cord blood was collected from HCA-exposed or control neonates (23-41 weeks gestation). Frequencies of Th17 and T regulatory (Treg) cells and assessments of Th17-type features in CD4 and Treg cells were determined by flow cytometric analysis.

Results—Cord blood samples from 31 PT and 17 term neonates were analyzed by flow cytometry. A diagnosis of HCA in extremely PT (EPT, GA ≤ 30 wk) gestations was associated with the highest cord blood frequencies of progenitor (pTh17, $CD4^+CD161^+$) and mature (mTh17, $CD4^+CD161^+CCR6^+$) Th17 cells. Preterm neonates exposed to HCA also exhibited elevated cord blood frequencies of IL-17^+ Treg cells, as well as T cells with effector memory phenotype (TEM) that co-expressed Th17-type surface antigens.

Conclusion—Th17-type responses are amplified in preterm neonates exposed to HCA. We speculate that a Th17 bias may potentiate the inflammatory responses and related morbidity observed in preterm neonates whose immune systems have been 'primed' by HCA exposure.

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INTRODUCTION

Histologic chorioamnionitis (HCA) is a neutrophil-driven inflammation of the placental membranes. A common antecedent of preterm delivery, the incidence and severity of HCA are inversely proportional to gestational age (1, 2). Extremely preterm neonates born after HCA exposure may also be at greater risk for chronic inflammatory disorders (3-7). However, the mechanisms leading to exaggerated inflammatory responses in these infants are incompletely understood. We recently reported that antenatal inflammation in a murine model induces exaggerated systemic inflammatory immune responses in exposed offspring, including enhanced lung expression of pro-inflammatory Th17 cells (3).

Th17 cells are a unique T helper subset that bridges adaptive and innate immune responses. While Th17 cells are critical to antimicrobial immunity, they can also mediate pathogenic inflammatory processes (reviewed in (4)). Ontogenic studies suggest a skewing of CD4 cell differentiation towards Th17-type immunity in human preterm neonates and in the offspring of immune-stimulated pregnant mice (3, 5, 6). Despite their importance to health and illness, however, the biology of Th17 cells in neonates and their role in inflammation remain incompletely defined.

We hypothesized that fetal exposure to inflammation during critical developmental windows can influence immune programming to augment inflammatory neonatal responses. To begin to test this, the goal of the present study was to determine Th17-type responses in preterm and term neonates exposed to intrauterine inflammation.

RESULTS

Patient characteristics/demographics

Forty-eight cord blood samples were variously analyzed by multi-parameter flow cytometry: 31 preterm (PT: 17 exposed to HCA, 14 unaffected controls, Ctrl) and 17 term (7 HCA, 10 Ctrl) (**Table 1**). The incidence of prolonged membrane rupture (PROM, >18 h prior to delivery) was more common in gestations complicated by HCA for either gestational age group, consistent with previous reports (7, 8). All women enrolled in this study with impending preterm delivery received antenatal steroids. Gestational and perinatal characteristics were generally similar for HCA-exposed neonates and their age-matched controls. In PT gestations with a diagnosis of HCA, only 5 of 17 (29%) of pregnant women had symptoms suggestive of clinical chorioamnionitis, while all term gestations with HCA had presumptive clinical chorioamnionitis.

Th17 cell populations

Higher frequencies of progenitor Th17 cells (pTh17, $CD161^+$) within gated $CD4^+$ populations were found in the cord blood of PT *vs.* term control neonates (**Figure 1a**). In both HCA-exposed PT and term neonates, pTh17 frequencies were elevated relative to age-matched controls. The absolute numbers of circulating pTh17 cells were also higher in HCA-exposed PT neonates relative to PT controls (**Table 2**) and also as compared against HCA-exposed term neonates (PT, 0.71 ± 0.14 *vs.* Term, 0.42 ± 0.21 × 10^9/L; $P < 0.05$; X ± SEM). Frequencies of mature Th17 cells (mTh17, $CD161^+CCR6^+$) in gated CD4 cells were

higher in control PT *vs.* term neonates. In contrast to our observations in pTh17 cells, HCA exposure was not associated with altered mTh17 cell frequencies for either the PT group as a whole or in term neonates (**Figure 1b**), and no differences were observed in the absolute numbers of mTh17 cells between control and HCA-exposed PT neonates (**Table 2**). However, comparative analyses of gestational age subsets of HCA-exposed neonates showed an inverse relationship between pTh17 and mTh17 cell populations and gestational age, with the highest frequencies observed in HCA-exposed EPT (extremely PT, GA ≤ 30 wk) compared to MPT (moderately PT, GA 31– 34 wk) and term neonates (**Figure 1c**).

Treg populations

A higher proportion of cord blood $CD4^+$ cells were identified as Tregs ($CD25^{hi}CD127^{lo}$) in control and HCA-exposed PT *vs.* term neonates (**Figure 2a**). A diagnosis of HCA was associated with lower Treg proportions in $CD4^+$ cells in the PT group, while a non-significant trend towards lower Treg frequencies was observed in term HCA-exposed *vs.* control neonates (P=0.09). Comparative analyses of $CD4^+$ cells in the cord blood of PT subsets and term populations revealed a trend of decreasing Treg cell frequencies with advancing gestational age (**Figure 2b**). However, although Treg frequencies within CD4 populations differed between control and HCA-exposed PT neonates, absolute Treg numbers in the cord blood were similar between these groups (**Table 2**).

Th17:Treg ratio

The circulating Th17:Treg ratio has been reported to correlate with disease severity in adult inflammatory disorders (reviewed in (9)). For the present study, we calculated this ratio using frequencies of mTh17 or pTh17 cells and Treg cells in cord blood $CD4^+$ populations of PT and term neonates. In comparative analyses, elevated mTh17:Treg ratios were observed in HCA-exposed EPT (≤ 30 wk) neonates relative to EPT controls, while no differences in this ratio were observed between HCA-exposed and control term neonates (**Figure 2c**). In contrast, the mean pTh17:Treg ratio, while generally higher in EPT *vs.* term neonates, did not appear to be affected by HCA exposure for either gestational group (**Figure 2d**). In contrast, when absolute numbers of pTh17 and Treg cells were used for calculations, an over seven-fold elevation in the pTh17:Treg ratio was observed in HCA-exposed PT neonates compared to controls (**Table 2**).

TEM populations

The TEM phenotype has been recently described in a subset of neonatal CD4 cells (10). To examine a potential effect of HCA on this cell population, we determined the frequencies of CD4 cells with a $CD25^{lo}CD127^{hi}$ phenotype. Frequencies of TEM cells were higher in PT relative to term controls, and exposure to HCA was not associated with altered proportions in either group (**Figure 3a**). In subset analyses, the frequencies of TEM cells that co-expressed CCR6 alone (**Figure 3b**) or in combination with CD161 (**Figure 3c**) were highest in PT HCA-exposed neonates. In contrast, whereas the mean expression patterns of the $CD161^+CCR6^+$ subsets (**Figure 3c**) were higher in HCA-exposed *vs.* control PT neonates, this pattern was reversed in term subjects.

IL-17A and RORγt expression in CD4 populations

Cord blood studies in a small subset of neonates showed elevated frequencies of IL-17A$^+$ (hence referred to as IL-17$^+$) CD4 cells in HCA-exposed PT and term neonates *vs.* age-matched controls; frequencies of IL-17$^+$ CD4 cells were highest overall in the HCA-exposed PT group (**Figure 4a**). Similarly, absolute numbers of IL-17$^+$ CD4 cells were higher in the cord blood of HCA-exposed PT neonates relative to PT controls (**Table 2**) or compared with HCA-exposed term neonates (Term, $0.63 \pm 0.31 \times 10^9$/l, $P<0.05$; X ± SEM). Frequencies of CD4 cells that co-expressed both RORγt and IL-17 were also elevated in HCA-exposed PT and term neonates relative to controls, and highest in PT HCA neonates (**Figure 4b**).

To determine if HCA exposure was associated with a Th17-like phenotype in neonatal Treg cells, we next analyzed a small subset of CD4$^+$C25^{hi}Foxp3$^+$ Treg cells for intracellular expression of IL-17 or RORγt. Mean proportions of IL-17$^+$ Tregs were quadrupled in PT-HCA neonates *vs.* PT controls; differences in the proportions of IL-17$^+$ Tregs between HCA and control term neonates approached but did not reach significance ($P=0.06$; **Figure 4c**). The proportions of RORγt$^+$ Treg cells were also higher in PT *vs.* term controls (**Figure 4d**). Although Treg co-expression of RORγt was not different between control and HCA-exposed PT neonates, analyses of this small group of term neonates with HCA exposure showed increased proportions of RORγt$^+$ Tregs relative to age-matched controls.

DISCUSSION

The goal of the present study was to compare the potential effects of histologic chorioamnionitis (HCA) on the Th17 phenotype in PT and term neonates. We now report Th17-type responses in the cord blood of HCA-exposed neonates that appear to be particularly prominent in extremely PT neonates. To our knowledge, this is the first report of the prevalence of immune cells with Th17-like properties in human PT and term neonates in the context of HCA.

We observed relatively higher proportions of Th17 cell populations in PT relative to term neonates, consistent with the low Th17-type responses reported in normal term pregnancies (11). The higher levels of Th17 cells in HCA-exposed PT *vs.* term neonates suggest an influence of developmental stage, consistent with the intrinsic Th17 bias in PT neonates reported by Black *et al.* (5). Rueda *et al.* recently reported a similar elevation of CD4$^+$IL-17$^+$ Th17 cells in the spleens of LPS-exposed fetal rhesus macaques (12). In the present study, we observed particularly robust frequencies of the CD4$^+$CD161$^+$ (pTh17) cell subset in the cord blood of PT neonates, a finding which may have pathogenic implications for this population. Pertinently, high frequencies of CD161$^+$ Th17 cells have been correlated with inflammatory disease activity and recent evidence indicates that this cell population is both excessively inflammatory and resistant to Treg-mediated suppression (13, 14). Higher co-expression of the canonical Th17 nuclear transcription factor, RORγt, and IL-17 was also determined in cord blood CD4 cells of HCA-exposed PT neonates. Taken together, our findings parallel the elevated Th17 cell frequencies or Th17-type cytokine levels observed in human neonates exposed to maternal filarial infection, HIV, or preeclampsia (24-26) or in a non-human primate model of intrauterine inflammation (12). The increased proportions of

Th17 cells in HCA-exposed neonates also likely reflect the heightened Th17-type responses in placentas with chorioamnionitis (15, 16).

Higher Treg frequencies were determined in the cord blood of PT relative to term control neonates, in keeping with previous reports (17). Although Treg proportions were decreased in HCA-exposed PT neonates relative to age-matched controls, an observation consistent with the findings of Treg depletion in a fetal non-human primate model of chorioamnionitis (12), the absolute numbers of Tregs were similar in PT groups. These data suggest that the elevation of absolute Th17 numbers in HCA-exposed PT neonates occurs independently of an effect of HCA on Tregs. Given that elevation of the Th17:Treg ratio reflects disease severity in other Th17-associated inflammatory disorders (9), our findings suggest an enhanced Th17-mediated inflammatory response in PT neonates in the context of HCA.

Treg cells typically function to restrain activated T cells, including inflammatory Th17 cells (9, 18). However, Treg cells can also exhibit Th17-like phenotypes in the context of an inflammatory milieu (18). In the present study, we determined a subset of cord blood Treg cells with pro-inflammatory Th17-type characteristics in HCA-exposed neonates, a finding also reported in a fetal primate model of chorioamnionitis (12). Although the proportions of cord blood Treg cells that also co-expressed IL-17 were not different between PT and term controls, we observed that PT neonates exposed to HCA exhibited a particularly robust elevation of IL-17$^+$ Tregs. In contrast, RORγt$^+$ Treg frequencies were markedly higher in the cord blood of preterm *vs.* term control neonates, a finding that may reflect developmental regulation of RORγt and the common lineage shared by Tregs and Th17 cells (5, 19). Frequencies of RORγt$^+$ Tregs were not different between control and HCA-exposed PT neonates. In contrast, HCA exposure of term neonates was associated with higher RORγt$^+$ Treg proportions relative to age-matched controls. Although Tregs with Th17-type features potentially retain suppressive activity (20), high RORγt expression can contribute to loss of Treg anti-inflammatory function (21). In addition, RORγt expression can facilitate the conversion of Tregs to a Th17 phenotype during inflammation (21, 22). Thus, the high RORγt expression in Tregs of HCA-exposed PT and term neonates could contribute to compromised Treg suppressor function and the enhanced Th17-type responses that we now report. While recent data are supportive of this premise (12, 23), the effects of HCA exposure on Treg function in human neonates remain to be more fully elucidated.

Our studies included an analysis of cord blood T effector memory (TEM) cells, a recently characterized CD4 subset with a CD25^{lo}CD127^{hi} phenotype (10). Neonatal TEM express various Th17-related markers and produce IL-17 under inflammatory conditions (10). A higher proportion of TEM cells were observed in the cord blood of PT relative to term controls, and these were not altered with HCA exposure for either group. However, PT neonates exposed to HCA exhibited high frequencies of TEM subsets with Th17-type phenotypes (CD161 or CCR6 co-expression), observations that may also be relevant to amplified inflammatory function (24). While the clinical significance of these findings is at present unclear, the potential contribution of TEM cells to Th17-type inflammatory responses in PT neonates is deserving of further exploration.

The present study was not powered to identify infectious etiologies of HCA or to extrapolate experimental findings to HCA-associated neonatal outcomes (25, 26). In addition, only a limited number of gestations retrospectively met some, but not all, of the criteria needed for the newly-defined diagnosis of 'confirmed Triple I' (intrauterine inflammation or infection or both) (27)), thus we were unable to employ this designation in the analyses of the present studies. However, our data confirmed a reported association between PROM and HCA (7, 8). Despite our limited sample size, the significant inter-group differences in Th17-type responses that we now report suggest biological relevance. However, it will be important to confirm and extend these findings in studies involving larger neonatal populations in order to establish clinical significance.

In summary, fetal exposure to HCA is associated with enhanced Th17-type responses in the cord blood of PT and term neonates. The prominence of the Th17 phenotype in PT neonates may be due to a combined intrinsic Th17 bias and a unique sensitivity of the developing immune system to environmental alterations (28). Our findings of enhanced Th17-type responses in addition to reports of diminished Treg suppressive activity (12, 23) suggest a compelling explanation for the potentiated inflammatory responses reported in HCA-exposed preterm neonates (3-6). While enhanced Th17 responses in preterm neonates could potentially augment protective immunity (4), as suggested by some evidence of decreased late-onset sepsis in gestations with chorioamnionitis (29), exposure to HCA has also been associated with increased neonatal susceptibility to infection (30). Furthermore, Wynn *et al.* recently showed a striking contribution of IL-17 signaling to inflammation-related neonatal mortality in a murine model of sepsis (31). Thus, new understanding of how antenatal inflammation both 'primes' the neonatal immune system to promote chronic inflammatory conditions and influences protective immune mechanisms will be critical to the development of effective treatment approaches for this fragile population.

METHODS

Ethics statement

This prospective observational study was performed with the approval of a protocol and according to the policies of the Institutional Review Board for Human Studies of Saint Louis University, SSM Cardinal Glennon Children's Hospital (CGCH), and SSM St. Mary's Health Center. Informed, written consent was obtained for all study participants.

Human subjects

Study subjects and gestational age-matched controls consisted of neonates born to women admitted to the Labor & Delivery service at SSM St. Mary's Health Center, a large perinatal referral center in St. Louis, MO, from January, 2013 through June, 2014. Women with intrauterine pregnancies of 23-41 weeks of gestation were prospectively enrolled in the study if they presented in preterm labor and/or had a clinical diagnosis of suspected chorioamnionitis. Potential subjects were excluded from study if mothers or pregnancies were affected by inflammatory conditions or infection other than suspected chorioamnionitis, or if a potential for altered immunity related to congenital or genetic

conditions in the fetus or newborn existed. Demographic and clinical details were obtained from the electronic medical record.

Diagnosis of histologic chorioamnionitis

Placentas of all PT infants and of term infants with suspected clinical chorioamnionitis were examined by a clinical pathologist as part of routine clinical care. The diagnosis of histologic chorioamnionitis (HCA) has been typically defined by the extent of neutrophilic involvement of the chorionic plate, the subchorionic space, umbilical cord vessels and/or Wharton's jelly (32, 33). The diagnosis of HCA was based on placental findings consistent with a Stage 2 or higher maternal inflammatory response and/or any fetal inflammatory response (umbilical vasculitis, arteritis or funisitis) (33) . For the present study, gestations were diagnosed with HCA based on placental findings alone regardless of whether or not chorioamnionitis was clinically suspected. Preterm gestations were considered to be “no HCA” controls based on the absence of inflammatory infiltrates or their restriction to the chorionic plate, chorionic vessels, or subchorionic space (Stage 1 maternal inflammatory response). Gestations were also considered to be “no HCA” controls in the presence of presumptive clinical chorioamnionitis if placental pathology was not consistent with HCA.

Cord blood collection

Immediately following delivery, cord blood was drawn from the placental umbilical vein using antiseptic technique and collected into sterile syringes or cord blood collection bags containing anticoagulant. Cellular analyses of blood samples were performed within 12 h of collection.

Antibodies and reagents

For multicolor flow cytometric analyses, the following fluorochrome-labeled Ab and IgG subset controls (all purchased from Becton-Dickinson (BD, Franklin Lakes, NJ, unless otherwise indicated) were utilized for surface or intracellular staining of cells: CD3-Alexa Fluor 700 (clone UCHT1), CD3-PerCPCy5.5 (5k7), CD4-V450 (RPA-T4), CD4-PE (RPA-T4), CD4-FITC (RPA-T4), CD4-V500 (RPA-T4), CD25-BV605 (2A3), CD25-PE (2A3), CD127-PE (hIL-7R-M21), CD127-PerCPCy5.5 (hIL-7R-M21), CD196-BV605 (11A9), IL-17A-Alexa Fluor 700 (N49-653), RORγt-PE (Q21-555), FoxP3-V450 (259D/C7). Foxp3-APC (PCH101) was a component of the Anti-Human Foxp3 Staining Set (eBioscience, San Diego, CA). FacsLysis was purchased from BD. The following reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO): RPMI-1640, phosphate-buffered saline (PBS), fetal bovine serum, paraformaldehyde, phorbol 12-myristate 13-acetate, ionomycin, brefeldin A.

Cell preparation and flow cytometry

To determine surface antigen expression in cord blood samples, whole blood (WB), or in some cases mononuclear cell (MNC) fractions, samples of adequate volume were stained with optimized mAb concentrations followed by fixation with paraformaldehyde, as described (3). For intracellular cytokine detection, samples were first stimulated with PMA (50 μg/ml), ionomycin (1 μg/ml), and brefeldin A (5 μg/ml) for 4 h at 37° C (or for

unstimulated samples, incubated with PBS alone), followed by staining with mAb against surface antigens (CD4, CD25). After fixation and permeabilization procedures (Anti-Human Foxp3 Staining Set, eBioscience), cells were stained with fluorochrome-labeled mAb directed against intracellular antigens. For nuclear transcription factor studies without cytokine expression, samples were processed for intracellular staining without stimulation, as described. Type-specific, fluorochrome-labeled IgG controls for each mAb used were included in all surface or intracellular antigen studies, under stimulated or unstimulated conditions as appropriate. Stained and fixed replicate samples were acquired within 24 h of staining using a 16-color BD LSR II Flow Cytometer. Compensation settings were based on the analysis of samples with single color staining. Unstained samples were used to exclude intrinsic background fluorescence, which was minimal in all studies examined.

Acquired samples were analyzed using the FlowJo 7.2.2 software (Tree Star, Ashland, OR). Initial gates were set on the lymphocyte population using forward- and side-scatter characteristics, and specific lymphocyte subsets were identified within $CD3^+CD4^+$ populations using fluorochrome-labeled mAb and IgG-subtype specific controls. In surface staining studies, Th17 cells were identified in $CD4^+$ cells with surface expression of CD161 (pTh17, progenitor populations (34) or that were $CD161^+CCR6^+$ (mTh17, mature populations (35). Within the gated $CD4^+$ population Tregs were identified by $CD25^{hi}$ $CD127^{lo}$ expression, and T effector memory cells (TEM) were identified by $CD25^{lo}CD127^{hi}$ expression (10). For intracellular antigen analyses studies of gated CD4 cells, Th17 cells were identified in populations that were $ROR\gamma t^+IL\text{-}17A^+$ while Treg cells were identified by the $CD25^{hi}Foxp3^+$ population. Specific gating strategies are summarized in supplemental data (**Supplemental Figure S1 and S2, online)**.

Determination of absolute cell numbers

Complete blood counts were obtained on study subjects at the discretion of the medical team. Absolute lymphocyte numbers were calculated from the corrected white blood cell count only if a CBC had been obtained in the first 6 h of life. The proportions of CD4 cells and their subsets as determined by flow cytometry were used to calculate absolute cell numbers.

Statistical analysis

Experimental data were compared using the Mann-Whitney rank test or the unpaired Student's *t*-test as appropriate, using Prism v6.03 (GraphPad Software, Inc., La Jolla, CA). A P value ≤ 0.05 was considered statistically significant. For demographic analyses, nominal variables are reported as frequencies, continuous variables are reported as means ± standard deviation (SD; parametric) or as medians (25^{th}-75^{th} percentiles; non-parametric). Nominal variables were compared using Chi-square analysis or Fisher's exact test using PASW (SPSS, v23.0; IBM, Armonk, NY).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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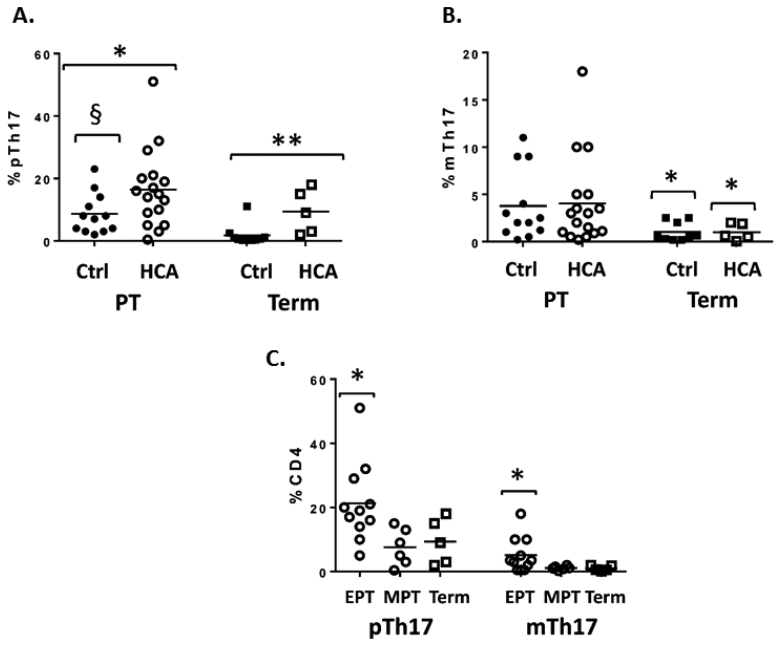

Figure 1. Gestational age and HCA effects on Th17 populations

Cord blood samples of control (Ctrl) or HCA-exposed PT (n = 29) and term (n =12) neonates were analyzed by flow cytometry to determine the frequencies of Th17 cell subsets within gated $CD3^+CD4^+$ cells (**a-c**). For the scatter plots with means presented here and in subsequent Figures, each data point represents the average value of replicate studies for an individual subject. **a.** Frequencies of Th17 progenitor cells (pTh17, $CD161^+$). *P<0.05, PT Ctrl *vs.* PT HCA; ** P<0.01, Term Ctrl *vs.* HCA; §P<0.001, PT Ctrl *vs.* Term Ctrl. **b.** Frequencies of mature Th17 cells (mTh17, $CD161^+CCR6^+$). *P<0.05, Term *vs.* PT. **c.** Comparative frequencies of pTh17 and mTh17 cell populations in PT subsets (EPT ≤ 30 wk GA; MPT, moderately PT, 31-34 wk GA) or in term neonates exposed to HCA. *P<0.05, EPT *vs.* Term (pTh17); EPT *vs.* MPT, Term (mTh17).

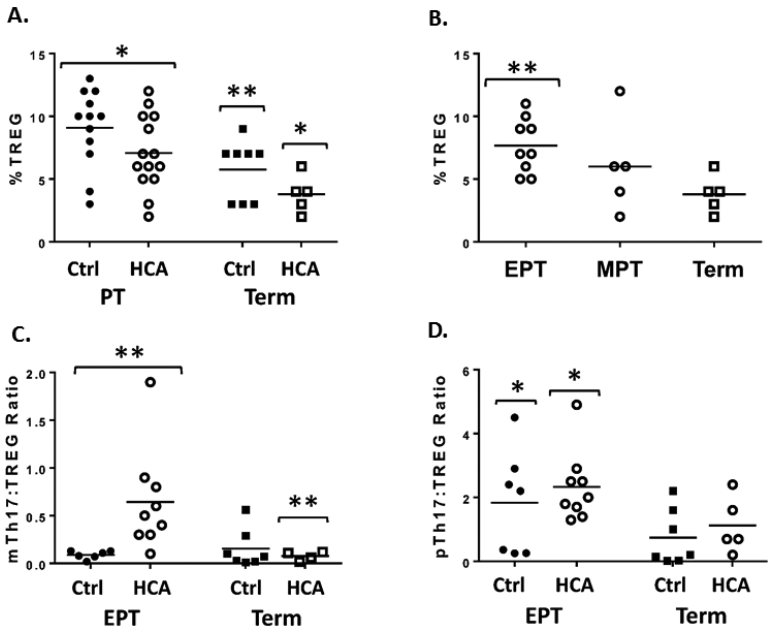

Figure 2. Gestational age and HCA influences Treg cell populations

Cord blood Treg cells ($CD25^{hi}CD127^{lo}$) within $CD3^{+}CD4^{+}$ gated populations were identified by flow cytometry (**a-d**). The scatter plot graphs shown represent the proportions of Treg cells in gated $CD4^{+}$ cells of PT and term neonates. **a.** Frequencies of Treg cells in Ctrl and HCA-exposed PT (*n* = 28) and term (*n* = 13) neonates. *P<0.05, PT (Ctrl *vs.* HCA); Term HCA *vs.* PT HCA. **P<0.01, Term Ctrl *vs.* PT Ctrl). **b.** Comparative Treg frequencies in HCA-exposed EPT (≤ 30 wk), MPT (31 – 34 wk), and term neonates. **P<0.01, EPT *vs.* Term. **c,d.** Comparative mTh17:Treg (**c.**) and pTh17:Treg (**d.**) ratios in EPT (*n* = 16) and term (*n* = 12) neonates. *P<0.05, EPT *vs.* Term. **P<0.01, EPT (Ctrl *vs.* HCA); Term HCA *vs.* EPT HCA.

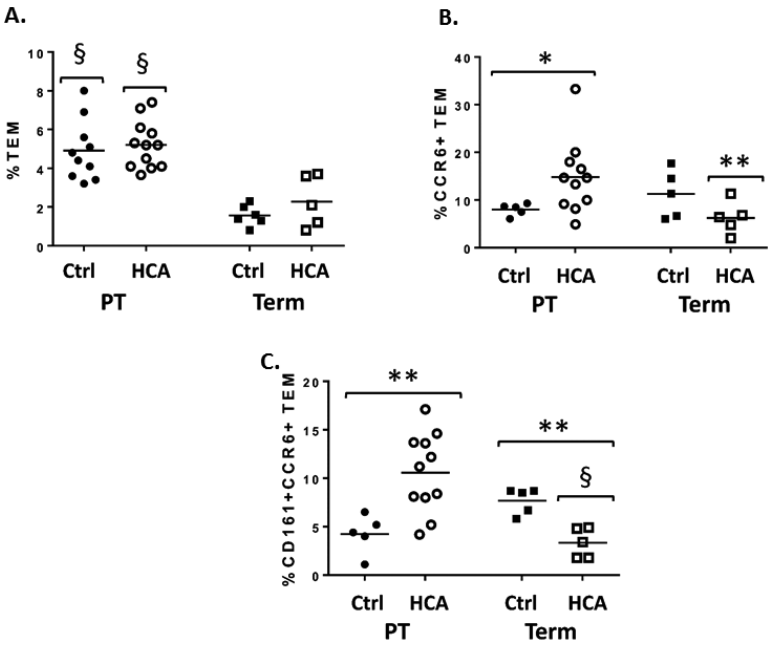

Figure 3. Effect of gestational age and HCA on TEM cells

TEM cells ($CD25^{lo}CD127^{hi}$) were identified within $CD3^{+}CD4^{+}$ gated populations in cord blood samples of PT (n = 16-22) and term (n = 10-16) neonates. The scatter plot graphs shown represent the frequencies of TEM cells in cord blood $CD4^{+}$ populations of PT and term neonates (**a**), or the proportions of specific cell subsets within the TEM gate (**b, c**) for each gestational age group. **a.** TEM cell frequencies in $CD4^{+}$ cells. §P<0.001, PT *vs.* Term. **b.** Frequencies of $CCR6^{+}$ cells in TEM populations. *P<0.05, PT (Ctrl *vs.* HCA). **P<0.01, Term HCA *vs.* PT HCA. **c.** Frequencies of $CD161^{+}CCR6^{+}$ cells in TEM populations. **P<0.01, Ctrl *vs.* HCA.

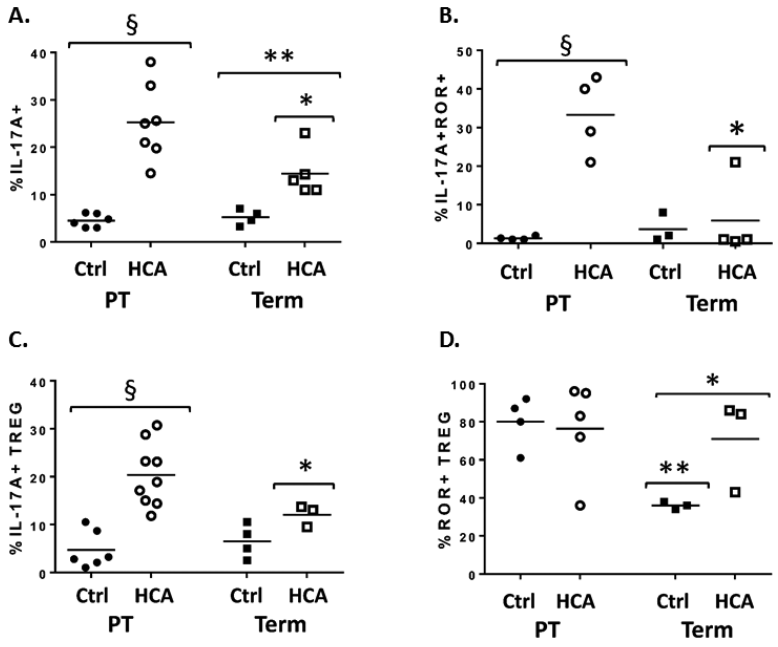

Figure 4. IL-17 expression in gated CD4 cells and Treg cells

The frequencies of cells that co-expressed IL-17A (IL-17) and/or RORγt within gated $CD4^+$ or Treg ($CD4^+CD25^+FoxP3^+$) populations were identified by flow cytometry in the cord blood of PT (n = 8-15) and term (n= 6-9) neonates with HCA or in Ctrl. Data are presented as scatter plots of the mean values from individual subjects (**a-d**). **a.** Frequencies of $IL\text{-}17^+$ cells in gated $CD4^+$ populations. *P<0.05, Term HCA *vs.* PT HCA. **P<0.01, Term (Ctrl *vs.* HCA). §P<0.001, PT (Ctrl *vs.* HCA). **b.** Frequencies of $CD4^+$ cells co-expressing both IL-17 and the Th17 nuclear transcription factor, RORγt. *P<0.05, Term HCA *vs.* PT HCA. §P<0.001, PT (Ctrl *vs.* HCA). **c**. Frequencies of $IL\text{-}17^+$ cells within gated Treg populations. *P<0.05, Term HCA *vs.* PT HCA. §P<0.001, PT (Ctrl *vs.* HCA). **d.** Frequencies of $ROR\gamma t^+$ cells within Treg populations. *P<0.05, Term (Ctrl *vs.* HCA). **P<0.01, Term Ctrl *vs.* PT Ctrl.

Table 1

Gestational and perinatal demographics.

	PT-Ctrl (*n*=14)	PT-HCA (*n*=17)	*P*	T-Ctrl (*n*=10)	T-HCA (*n*=7)	*P*
Maternal Age (yr)	25.0 ± 10.0	26.0 ± 8.0	NS	25.5 ± 14.0	20.0 ± 12.0	NS
BMI	28.3 ± 9.4	30.7 ± 11.5	NS	32.7 ± 4.5	30.9 ± 8.8	NS
Anesthesia			NS			NS
General/IV	3 (21%)	4 (24%)	NS	1 (10%)	1 (14%)	
Spinal/Epidural	7 (50%)	10 (58%)	NS	9 (90%)	6 (86%)	
Local/None	4 (29%)	3 (18%)	NS	0	0	
GBS+	3 (21%)	3 (18%)	NS	0	1 (14%)	NS
Antenatal steroids	14 (100%)	17 (100%)	NS	0	0	NS
PROM	**3 (21%)**	**13 (77%)**	**<0.01**	1 (10%)	2 (29%)	NS
Clinical chorioamnionitis	1 (7%)	5 (29%)	NS	**2 (20%)**	**7 (100%)**	**<0.01**
GA	29.4 ± 2.5	29.2 ± 3.6	NS	38.0 ± 0.94	40.0 ± 0.82	NS
BW	1213 ± 274	1523 ± 738	NS	3217 ± 435	2922 ± 790	NS

BMI (body mass index), GBS (group B *Streptococcus*) antenatal screening culture, PROM (prolonged rupture of membranes ±18 h), GA (weeks), BW (grams). Data are shown as mean ± SEM.

Table 2

Circulating Th17 and Treg cell numbers in preterm cord blood.

	Preterm		
	Controls *(n)*	**HCA *(n)***	**P value Ctrl *vs.* HCA**
Lymphocytes (× 10^9/L)	4.13 ± 0.40 *(14)*	5.11 ± 0.54 *(17)*	NS
CD4% (× 10^9/L)	64.6 ± 2.7 *(13)*	67.8 ± 2.4 *(17)*	NS
CD4 (× 10^9/L)	2.67 ± 0.34 *(13)*	3.32 ± 0.39 *(17)*	NS
pTh17 (× 10^9/L)	**0.24 ± 0.08** *(12)*	**0.71 ± 0.14*** *(17)*	**<0.05**
mTh17 (× 10^9/L)	0.15 ± 0.05 *(12)*	0.20 ± 0.08 *(17)*	NS
IL-17+ (× 10^9/L)	**0.29 ± 0.14** *(6)*	**1.38 ± 0.41*** *(7)*	**<0.01**
Treg (× 10^9/L)	0.26 ± 0.04 *(12)*	0.22 ± 0.03 *(14)*	NS
Th17:Treg ratio	**0.59 ± 0.19** *(12)*	**4.25 ± 0.95** *(14)*	**<0.001**

This Table summarizes Th17-type responses in CD4 lymphocyte subsets of preterm cord blood. The absolute lymphocyte count (ALC) was derived from the first postnatal CBC, if obtained within the first 6 h of life. Mean CD4 percentages represent the proportion in gated CD3+ lymphocyte populations. Mean percentages of Th17 and IL-17+ subsets represent their proportions within gated CD4 populations. Data are shown as mean ± SEM.

*P<0.05, PT HCA *vs.* Term HCA.