



Published in final edited form as:

*Newborn (Clarksville)*. 2023 ; 2(2): 133–141. doi:10.5005/jp-journals-11002-0064.

## Linked Th17 and Calgranulin Responses in Maternal-cord Blood Dyads of Preterm Gestations with Histologic Chorioamnionitis

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### Abstract

**Introduction:** Maternal–fetal immune crosstalk mechanisms are increasingly identified in the pathogenesis of gestational disorders, including histologic chorioamnionitis (HCA). Although an inflammatory Th17 immune phenotype has been described in preterm neonates with HCA, the associated maternal Th17 response is relatively unknown. To refine our understanding of Th17 biology in this context, we examined Th17 responses in maternal-cord blood dyads of preterm gestations.

**Materials and methods:** Paired maternal and cord blood (CB) samples were prospectively collected from preterm gestations (23–34 weeks) with HCA or controls. Th17-linked cell frequencies and plasma calgranulin (S100A8, S100A12) levels were determined by flow cytometry and enzyme-linked immunoassay, respectively.

**Results:** Analyses of 47 maternal-cord blood pairs showed striking parallel increases in Th17 cell frequencies as well as plasma calgranulin levels in the presence of fetal inflammation. Cord blood S100A12 levels were directly correlated with Th17 cell frequencies. In CB cultures, rh-S100A12 promoted *in vitro* propagation of Th17-type CD4<sup>+</sup> cells.

**Conclusions:** Maternal and CB Th17-linked responses are dually amplified in gestations with HCA, supporting a biological role for maternal–fetal interactions in this disorder. In addition to

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Author Contributions

Authors CQB and MLL contributed equally to this work.

**Conflict of interest:** None

advancing current knowledge of neonatal Th17 mechanisms, these data shed new light on their association with maternal inflammation.

### Keywords

Fetal inflammation; Gamma–delta T cells; Maternal inflammation; S100; S100A8; S100A12; Treg cells

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### INTRODUCTION

Preterm birth is a significant and increasing global health concern. The Centers for Disease Control reported that over 1 in 10 deliveries were preterm in the United States and that this number is increasing.<sup>1,2</sup> Of these, 70% were spontaneous, the result of preterm labor or preterm premature rupture of membranes (PPROM). Histologic chorioamnionitis (HCA) is a placental inflammation closely linked to spontaneous preterm birth.<sup>3</sup> Fetal exposure to HCA can result in adverse outcomes in preterm neonates, including brain injury, sepsis, necrotizing enterocolitis, and chronic lung disease, as detailed in a recent elegant review.<sup>4</sup> A postnatal diagnosis of HCA based on placental pathology is common in extremely preterm gestations, even in the absence of maternal or fetal symptoms associated with intra-amniotic infection (IAI).<sup>5,6</sup>

T-helper (Th) 17 cells represent a unique lymphocyte subset that can bridge adaptive and innate immune responses to protect the host against microbial pathogens.<sup>7,8</sup> Conversely, dysregulated Th17 cells may mediate pathologic processes leading to chronic inflammation of various organs, particularly affecting the brain in neonates.<sup>9</sup> Mounting evidence points to a role for Th17 cells in modulating immune function during normal pregnancy and in healthy neonates; however, much remains unknown, representing a significant knowledge gap.<sup>10,11</sup> In contrast, recent observations suggest potential contributions of Th17 cells to pathologic gestational processes including those leading to preterm delivery.<sup>12–15</sup> Also, Th17 cells may be functionally linked to members of the S100 family of proteins, including the calgranulins (S100A8, S100A9, S100A12).<sup>16,17</sup> While elevated calgranulin expression levels in association with HCA have been reported in placentas, amniotic fluid, and in preterm cord blood (CB) and neonatal blood,<sup>18–21</sup> a link between calgranulins and Th17 cells in the setting of HCA has not been established.

A growing body of literature suggests the importance of maternal–fetal immune crosstalk mechanisms to the pathogenesis of certain placental disorders, including chorioamnionitis.<sup>22</sup> However, whether systemic maternal Th17-type responses mimic the expression patterns found in their neonates, and whether such responses contribute to the pathogenesis of HCA, is presently unknown. Such observations could also be relevant to evidence that women who deliver a preterm infant are at increased risk of future heart disease,<sup>23,24</sup> particularly given the connection between enhanced Th17 responses and cardiovascular disorders.<sup>25</sup> We designed the present study to test the hypothesis that HCA is associated with enhanced circulating Th17 cell frequencies and Th17-linked calgranulin levels in affected pregnant women that parallel responses in the CB of their preterm neonates.

## MATERIALS AND METHODS

### Human Subjects

From December 2016 to March 2019, eligible pregnant women admitted to the labor and delivery service at a large perinatal center in St. Louis, Missouri, USA were approached for their own enrollment and that of their delivered preterm neonates. Eligibility criteria included singleton or uncomplicated twin gestations, preterm labor and/or PPROM, and delivery between 23<sup>0</sup> and 34<sup>6</sup> gestational weeks. Potential subjects were excluded from study if mothers or pregnancies were affected by inflammatory conditions or infection other than suspected clinical 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. 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 Health Cardinal Glennon Children's Hospital (CGCH), and SSM Health St. Mary's Health Center (SMHC). Informed, written consent was obtained for all study participants.

### Diagnosis of Histologic Chorioamnionitis

All placentas were examined by a clinical academic pathologist as part of routine clinical care (Redline criteria<sup>3</sup>). Diagnosis and staging of HCA were based on the involved compartment (maternal and fetal) and the extent of neutrophil invasion.<sup>3,26</sup> A diagnosis of maternal HCA (MHCA) was based on neutrophil infiltration at or below the chorionic plate; fetal HCA (FHCA) was identified by neutrophil invasion of veins or arteries in the chorionic plate and/or of the umbilical CB vessels.<sup>3</sup> Chronic inflammation was diagnosed in placentas with lymphocytic infiltration of the chorionic villi (*chronic villitis*), chorioamniotic membranes/plate (*chronic chorioamnionitis*), or basal plate (*chronic deciduitis*).<sup>27</sup> Gestations were defined as "controls" in placentas without evidence of HCA or chronic chorioamnionitis, or other significant pathology. Medical records were reviewed for maternal or fetal evidence of IAI or clinical chorioamnionitis.<sup>28,29</sup>

### Blood Sample Collection

Anticoagulated (citrate phosphate) maternal blood samples were obtained from pregnant women by peripheral venipuncture within 24 hours prior to delivery. For cord blood (CB) samples, anticoagulated blood was aspirated from the placental umbilical vein (cleansed of maternal blood) immediately following delivery. Whole blood was processed for flow cytometric analysis and for the collection of plasma aliquots as described.<sup>13</sup> Plasma samples were stored at -80°C until batch analysis. For *in vitro* studies, anonymous CB samples (collected less than 12 hours postdelivery) were obtained from the SSM Health St. Louis cord Blood Bank.

### Flow Cytometric Analyses of Patient Samples

Multiparameter flow cytometric analyses of antibody-stained whole blood samples were used to identify specific immune cell subsets. Briefly, samples stained with fluorochrome-labeled mAb or type-specific immunoglobulin G (IgG) controls were acquired within 24

hours of staining using a BD LSR-II Flow Cytometer, and were analyzed with the FlowJo 7.2.2 software (Tree Star, Ashland, Oregon, USA), as previously described.<sup>13</sup> Within the viable gated CD3<sup>+</sup> lymphocyte population, Th17 cells were identified in CD4<sup>+</sup> cells with surface expression of CD161<sup>+</sup> (progenitor Th17 cells [pTh17])<sup>30</sup> or both CD161<sup>+</sup> and CCR6<sup>+</sup> (mature Th17 cells [mTh17]) (7). The T regulatory (Treg) cells were identified in CD4<sup>+</sup> cells expressing the CD25<sup>hi</sup>CD127<sup>lo</sup> phenotype.<sup>31</sup> The TCR $\gamma\delta$ <sup>+</sup> T cells were identified within the gated CD3<sup>+</sup> cell population.<sup>32</sup> Furthermore, the Th17:Treg ratios were determined by calculating the ratios of pTh17 or mTh17 cell frequencies, respectively, to those of Treg cells.

### Determination of Plasma Calgranulins

Calgranulin levels were determined in batched duplicate plasma samples using commercial ELISAs (CircuLex S100A8/MRP8, catalog No. CY-8061; CircuLex S100A12/EN-RAGE, catalog No. CY-8058; Circulex; MBL International Corporation, Woburn, Massachusetts, USA). Readings (405 nm) were compared against an internal standard curve, and the concentrations of S100A8 or S100A12 in each sample were calculated by plotting against a four-parameter logistic equation. Assay limits for detection were: S100A8, 43.4 pg/mL; S100A12, 8.2 pg/mL. Due to variability in plasma sample volumes, S100A8 and/or S100A12 levels were not determined in all subjects.

### Th17 Cell Propagation in CB Cultures

For these studies, CB CD4<sup>+</sup> cells were purified from mononuclear cells, as we described.<sup>33</sup> Briefly, CD4<sup>+</sup> T cells were isolated by negative selection (EasySep<sup>TM</sup> Human Naïve CD4<sup>+</sup> T Cell Isolation Kit (Catalog 19555), STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. Purified CD4<sup>+</sup> cells ( $2 \times 10^6$  cells) were suspended in CTCM (2 mM glutamine, 50- $\mu$ M  $\beta$ -mercaptoethanol, 10% heat-inactivated human AB type serum, 100-U penicillin/100  $\mu$ g streptomycin/mL) and cultured either in CTCM alone, or in CTCM containing a Th17-propagating cocktail (10 ng/mL: Interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-23; 3 ng/mL: TGF $\beta$ ) or varying concentrations of rh-S100A12. Cell suspensions were added to 24 well plates coated with anti-CD3 Ab (2  $\mu$ g/mL) and in the presence of IL-2 (50 U/mL) at 37°C, 5% CO<sub>2</sub>. Following a 72-hour culture, cells were harvested and stimulated for intracellular staining, including viability, as described.<sup>33</sup> Samples were acquired within 24 hours of staining using a 16-color BD LSRII flow cytometer. Acquired samples were analyzed using the FlowJo 7.2.2 software (Tree Star, Ashland, OR). Within the gated CD4<sup>+</sup> T cell population, Th1 cells were identified by the intracellular expression of the nuclear factor, Tbet, and Th17 cells were identified by expression of the nuclear factor, ROR $\gamma$ t, or IL-17A. Tregs were identified in CD4<sup>+</sup> cells with intracellular expression of the nuclear factor, Foxp3.

### Antibodies and Reagents

Fluorochrome-labeled mAb (all, Becton-Dickinson, Franklin Lakes, New Jersey, USA) were used for surface staining: CD3-FITC (clone SK7), CD4-Alexa Fluor 700 (RPA-T4), CD25-PE (2A3), CD45-V450 (HI30), CD127-BV650 (HIL-7R-M21), CD161-APC (DX12), CD196-PerCPcy5.5 (IIA9), and TCR $\gamma\delta$ -BV605 (B1). The vital stain, Live/Dead Aqua, was purchased from Invitrogen/Thermo Fisher Scientific (Waltham, MA; catalog No. L34957).

Recombinant human (rh) cytokines were purchased from BD Biosciences, San Jose, CA (rhIL-2), Peprotech, Inc., Rocky Hills, New Jersey, USA (rhIL-1 $\beta$ , rhIL-6), and R&D Systems, Minneapolis, Minnesota, USA (rhIL-23, rhTGF $\beta$ , rhS100A12).

**Statistical Analyses**—Experimental data were analyzed using the non-parametric Kruskal–Wallis test for intragroup comparisons across conditions; the non-parametric Mann–Whitney *U* test or independent Student’s *t*-test for comparisons of unpaired data; and the non-parametric Wilcoxon rank test for paired data analyses (Prism v7; GraphPad Software, La Jolla, California, USA). Demographic data were compared using the Mann–Whitney *U* test or independent Student’s *t*-test for continuous data, or Fisher’s exact test for categorical data using Statistical Package for the Social Sciences (SPSS) (v23; IBM, Armonk, New York, USA) or Prism. Correlations between variables were calculated using the Pearson correlation coefficient;  $p < 0.05$  was considered significant.

## RESULTS

### Subject Characteristics and Demographics

We studied 47 women in preterm labor who were enrolled at the time of their admission to labor and delivery, and their delivered neonates. Placental analyses identified 37 gestations with HCA (12 MCHA; 25 FHCA). Seven placentas were unaffected by HCA or other identified placental pathology, and served as controls. Placentas of three gestations were diagnosed with chronic inflammation only (villitis or deciduitis); these were analyzed separately from the HCA or control groups. Key baseline maternal and perinatal characteristics, including clinically suspected IAI, were not different between groups (Table 1). Neonates with HCA were delivered at earlier gestational ages relative to controls (Table 2), especially in the presence of FHCA ( $29.4 \pm 3.1$  weeks,  $p = 0.02$ ); however, no age differences were observed between MHCA and FHCA gestations. Birth weights were lower in HCA relative to controls, particularly in the presence of FHCA ( $1471 \pm 542$  gm,  $p = 0.01$ ). In contrast, the three neonates with chronic inflammation had higher birth weights ( $2166 \pm 465$  gm) relative to CB with any HCA ( $p = 0.02$ ) and FHCA ( $p = 0.01$ ). No differences were observed between groups for the remainder of neonatal outcomes, including early- or late-onset sepsis.

### Immune Cell Responses

In flow cytometric studies of maternal peripheral blood and preterm CB, we examined the proportions of Th17 cells subsets with progenitor or mature phenotypes, as these have been variably linked to the pathogenicity of chronic inflammatory disorders.<sup>7,34–36</sup> We determined the highest frequencies of progenitor (p)Th17 cells in FHCA gestations for both mothers and in CB relative to controls, while these were significantly elevated only for mothers in MHCA (Fig. 1A). In paired comparisons, pTh17 cell frequencies were higher in mothers in control gestations ( $p = 0.02$  vs CB); frequencies were similar between mothers and in CB with MHCA ( $p = 0.42$ ) or FHCA ( $p = 0.61$ ). In studies of mature (m)Th17 cells, we also observed the highest maternal and CB frequencies in FHCA gestations, similar to our observations for pTh17 cell subsets (Fig. 1B). Maternal and CB mTh17 cell frequencies were also both elevated in MHCA relative to control gestations. In paired studies, maternal

mTh17 cell frequencies were higher than in the CB of their neonates in FHCA gestations; however, frequencies were not significantly different between pairs in MHCA ( $p = 0.28$ ) or control ( $p = 0.06$ ) gestations.

Quantitative alterations in Treg cells, which have the capacity to suppress Th17 responses,<sup>37</sup> have been reported in pregnancy-related inflammatory disorders, including in women with preeclampsia.<sup>15</sup> We examined frequencies of Treg cells in paired maternal-CB blood samples in gestations with or without HCA (Fig. 1C). We observed lower circulating Treg cell frequencies in mothers with MHCA ( $p < 0.05$  vs controls), while no differences in CB Treg frequencies were determined between HCA and control gestations. In paired comparison studies, maternal Treg cell frequencies were also lower compared with CB in MHCA gestations but were similar to CB Treg cell frequencies in gestations with FHCA ( $p = 0.89$ ) or in controls ( $p = 0.05$ ).

Gamma-delta T cells represent a lymphocyte subset that may contribute to inflammatory pathology in part through the release of IL-17.<sup>38</sup> To more fully define Th17-type responses in HCA, we compared maternal and CB  $\gamma\delta$  T cell frequencies in affected and control gestations (Fig. 1D). No differences in  $\gamma\delta$  T cell frequencies were determined for either mothers or in CB for any HCA condition relative to controls. However, in pairwise assessments, higher  $\gamma\delta$  T cell frequencies were determined in mothers vs CB in FHCA, while frequencies were similar between pairs in both MHCA ( $p = 0.17$ ) and control gestations ( $p = 0.19$ ).

The Th17:Treg ratio has been correlated with disease severity in a variety of inflammatory disorders.<sup>39</sup> We observed elevated pTh17:Treg ratios for both mothers and in CB in FHCA vs control gestations, while only maternal pTh17:Treg ratios were elevated in MHCA gestations (Fig. 2A). We also determined elevation of mTh17:Treg ratios for mothers and in CB in gestations with either HCA condition vs relative controls (Fig. 2B). In paired analyses, pTh17:Treg ratios were not different between mothers and CB for a specific HCA condition (MHCA,  $p = 0.07$ ; FHCA,  $p = 0.55$ ), while maternal ratios were higher than CB ratios in controls (Fig. 2A). In contrast, mTh17:Treg ratios in mothers were higher than in the CB of their neonates for any gestational condition (Fig. 2B). In analyses that compared controls with combined MHCA and FHCA ratios (any HCA), maternal and CB ratios were higher in gestations with any HCA (MHCA + FHCA) for both Th17 subsets: pTh17 (maternal,  $p = 0.01$ , CB,  $p = 0.02$ ); mTh17 (maternal,  $p = 0.04$ , CB,  $p = 0.03$ ).

### **S100A8 and S100A12 Levels**

As calgranulins and other S100 proteins have been linked to Th17-mediated inflammatory disorders (16, 17), we performed comparison studies of maternal and CB calgranulin levels in HCA or control gestations (Fig. 3). In gestations with FHCA, S100A8 levels in CB were increased three-fold relative to controls while maternal levels were doubled (Fig. 3A). In contrast, in MHCA gestations, S100A8 levels were not elevated for either mothers or CB. In studies of S100A12 expression, in FHCA gestations, CB levels were quadrupled relative to controls, while maternal S100A12 levels were nearly twice those of controls (Fig. 3B). In MHCA gestations, elevation of S100A12 levels were significantly elevated only for CB. Paired analyses showed similar expression levels of S100A12 between mothers and preterm

CB for all gestational conditions (controls,  $p = 0.21$ ; MHCA,  $p = 0.16$ ; FHCA,  $p = 0.81$ ). Paired CB comparisons of S100A12 levels and Th17 cell frequencies showed a correlation for pTh17 frequencies ( $r = 0.62$ ,  $p < 0.02$ ), with a relationship also observed for mTh17 cell frequencies ( $r = 0.44$ ,  $p < 0.02$ ).

### rhS100A12 Promotes Th17 Cell Propagation in CB Cultures

The prominent CB S100A12 levels observed in association with fetal inflammation in this study led us to hypothesize that S100A12 might serve as a “transducer” between fetal neutrophil and Th17 responses. In preliminary *in vitro* studies, coculture of term CB CD4<sup>+</sup> cells with rhS100A12 resulted in a higher proportion of cell populations expressing the nuclear factor, ROR $\gamma$ t (a Th17 cell marker), as well as the Th1 nuclear factor, Tbet (Fig. 4A), compared to CD4<sup>+</sup> cells cultured in media alone. Cultures containing either rh-S100A12 alone or only a potent Th17-propagating cytokine cocktail induced IL-17<sup>+</sup> CD4<sup>+</sup> cells to a similar degree ( $p = 0.31$ ) (Fig. 4B). However, the S100A12 effect appeared to be specific for Th17 cell induction, as increased Treg (CD4<sup>+</sup> FoxP3<sup>+</sup>) cell frequencies were observed in cultures containing a Th17-propagating cocktail but not in the presence of S100A12 alone (Fig. 4B).

### DISCUSSION

The primary goal of the present study was to determine maternal Th17-type responses relative to those in the CB of their neonates in preterm gestations with HCA. We determined concurrently elevated circulating expression levels of Th17 cells in whole blood and the plasma Th17-associated calgranulins, S100A8 and S100A12, in both mothers and in preterm CB, particularly in the presence of fetal inflammation. To our knowledge, this is the first report describing these combined Th17-related responses in both mothers and in preterm CB in HCA gestations.

Our findings provide added evidence supporting the role of maternal–fetal crosstalk mechanisms and extend existing information regarding Th17 responses in pregnant women and in neonates with HCA gestations.<sup>13,14,40</sup> We observed elevations in circulating Th17 cell subset frequencies in both pregnant women in preterm labor and in the CB of their preterm neonates especially in gestations with fetal inflammation (FHCA). Notably, while elevated in both groups, mTh17 cell frequencies were higher in mothers relative to those in the CB of their neonates in gestations with fetal inflammation. This latter observation may reflect a specific maternal inflammatory response to fetal inflammation, as a strong association between elevated mTh17 cell responses and inflammatory status has been reported in other disorders.<sup>41</sup> Our results differ from a recent report showing higher maternal Th17 frequencies in term vs preterm gestations.<sup>40</sup> However, our results may reflect the focus of our study on the comparison of preterm gestations with a diagnosis of HCA vs those with absent placental or infectious pathology. That report also described elevated circulating maternal IL-6 levels in preterm gestations as a group, although whether maternal IL-6 levels correlated with placental inflammation was not clear (and fetal inflammation was not specifically identified). However, this finding is supportive of increased Th17-linked responses, as IL-6 is critical to the preferential propagation of Th17 cells over

anti-inflammatory Treg cells.<sup>42</sup> In our studies of  $\gamma\delta$  T cells, an immune cell that is an important source of IL-17,<sup>34</sup> we found higher frequencies in mothers than in the CB of their neonates specifically in gestations with fetal inflammation. In light of an established association between Th17-type responses and inflammatory disorders,<sup>25</sup> these maternal Th17-type responses are consistent with the possibility that maternal exposure to fetal inflammation could set the stage for later maternal metabolic or cardiovascular disease.<sup>43</sup>

Enhanced Th17 responses have been ascribed to neuroinflammation in neonates,<sup>9</sup> and have been associated with the severity of chronic inflammation in adults.<sup>39</sup> Prominent circulating Th17 cell populations have also been observed in women with recurrent pregnancy loss and pre-eclampsia, gestational disorders also associated with inflammation.<sup>44,45</sup> Our present findings extend previous observations of elevated Th17 responses in preterm neonates with HCA<sup>13,14</sup> and provide new evidence of a potential involvement of maternal Th17 cells in its pathogenesis.<sup>46,47</sup> In addition, the observed imbalances between Th17 cells and Tregs could enhance tissue Th17 responses that amplify the inflammatory cascade<sup>8,48</sup> in mothers, in neonates, or in both. Some data suggest that pathologic Th17 cells contribute to preterm labor through processes involving fetal immune activation against maternal antigens.<sup>12,49</sup> In addition, imbalances in Th17 and Treg cell expression levels (such as in this study and in other<sup>13,14</sup> studies) as well as Th17-calgranulin interactions have been observed in conjunction with immune rejection processes.<sup>15,17</sup> Notably, rejection has been identified as a potential mechanism associated with preterm birth<sup>22,49</sup> In addition to its possible role in preterm delivery, intrauterine Th17-mediated inflammation could “imprint” the immune system of the developing fetus, leading to altered postnatal responses.<sup>50,51</sup> Taken together, our present observations highlight a need for improved understanding of the roles of pathogenic Th17 processes and Th17 cell heterogeneity to the development of placental inflammation.<sup>8</sup> Such information could guide the development of novel prenatal therapeutic approaches,<sup>52</sup> for example, by targeting elements of the Th17 pathway.<sup>53</sup>

Our studies included analyses of circulating maternal and CB levels of the calgranulin proteins, S100A8 and S100A12. We found marked elevations of plasma calgranulin levels in mothers and especially in the CB of their preterm neonates in the context of fetal inflammation. Our findings are consistent with the increased S100A12 blood levels previously reported in a small subset of neonates born after HCA,<sup>21</sup> as well as recently described neonatal monocyte and blood expression levels of S100 proteins in chorioamnionitis.<sup>20</sup> While the role of calgranulins in the pathogenesis of fetal inflammation has not been discerned, evidence suggests a link between inflammatory neutrophils, a driving force in this disease process<sup>54,55</sup> and their contribution to circulating S100 proteins.<sup>56</sup> Pertinently, neutrophils can promote Th17 cell propagation and function,<sup>33,57</sup> a process that may involve neutrophil-derived calgranulin mediators (in this study and in other<sup>58,59</sup> studies). Conversely, calgranulins modulate inflammatory responses by promoting neutrophil production and activation.<sup>60,61</sup> Additionally, crosstalk between neutrophils and Th17 cells can amplify the inflammatory cascade.<sup>62</sup> Our finding that rhS100A12 also promoted the expression of Tbet, a canonical nuclear transcription factor for Th1 cells,<sup>63</sup> is consistent with the Th1 polarization bias observed in exometabolomic studies of HCA-exposed preterm neonates.<sup>64</sup> Our preliminary observations in the context of existing data hint at feed-forward mechanisms involving interactions between neutrophils, calgranulins,



and Th17 cells in the pathophysiology of fetal inflammation. However, our findings are based on *in vitro* studies that targeted healthy CB CD4<sup>+</sup> cells of term gestations. Future studies involving preterm CB cells and *in vivo* models may provide important clues to understanding whether these “pieces fit the puzzle” of mechanisms that drive fetal inflammation and preterm birth and as well as postpartum maternal inflammatory disease.<sup>65</sup>

Our observations suggest an attributable risk of HCA in association with elevated Th17 cell frequencies and calgranulin levels, despite the limited size of our study sample. Our findings also support the potential utility of calgranulin levels in identifying clinically “silent” fetal inflammation, an important task given its association with preterm delivery and adverse neonatal outcomes.<sup>20,66</sup> Such information could also facilitate anticipatory clinical management of HCA and guide effective postpartum and postnatal interventions.<sup>52,67,68</sup> However, studies in much larger, diverse populations are clearly warranted to define the use of calgranulins in this context.

A major strength of this study lies in its prospective design with stringent inclusion and exclusion criteria that minimize the presence of confounding factors associated with other perinatal inflammatory disorders.<sup>69</sup> Thus, despite our limited sample size, the significant intergroup differences in Th17 responses that we now report in maternal-CB dyads with fetal inflammation support the biological relevance of our findings. However, our study was neither powered to identify infectious etiologies nor to specifically correlate our findings with adverse maternal or neonatal outcomes.

In summary, the enhanced Th17-linked response patterns observed in pregnant women parallel those in their preterm neonates especially in the context of fetal inflammation. These findings provide compelling supportive evidence of maternal–fetal crosstalk mechanisms that may influence gestational inflammatory or immune processes.<sup>27</sup> While our understanding of perinatal Th17 responses in HCA continues to evolve, their contributions to maternal and neonatal health and disease remain critical knowledge gaps that will benefit from continued investigations in this area.

## Acknowledgments

This work was presented in part at annual meetings of the Pediatric Academic Societies (5–8 May 2018, Toronto, Canada; 24 July 2020; Summer Webinar Series), and the Society for Maternal–Fetal Medicine (6–8 February 2020, Grapevine, Texas, USA). We thank the Labor and Delivery and Neonatal Intensive Care Unit medical staff for their helpful assistance with the success of this project. We thank Dr. Gilad Gross for useful discussions during the course of these studies. We very much appreciate Dr. David Carlton’s insightful and thoughtful review of the manuscript.

### Source of support:

This work was supported in part by funding from the Gerber Foundation, the National Institutes of Health, a private institutional foundation, and the institution’s Department of Pediatrics. No funding source had involvement in study design, data analysis, or publication decisions. NIH R21 AI094478 (to JMK).

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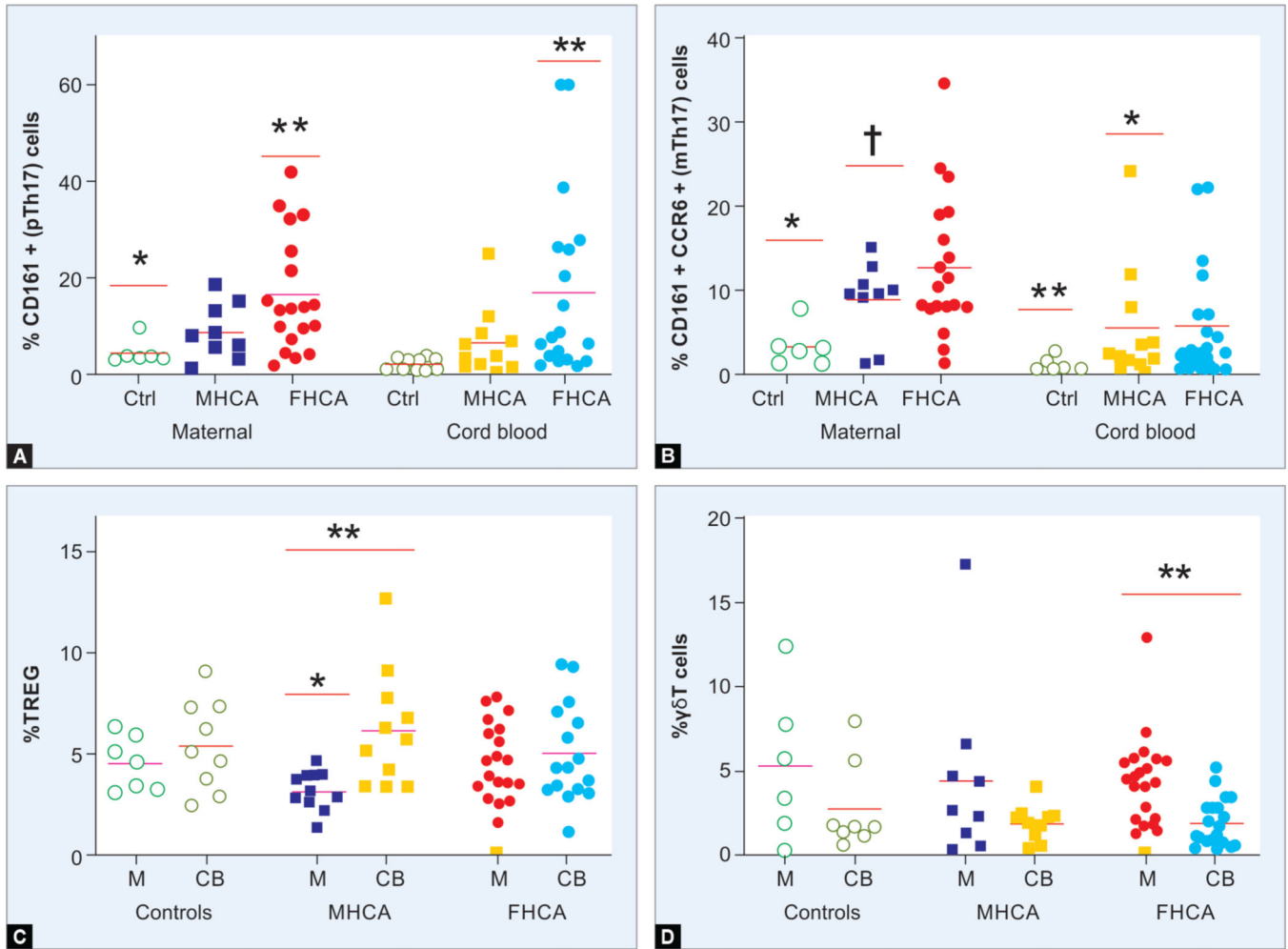
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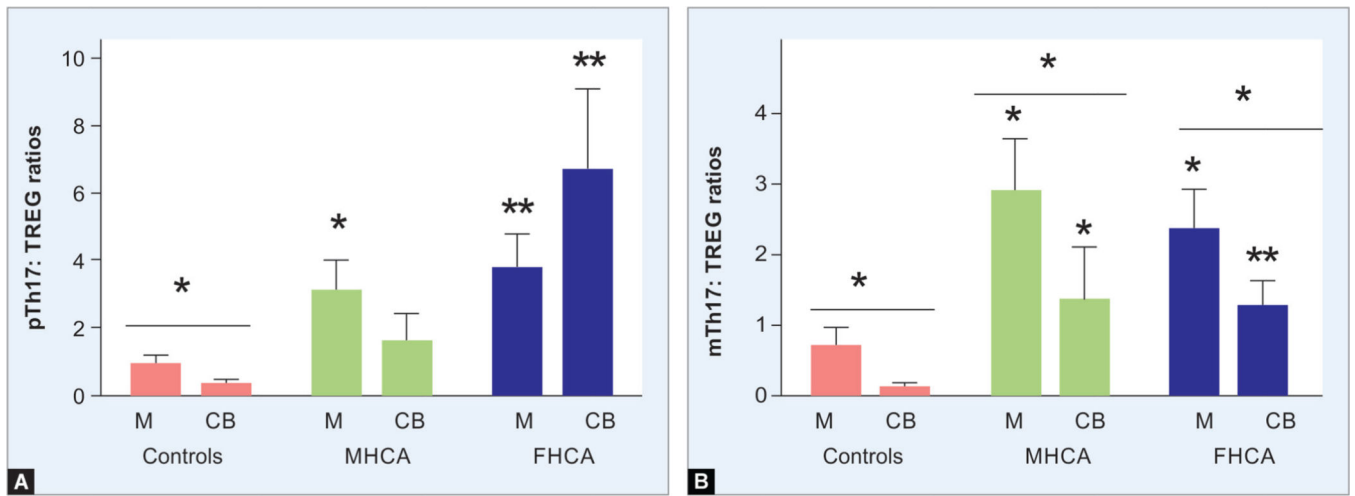
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**HIGHLIGHTS**

- The key message of our study is that certain parallel T-helper (Th) 17 cells and calgranulin responses can be found in mothers and cord blood (CB) of preterm gestations with histologic chorioamnionitis (HCA), particularly in the presence of fetal inflammation and despite the absence of maternal clinical symptoms.
- The effects of fetal inflammation on maternal and CB Th17 responses support mounting evidence of maternal–fetal inflammatory and immune crosstalk mechanisms.
- Calgranulins may be key mediators of perinatal inflammation modulated by the Th17 pathway.
- Our findings advance still limited understanding of the contributions of Th17 and calgranulin biology to placental, maternal, and CB inflammatory processes.
- This knowledge could be important to the targeted development of strategies to mitigate the pathogenesis of perinatal and neonatal inflammation.

**Figs 1.**

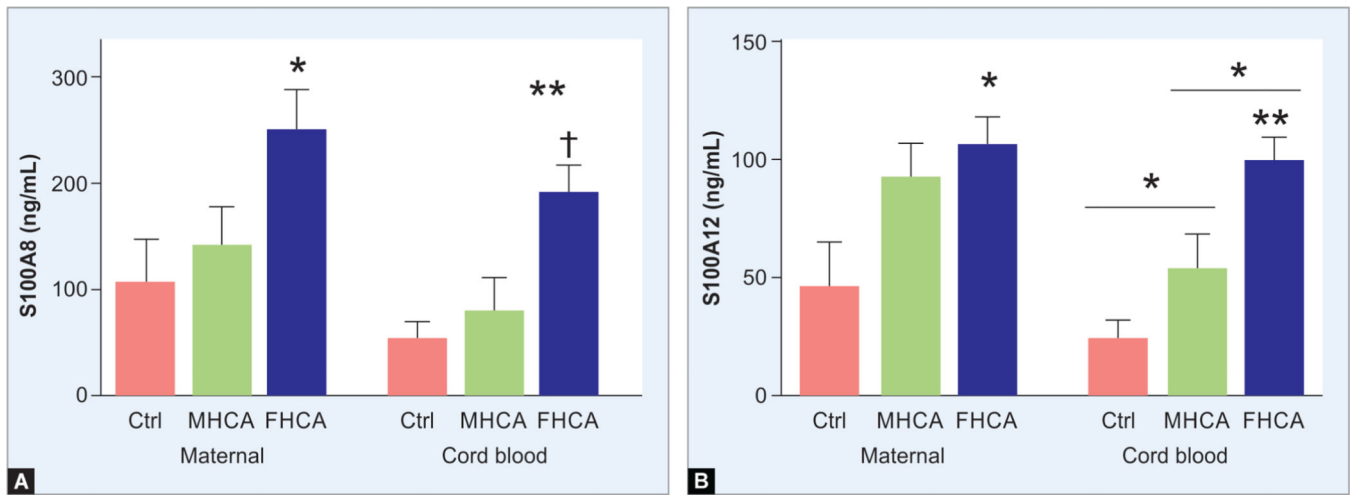
**A to D:** Immune cell frequencies. Gated CD4<sup>+</sup> cells in maternal peripheral blood and CB (cord blood) samples from preterm gestations with HCA or from unaffected preterm controls were analyzed for circulating frequencies of Th17 and Treg cell populations by multi-parameter flow cytometric analysis. The analysis of  $\gamma\delta$  T cell populations was performed within gated CD3<sup>+</sup> cells. Each symbol represents a single subject. Horizontal bars represent mean  $\pm$  SEM. (A) Progenitor (p)Th17 cells. Mean frequencies of CD4<sup>+</sup>CD161<sup>+</sup> populations. Maternal (M)-CB pairs: Controls (Ctrl),  $n = 6$ ; MHCA,  $n = 11$ ; FHCA,  $n = 19$ . \* $p < 0.05$  vs Ctrl; \*\* $p < 0.01$  vs Ctrl; (B) Mature (m) Th17 cells. Mean frequencies of CD4<sup>+</sup>CD161<sup>+</sup>CCR6<sup>+</sup> populations. M-CB pairs: Ctrl,  $n = 7$ ; MHCA,  $n = 11$ ; FHCA,  $n = 19$ . \* $p < 0.05$  vs Ctrl; \*\* $p < 0.01$  vs Ctrl; † $p < 0.001$  vs Ctrl; (C) Treg cells. Frequencies of CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup> Treg populations. M-CB pairs: Ctrl,  $n = 7$ ; MHCA,  $n = 11$ ; FHCA,  $n = 16$ . \* $p < 0.05$  vs Controls; \*\* $p < 0.01$ , M vs CB; (D)  $\gamma\delta$  T cells. Mean frequencies of CD3<sup>+</sup>TCR<sup>+</sup> $\gamma\delta$ <sup>+</sup> cell populations. M-CB pairs: Ctrl,  $n = 7$ ; MHCA,  $n = 11$ ; FHCA,  $n = 18$ . \*\* $p < 0.01$ , M vs CB



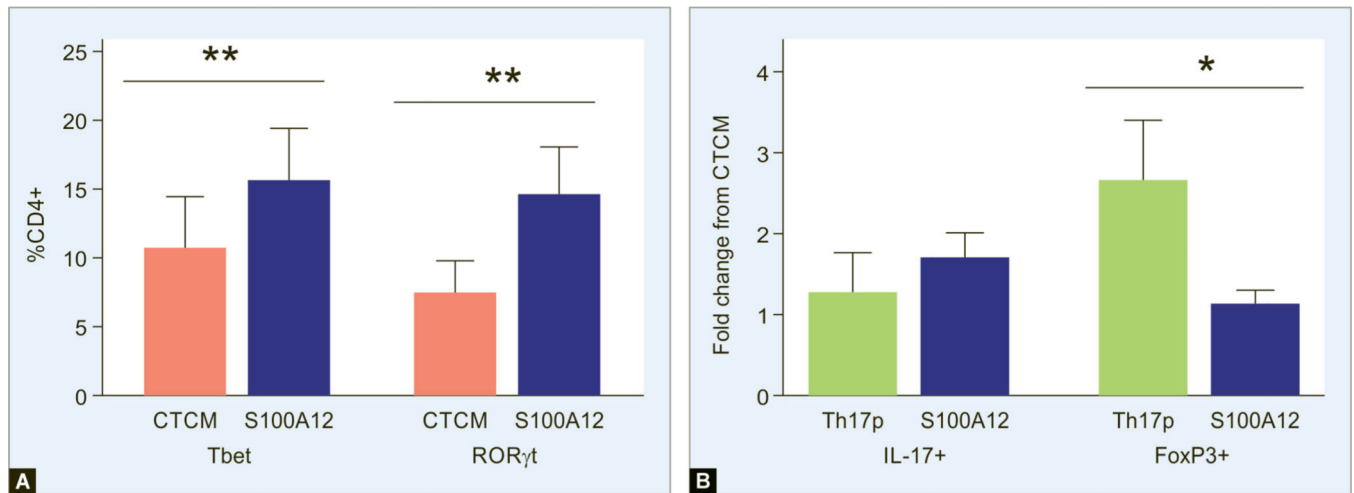
**Figs 2.**

**A and B:** The Th17:Treg ratios. The Th17:Treg ratio was calculated by dividing individual frequencies of pTh17 or mTh17 cells by Treg cell frequencies. Maternal (M)-CB pairs: Ctrl,  $n = 7$ ; MHCA,  $n = 11$ ; FHCA,  $n = 13$ . (A) pTh17:Treg ratio. \* $p < 0.05$ , M vs CB;  $p < 0.05$  vs Controls; \*\* $p < 0.01$  vs Ctrl;  $p < 0.01$ , M vs CB; (B) mTh17:Treg ratio. \* $p < 0.05$ , M vs CB;  $p < 0.05$  vs Ctrl; \*\* $p < 0.001$  vs Ctrl



**Figs 3.**

**A and B:** Maternal and CB S100A8 and S100A12 plasma levels. Plasma levels of S100A8 or S100A12 were determined in maternal (M)-CB pairs from HCA or control gestations. (A) S100A8 levels. Ctrl,  $n = 9$ ; MHCA,  $n = 10$ ; FHCA,  $n = 18$ . \* $p < 0.05$  vs Ctrl; \*\* $p < 0.01$ , MHCA vs FHCA; † $p < 0.001$  vs Ctrl; (B) S100A12 levels. Ctrl,  $n = 9$ ; MHCA,  $n = 10$ ; FHCA,  $n = 20$ . \* $p < 0.05$  vs Ctrl; MHCA vs FHCA; \*\* $p < 0.001$  vs Ctrl



**Figs 4.**

**A and B:** The rh-S100A12 and Th17 propagation in term CB cultures. CD4<sup>+</sup> T cells isolated from banked CB were incubated for 72 hours in the presence of complete medium (CTCM) containing either S100A12 (1000 ng/mL) or Th17-propagating cocktail (Th17p), then analyzed by flow cytometry. Data represent the results of about four to six individual studies. \* $p < 0.05$ ; \*\* $p < 0.01$ . (A) rh-S100A12 vs CTCM. rh-S100A12 induced greater propagation of Tbet<sup>+</sup> and RORγt<sup>+</sup> CD4 cells relative to culture with CTCM alone; (B) S100A12 vs Th17 propagating cocktail (Th17p). The induction of CD4<sup>+</sup>FoxP3<sup>+</sup> cells by rh-S100A12 alone was only half of those determined in the presence of Th17p

**Table 1:**

## Maternal characteristics

Parameter	HCA (n = 37)	Controls (n = 7)	p-value
Age (year)	26.8 ± 6.5	26.1 ± 7.0	0.80
BMI (kg/m <sup>2</sup> )	30.2 ± 6.8	30.6 ± 5.9	0.86
African-American	19 (51%)	3 (43%)	>0.99
Caucasian	18 (49%)	4 (57%)	>0.99
Multiparous	22 (59%)	6 (86%)	0.39
Prior preterm delivery	9 (24%)	4 (57%)	0.17
History of smoking	8 (22%)	3 (43%)	0.34
PPROM	31 (84%)	6 (86%)	>0.99
Suspected IAI	6 (16%)	1 (14%)	>0.99
Antenatal antibiotics	37 (100%)	7 (100%)	>0.99
Antenatal steroids	37 (100%)	7 (100%)	>0.99

Data are shown either as mean ± standard deviation (SD) or as *n* (%). BMI, body mass index; IAI, intraamniotic infection; PPRM, prolonged preterm rupture of membranes

**Table 2:**

## Neonatal outcomes

Parameter	HCA (n = 37)	Controls (n = 9)	p-value
Gestational age at delivery (weeks)	29.9 ± 3.0	31.8 ± 2.6	0.04
Birth weight (gm)	1546 ± 612	1898 ± 533	0.03
SGA	2 (5%)	0 (0%)	>0.99
EOS	8 (22%)	1 (11%)	0.66
LOS	3 (8%)	0 (0%)	>0.99
IVH	3 (8%)	0 (0%)	>0.99
NEC	2 (4%)	0 (0%)	>0.99
BPD	5 (14%)	1 (11%)	>0.99
Death	4 (11%)	0 (0%)	0.57

Data are shown as either mean ± SD or as *n* (%). Death at delivery or at any time prior to hospital discharge. BPD, bronchopulmonary dysplasia; EOS, early-onset sepsis; IVH, intraventricular hemorrhage; LOS, late-onset sepsis; NEC, necrotizing enterocolitis; SGA, small for gestational age