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Effect of chorioamnionitis on regulatory T cells in moderate/late preterm neonates★

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Abstract

Regulatory T-cells (Treg) have a protective role for the control of immune activation and tissue damage. The effects of chorioamnionitis (chorio) on Treg in moderate/late preterm newborns are not known. We hypothesized that infants exposed to chorio would have decreased Treg frequency and/or function.

We isolated mononuclear cells from adult peripheral blood and cord blood from term and moderate/late preterm infants who were classified for severity of chorio exposure. Mononuclear cells were analyzed by flow cytometry for Treg frequency and phenotype. Treg suppression of activation of conventional T-cells (Tcon) was also quantified.

Treg frequencies were similar in all groups of neonates, but lower than that found in adults. Newborn Treg had a naïve phenotype, with decreased levels of CD45RO, HLA-DR, CD39 and TIGIT compared to adult Treg and chorio did not affect the phenotype. Treg from preterm newborns exposed to severe chorio had higher expression of Ki67 compared to the other groups. Treg from preterm newborns were less suppressive than Treg from adults or term, and the level of suppression was reduced with severe chorio.

Relative to term, Treg frequency and phenotype were not affected by prematurity and chorio but their functionality was decreased. Lower Treg activity may contribute to inflammation in newborns that is often associated with chorioamnionitis.

Keywords

Regulatory T cell; Fetal inflammation; Full term newborn; Prematurity; Suppression

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.humimm.2014.10.016>.

1. Introduction

Moderate/late preterm infants (32–36 weeks of gestational age, GA) are approximately 85% of all premature infants [1]. These infants have higher mortality and morbidity compared to full term infants [2–5]. Chorioamnionitis (chorio) is associated with high morbidity in extremely premature infants [6]. Although the outcomes of moderate/late preterm infants exposed to chorio have not been extensively studied, an increase in adverse neonatal outcomes, including asthma, during later childhood has been reported [7,8].

During chorio exposure, inflammatory cells and cytokines in the amniotic fluid directly contact the fetus through the skin, lungs and gut. In fact, previous studies have shown an increase of activation/memory markers (CD45RO, CD69, HLA-DR, CD25) from total cord blood (CB) CD4⁺ cells in early preterm neonates (27–31w) exposed to chorio compared to healthy full term neonates [9,10]. Interestingly, fetal sheep exposed to chorio have inflammatory changes in many organ systems both in utero and after birth [11,12]. Notably, intraamniotic IL-1 α decreases the number of regulatory T-cells (Treg) in the fetal gut [13]. Treg are a subset of CD4⁺ T-cells critical for immunological self-tolerance and immune homeostasis [14]. Levels of the transcription factor FOXP3 are associated with Treg development and suppressive function [15]. The immune mechanisms underlying the long term morbidities of preterm infants and chorio have been minimally explored. Because of the correlation between asthma and Treg [16], and low Treg frequency in animal models of chorio, we hypothesized that premature infants exposed to chorio would have decreased Treg frequency and/or function. We focused on moderate/late preterm newborns (32–36 weeks GA) because they are the most abundant preterm group.

2. Materials and methods

2.1. Patient recruitment

Mothers delivering moderate/late preterm infants (32–36 weeks gestation) and mothers delivering term infants (39–40 weeks gestation) were consented for collection of pregnancy related information, placental tissues and cord blood (CB) with a protocol approved by the IRB of Good Samaritan Hospital (GSH), Cincinnati, OH and Cincinnati Children's Hospital Medical Center. Subsets of patients were randomly selected from a cohort of 557 moderate/late preterm and full term newborns. Mother and infant demographics were collected by chart review. Seven healthy adults were recruited by the Hoxworth Blood Center (Cincinnati, OH).

2.2. Diagnosis of chorioamnionitis

Placenta and umbilical cord were assessed for antenatal inflammation using a standardized procedure. Histologic sections of chorioamnion, umbilical cord and placenta were stained with hematoxylin and eosin and preterm and term infant samples were scored for chorio based on Redline's criteria [17]. All placentas were reviewed blindly by physician co-authors (TG and SGK) who have expertise in evaluating placenta for chorioamnionitis. Final diagnosis of chorioamnionitis required a consensus between the two researchers. In addition, most placentas were evaluated by a pathologist for clinical reports. There was a high

concordance between the pathologist's diagnosis and our diagnosis. Chorio groups were differentiated into mild and severe defined as: mild, denoted stage 1 and grade 1 chorio and severe, denoted > stage 1 and/or > grade 1 chorio. According with Redline et al. 2003, the grade 1 (mild chorio) include small clusters of maternal neutrophils diffusely infiltrating amnion, chorionic plate, chorion laeve, and/or subchorionic fibrin. Grade 2 (severe chorio) is confirmed by three or more chorionic microabscesses (microabscesses = confluent neutrophils measuring at least 10×20 cells in extent) between chorion and decidua in the membranes and/or under the chorionic plate or a continuous band of confluent chorionic polymorphonuclear leukocytes (more than 10 cells in width) occupying more than half of the subchorionic fibrin or one revolution of the membrane role. Fetal inflammation was identified by funisitis, defined as neutrophilic infiltration surrounding the umbilical cord vessels or into the Wharton's jelly.

2.3. Collection of umbilical cord blood and adult peripheral blood

Following delivery of the infant and before delivery of the placenta, the umbilical cord vein was cannulated using sterile procedures. The CB was allowed to flow freely, assisted by gravity, into a sterile pouch (Pall Medical, Port Washington, NY) containing the anticoagulant citrate phosphate dextrose. Peripheral blood from healthy adults was collected as an external control group (median age: 34; female: 71%; African American: 17%). Mononuclear cells from cord blood (CBMC) and adult peripheral blood (P BMC) were isolated with Ficoll-Hypaque gradients (GE Healthcare, Fairfield, CT). Aliquots of mononuclear cells were frozen in fetal calf serum (FCS) +10% DMSO and stored in liquid nitrogen.

2.4. Multiplex ELISA

Cytokine/chemokine concentrations in CB plasma were determined by Luminex using MILLIPLEX MAP Human Cytokine/Chemo-kine Magnetic Bead Panel (Millipore, Billerica, MA) according to the manufacturer's protocol and analyzed with a Bio-Plex® 100 System (Bio-Rad, Hercules, CA). Concentrations were calculated from standard curves using recombinant proteins and expressed in pg/ml.

2.5. Flow cytometry

CBMCs and PBMCs were thawed and washed with FCS followed by a wash with PBS. Erythrocytes were lysed using Pharm Lyse buffer (BD Biosciences, San Jose, CA) and then washed with PBS. Cells were treated with human IgG to block Fc-receptors and stained for surface markers. Cells were then washed and fixed with Fixation/Permeabilization Buffer (eBioscience, San Diego, CA) followed by Permeabilization Buffer (eBioscience). Cells were finally stained for intracellular markers and analyzed on FACS LSR-II (BD Biosciences). Live cells were gated based on forward- and side-scatter properties and the absence of fluorescence in the LIVE/DEAD® Fix-able Dead Cell Stain kit (Invitrogen, Grand Island, NY). Samples analyzed had >85% viability. Flow cytometry analysis was performed using FACSDiva software version 6.1.2 (BD Biosciences).

Human antibodies included anti-CD3-V500 (UCHT1), anti-CD45RO-FITC (UCHL1), anti-CD25-APC-H7 (M-A251), anti-CD8-PerCP-Cy5.5 (SK1) and anti-Ki67-PerCP-Cy5.5 (B56)

(BD Biosciences); anti-CD4-AF700 (RPA-T4), anti-FOXP3-AF647 (259D), anti-CD279-FITC (PD-1; EH12.2H7), and anti-CD45RO-PB (UCHL1) (BioLegend, San Diego, CA); anti-CD45RA-PE-Cy7 (H100), anti-HLA-DR-PE-Cy7 (LN3), anti-FOXP3-eFluor660 (PCH101), anti-CD152-PE (CTLA-4; 14D3), anti-CD39-PE (eBioA1), anti-TIGIT-PE (MBSA43) and anti-CD127-eFluor450 (eBioRDR5) (eBioscience). FOXP3 clones 259D and PCH101 were used in combination in order to capture all FOXP3⁺ cells in a given sample [18]. Antibody combinations are shown in Supplementary Table 1.

Compensation for 7 and 9 color stain sets was accomplished based on either antibody-capture beads (CompBeads, BD) or PBMCs stained with the above-described antibodies. Then, single-stained cells with different mAbs used in the study were run to ensure that each stain was the brightest in its own channel before acquisition of 10,000 events per tube. Manual compensation was used, as it allowed for an optimal adjustment of the spectral overlap between the different fluorochromes. For the positivity gate, we used a negative biological population (CD3⁻CD4⁻ or CD3⁺CD4⁻), as previously reported [18]. Appropriate isotype controls were used for each antibody related with Treg phenotype.

2.6. Treg purification

Resting CD4⁺ T-cells were purified from CBMC and PBMC by negative selection using magnetic beads, according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Briefly, CD8⁺ T cells, $\gamma\delta$ T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells, were labeled by using a cocktail of biotin-conjugated antibodies (Miltenyi Biotec, Auburn, CA, cat 130-091-155). These cells were subsequently magnetically labeled with Anti-Biotin MicroBeads for depletion. The viability after CD4 isolation was 100%. Purified CD4⁺ T-cells were stained with anti-CD8-FITC (RPT-T8), anti-CD25-APC (M-A251) (BD Biosciences), and anti-CD127-PE (eBioRDR5; Beckman Coulter, Fullerton, CA). Treg were separated by cell sorting using a FACS Aria flow cytometer (BD Biosciences). The purity of Treg was confirmed post sorting by intracellular staining of FOXP3. Purity of the sorted populations was $\geq 90\%$.

After the sorting, the cells were washed to remove the sorting buffer, and re-suspended in culture medium. Purified Treg (CD8⁻CD25⁺CD127^{Low/-}) were activated for 4 days, using anti-CD3/CD28 beads (Invitrogen) at a 1:1 bead-cell ratio, in RPMI medium supplemented with 10% FCS, and 100 U/mL IL-2 (provided by the National Institutes of Health AIDS Research and Reference Reagent Program, Bethesda, MD). Anti CD3/CD28 beads were removed with a magnet. Activated Treg were treated with human IgG to block Fc-receptors and stained for surface marker with anti-CD4-AF700 (RPA-T4), anti-CD25 APC (M-A251) (BD Biosciences). Cells were then washed and fixed with Fixation/Permeabilization Buffer (eBioscience, San Diego, CA) followed by incubation in the Permeabilization Buffer (eBioscience). Cells were finally stained with anti-Ki67 PerCP-Cy5.5 (B56) (BD Biosciences).

2.7. Suppression assays

Treg suppression was measured using allogeneic Tcon cells as targets. To diminish inter-individual variability, these Tcon cells came from a single healthy adult donor. They were

purified from mononuclear cells by CD4 negative selection using magnetic beads (Miltenyi Biotec), followed by cell sorting, as described above for Treg. Purified Tcon cells (CD8⁺CD25⁻) were cryopreserved in aliquots until use as the target in suppression assays. After thawing, they were stained with 0.3 μ M CFSE (Molecular Probes) and activated for 3 days using anti-CD3/CD28 beads (1bead: 5cells), in the presence or absence of activated Treg from neonate or adult donors. Before the suppression assays, Treg were stained with calcein blue-AM (3 μ M), following the manufacturer's instructions (Invitrogen).

To determine Treg suppression on Tcon production of cytokines, cells were washed with DPBS, fixed/permeabilized with Cytofix/Cytoperm™ solution kit (BD Pharmingen), and stained intracellularly for TNF- α -APC (Mab11) and IFN- γ -PE (25723.11; BD Pharmingen). Live cells were gated based on forward- and side-scatter properties and the absence of fluorescence in the Live/Dead Fixable Dead Cell Stain kit. Tcon were gated based on CFSE staining.

2.8. Data analysis

Demographic data for the preterm and term infants are reported as percent of the population or mean $\pm$ SEM. Comparisons among 3 or more groups were performed by chi-squared tests or Kruskal-Wallis. Comparison of 2 groups was by chi-squared tests or Mann-Whitney U-test using GraphPad Prism, version 5.0 for Windows (LaJolla, CA). Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Demographic and clinical characteristics of neonates

CBMC from six full term infants (GA 39.2w), without histologic chorio ("term"), and 28 preterm infants were studied (Table 1). The preterm infants consisted of 3 groups: (a) 10 preterms without chorio (mean GA 34.5w), (b) 6 preterms exposed to mild chorio (mean GA 35.5w) and (c) 12 preterms exposed to severe chorio (mean GA 34.7w). GA among the 3 groups of preterm infants were similar. There were no significant differences among the groups regarding gender (50–67% female) and mode of delivery (50–100% vaginal delivery). A higher percentage of African American preterms had severe chorio compared to the other neonate groups. Funisitis was present only in the severe chorio group (42%). There were no significant differences among the groups in either need for supplemental oxygen or length of hospital stay. Premature rupture of membranes (PROM) and hypertension (HTM) were the most prevalent in preterm and mild chorio (Table 1). In severe chorio, PROM was the main reason for preterm delivery (Table 1). Around 50% of mothers from preterm and chorio newborns received prenatal steroids treatment, which was not significantly different between the groups (data not shown). In addition, the proportion of mothers who received antibiotics during the admission were 70%, 83%, and 50% in preterm, mild and severe chorio groups, respectively.

3.2. Pro-inflammatory cytokines in cord blood plasma

The preterm infants exposed to severe chorio were the only group to have significantly increased IL-8 levels, compared to either term (50 vs. 12 pg/ml; $p = 0.03$) or no chorio

neonates (50 vs. 27 pg/ml; $p = 0.04$) (Fig. 1A). A similar trend was found for IL-6 levels in severe chorio compared to term (31 vs. 3.5 pg/ml; $p = 0.07$) and no chorio neonates (31 vs. 1.4 pg/ml; $p = 0.01$) (Fig. 1B). There was a significant variability in CB cytokine levels in severe chorio infants: only 6 of the 10 plasmas from preterms exposed to severe chorio had high levels of IL-6 and IL-8. However, this variability was not caused by presence or absence of funisitis (data not shown). There was a positive correlation between the levels of IL-6 and IL-8 in the severe chorio group ($r=0.74$; $p = 0.01$). MCP-1 levels were comparable among the neonate groups ($p = 0.45$; Fig. 1C).

3.3. Frequency of circulating regulatory T-cells

Some studies have shown changes in the frequency, phenotype or function on Treg after cryopreservation [19,20]. However, in our hands, Treg retain their suppressive capacity and phenotype after cryopreservation (data not shown). Similar findings have been described by other authors [21–25]. We quantified peripheral Treg frequency in CBMC from the different groups of infants and PBMC from adults as an external control group. We analyzed the frequency of Treg using different strategies: $CD4^+FOXP3^+$; $CD4^+FOXP3^+CD127^{Low/-}$ and $CD4^+CD25^+CD127^{Low/-}$. As shown in the representative Fig. 2A and Supplementary Table 2, similar percentages of Treg were detected irrespective of the gating strategy. In fact, there was a strong correlation between the frequency of $CD4^+FOXP3^+$ and $CD4^+FOXP3^+CD127^{Low/-}$ cells ($r = 0.97$, $p < 0.001$), as well as between $CD4^+FOXP3^+$ and $CD4^+CD25^+CD127^{Low/-}$ cells ($r = 0.96$, $p < 0.001$). We therefore used $CD4^+FOXP3^+$ as the Treg definition and $CD4^+FOXP3^-$ as the Tcon definition for this study.

Circulating Treg frequencies were significantly lower in no chorio (2.6%), mild chorio (1.6%) and severe chorio (2.1%) infants compared to adults (3.8%) (Fig. 2B). Treg frequencies were not different among the preterm infant groups, regardless of exposure to chorioamnionitis or prematurity (Fig. 2B). However, infants with severe chorio had more intragroup variability in Treg frequencies, which was not associated with the level of plasma pro-inflammatory cytokines or funisitis ($p = 0.75$ and 0.53 , respectively).

3.4. Percentage of Treg in cell cycle

The percentage of Treg in cell cycle as determined by Ki67 expression was significantly decreased in no chorio and mild chorio infants compared to adults (Fig. 3A). However, Treg from infants exposed to severe chorio had higher expression of Ki67 compared to the other groups of infants (median of 21%; Fig. 3A and B). Similar differences in Ki67 expression were measured for $CD4^+CD25^+CD127^{Low/-}$ and $CD4^+FOXP3^+CD127^{Low/-}$ (data not shown). Overall, frequency of $Ki67^+$ Treg positively correlated with CB plasma IL-6 levels ($p = 0.04$, $r = 0.36$) (Fig. 3C). We therefore analyzed whether this activation was specific for Treg by measuring Ki67 expression in Tcon ($CD4^+FOXP3^-$). As expected based on previous studies [26], Ki67 expression in Tcon (Fig. 3D) was lower compared to Treg (Fig. 3A) in each group. The frequency of $Ki67^+$ Tcon was significantly, albeit modestly, increased in all infant groups compared to adults (all $p < 0.01$) (Fig. 3D). Tcon from infants exposed to severe chorio had the highest frequency of Ki67 expression (median of 5%), but it was not significantly different from that of the other infant groups because of the range of values in the severe chorio group (1–10%). $Ki67^+$ Tcon frequency was not correlated with

IL-6 levels ($p = 0.86$, $r = 0.03$) (Fig. 3E). Increased expression of Ki67 in Treg and Tcon from severe chorio was not associated with funisitis or prenatal steroids.

3.5. Treg expression of molecules associated with their function

Treg express an arsenal of molecules associated with a pheno-type of memory and activation; some of these molecules are also used by Treg as mechanism of suppression. The phenotype of circulating Treg has not been thoroughly examined in neonates, particularly in those exposed in utero to chorio. We thus compared Treg phenotype in the different groups of neonates. As expected, the frequency of memory Treg (expressing CD45RO) was lower for term, no chorio, mild and severe chorio neonates compared to adults, but was similar among all infant groups (Table 2 and Fig. 4 shows a representative experiment of each stain). Similarly, the proportions of Treg expressing the activation/memory marker HLA-DR and the suppressive molecules CD39 and TIGIT were decreased significantly in infants compared to adults, but were comparable in samples from term, no chorio, mild and severe chorio neonates (Table 2 and Fig. 4). Interestingly, when we analyzed HLA-DR, CD39 and TIGIT separately in naïve (CD45RA⁺) and memory (CD45RO⁺) Treg, we found that frequencies of each marker were lower in naïve Treg compared to memory Treg, but were at similar levels in adults and neonates. This suggests that the difference in frequencies of adult and neonate Treg expressing these markers is primarily explained by their proportion of naïve/memory Treg. This is supported by the fact that frequencies of expression of HLA-DR, CD39 and TIGIT by Treg correlated to their expression of CD45RO (all $p < 0.05$, data not shown).

In contrast to HLA-DR, CD39 and TIGIT expression, the frequency of Treg expressing the inhibitory molecule programmed death-1 (PD-1) was higher in all the groups of infants compared to adults, although PD-1 was expressed by a very small percentage of Treg in all groups (Table 2). There was a high variability of PD-1 expression in infants with chorio, both mild and severe. Treg CTLA-4⁺ frequency was similar in adults and infants (46–67%) (Table 2).

3.6. Treg suppression

Because the phenotypic evaluation of regulatory molecules expressed by Treg does not directly estimate their functional activity, we analyzed their suppressive capacity, using a classic *in vitro* assay. Infants that did not receive prenatal steroids were included in these functional assays. We evaluated the expression of Ki67 after activation; the frequency of Ki67⁺ Treg was not different in preterm, term and adult Treg (~80%) (data not shown). Then, we compared the effect of Treg on Tcon activation in response to CD3/CD28 stimulation. To normalize Treg suppression, we used Tcon from one healthy adult as target T-cells in all assays. Treg significantly decreased Tcon proliferation (as evidenced by decreased percentage of CFSE^{Low} cells by ~90%) as well as their TNF- α production (decreased percentage of TNF- α ⁺ cells by ~50%), as shown in a representative experiment in Fig. 5A. Likewise, Treg decreased Tcon expression of Ki67 and production of IFN- γ (data not shown).

Treg from term newborns and adults similarly suppressed Tcon proliferation. Treg from no chorio preterm neonates were modestly but significantly decreased compared to term Treg (Fig. 5B and C). Interestingly, inflammation further decreased Treg activity as Treg from preterm exposed to severe chorio were significantly less suppressive than those of preterm unexposed to chorio (Fig 5B and C). Treg from preterm exposed to mild chorio appeared to have a lower suppressive capacity, though not significantly different, than those of preterm unexposed to chorio, suggesting that the severity of inflammation is an important factor in the modulation of Treg suppressive capacity. CB plasma IL-8 levels correlated negatively with Treg suppression ($r = -0.5$, $p = 0.02$), with a similar trend for CB plasma IL-6 levels ($r = -0.4$, $p = 0.07$). In contrast, Treg from all groups similarly suppressed Tcon production of TNF- α (Fig. 5D) and IFN- γ (data not shown). In addition, the percentage of viable Treg (calcein⁺) and Tcon (CFSE⁺) in the co-cultures ranged from 81% to 92%, and was similar in the different groups (data not shown).

4. Discussion

Immune activation with large inflammatory responses during infections usually cause compensatory regulatory mechanisms [27]. Treg control excessive immune activation and reduce tissue damage. However, the effect of chorio on Treg had not been described. During chorio, increased inflammatory cells and cytokines/acute phase reactants in the CB were associated with injury responses to organs such as fetal brain, lung, gastrointestinal tract and skin [28]. In concordance with previous evidence, we detected high levels of IL-6 and IL-8 in CB in newborns exposed to severe chorio but not to mild chorio [29].

There is still some controversy concerning Treg characterization in humans. Some reports identified Treg based on high levels of CD25 expression. A better characterization is achieved by determining the expression of FOXP3, the best marker identified so far to characterize this cell subset. Absence or low expression of the alpha chain of the IL-7 receptor, CD127, along with the expression of the transcription factor FOXP3, are also considered good criteria to define this cell subpopulation [30,31]. In this study, we compared three different gating strategies (Foxp3⁺; CD25⁺CD127^{Low/-}, Foxp3⁺ CD127^{Low}) to characterize Treg during chorioamnionitis, and found very similar frequencies. These data are in agreement with previous studies of PBMC from adults, children and cord blood from neonates, having similar percentages of Treg when these cells were defined with the combination of these molecules (CD25⁺Foxp3⁺; CD25⁺CD127^{Low/-}, Foxp3⁺CD127^{Low}) [30].

In agreement with several studies in different cohorts, we did not find marked changes in the frequency of CB Treg from full term infants compared to peripheral blood Treg from adults [32–34]. During fetal life a substantial number of maternal cells cross the placenta to reside in lymphoid tissues, inducing the development of Treg that keep maternal-fetal tolerance [35,36]. This maternal alloantigen stimulus explains the presence of a high percentage of Treg in CB in early preterm (29w) compared to full term infants [33,34,37]. Treg prevalence then gradually decreases with GA, reaching a stable number at birth [34,37]. In our study, Treg frequencies did not differ significantly between moderate/late preterm and term neonates. The discrepancy with these previous studies could be related to the fact that very

preterm infants were the target population, while our study evaluated CBMC from moderate/late preterm infants. In addition, the moderate/late preterm neonates we studied comprised a relatively healthy group without significant neonatal complications.

However, one caution in interpreting these results is that immune responses occurring in tissues may not be reflected in the blood. In experimental models of chorio, intraamniotic LPS or IL-1 β decreased the frequencies of FOXP3⁺ cells in sheep thymus and lymph nodes [11,38,39]. In fetal rhesus macaques, a single intra-amniotic exposure of IL-1 β also decreased the frequency of Treg at 24 h in spleen and lymph nodes, but not blood [40].

A strength of our study is that it included a detailed phenotypic analysis of Treg from newborns, which had not yet been performed. Moderate/late prematurity or chorio did not significantly modify Treg phenotype. As reported earlier, we found that infant Treg have a predominantly naïve phenotype [34,41–43]. Newborn Treg also had less HLA-DR expression compared to adult, as previously reported [34,43]. Newborn Treg (term and pre-term) also had lower frequencies of CD39 and TIGIT due to the absence of antigen exposure in naïve cells. In contrast, neonate Treg and adult Treg expressed high levels of CTLA-4, confirming a previous study that evaluated CTLA-4 mRNA [34].

Ki67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis) and is associated with cell proliferation. As reported earlier in adults, the expression of Ki67 in Treg was higher than in Tcon [26,40] and the percentages of Ki67⁺ Treg correlated modestly with the levels of plasma IL-6. Although IL-6 could stimulate the cell cycle progression and survival [44,45], we cannot rule out the possibility that other molecules are involved in this process. In contrast to Ki67 expression, CD45RO/HLA-DR/CD39/CTLA-4 expression was not different in Treg from chorio compared to no chorio preterm newborns. These results are reminiscent of the phenotypic profile of Treg we reported in a model of IL-1 β -induced chorio in rhesus monkeys, in which we found increased Ki67 but no changes in CD39 or CTLA-4 expression in Treg [40].

Resting Treg from term newborns are completely functional in their ability to suppress Tcon proliferation and cytokine production [34,46]. However, compared to adults these cells are less potent due to their phenotypic immaturity [47]. To avoid differences in the Treg suppression capabilities between adults and newborns as a consequence of their naïve phenotype, we pre-activated Treg before adding Tcon and measured suppressive activity. Pre-activated term Treg were fully suppressive, similar to previous data [41,47–49]. However, in contrast to the data reported by Fan et al., we did not find Treg from term CB to be more suppressive than adult Treg [48]. This difference could be explained by the long period of pre-activation (2 cycles of 6 days with polyclonal stimulus) used by Fan et al., 2012, compared with the 4-days stimulation in our experiments. We found that moderate/late preterm Treg had slightly reduced Tcon suppressive activity compared to term, as previously reported in a study comparing preterm (29–35w) with term (37–42w) newborns [50]. In this study, the low level of FOXP3 expression in Treg from preterm neonates correlated with low suppressive activity [50]. However, in our study, similar FOXP3 MFI was found in the different groups. Future studies will be needed to determine the mechanisms underlying decreased suppressive activity.

Importantly, we report for the first time that Treg from pre-terms exposed to chorio have reduced suppressive activity. This reduction in Treg suppression could be explained by TLR-2 and pro-inflammatory cytokine signals: these stimuli induce divergent chromatin changes at the FOXP3 locus, downregulating function while increasing the expression of IL-6 and RORC/IL-17 in Treg subsets [51–53]. Th17 frequency was not measured in this study but we have shown an increased Th17 proportion in IL1- β -induced chorioamnionitis in macaques [40]. Characterization of IL-17⁺ cells will be warranted in future studies of chorio-exposed neonates. Some limitations of our approach are the low number of subjects enrolled in our functional experiment, and the fact that magnesium sulfate usage, which has recently been associated with Treg phenotypic alterations (such as the percentage of CD31⁺ Treg) was not recorded [54]. Future studies will be needed to clarify potential differences between Treg from mild and severe chorio and the effect magnesium sulfate treatment.

In conclusion, our data demonstrate that CB Treg frequency and phenotype is not significantly modified by late prematurity and chorio, yet activated Treg are not equally suppressive on a per cell basis in chorio exposed newborns. Treg activity may not be sufficient to control the excessive inflammation and tissue damage induced by chorio. Additional studies will be required to address the mechanisms driving this Treg dysfunction and to test whether strategies designed to boost regulatory responses may limit immune activation in preterm infants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Treg	regulatory T cell
Chorio	chorioamnionitis
PBMC	peripheral blood mononuclear cell
CBMC	cord blood mononuclear cell
Tcon	conventional T cells
CB	cord blood

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Fig. 1. Plasma levels of pro-inflammatory cytokines in newborns. Concentrations of (A) IL-8, (B) IL-6 and (C) MCP-1 were measured in samples from full term neonates ($n = 6$), preterm neonates without chorio ($n = 10$), preterm exposed to mild chorio ($n = 6$) and preterm exposed to severe chorio ($n = 10$). Bars show the median of the group. Groups were compared using Mann–Whitney U tests.

Fig. 2.

Frequency of Treg in peripheral blood from adults and cord blood from newborns. (A) Flow cytometry data from one representative experiment show the Treg characterization in a newborn sample. Three different ways of defining Treg were analyzed in the CD3⁺CD4⁺ gate: CD4⁺FOXP3⁺CD127^{Low/-} (upper panel), CD25⁺CD127^{Low/-} (middle panel) and CD4⁺FOXP3⁺ (lower panel). (B) Treg frequency (CD4⁺FOXP3⁺) from adult PBMC and CBMC. Bars show the median of the group. Groups were compared using Mann–Whitney *U* tests.

Fig. 3.

Frequency of Treg and Tcon subsets from adults and newborns in cell cycle. (A) Percentage of Treg expressing Ki67 within the Treg population (CD4⁺FOXP3⁺). (B) Flow cytometry data from one representative experiment show the expression of Ki67 on CD4⁺FOXP3⁺ T cells in term and chorio severe newborns. (C) Positive correlation between plasma IL-6 levels and percentage of Ki67⁺ Treg in newborns. (D) Percentage of Tcon expressing Ki67 within the Tcon population (CD4⁺FOXP3⁻). (E) No correlation between IL-6 levels and percentage of CD4⁺FOXP3⁻Ki67⁺ in newborns. Spearman correlation test.

Fig. 4. Phenotype of Treg in peripheral blood from adults and severe chorio newborns. Flow cytometry data from one representative experiment show the expression of CD45RO, HLA-DR, CD39, TIGIT, PD-1 and CTLA-4 by Treg (CD4⁺FOXP3⁺).

Fig. 5.

Treg suppression of Tcon proliferation and cytokine production. (A) Allogeneic Tcon proliferation (CFSE^{Low}) and TNF-α production were analyzed in Tcon alone (upper panels) and cocultured with Treg (lower panels) (data from one representative term donor). Values represent the percentage of activated cells (% CFSE^{Low} and % TNF⁺). (B) Representative figure represent the activity Treg suppression of one donor from term, preterm and severe chorio. Values represent the percentage of activated cells (% CFSE^{Low}). (C) Treg suppression of Tcon proliferation, mean % ± SEM suppression are shown. (D) Treg suppression of TNF-α production. % suppression was calculated in relation to Tcon alone (100%-[response of Tcon in coculture/response of Tcon alone]). Full term ($n = 4$), preterm without chorio ($n = 8$), preterm exposed to mild chorio ($n = 3$) and preterm exposed to severe chorio ($n = 3$).

Table 1

Comparison of clinical characteristics of infants at birth.

	Term (<i>n</i> = 6)	Preterm no chorio (<i>n</i> = 10)	Preterm mild chorio (<i>n</i> = 6)	Preterm severe chorio (<i>n</i> = 12)
Gestational age (wks) (mean ± SEM)	39.2 ± 0.2*	34.5 ± 0.5	35.5 ± 0.3	34.7 ± 0.4
Birth weight (g) (mean ± SEM)	3400 ± 226 ^t	2333 ± 178	2815 ± 219	2355 ± 124
Sex (% <i>F</i>)	50	60	67	50
Race (% African Am)	33	30	33	67
% Vaginal delivery	50	60	100	67
Funisitis (%)	0	0	0	42
Any supplemental oxygen (%)	17	30	33	25
Hospital length of stay (days)	1.8 ± 0.3	9.1 ± 2.8	3.0 ± 0.7	6.5 ± 2.0
Reason for preterm birth (<i>n</i>)	NA	3 PROM 3 HTN 3 MP 1A	3 PROM 2 HTN 1 PTL	8 PROM 1 HTN 1 MP 1 PTL 1 Chorio

PROM, premature rupture of membranes; HTN, hypertension; MP, multiple pregnancy; PTL, preterm labor; A, abruption.

* $p < 0.05$ vs. all groups.^t $p < 0.05$ vs. no chorio and severe chorio.

Table 2

Phenotypic analysis of Treg.

% Treg expressing*	Adult	Term	Preterm no chorio	Preterm mild chorio	Preterm severe chorio
CD45RO	71 ^f (60–77)	11 (9–16)	14 (8–16)	6 (3–15)	15 (8–21)
HLA-DR	39 ^f (38–44)	6 (6–9)	5 (3–8)	6 (5–10)	8 (7–15)
CD39	49 ^f (37–60)	14 (12–21)	16 (9–18)	16 (7–21)	22 (13–33)
TIGIT	74 ^f (70–81)	34 (29–41)	37 (28–41)	28 (21–36)	33 (30–36)
PD-1	2 ^f (1–3)	4 [#] (3–8)	9 (8–11)	10 (6–17)	8 (3–11)
CTLA-4	50 (32–54)	67 (42–69)	53 (48–63)	45 (41–59)	60 (47–68)
FOXP3 MFI	7215 (4799–9603)	5110 (3636–7559)	5665 (3878–9126)	6627 (3426–10,394)	5430 (4452–11,653)

* Data are expressed as median (range) percentage of Treg (CD4⁺FOXP3⁺) expressing each marker or median (range) MFI.^f <0.05 vs. all the neonate groups.[#] <0.05 vs. no chorio and mild.