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Chorioamnionitis in Rats Precipitates Extended Postnatal Inflammatory Lymphocyte Hyperreactivity

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Abstract
Preterm birth is an important cause of perinatal brain injury (PBI). Neurological injury in extremely preterm infants often begins in utero with chorioamnionitis (CHORIO) or inflammation/infection of the placenta and concomitant placental insufficiency. Studies in humans have shown dysregulated inflammatory signaling throughout the placental-fetal brain axis and altered peripheral immune responses in children born preterm with cerebral palsy (CP). We hypothesized that peripheral immune responses would be altered in our well-established rat model of CP. Specifically, we proposed that isolated peripheral blood mononuclear cells (PBMCs) would be hyperresponsive to a second hit of inflammation throughout an extended postnatal time course. Pregnant Sprague-Dawley dams underwent a laparotomy on embryonic day 18 (E18) with occlusion of the uterine arteries (for 60 min) followed by intra-amniotic injection of lipopolysaccharide (LPS, 4 μg/sac) to induce injury in utero. Shams underwent laparotomy only, with equivalent duration of anesthesia. Laparotomies were then closed, and the rat pups were born at E22. PBMCs were isolated from pups on postnatal day 7 (P7) and P21, and subsequently stimulated in vitro with LPS for 3 or 24 h. A secreted inflammatory profile analysis of conditioned media was performed using multiplex electrochemiluminescent immunoassays, and the composition of inflammatory cells was assayed with flow cytometry (FC). Results indicate that CHORIO PBMCs challenged with LPS are hyperreactive and secrete significantly more tumor necrosis factor α (TNFα) and C-X-C chemokine ligand 1 at P7. FC confirmed increased intracellular TNFα in CHORIO pups at P7 following LPS stimulation, in addition to increased numbers of CD11b/c immunopositive myeloid cells. Notably, TNFα secretion was sustained until P21, with increased interleukin 6, concomitant with increased expression of integrin β1, suggesting both sustained peripheral immune hyperreactivity and a heightened activation state. Taken together, these data indicate that in utero injury primes the immune system and augments enhanced inflammatory signaling. The insidious effects of primed peripheral immune cells may compound

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PBI secondary to CHORIO and/or placental insufficiency, and thereby render the brain susceptible to future chronic neurological disease. Further understanding of inflammatory mechanisms in PBI may yield clinically important biomarkers and facilitate individualized repair strategies and treatments.

Introduction

In the USA and worldwide, prematurity is a major cause of infant mortality and long-term disability in children [1]. Survivors of very preterm birth can have numerous neurological disorders and cognitive impairment, including cerebral palsy (CP), epilepsy, intellectual disability, impaired sensory processing, and attentional disorders [2–4]. Epidemiological, clinical, and preclinical studies support a placental-fetal brain axis in neurological development, with alterations or disruptions in this axis leading to brain injury [2, 5–7]. Chorioamnionitis (CHORIO) is the most common abnormality found in placentas from very preterm infants and a principle cause of preterm birth [8–11]. Defined by inflammation and hypoxia-ischemia (HI), CHORIO disrupts the maternal-placental interface and directly impacts the fetal microenvironment [12, 13]. It is well established that injuries during these critical periods of development have long-term effects on growth, metabolism, cognitive function, motor performance, and inflammatory response [5, 6, 12, 14–16].

Changes in the intrauterine microenvironment, and subsequent fetal and neonatal events, impact development and life-long susceptibility to illness. Indeed, environmental stressors can negatively affect the development of fundamental physiological processes, including those of the immune and central nervous systems. CHORIO is a significant risk factor for CP in term, near-term, and preterm infants [8, 17–22]. Perinatal inflammatory responses have been implicated in the pathophysiology of CP [14, 23, 24]. A fetal inflammatory response syndrome (FIRS), the fetal equivalent of systemic inflammatory response syndrome (SIRS), is frequently present in neonates born as a result of spontaneous preterm labor secondary to CHORIO [25–28].

Like the central nervous system, the fetal immune system develops and matures over the course of gestation [26]. Similar to neural cells, fetal and neonatal leukocytes are uniquely responsive to their environment [26, 29]. Indeed, delineation of leukocyte responses following injury may serve as an important clinical and scientific biomarker. Minimizing the impact of perinatal brain injury (PBI) due to preterm birth is dependent on the successful identification of critical pathways essential to the developmental programs amongst neural-immune cells in the placental-fetal brain axis [30]. While the interactions between innate and adaptive immune responses following PBI are relatively unknown, human studies have shown that preterm children with CP have altered inflammatory responses at school age [14]. Specifically, peripheral blood mononuclear cells (PBMCs) from children with CP are hyperresponsive to lipopolysaccharide (LPS) stimulation compared to age-matched typically developing preterm controls. Previously, we found a robust FIRS in our rat model of CP secondary to placental inflammation and insufficiency, along with lasting cognitive and motor impairment, and significant alterations in the placental-fetal brain axis [25, 31–33]. Thus, given that developmental plasticity is altered by perinatal injury and may have long-term effects on the inflammatory responses of circulating leukocytes, we hypothesized that peripheral immune responses would be altered through a prolonged period of development in our rat model of CP.

Materials and Methods

Animals

All procedures were performed consistent with National Research Council guidelines, and with the approval of the Institutional Animal Care and Use Committee at the University of New Mexico Health Sciences Center. ARRIVE guidelines were followed [34].

Prenatal Insult

Pregnant Sprague-Dawley rat dams underwent abdominal laparotomy on embryonic day 18 (E18), consistent with previous reports [25, 31–33, 35–38]. To induce prenatal injury similar to CHORIO, bilateral uterine arteries were transiently occluded for 60 min to induce placental insufficiency, followed by an intra-amniotic injection of LPS 0111:B4 (4 μg/sac; Sigma-Aldrich, St. Louis, MO, USA) as previously published [25, 30–33]. Laparotomies were closed, and the rat pups were born at term on E22. Sham dams underwent laparotomy with equivalent exposure to anesthesia. Male and female pups were used and randomly assigned to all outcome measures, and they represented the offspring from at least 4 different dams per condition. Previously, we published the placental pathology with robust neutrophilia and cytokine levels consistent with histological CHORIO, FIRS, neuroinflammatory responses, as well as MRI outcome and the long-term cognitive and motor functional abnormalities in this model [25, 31–33].

PBMC Isolation

PBMCs from sham or CHORIO pups were isolated on postnatal day 7 (P7) and P21 using a Ficoll gradient separation [14]. Specifically, venous blood was collected from the right atrium in pyrogen-free, heparinized, K2 EDTA vacutainer tubes (Becton Dick-

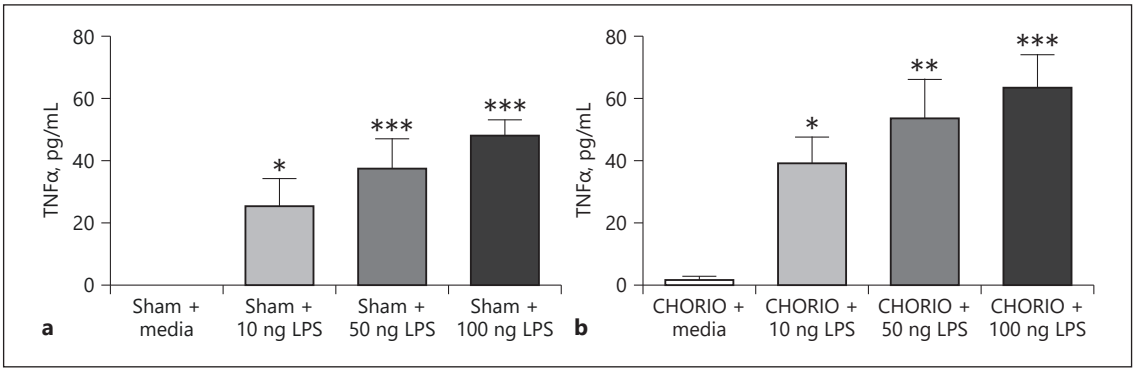

Fig. 1. LPS induces dose-responsive increases in TNFα. Peripheral blood mononuclear cells isolated from postnatal day 21 pups were stimulated with control or LPS (10, 50, and 100 ng/mL) for 24 h in sham (**a**) and CHORIO (**b**) pups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

inson, Franklin Lakes, NJ, USA). Blood was pooled from P7 pups but not P21 pups. Two milliliters of blood plus 2 mL RPMI 1640 media (Gibco, Waltham, MA, USA), were then added to a 15-mL conical tube with mixing by inversion. The blood mixture was layered on 3 mL of well-mixed Ficoll-Paque Plus 1084 (GE Healthcare, Chicago, IL, USA) media, and then centrifuged at 400 *g* for 30 min at room temperature with the centrifuge brake in the "off" position. Using a sterile pipette, the upper layer containing plasma and platelets was drawn off, leaving the mononuclear cell layer undisturbed at the interface. Mononuclear cells were then transferred to a sterile centrifuge tube using a sterile pipette, and 3 volumes (approx. 6 mL) RPMI media were added. Cells were suspended by gently drawing them in and out of a pipette and then centrifuged at 400 *g* for 10 min at room temperature with the centrifuge brake on. Subsequently, supernatant was removed, and the cells were resuspended in 6 mL RPMI media. After an additional round of centrifugation as described above, supernatant was discarded, and the cell pellet resuspended in 6–8 mL RPMI media and prepared for plating.

PBMC Treatment with LPS

PBMCs at P7 or P21 were plated in 3.5-cm culture dishes at a density of 2×10^6 cells per dish (1×10^6 cells/mL, 2 mL each). PBMCs were then stimulated without or with LPS (10, 50, or 100 ng/mL) for 3 or 24 h and the supernatants collected, consistent with prior reports [14, 39]. Each experimental condition was performed in triplicate. Notably, treatment of PBMCs from both CHORIO and sham pups with differing doses of LPS yielded a dose response of TNFα secretion (Fig. 1). Given the robustness of the response, and consistent with previous reports, 100 ng/mL of LPS was used for all subsequent experiments [40–42].

Multiplex Electrochemiluminescent Immunoassay

A secreted cytokine and chemokine profile analysis was performed on supernatants from cultured PBMCs (n = 6–7/group) using a V-plex rat proinflammatory panel for TNFα, interleukin (IL)-1β, C-X-C chemokine ligand 1 (CXCL1) and IL-6 (MesoScale Discovery, Gaithersburg, MD, USA). Specifically, conditioned media was loaded (diluted 1:4) in duplicate on a 96-well plate, consistent with the manufacturer's specification and also numerous prior preclinical and clinical studies [25, 43–48]. Plates were read on a Quickplex SQ 120 Imager. This system has high content validity and an interassay variation of <12% in our laboratory.

Flow Cytometry

PBMCs from CHORIO and sham groups were isolated and plated as described above. All antibodies were purchased from Thermo Fisher Scientific-eBioscience (Waltham, MA, USA) and were used at 0.125–0.5 μg per 1×10^6 cells, as recommended by the manufacturer. Four pups per condition (sham or CHORIO) were used for each experiment, resulting in a total of 8 pups at both P7 and P21. Cells for each condition were plated in 2 replicate wells. Cells from one of the replicate wells were used for the surface staining of CD45 (common leukocyte marker), CD11b/c (integrin α-M, pan marker for myeloid cells), MHC2 (immune activation marker), ED2-like antigen (rat macrophage marker, HIS36), and CD29 (integrin β1). Cells from other replicate wells were used for surface staining of CD45 and CD11b/c, followed by intracellular staining for the cytokine TNFα. Cells that were assayed for intracellular cytokine detection were treated with a 2 μL/mL protein transport inhibitor cocktail (containing brefildin A and monensin; Thermo Fisher Scientific-eBioscience) that was added simultaneously with media or LPS at the beginning of the cultures. The protein transport inhibitor cocktail inhibits the intracellular protein secretory/transport pathway, resulting in the accumulation of secreted proteins/cytokines in the lumen of the endoplasmic reticulum and in the Golgi apparatus which can be detected by intracellular staining and flow cytometric analysis [49].

Staining for surface antigens and intracellular cytokines was conducted as described in Noor et al. [50]. Briefly, following 24 h of stimulation with LPS or media, cells were transferred into separate FACS tubes (BD Falcon™, Becton Dickinson) and pelleted by centrifugation at 300 *g* for 10 min at 4 °C, with the supernatant discarded. Cells were then resuspended in 1 × PBS (without calcium and magnesium; Sigma-Aldrich) and stained with Viability Dye eFluor® 450 (Thermo Fisher Scientific-eBioscience) for 30 min, washed with FACS buffer (1× PBS containing 1.0% BSA and 1 mM EDTA). Cells were then incubated with a saturating solution of Fc block (BD Biosciences, San José, CA, USA) for 10 min, followed by staining with fluorochrome-conjugated antibodies against surface antigen or appropriate isotype controls for 30 min.

All of these steps were conducted in the dark on ice. Following surface antibody staining, cells were washed and resuspended in 250 μL FACS buffer, and then passed through a 40-μm cell strainer immediately prior to analysis to avoid cell clumping.

For intracellular staining of TNFα, cells were washed with PBS and stained with viability dye and surface markers (CD45 and CD11b/c), as described above. Cells were then fixed with 4% PFA (Sigma-Aldrich) for 20 min at room temperature, washed with FACS buffer and permeabilized with 0.3% saponin (Sigma-Aldrich) in FACs buffer, followed by incubation with anti-rat TNFα for 40 min on ice in the dark. Cells were then washed in saponin-FACs buffer, resuspended in FACS buffer, and then proceeded to flow cytometer data acquisition, as described previously [32, 50].

For flow cytometry (FC) analysis at P7, UltraComp eBeads (Thermo Fisher Scientific-eBioscience) were used for generating compensation controls; for P21 FC, blood leukocytes with single fluorochrome stains were used for compensation controls. For P21 FC analysis, viability dye was not included; live cells were identified by their size and granularity. Data were acquired using the BD LSR Fortessa cell analyzer (BD Biosciences) and analyzed using FlowJo software v8.7.4 (FlowJo LLC, Ashland, OR, USA). Cells were gated first on size and granularity (FSC vs. SSC), followed by gating on single cells (SSC-A vs. SSC-H and FSC-A vs. FSC-H). Viable (verified by viability dye staining) and CD45-positive (CD45+) cells were identified, as described before [43, 50]. To identify myeloid cells, only CD45+ cells were analyzed for CD11b/c+ expression. CD45+CD11b/c+ cells were then further analyzed for MHC2, ED2-like antigen, CD29, or TNFα expression. Mean fluorescence intensity (MFI) was measured.

Statistical Analyses

Data are represented as mean ± standard error of the mean (SEM). Parametric statistical differences between 2 groups were compared with Student's *t* test, and between 3 groups with a one-way ANOVA with Tukey's post hoc correction. $p < 0.05$ was considered statistically significant.

Results

In utero Insult Yields Altered Inflammatory Responses at P7

Beginning on P7, we isolated PBMCs from pups exposed to sham conditions or in utero CHORIO insult. The levels of TNFα in the supernatant of nonstimulated PBMCs from sham and CHORIO pups were comparable after 3 h (0.22 ± 0.7 vs. 0.30 ± 0.06 pg/mL, respectively), although a baseline difference in the levels of IL-6 was detected with CHORIO PBMCs secreting 2.6-fold more IL-6 than sham PBMCs at 3 h ($p < 0.001$). LPS stimulation of the PBMCs resulted in significant increases of TNFα in the supernatants of both groups (sham: 0.22 ± 0.7 vs. 11.02 ± 0.7 pg/mL; CHORIO: 0.30 ± 0.06 vs. 26.66 ± 6.8 pg/mL, $p < 0.001$ for both). However, CHORIO PBMCs had significantly higher levels of secreted TNFα in response to LPS stimulation than the PBMCs from the sham pups (Fig. 2a) as well as significantly increased CXCL1 secretion (Fig. 2b). No additional changes in IL-6 levels were observed with LPS challenge. These increased responses compared to sham demonstrates enhanced immune reactivity. This pattern held at 24 h of LPS stimulation, with CHORIO PBMCs continuing to secrete more TNFα and CXCL1 than sham-stimulated PBMCs (Fig. 2c–d), but not more IL-6 (114 vs. 91 pg/mL, $p > 0.05$). Interestingly, by 24 h, the levels of TNFα in the supernatant of nonstimulated PBMCs from sham and CHORIO pups were significantly different. In this nonstimulated condition, TNFα secretion was increased by 84% in CHORIO pups compared to sham controls (Fig. 2e, $p < 0.05$). Furthermore, FC analyses confirmed increased intracellular TNFα in CHORIO PBMCs compared to sham PBMCs (Fig. 2f–g). Together, these data indicate that the PBMCs from the CHORIO pups were hyperreactive in response to a second LPS stimulus during the first postnatal week.

To test whether differences in cytokine secretion and PBMC hyperreactivity were a result of fundamental changes in white blood cell populations after CHORIO, we performed FC for common cell-surface, cell-specific markers. At baseline and without LPS stimulation, CHORIO PBMCs were defined by significantly higher percentages of CD11b/c- and ED-2/CD163-expressing cells (Fig. 3a, b), indicating increased numbers of circulating myeloid and mature macrophages at P7 in CHORIO pups compared to sham pups. However, at baseline, there were no differences in integrin β1, a marker of activation (Fig. 3c). Interestingly, LPS stimulation sustained the increase in CD11b/c+ cells in CHORIO pups compared to sham pups (Fig. 3d). This alteration, however, occurred in the absence of any difference in MHC2 or integrin β1 expression (Fig. 3e, f). Taken together, these data show that CHORIO changes the population of white blood cells and that a secondary hit of LPS sustains the number of myeloid cells when compared to nonstimulated LPS controls.

In utero Insult Yields Sustained Changes in Inflammatory Responses at P21

After establishing changes in peripheral inflammatory reactivity at P7, or 2 weeks after in utero insult, we determined whether changes in immune cell reactivity and response had been sustained. Thus, we assessed PBMCs for the secretion of proinflammatory cytokines and chemokines at P21, 4 weeks after CHORIO, equivalent to toddler age in humans. Similar to what was observed at P7, sham and CHORIO PBMCs secreted similar levels of TNFα in the absence of LPS (0.14 ± 0.07 vs. 0.16 ± 0.12

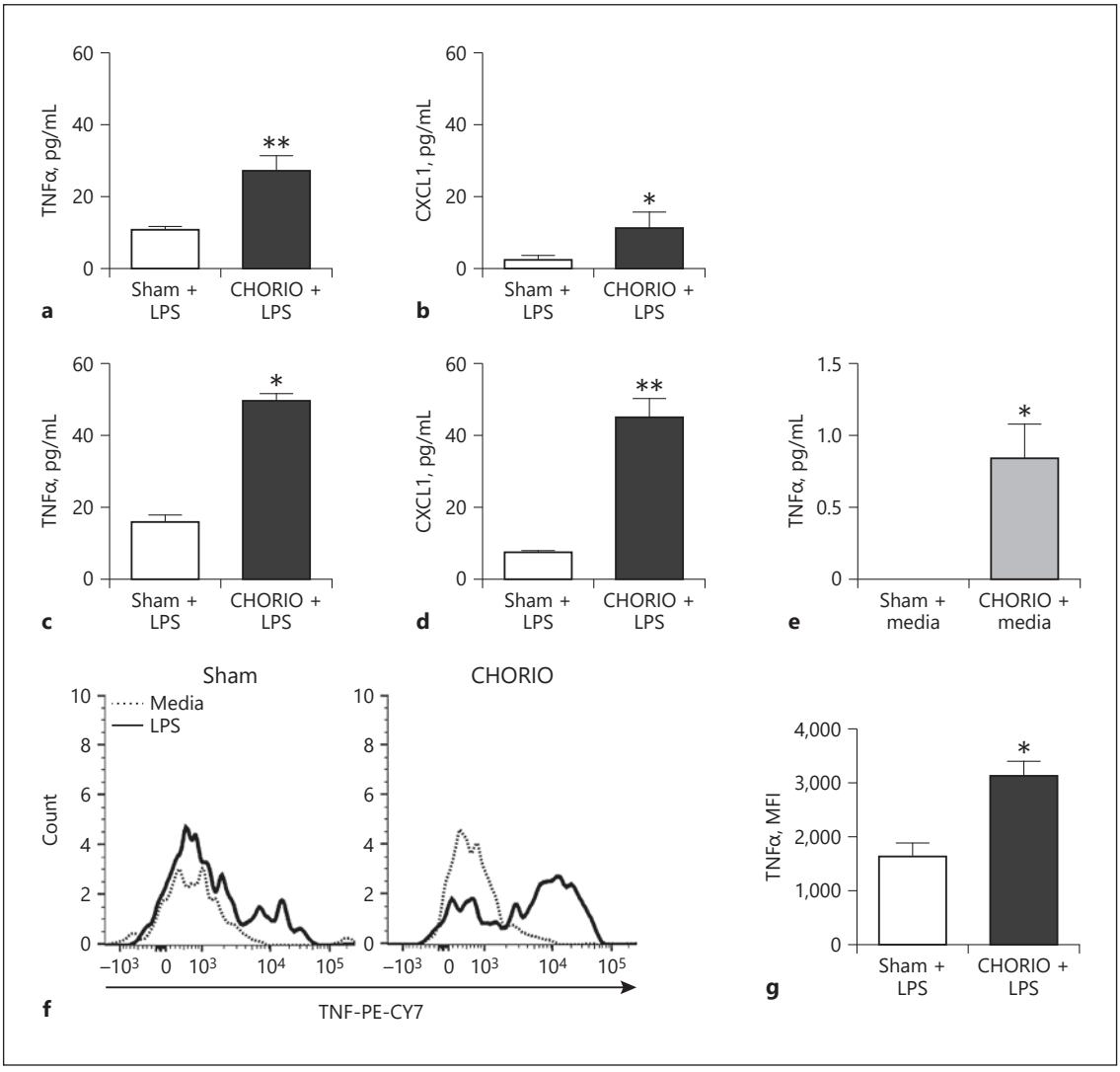

Fig. 2. CHORIO peripheral blood mononuclear cells (PBMCs) are primed and augment proinflammatory cytokine secretion following LPS stimulation. PBMCs were isolated from postnatal day 7 sham or CHORIO pups and stimulated with control or LPS for 3 h (**a**, **b**) or 24 h (**c–g**). Secreted levels of TNFα (**a**) and CXCL1 (**b**) were significantly increased in CHORIO PBMCs challenged with LPS compared to sham control PBMCs. At 24 h, CHORIO PBMCs also secreted more TNFα (**c**) and CXCL1 (**d**) than sham PBMCs in response to LPS challenge. **e** Notably, CHORIO PBMCs secreted more TNFα in the absence of LPS challenge and at baseline when compared to sham cells. **f**, **g** Flow cytometry confirmed increased intracellular TNFα in LPS-stimulated CHORIO PBMCs. * $p < 0.05$, ** $p < 0.01$.

pg/mL). Additionally, both sham and CHORIO cells responded to LPS by increasing TNFα (sham: 0.14 ± 0.07 vs. 10.44 ± 2.3 pg/mL TNFα; CHORIO: 0.16 ± 0.12 vs. 23.32 ± 3.8 pg/mL, $p < 0.001$ for both). Notably, in the presence of LPS for 3 h, PBMCs from P21 CHORIO rats hypersecreted TNFα and IL-6, compared to sham PBMCs, but not CXCL1 (Fig. 4a–c). This effect was also observed when the PBMCs were stimulated with LPS for 24 h (Fig. 4d–f). Interestingly, by 24 h, the levels of TNFα in the supernatant of nonstimulated PBMCs from sham and CHORIO pups were also significantly different (0.16 ± 0.07 vs. 0.99 ± 0.13 pg/mL, $p < 0.001$, Fig. 4g), confirming a long-term change in PBMC secretion of TNFα and immune reactivity even at baseline. FC performed on P21 cells corroborated a trend to increased intracellular TNFα (Fig. 4h, $p = 0.06$). These data indicate persistent peripheral hyperimmune reactivity following CHORIO and in response to LPS stimulation.

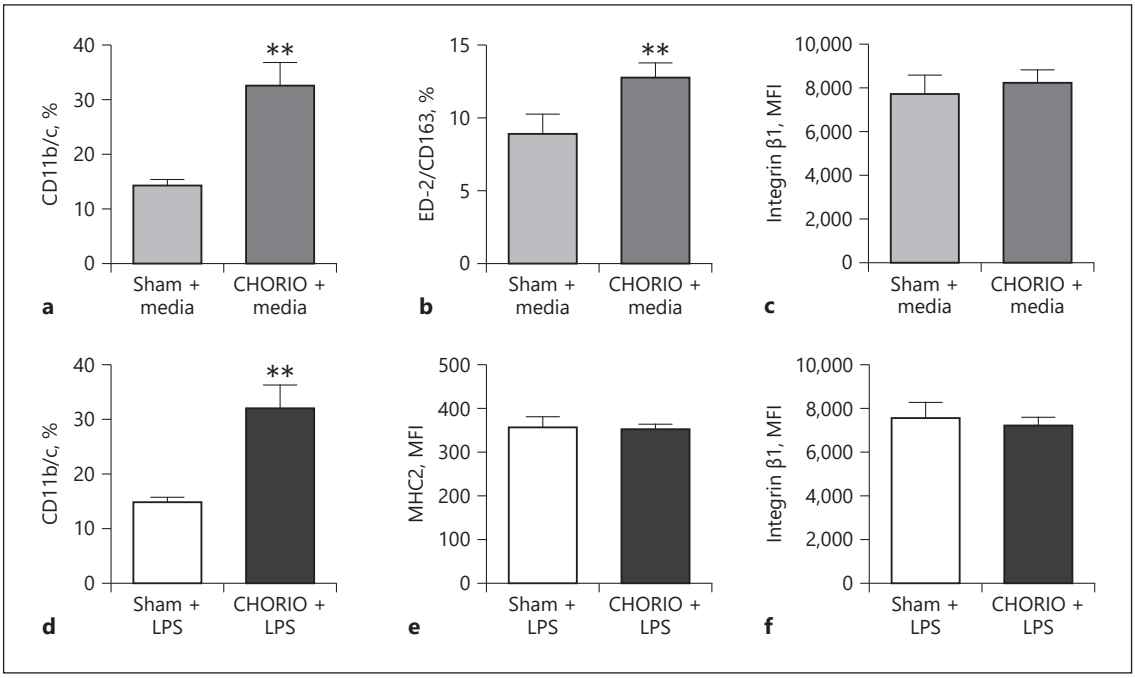

Fig. 3. CHORIO increases circulating myeloid cells. Peripheral blood mononuclear cells (PBMCs) isolated from postnatal day 7 pups were stimulated with control or LPS (100 ng/mL) for 24 h. Flow cytometry analysis was used to identify the proportions of myeloid cells and their activation levels. **a** Notably, proportions of viable (identified by size, granularity and viability dye staining) myeloid (CD45+CD11b/c+) cells were increased in the PBMCs of CHORIO pups compared to sham controls at baseline. **b** Similarly, the proportion of cells showing positive expression for the mature macrophage marker (ED-2/CD163-like antigen) among viable myeloid cells even without LPS stimulation was also increased in CHORIO pups compared to sham controls. **c** When viable myeloid cells at baseline were further analyzed for the adhesion molecule integrin β1, however, there were no differences in expression (mean fluorescence intensity, MFI), indicative of equivalent activation. **d** With LPS challenge, proportions of CD11b/c+ cells remained increased compared to sham PBMCs stimulated with LPS. However, no differences in MFI for MHC2 (**e**) or integrin β1 (**f**) were observed. ** $p < 0.01$.

Next, we examined whether PBMC hyperreactivity was associated with changes in immune cell markers using FC with surface antigen staining. Interestingly, despite changes in proinflammatory secretion, we found similar levels of CD11b/c cells in CHORIO and sham cells treated with LPS (Fig. 5a), as well as an equivalent expression of MHC2 (Fig. 5b). However, integrin β1 (CD29) was significantly elevated on LPS-stimulated CHORIO PBMCs compared to sham-stimulated cells, consistent with an increased activation state (Fig. 5c). Notably, baseline levels of integrin β1 at P21 were equivalent in sham and CHORIO cells (Fig. 5d).

Discussion

Preterm infants exposed to intrauterine inflammation are at an increased risk of neurodevelopmental disorders, and adverse outcomes are more strongly associated with a combination of antenatal and postnatal inflammation than either circumstance alone [51]. In this report, we provide the first evidence that levels of TNFα, CXCL1, and IL-6 released from LPS-stimulated PBMCs are significantly higher in term-equivalent P7 and toddler-equivalent P21 rats exposed to prenatal placental inflammation and insufficiency concomitant with acute changes in inflammatory cell composition and enduring alterations in their systemic inflammatory response. Together, these data suggest that rats with in utero injury have sustained peripheral immune hyperreactivity (SPIHR). These data corroborate reports on preterm children with CP [14] and our own previous reports of elevated serum proinflammatory cytokines, enhanced inflammatory signal transduction through the maternal-placental-fetal axis, and CP-like motor phenotypes in this model [25, 32]. Indeed, immune plasticity altered by in utero insults may have long-term effects on the inflammatory responses of circulating leukocytes, which may

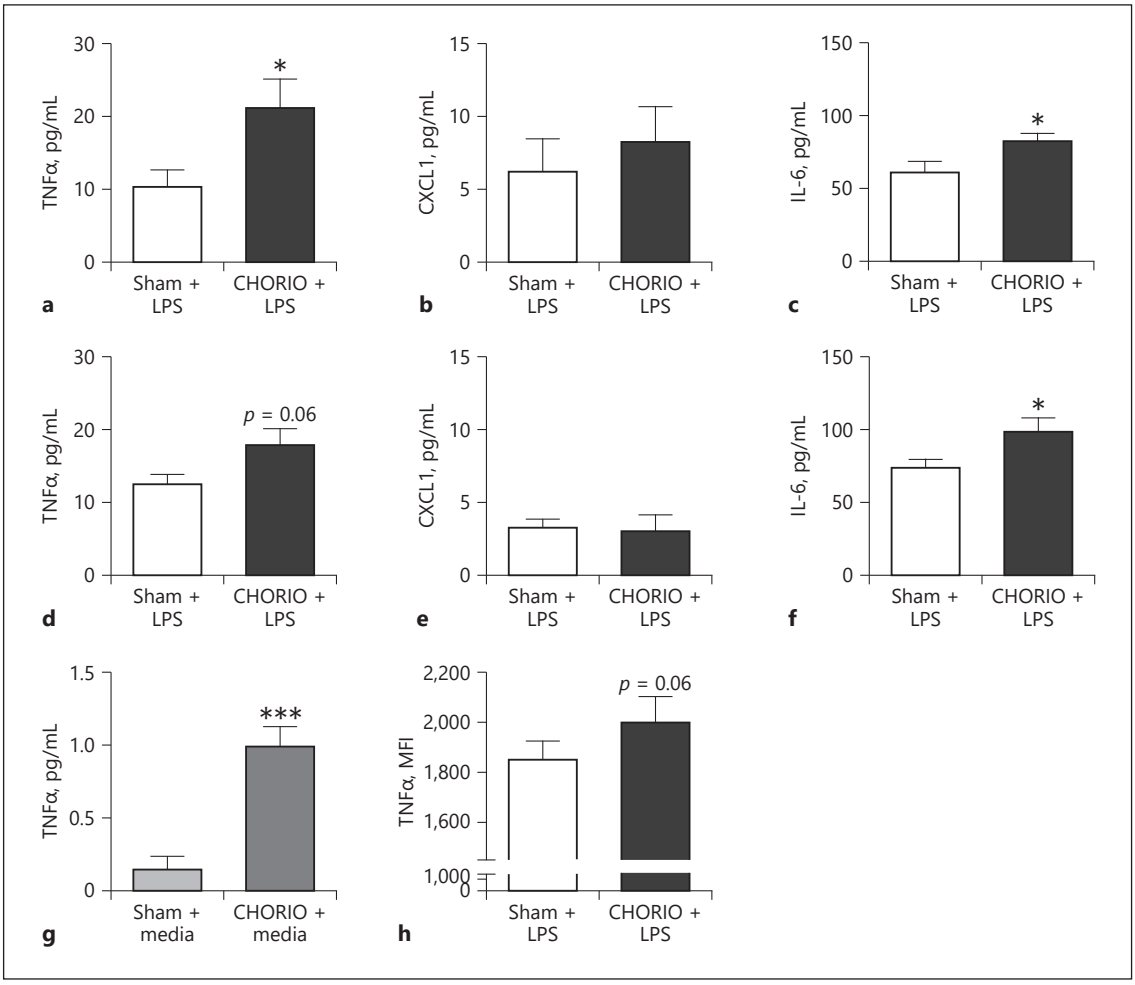

Fig. 4. CHORIO-induced hyperactivation of peripheral blood mononuclear cells (PBMCs) is sustained until postnatal day 21 (P21). PBMCs were isolated from P21 sham or CHORIO pups and stimulated with control or LPS for 3 h (**a–c**) or 24 h (**d–h**). Secreted levels of TNFα (**a**) and IL-6 (**c**) were significantly increased in CHORIO PBMCs challenged with LPS compared to sham control PBMCs, but the CXCL1 level remained unchanged. At 24 h, CHORIO PBMCs also secreted more TNFα (**d**) and IL-6 (**f**) than sham PBMCs in response to LPS challenge. **g** CHORIO P21 PBMCs secreted more TNFα in the absence of LPS challenge and at baseline compared to sham cells. **h** Flow cytometry confirmed increased intracellular TNFα in LPS-stimulated CHORIO PBMCs. * $p < 0.05$, *** $p < 0.001$.

serve as a biomarker of persistent or prior neuroinflammation and brain injury [52, 53]. Notably, preterm newborns that have elevated levels of biomarkers of systemic inflammation on 2 occasions 1 week apart are at a higher risk of brain injury and impaired neurodevelopment [47, 48, 51, 54]. Thus, the insidious effects of primed peripheral immune cells may compound PBI secondary to CHORIO and increase susceptibility to future chronic onset neurological diseases.

The mechanisms for how remote maternal infections or CHORIO facilitate PBI are unknown. Numerous studies have reported that higher levels of proinflammatory cytokines such as TNFα in the amniotic fluid, plasma, and umbilical cord blood are associated with CP in children who are born preterm [14, 55–59]. Circulating proinflammatory cytokines can directly induce damage [56]. Similarly, immune cells can also be directly involved in injury [60–62]. The immature brain expresses CXC chemokines that promote cellular infiltration [25, 32, 56, 60], and lymphocytes expressing TNFα and IL-6 have been identified within lesions in the preterm human brain. Undoubtedly, the role of TNFα is multifactorial, as it is secreted by numerous cells, including microglia and macrophages in the periphery [63]. T helper cells, including

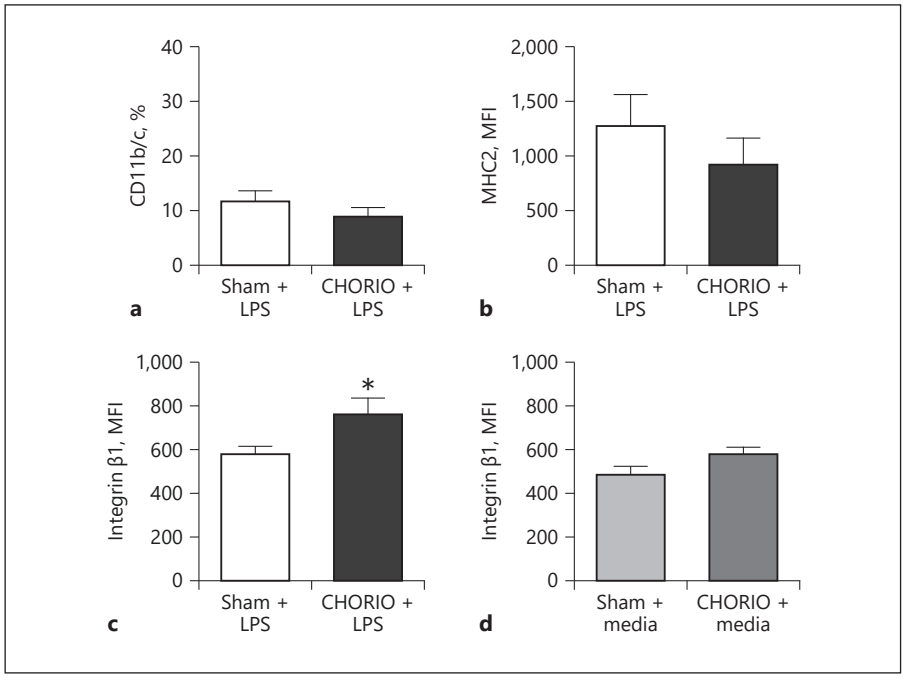

Fig. 5. CHORIO-induced hyperactivation of myeloid cells continues at least until postnatal day 21 (P21). Flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) isolated from P21 pups revealed equivalent proportions of viable myeloid (CD45+CD11b/c+) cells in PBMCs from sham and CHORIO pups following LPS stimulation (**a**), and similar MHC2 mean fluorescent intensity (MFI) (**b**). **c** However, when viable myeloid cells from LPS-stimulated groups were further analyzed for the adhesion molecule, integrin β1, CHORIO cells challenged with LPS had significantly increased expression (MFI) compared to sham cells challenged with LPS, indicative of enhanced activation. **d** Levels of integrin β1, and thus activation, are similar between sham and CHORIO cells at baseline. * $p < 0.05$.

TH1, also secrete TNFα [64]. Previously, we documented transient, acute elevations in TNFα and sustained elevations in CXCL1, a potent neutrophil trafficking chemokine, in the serum of pups with CHORIO [25, 32]. The mechanism(s) for the elevated serum levels in CHORIO rats may be in part related to the increased secretion of TNFα and CXCL1 from PBMCs, as shown here. Together these data indicate augmented immune function by prior exposure to inflammation during development. Our data are consistent with previous studies in preterm sheep, where responses to LPS in the monocytes of sheep 7–14 days after exposure to intra-amniotic endotoxin tended to exceed those of adults and preterm controls [14, 65]. These data are also consistent with an altered inflammatory cytokine network, hallmarked by increased TNFα and IL-6, in a mouse model of intrauterine infection [64, 66] and a porcine model of maternal infection during pregnancy [67]. Taken together, these data support that the secretion of cytokines and chemokines facilitates a damaging cellular inflammatory response, including the maturation and migration of immune cells.

The underlying causes of altered cellular inflammatory responses in preterm children and those who develop CP remain unknown. While there are many converging genetic and environmental factors that warrant consideration, the increased LPS sensitivity of PBMCs, noted in both children with CP and our rats exposed to CHORIO, suggests that inflammation during both the perinatal and postnatal periods have a yet-to-be-defined programming effect, yielding a lasting change in immune response. Indeed, neural-immune communication and programming has been reported in several disorders, including chronic pain, fetal alcohol syndrome, stroke, schizophrenia, Alzheimer's disease, and autism spectrum disorders [50, 52, 53, 68–71]. Notably, fetal white blood cell counts change with gestational age, with lymphocytes being the most prevalent leukocyte up to 37 weeks' gestation [26, 72]. While lymphocytes increase linearly with gestational age, neutrophils increase exponentially after 31 weeks' gestation and become the predominant lymphocyte at term [26]. CHORIO induces circulating CD45RO+ effector/memory T cells associated with brain injury in preterm neonates [20, 55]. Here, we found increased TNFα expression by myeloid cells concomitant with increased CD11b/c cells, a marker of dendritic cells, monocytes, macrophages, and neutrophils, and indicative of hyperimmune activation following LPS stimulation in CHORIO pups. We also observed increased numbers of CD11b/c cells and ED-2/CD163+ macrophages in CHORIO pups alone compared to sham pups in the absence of a second LPS hit. Previously, we reported increased placental and cerebral neutrophils in this model, with elevated CXCR2 and MHC2 expression supporting a global change in immune action following CHORIO. While

these changes in immune cell composition had normalized by P21, the markers of immune activation, including elevated integrin β1 expression, remained in CHORIO PBMCs stimulated with LPS when compared to sham-stimulated cells, supporting a persistently increased activation state. Undoubtedly, future investigations must focus on the developmental expression of cytokines, immune markers, and activation states of the circulating and resident leukocyte populations, including neutrophils, as we observed changes in cytokine levels, and leukocyte activation and maturation markers between P7 and P21, with and without additional LPS stimulation.

Understanding the dynamics of altered immune responses and persistent inflammation through the placental-fetal brain axis is a prerequisite for the rational design of therapeutic interventions in this population of vulnerable patients [73]. Lymphocytes are found in the brain after injury in both rodents and humans, and a lack of mature lymphocytes protects from HI-induced white-matter injury [73]. Other groups have shown that a cerebral influx of TH17-like lymphocytes coordinates neuroinflammatory responses, and documented an elevated expression of the early TH17 lymphocyte marker, IL-23R, in PBMCs in infants with confirmed histological CHORIO and in rodents with LPS-sensitized HI injury [20]. Interestingly, in the preclinical rat experiments, administration of FTY720 (fingolimod) blocked leukocyte trafficking and acute induction of NF-κβ signaling in the developing brain [20], attenuating blood-brain barrier damage and proinflammatory cytokine expression with improved white-matter health [20]. Similarly, ELGAN studies have demonstrated the capacity of infants to respond to in utero inflammation with a proinflammatory TH1/TH17 phenotype [13]. While these responses may be protective against pathogens, they may also promote a sustained fetal and neonatal inflammatory response syndrome, including SPIHR, which involves multiorgan inflammation and injury [13, 45].

In conclusion, this study supports the notion that an inflammatory process that starts in utero may continue through childhood and beyond. Dysfunction throughout the placental-fetal brain axis, including inflammation and HI, may sensitize and program immune cells and associated cytokine networks to respond more vigorously, and for a longer period of time, to a stimulus that would not otherwise have evoked such an intense response [51]. To this end, SPIHR and the activation of immunological memory stemming from in utero insults may be associated with impaired neurodevelopment [55]. The durable changes in PBMC reactivity demonstrated here and elsewhere [14] may prove to be an effective biomarker of perinatal brain injury, and the clinical utility may prove to be high, given the ease of access to these cells and to well-defined stimulation protocols. Additional investigations are required to further understand the homeostatic regulation of central and peripheral inflammatory cells in infants with CHORIO as well as the long-term consequences of its dysregulation.

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Statement of Ethics

The study was performed with the approval of the Institutional Animal Care and Use Committee at the University of New Mexico Health Sciences Center.

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