

Heme-oxygenase-1 (HO-1) in CCR2/CCL2/CD200 brain mononuclear cells contribute to neuroinflammation in young adulthood after chorioamnionitis

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Article

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Abstract

Chemokine receptor 2⁺ (CCR2⁺) mediates peripheral mononuclear cell infiltration into the brain via its ligand C motif ligand 2 (CCL2), whereas Immune inhibitory ligand CD200, suppresses mononuclear cell activation. Whether sustained alterations in CCR2/CCL2/CD200-expressing mononuclear cell populations and their Heme-oxygenase-1 (HO-1) expression contribute to persistent neuroinflammation following perinatal chorioamnionitis (CHORIO) remains unknown. We hypothesized that HO-1 expression within CCR2/CCL2/CD200 mononuclear cell subsets is altered in adulthood following perinatal CHORIO compared with sham controls. Brains were collected at postnatal day (P) 21 and P60 (n = 6–8/group), dissociated and surface and intracellular markers used to identify inflammatory and oxidative mononuclear cell subsets with flow cytometry. At P60, CHORIO brains exhibited fewer CCR2⁺CD11bc^{L/INT} cells (p = 0.008), increased intracellular HO-1 expression, (p = 0.01) and a reciprocal increase in CCR2[−]CD11bc^{L/INT} cells (p = 0.008). In addition, CCL2⁺/CCR2⁺CD11bc^{L/INT} (p = 0.04) and CD200⁺/CCR2⁺CD11bc^{L/INT} (p = 0.005) ratios were also increased in CHORIO. HO-1 expression in CCR2⁺CD11bc^{L/INT} cells was lower at P21 than at P60, suggesting an age-dependent phenotypic shift after CHORIO. Here we demonstrate that alterations in CCR2/CCL2/CD200 signaling and HO-1 expression in adult brain mononuclear cells following perinatal CHORIO are sustained, suggesting long-term inflammatory and oxidative immune signaling that may contribute to chronic neuroinflammation.

Introduction

Fetal inflammatory response syndrome (FIRS) can result in neonatal morbidities. Inflammation can be either physiologic or pathological.[1] Physiologic inflammatory responses restore homeostasis, promote tissue repair and/or generate immune memory.[1] These responses are essential to normal development including placentation, cell proliferation and synaptic pruning.[2, 3] Innate immune cells are at the core of inflammatory resolution and post-inflammatory memory[1], however, when inflammation fails to resolve, it becomes sustained and damaging to the fetus including the brain.[1, 4–8]

Heme oxygenase-1 (HO-1) is an inducible isozyme with crucial functions across species.[9] HO-1 is present at low levels in the healthy adult brain[10] but is upregulated in response to inflammation.[11] Because it is a key regulator of the immune system and pivotal for optimal cell function, HO-1 levels are tightly controlled.[9, 12, 13] We have shown that HO-1 is upregulated in the brain in the immediate perinatal period in the setting of chorioamnionitis (CHORIO) with increased brain mononuclear cells at P7,[14] altered cerebral and peripheral cytokine signatures across pre- and postnatal life,[7] a persistent T cell neuroinflammation through adulthood [6, 7] and a cerebral palsy (CP) phenotype.[15–17]

While upregulation of HO-1 can be protective, sustained, or uncontrolled upregulation is detrimental, and promotes a pro-inflammatory milieu with lipid peroxidation, oxidative injury [18] cytotoxicity and cell death[19, 20]. HO-1 also regulates mononuclear cell differentiation and polarization.[21] A relationship between oxidative microenvironments and macrophage Mox (HO-1 high)/M1 (HO-1 low) polarization is seen in adult cardiovascular atherosclerosis models.[21] Whether an analogous HO-1-driven polarization

occurs in brain myeloid cells following perinatal inflammation had not been investigated, and was the goal of the present study.

We hypothesized that CHORIO generates a sustained disruption of HO-1 homeostasis in brain myeloid cells through adulthood, with inflammatory (M1) and oxidative (Mox) mononuclear cell phenotypes predominating in adulthood. Our objective was to determine whether inflammatory myeloid populations differentially contribute to neuroinflammation in CHORIO and to define HO-1-driven M1/Mox continuums. To this end, we delineated distinct mononuclear cell phenotypes, and their associated chemokine/receptor expression, then determined intracellular HO-1 expression in each population, to establish M1 or Mox status.

Materials and Methods

Procurement and housing of animals

Timed-pregnant Sprague-Dawley rats at Embryonic day (E)16 were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were acclimated and housed in a 12-hr light/12-hr dark cycle in a temperature controlled (23–25 °C) central facility with ad libitum food and water. All studies and procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee (JHU IACUC Approval Number: RA25M145). All experiments, procedures and methods were performed in accordance with the National Institutes of Health (NIH) for care and use of laboratory animals and Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines and regulations.[22]

Chorioamnionitis rat model

Briefly, at E18 an abdominal laparotomy was performed under isoflurane anesthesia as previously described.[6, 7, 14, 23–25]. In the CHORIO group, aneurysm clips were placed on bilateral uterine arteries for 60 min to induce transient systemic hypoxia-ischemia, followed by intraamniotic injection of lipopolysaccharide (LPS, *Escherichia coli* (*E. Coli*), serotype O111:B4, Sigma, St Louis, MO, 4 µ/sac) and abdominal closure. Sham controls underwent laparotomy. Uterine sacs were visualized and abdomen closed after equivalent duration of anesthesia. All dams delivered pups vaginally at E21. Figure 1a.

Experimental design, brain tissue collection and dissociation

Offspring from sham and CHORIO groups were housed with dams until weaning at postnatal day (P)21. A subset of pups of both sexes from each group were randomized to P21 (n = 7 sham, 7 CHORIO) and P60 (n = 6 sham and 8 CHORIO) timepoints. At tissue harvest, animals were sedated with 4% isoflurane for induction, followed by ketamine (100 mg/kg) and xylazine (5 mg/kg), blood was collected by intracardiac puncture. Then, brain were collected after decapitation and placed immediately in cold D-PBS. Cerebellums and frontal cortexes were removed. Remaining bilateral cerebral hemispheres then

were processed for fresh tissue flow cytometry as follows: Tissue was dissociated into single-cell suspensions using the adult brain dissociation kit (Miltenyi Biotec, Auburn, CA, USA) per manufacturer's protocol combining enzymatic digestion (Buffer Z, Y, enzyme P and A at 37 °C) with mechanical dissociation (gentleMACS Dissociator and MACS Mix Tube rotator, Miltenyi Biotec). Cell suspensions were filtered through a 70-micron MACS Smartstrainer and resuspended in cold D-PBS. Cells were centrifuged, resuspended with D-PBS, mixed with debris removal solution and appropriate volume of D-PBS was overlaid and centrifuged. After removal of the top 2 phases, D-PBS was added, red blood cells were removed. Cells were counted by a Countess™ III FL Automated Cell Counter (Thermo Fisher Scientific) and cryopreserved in MACS^R Freezing Solution (10⁷ cells/ml) and stored at -80 °C until analysis.

Multiparameter flow cytometry panel staining and analysis

A multiparameter flow staining panel was developed for Cytex Aurora Spectral Flow Cytometer using antibodies from Miltenyi (Auburn, CA, USA), BD Bioscience (San Jose, CA, USA), Biolegend (San Diego, CA, USA), Novus Biologicals (Littleton, CO, USA), Enzo Life Sciences (Farmingdale, NY, USA) and Thermo Fisher (Carlsbad, CA, USA): anti-CD45 APC Cy7, CD11b/c BV605, CCR2 biotin (PA5-23038), CCL2 (MCP-1) APC (130-107-536), CD200 PEVio770 (130-108-093), Streptavidin BV421 and intracellular HO-1 FITC. Purified mouse anti-rat CD32 and Live/Dead Fixable Aqua Dead Cell Stain Kit were used for Fc blocking and dead cell exclusion, respectively (panel complexity index Fig. 1b.)

Cells were thawed in a bead bath, washed, resuspended carefully in 37 °C sterile PBS and counted (Countess™ III FL Automated Cell Counter, Thermo Fisher Scientific) before staining. All staining steps were performed on ice. Cell viability was > 85%. After the addition of viability dye and Fc block, 1x10⁶ cells were surface stained at 1:100 dilution with myeloid cell surface makers in MACS buffer, then fixed, permeabilized (intracellular permeabilization fixation buffer set, eBioscience, Thermo Fisher Scientific) for intracellular HO-1 staining per manufacturer recommended protocol. Single stained compensation controls (OneComp eBeads) and a viability compensation control Arc Amine Reactive Compensation Bead Kit (Thermo Fisher) were included with each run. Data was acquired on a 5-laser Cytex Aurora Spectral Flow Cytometer (Cytex^R Biosciences, Fremont, CA) with autofluorescence extraction enabled and analyzed with FCS Express Plus 7 software (De Novo Software, Pasadena, CA, USA). After excluding debris and identifying single cells by gating forward scatter height versus side scatter height, we sequentially gated on live cells, CD45⁺, CD11bc⁺ cells, distinct myeloid subpopulations based on their respective surface and intracellular markers (representative gating strategy, Fig. 1c).

Brain RNAseq database interrogation for myeloid markers and Hmox-1 in rodent and human at baseline steady state

To evaluate cross-species relevance of the markers used to phenotype brain myeloid cells, we searched two publicly available RNAseq databases [26, 27] for myeloid cell surface markers, chemokine receptors,

chemokines and for *Hmox1*, the gene encoding HO-1 Normalized expression matrices from brainRNAseq.org database were used without reprocessing of raw reads. Data are represented as Fragments per Kilobase of transcript per million mapped reads (FPKM).

Statistical Analyses

GraphPad Prism v10.6.1 (San Diego, CA, USA) was used for all statistical analysis. Outlier analyses were performed using Grubbs' test, and normality using Shapiro-Wilk test. Normally distributed data were analyzed by 2-tailed Student's t-test with Welch correction and not normally distributed data with Mann Whitney test. Differences in baseline HO-1 expression across myeloid cell subsets in sham controls through adulthood were evaluated by 2-way-ANOVA with Tukey's multiple comparisons test. Data were presented as mean $\pm$ standard error of the mean (SEM). Results were deemed significant at $p < 0.05$.

Results

Mononuclear cell marker specificity in naïve mouse and human brain

To confirm that CCR2, CCL2, CD200 and *Hmox1* are canonical, cross-species markers of brain myeloid cells, we examined their baseline expression in a publicly available naïve mouse and human brain RNA-seq datasets.[26, 27] This was necessary to validate their use as myeloid specific readouts in this study. We found that all four markers, were predominantly expressed by brain myeloid cells relative to other glia and neuronal populations in both species. Figure 2a **and b**.

CCR2 pro-inflammatory and CCR2 reparative mononuclear cell populations

After gating on $CD45^+CD11bc^{L/INT}$ and $CD45^+CD11bc^H$ mononuclear cell populations we defined mononuclear cell subtypes based on their pro-inflammatory (CCR2, CCL2) or immune inhibitory ligand (CD200) expression. Myeloid cells positive for CCR2 are classically identified as M1-like cells with functional properties such as propagating inflammation and/or migration in adult brains and are present in the central nervous system (CNS) following injury.[28–32].[32] In contrast, $CCR2^-$ cells are either M2-like macrophages involved in reparative phases of inflammation or microglia.[33–35] We examined the distribution of the myeloid cell pool in adulthood after a perinatal CHORIO exposure to determine whether peripheral mononuclear cell populations differentially contribute to perinatal brain injury seen in CHORIO.

At P21 and P60, we identified 2 distinct $CCR2^+$ phenotypes, $CCR2^+ CD45^+ CD11bc^{L/INT}$ and $CCR2^+ CD45^+ CD11bc^H$. At P21, the percentage of $CCR2^+ CD45^+ CD11bc^{L/INT}$ and $CCR2^+ CD45^+ CD11bc^H$ cells were

similar in CHORIO and sham, Table 1. At P60, CCR2⁺ CD45⁺ CD11bc^{L/INT} cells were decreased in CHORIO (p = 0.008) compared to sham while CCR2⁺ CD45⁺ CD11bc^H remained similar in the two groups Table 1 commensurate with a decrease in the CCR2⁺ CD11bc^{L/INT} mononuclear cell subset in at weaning age.

Table 1

CCR2, CCL2, CD200 Mononuclear cell subsets in brains of CHORIO exposed animals at P21 and P60.

AGE	P21		P60	
GROUPS	Sham	CHORIO	Sham	CHORIO
CCR2 ⁺ CD11bc ^{L/INT}	25.0 ± 2.4	24.2 ± 4.2	61.4 ± 6.6[†]	36.3 ± 4.7[†]
CCR2 ⁺ CD11bc ^H	38.1 ± 9.8	40.6 ± 12.8	91.5 ± 3.0	80.0 ± 6.7
CCL2 ⁺ CD11bc ^{L/INT}	1.9 ± 0.9	1.3 ± 0.6	2.4 ± 2.0	3.8 ± 1.1
CCL2 ⁺ CD11bc ^H	8.8 ± 5.9	1.4 ± 0.7	4.2 ± 1.4	7.9 ± 1.5
CD200 ⁺ CD11bc ^{L/INT}	0.5 ± 0.1	0.6 ± 0.1	46.6 ± 4.1	54.9 ± 4.7
CD200 ⁺ CD11bc ^H	25.4 ± 5.3	32.2 ± 11.8	44.0 ± 7.9	23.0 ± 6.1
CCR2 ⁻ CD11bc ^{L/INT}	73.3 ± 2.4	74.2 ± 4.5	36.8 ± 6.4*	62.1 ± 4.6*
CCR2 ⁻ CD11bc ^H	59.7 ± 9.9	57.5 ± 13.3	7.8 ± 2.9	18.8 ± 6.5
CCL2 ⁻ CD11bc ^{L/INT}	0.99 ± 0.3	0.8 ± 0.3	97.1 ± 2.3	95.7 ± 1.2
CCL2 ⁻ CD11bc ^H	89.3 ± 6.6	97.9 ± 0.9	94.8 ± 1.7	89.8 ± 1.9
CD200 ⁻ CD11bc ^{L/INT}	42.4 ± 7.8	29.3 ± 8.5	51.8 ± 4.1	42.4 ± 4.2
CD200 ⁻ CD11bc ^H	71.8 ± 5.9	65.6 ± 12.0	53.5 ± 6.9	73.8 ± 6.2
All values are expressed as mean ± SEM. Immune cell population expressed as %CD45 ⁺ CD11bc ⁺ .p < 0.05, by unpaired two-tailed Student's t-test or Mann Whitney test; At P60, CCR2 ⁺ CD11bc ^{L/INT} cells were decreased in CHORIO compared to sham, ([†] p = 0.008) and CCR2 ⁻ CD11bc ^{L/INT} cells were increased, (*p = 0.008).				

Next, we defined the CCR2⁻ population, in the context that microglia are traditionally considered CCR2 negative.[35] We detected 2 distinct CCR2⁻ phenotypes within the myeloid cell pool. Again, at P21, neither CCR2⁻ CD45⁺ CD11bc^{L/INT} nor CCR2⁻ CD45⁺ CD11bc^H percentages were altered by CHORIO Table 1. At P60 however, CCR2⁻ CD45⁺ CD11bc^{L/INT} cells, were elevated in CHORIO brains (p = 0.008) compared to sham Table 1. while CCR2⁻ CD45⁺ CD11bc^H remained similar in CHORIO and shams at P60 Table 1.

CCL2⁺ pro-inflammatory and CCL2⁻ mononuclear cell populations

Previously, we reported sustained peripheral immune hyperreactivity, with elevated serum CCL2 in CHORIO compared to sham at P60 and P120 but not at P21.[6] Thus, we examined CCL2 expression in the CD45⁺ myeloid population in the brains at P21 and P60. At neither P21 nor at P60, CCL2⁺ CD45⁺ CD11bc^{L/INT} and CCL2⁺CD45⁺CD11bc^H cells differ between CHORIO and sham Table 1.

At P21 and at P60, CCL⁻ CD45⁺ CD11bc^{L/INT} and CCL2⁻ CD45⁺ CD11bc^H cell percentages were also similar between CHORIO and sham Table 1. Hence, intracellular CCL2 expression in myeloid cells did not differ between CHORIO and sham at either P21 or P60. As chemokines are rapidly secreted and are not stored intracellularly, this dissociation between elevated serum CCL2 and unchanged cerebral CCL2 expression suggests a serum-to-brain chemokine gradient at P60 that may contribute to ongoing mononuclear cell recruitment and inflammation.

CD200⁺ immune inhibitory ligand and CD200⁻ mononuclear cell populations

CD200 and its receptor CD200R is an inhibitory ligand-receptor pair that acts as an immune checkpoint by limiting monopoiesis, monocyte recruitment and macrophage activation to alleviate inflammation. [36–38] A decrease in CD200 would therefore be expected to exacerbate inflammation via increased leukocyte activation.[37] At P21 and at P60, CD200⁺ CD45⁺ CD11bc^{L/INT} and CD200⁺ CD45⁺ CD11bc^H cell percentages were similar between CHORIO and sham Table 1.

At P21, CD200⁻ CD45⁺ CD11bc^{L/INT} and CD200⁻ CD45⁺ CD11bc^H cell percentages in CHORIO and sham brains were not different Table 1. At P60, CD200⁻ CD45⁺ CD11bc^{L/INT} cells were similar between CHORIO and sham Table 1., while CD200⁻ CD45⁺ CD11bc^H cells showed a trend towards an increase in CHORIO, $p = 0.0517$ vs shams, Table 1.

Relative abundance of CCR2⁺, CCL2⁺ and CD200⁺ mononuclear cell subsets in CD11bc^{L/INT} and CD11bc^H cells in sham brains at P21 and P60

When we compared the relative abundances of CCR2⁺ and CCL2⁺, pro-inflammatory and CD200⁺ immune inhibitory ligand positive mononuclear cell subsets, within CD11bc^{L/INT} and CD11bc^H cell populations in sham brains, at P21, CCR2⁺ cells were the predominant subset within the CD11bc^{L/INT} population in sham brains, compared to CCL2⁺ ($p < 0.0001$) and CD200⁺ ($p < 0.0001$) cells Fig. 3a. At P21, CCR2⁺ CD11bc^H cell subsets in sham brains were higher ($p = 0.0058$) compared to CCL2⁺ but not different than CD200⁺ Fig. 3b. Within the CD11bc^{L/INT} population at P60, Sham brains had higher proportion of CCR2⁺ ($p < 0.0001$) and CD200⁺ ($p < 0.0001$) cells compared to CCL2⁺ cells Fig. 3a. Both

CCR2⁺ (< 0.0001) and CD200⁺ (p = 0.0005) cell subsets within CD11bc^H population was increased compared to CCL2⁺ cells at P60 sham brains Fig. 3b.

Altered CCR2, CCL2, and CD200 ratios in the brain following perinatal CHORIO

Next, we examined whether CHORIO altered the relative abundance of inflammatory recruitment (CCR2 and CCL2) and inhibitory ligand (CD200) signaling within brain microenvironment. At P21, CCL2⁺/CCR2⁺ ratios within the CD45⁺ CD11bc^{L/INT} population were similar between CHORIO and sham brains Table 2. In contrast, at P60, the CCL2⁺/CCR2⁺ ratio increased by 76% in CHORIO brains (p = 0.0426) compared to sham, suggesting a relative abundance of CCL2⁺ cells within the CD45⁺ CD11bc^{L/INT} population Table 2. These findings confirm that CHORIO promotes a sustained pro-inflammatory microenvironment in the CNS.

Table 2
CCR2, CCL2, CD200 Ratios in the brain following perinatal chorioamnionitis.

AGE	P21		P60	
GROUPS	Sham	CHORIO	Sham	CHORIO
CCL2 ⁺ /CCR2 ⁺ CD11bc ^{L/INT}	0.07 ± 0.03	0.09 ± 0.04	0.065 ± 0.060 ^a	0.1156 ± 0.03549^a
CCL2 ⁺ /CCR2 ⁺ CD45 ⁺ CD11bc ^H	0.03 ± 0.02	0.03 ± 0.02	0.046 ± 0.016	0.115 ± 0.032
CD200 ⁺ /CCR2 ⁺ CD11bc ^{L/INT}	0.02 ± 0.004	0.02 ± 0.005	0.79 ± 0.086 [#]	1.65 ± 0.22[#]
CD200 ⁺ /CCR2 ⁺ CD11bc ^H	0.31 ± 0.07	0.48 ± 0.21	0.48 ± 0.085	0.29 ± 0.08
CD200 ⁻ /CCR2 ⁻ CD11bc ^{L/INT}	0.59 ± 0.12	0.39 ± 0.11	1.55 ± 0.16 ^δ	0.73 ± 0.11^δ
CD200 ⁻ /CCR2 ⁻ CD11bc ^H	1.59 ± 0.41	1.14 ± 0.37	12.53 ± 4.57	11.33 ± 3.53
All values are expressed as mean ± SEM. Immune cell population expressed as %CD45 ⁺ CD11bc ⁺ . p < 0.05, by unpaired two-tailed Student's t-test or Mann Whitney test; At P60, CCL2 ⁺ /CCR2 ⁺ CD11bc ^{L/INT} ratio was increased in CHORIO (^a p = 0.0426) compared to sham. At P60, CD200 ⁺ /CCR2 ⁺ CD11bc ^{L/INT} ratio was increased in CHORIO brains compared to sham, ([#] p = 0.0054). At P60, CD200 ⁻ /CCR2 ⁻ CD11bc ^{L/INT} ratio was decreased in CHORIO compared to sham (^δ p = 0.0023).				

At P21 and P60, ratio of CCL2⁺/CCR2⁺ CD45⁺ CD11bc^H cells in CHORIO and sham were similar Table 2.

At P21, CD200⁺/CCR2⁺ ratio within the CD45⁺ CD11bc^{L/INT} mononuclear cell population was similar between CHORIO and sham. At P60, the CD200⁺/CCR2⁺ ratio was increased by 108.797 percent in CHORIO compared to sham (p = 0.0054) Table 2. This suggests an age dependent shift towards a relative abundance of CD200⁺ cells within the CD45⁺ CD11bc^{L/INT} population following perinatal CHORIO.

At P21 and at P60, CD200⁺ /CCR2⁺ ratio within the CD45⁺ CD11bc^H population was similar between CHORIO and sham brains Table 2. The ratio of CD200⁻/CCR2⁻ CD11bc^{L/INT} population was similar at P21 between CHORIO and sham brains. However, at P60, this ratio was significantly reduced by 52.9% in

CHORIO brains compared to sham ($p = 0.0023$) Table 2. $CD200^-/CCR2^-$ ratio within the $CD11bc^H$ population did not differ between CHORIO and sham brains at either P21 or P60 Table 2. Collectively, these data indicate persistent alterations in mononuclear cell homeostasis and dysregulated CCR2, CCL2, and CD200 signaling within the brain microenvironment following perinatal CHORIO in adulthood.

HO-1 intracellular protein expression in brain mononuclear cells under normal physiologic conditions across postnatal ages

We determined whether under normal physiologic conditions intracellular HO-1 expression differed among brain mononuclear cell subsets at P21 and P60 in sham controls. $CCR2^+ CD11bc^{L/INT}$ cells had higher intracellular HO-1 expression at P21 (906.5 ± 186 , $p = 0.0025$) compared to P60 (19.48 ± 6.437) sham brains Fig. 4a. $CCL2^+ CD11bc^{L/INT}$ (60022 ± 22346 vs. 4281 ± 1066 , $p = 0.0221$) Fig. 4b., and $CD200^+ CD11bc^{L/INT}$ (313.6 ± 43.91 vs. 29.45 ± 12.26 , $p = 0.0025$) cells also had higher intracellular HO-1 expression at P21 compared to P60 Fig. 4c. In the $CCR2^+ CD11bc^H$ population, intracellular HO-1 expression in $CCR2^+ CD11bc^H$ cells did not differ in sham brains at P21 (859.7 ± 391.3) and P60 (12.04 ± 4.086) Fig. 4d. However, we detected an increased intracellular HO-1 expression in $CCL2^+ CD11bc^H$ (38449 ± 10868 vs. 752.4 ± 185.9 , $p = 0.0133$) Fig. 4e., and $CD200^+ CD11bc^H$ (488.4 ± 78.12 vs. 12.78 ± 3.87 , $p = 0.0009$) cells at P21 compared to P60 Fig. 4f. These data highlight that in young adult brains HO-1 expression is low under normal physiologic conditions.

Relative abundance of HO-1 in brain mononuclear cell subsets under normal physiologic conditions

Both postnatal age and cell subtype significantly affected HO-1 expression in sham. At P21, within the $CD11bc^{L/INT}$ population, $CCL2^+$ cells had the highest expression compared with $CCR2^+$ ($p = 0.0005$) and $CD200^+$ ($p = 0.0005$) cells Fig. 5a. Similarly, within the $CD11bc^H$ population, $CCL2^+$ cells had the highest expression compared with both $CCR2^+$ ($p < 0.0001$) and $CD200^+$ ($p < 0.0001$) cells Fig. 5b.

Brain mononuclear cell intracellular HO-1 expression shifts from a proinflammatory (M1-like) to an oxidative (Mox-like) between P21 and P60 following perinatal CHORIO

At P21, intracellular HO-1 expression in $CCR2^+ CD45^+ CD11bc^{L/INT}$ cells decreased by 57% in CHORIO (389.5 ± 82.6) compared to sham (906.5 ± 186.0 , $p = 0.034$) Fig. 6a., consistent with literature attributing lower HO-1 expression to an M1-like pro-inflammatory phenotype.[39] At P60, $CCR2^+ CD45^+ CD11bc^{L/INT}$ cells showed a 7.43 fold increase in intracellular HO-1 in CHORIO (164.2 ± 40.48) compared to sham (19.48 ± 6.43 , $p = 0.0101$), Fig. 6b., consistent with a shift toward an oxidative Mox-like pro-inflammatory phenotype by adulthood after CHORIO.

CCR2⁺ CD45⁺ CD11bc^H cells showed similar HO-1 expression between CHORIO (1198 ± 642.2) and sham (859.7 ± 391.3) at P21 Fig. 6c., and a trend toward increase in CHORIO at P60 (72.18 ± 33.25) compared to sham (12.04 ± 4.086, p = 0.0593) brains Fig. 6d.

At P21, intracellular HO-1 expression in CCL2⁺ CD45⁺ CD11bc^{L/INT} cells was similar between CHORIO (49159 ± 22252) and sham (60022 ± 22346) Fig. 6e. HO-1 expression in CCL2⁺ CD11bc^H cells between CHORIO (69034 ± 30313) and sham (38449 ± 10868) was also similar at P21 Fig. 6g. At P60, HO-1 expression remained similar between CHORIO and sham in both CCL2⁺ CD45⁺ CD11bc^{L/INT} (5301 ± 1655 and 4281 ± 1066) and CCL2⁺ CD45⁺ CD11bc^H (1350 ± 542.5 and 752.4 ± 185.9) cells Fig. 6f **and h**.

Within the CD200⁺ CD45⁺ CD11bc^{L/INT} subset intracellular HO-1 expression decreased by 52.1365% in CHORIO at P21 (150.1 ± 21.6) compared to sham brains (313.6 ± 21.6, p = 0.009) Fig. 6i, consistent with an M1-like phenotype.[39] At P60, there was a 2.04-fold increase in intracellular HO-1 expression in CD200⁺ CD45⁺ CD11bc^{L/INT} in CHORIO (89.43 ± 17.22) compared to sham (29.45 ± 12.26, p = 0.0303) Fig. 6j. At P21, CD200⁺ CD45⁺ CD11bc^H cells did not differ in their intracellular HO-1 expressions between CHORIO (342.5 ± 101.1) and shams (488.4 ± 78.12) Fig. 6k but showed a 13.44-fold increase in CHORIO at P60 (184.6 ± 69.4) compared to shams (12.78 ± 3.87, p = 0.0186) Fig. 6l.

The CCR2⁻ mononuclear cell/macrophage population lacked intracellular HO-1 expression **Suppl Fig. 1**. Although the proportion of CD200⁻ CD45⁺ CD11bc^H cells did not differ between CHORIO and sham at P60, HO-1 expression increased marking oxidative stress within these cells in CHORIO compared to sham brains **Suppl Fig. 1**. Collectively, these data indicate a shift from a pro-inflammatory M1-like, HO-1 low phenotype at P21 toward an oxidative Mox-like, HO-1 phenotype by P60 in mononuclear cell populations following perinatal CHORIO.

Discussion

In this study, we hypothesized that CHORIO persistently disrupts HO-1 homeostasis in brain myeloid cells through early adulthood. The role of myeloid HO-1 in the pathogenesis of CHORIO-associated perinatal brain injury is unknown. HO-1 under- and over-expression are both detrimental to cells.[19, 21] Following CHORIO, at P21, there was reduced intracellular HO-1 expression consistent with a M1-like proinflammatory phenotype in CCR2⁺ and CD200⁺ cells, whereas by P60, HO-1 expression increased suggesting an oxidative, Mox-like phenotype. In addition, there were relative increase in CCL2⁺/CCR2⁺ and CD200⁺/CCR2⁺ CD11bc^{L/INT} ratios at P60, indicating an age-dependent shift in CCR2/CCL2/CD200 signaling after CHORIO. Collectively, our findings suggest that perinatal CHORIO results in a persistent, age-dependent changes in CCR2/CCL2/CD200 myeloid cells and a phenotypic transition from an M1-like proinflammatory state in early life to a Mox-like oxidative state in adulthood.

Following CHORIO, there is sustained peripheral and central T-cell inflammation, white matter loss, chronic microglia activation, and persistent gait and executive function deficits.[6, 7, 14, 23–25]. These

are all features of encephalopathy of prematurity. Thus, we chose it to investigate CHORIO mediated changes in HO-1. Our data show that, CCR2⁺ and CD200⁺ mononuclear cell subsets shift from a predominantly inflammatory HO-1 phenotype (M1, HO-1 low) at P21 to a predominantly oxidative HO-1 phenotype (Mox, HO-1 high) at P60. We speculate that CHORIO and the resulting HO-1 dysregulation may prime these cells toward an inflammatory to oxidative continuum from early life into young adulthood.

CCR2 is a chemokine receptor involved in macrophage recruitment.[30, 34, 40, 41] CCR2⁺ macrophages classically promote peripheral mononuclear cell recruitment, inflammatory cytokine release, oxidative damage and injury.[42–44] The distinction between CCR2⁺ bone marrow derived and CCR2[−] tissue resident macrophage pools is established in cardiac and other organ models.[43, 45] We applied this established framework to identify analogous populations in the CHORIO brain, where equivalent characterization had not been performed. CCR2⁺ mononuclear cell and macrophages in the brain almost exclusively originate from peripheral blood mononuclear cell pool while CCR2[−] cells traditionally comprise the majority of the tissue resident pool, originating from yolk sac and fetal monocyte progenitors and considered largely stable from early life.[43, 45–51] This resident cell pool can self-renew and peripheral monocytes can contribute to it in other disease models [44, 46, 52] while microglia do not upregulate CCR2 expression during inflammatory stimulus.[35] The CCR2[−] brain resident population was comprised of two subsets CCR2[−] CD11bc^H and CCR2[−] CD11bc^{L/INT}. CCR2[−] CD11bc^H cells were similar between sham and CHORIO, but CCR2[−] CD11bc^{L/INT} cells were significantly increased in CHORIO, suggesting expansion of the tissue resident pool, via proliferation or peripheral monocyte contribution as seen in other models,[44, 46, 52]. There was a reciprocal decrease in CCR2⁺ CD11bc^{L/INT} cells in CHORIO brains, further supporting this interpretation. CCR2[−] macrophages are associated with phagocytosis, efferocytosis, angiogenesis and repair peripherally, whether they behave similarly in brain is unclear.[42–44] We found these distinct CCR2⁺ and CCR2[−] brain myeloid pools are also altered following CHORIO.[33, 53] Together, sustained loss of CCR2[−] and CCR2⁺ Cd11bc^{L/INT} cell homeostasis is consistent with a persistent inflammatory milieu in the brain long after the inciting insult suggesting a complex immune dysregulation in the brain that warrants further investigation of the functional status of these myeloid cells.

CCL2, also known as monocyte chemoattractant protein (MCP-1), is the major chemokine ligand for CCR2.[32, 54, 55] The CCL2/CCR2 pathway's role in recruiting bone-marrow derived macrophages to the CNS and their subsequent differentiation and contribution to the resident Iba1⁺ microglia pool during inflammation is well established in the pain literature,[55] but has not been examined following CHORIO. CCL2 elevation is associated with mononuclear cell recruitment and neuroinflammation and worse outcomes in neuroinflammatory diseases.[54] At P60, the relative abundance of CCL2 compared to CCR2 in CD45⁺CD11bc^{L/INT} cells suggests that the mononuclear cell/microglia pool is primed towards a sustained pro-inflammatory, oxidative state after CHORIO, as is seen after adult TBI[32] and neuroinflammatory diseases.[56, 57] Given the association between elevated brain CCL2/CCR2 signaling

and cognitive impairment[56, 57], this shift may contribute to the cognitive deficits reported in this CHORIO model.[23]

Our results suggest that mononuclear cell populations may contribute to sustained neuroinflammation in young adulthood following perinatal CHORIO. We detected perturbed intracellular HO-1 in CCR2⁺CD11bc^{L/INT} cells in adulthood which suggests that the Mox oxidative phenotype was more prevalent at P60 brains after CHORIO. Sustained upregulation of HO-1 is linked to adverse effects in other neuroinflammatory models[9] and may indicate ongoing increased intracellular oxidative stress in the young adult brain. The significantly increased pool of Mox in CHORIO brains, as shown by CCR2⁺CD45⁺CD11bc^{L/INT} cells, may suggest that these migratory, inflammatory, oxidative cells originate preferentially from the bone marrow derived mononuclear cell pool, given that CCR2 has traditionally been reported to be absent on brain microglia but present on peripheral myeloid cells, however lineage was not traced in our study.

Innate inhibitory ligand CD200, with its canonical receptor CD200R, decreases inflammation through inhibition of macrophage and microglia activation in adult neuroinflammatory conditions.[36, 38, 58–61] After ischemic stroke, decreases in CD200 are associated with an increase in CD200R⁺ leukocytes in the CNS.[58] In EAE animal models, CD200 deficiency increases monocyte-derived macrophage infiltration into the CNS and heightens microglia activation by increased release of pro-inflammatory chemokines including CCL2.[38, 61, 62] High amniotic fluid CD200 levels in patients with PPRM, are associated with funisitis.[63] In this small study, neither neonatal blood CD200 levels nor any neonatal morbidities were reported. In our study, we showed a relative abundance of CD200 over CCR2 in CD11bc^{L/INT} subset and increased HO-1⁺ in CD200⁺ cells in CHORIO brains at P60 compared to sham, indicating elevated intracellular oxidative stress in these cells. Taken together, with human amniotic fluid CD200 data, our findings of elevated brain CD200 after CHORIO may have clinical relevance.

Our data expand the existing knowledge of the mononuclear cell/microglia-like population and add new findings by delineating HO-1 profiles of these cells following CHORIO in the adult brain. Because of the paucity of data on *Hmox1* in perinatal brain injury, we extrapolate from other disease models. Silencing of the *Hmox1* gene attenuates cerebral ischemia/reperfusion injury.[64] The only data on intracellular HO-1 in brain immune cell populations derives from glioma models.[65] Increased *Hmox1* expression in lower grade gliomas is associated with increased monocyte and macrophage infiltrates to the brain and tumor microenvironment, worse outcomes and poor survival.[65] Conversely, lower *Hmox1* expression correlated with more favorable responses to immunotherapy[65] Our findings of increased HO-1 in mononuclear cell subsets in CHORIO indicated that brain immune cells retain a lasting signature of perinatal inflammation.

Our results may have translational implications. Glia are the predominant cells expressing HO-1 in animals with focal cerebral ischemia or TBI.[66, 67] and prolonged microglia/macrophage intracellular HO-1 expression is reported in humans focal cerebral infarction or TBI and is largely absent in uninjured human brains.[68] These studies support the concept that sustained myeloid HO-1 expression may

accompany a chronic neuroinflammatory response after CNS injury. Altered HO-1 signaling in brain mononuclear cells warrants further investigation as a potential mechanism contributing to long-term neuroimmune dysfunction following perinatal inflammation. Immunohistochemistry characterization of CCR2/CCL2/CD200 and HO1 on brain mononuclear cells, functional studies and tracing immune cell lineage would be warranted in future studies. Whether this extends therapeutic relevance will need to be determined.

Summary/Conclusions

In summary, we define disrupted homeostasis in brain mononuclear/microglia-like cells in adult rats after perinatal CHORIO exposure, marked by unique immune signatures and significantly altered intracellular HO-1 expression (Fig. 7 summary). To our knowledge, this is the first study to characterize HO-1 perturbations in these cell populations following CHORIO, showing that the HO-1 signature present at early life differs from that in young adulthood. This previously uncharacterized brain M1/Mox population displays distinct HO-1 profiles suggestive of sustained inflammation and high oxidative stress. This may be relevant for identifying heightened brain injury phenotypes.

Declarations

Ethics approval and consent to participate – Studies were approved by JHU IACUC, protocol number, RA25M145.

Credit author contributions statement: Conceptualization and design: MO, supervision: MO, LJ, SR project administration and funding acquisition: MO, LJ, SR, investigation: PK, NW, MK, MO, methodology, resources: MO, LJ, SR, writing – review and editing: MO, PK, NW, LJ, writing – original draft preparation: MO, analysis and interpretation: PK, MO, correspondence and material request: MO. Validation, methodology, formal analysis: MO, visualization: MO. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials – All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Figures

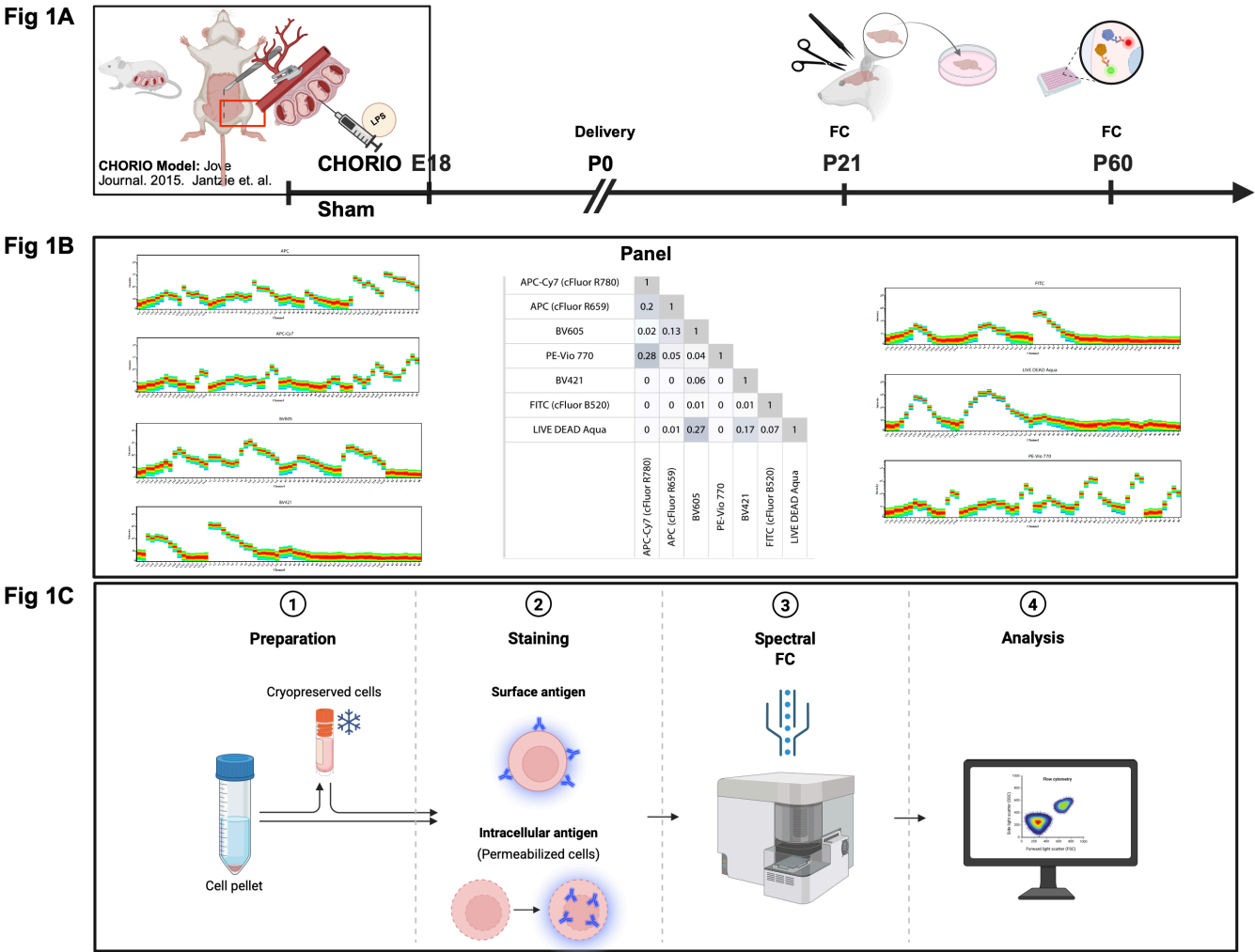


Figure 1

Experimental Design. **a.** At Embryonic day 18 (E18) pregnant dams were randomized to sham control and experimental CHORIO groups. Pups were delivered at P0 and allocated to P21 or P60 experimental groups. Brains were collected at P21 or P60. **b.**Spectral distribution of multiparameter flow cytometry markers and mononuclear cell panel complexity index (1.8) is shown. **c.** Flow cytometry workflow:

cryopreserved cells thawed, stained with surface and intracellular markers, acquired on a spectral flow cytometer and analyzed.

Figure 2

Brain RNAseq

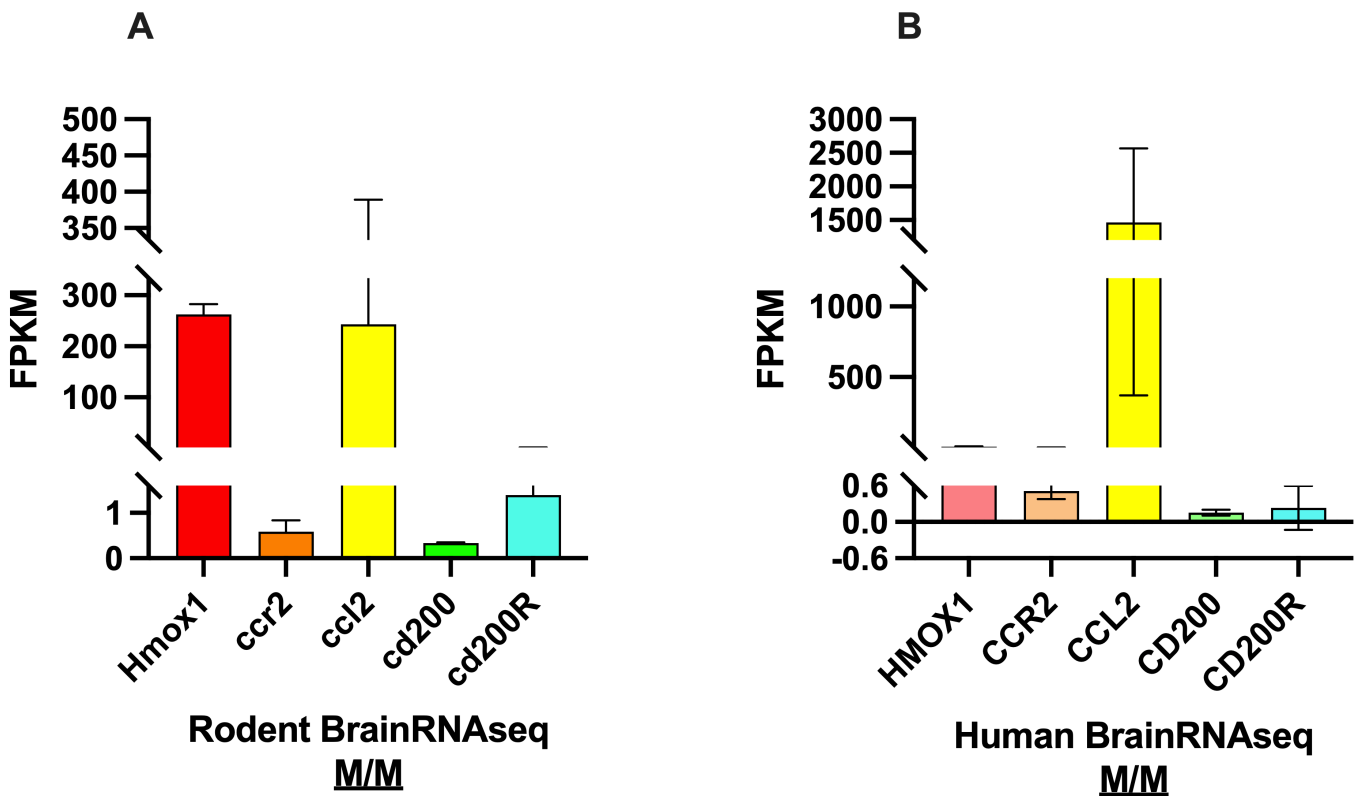


Figure 2

Interrogation of publicly available brain RNAseq datasets demonstrate expression of flow cytometry panel markers in rodent and human brain myeloid cells. **a.** Rodent brain RNAseq dataset showing expression of ccr2, ccl2, cd200, cd200R and Hmox1. **b.** Human brain RNAseq dataset demonstrating expression of CCR2, CCL2, CD200, CD200R and HMOX1. Expression is represented as Fragments per Kilobase of transcript per million mapped reads (FPKM).

Figure 3

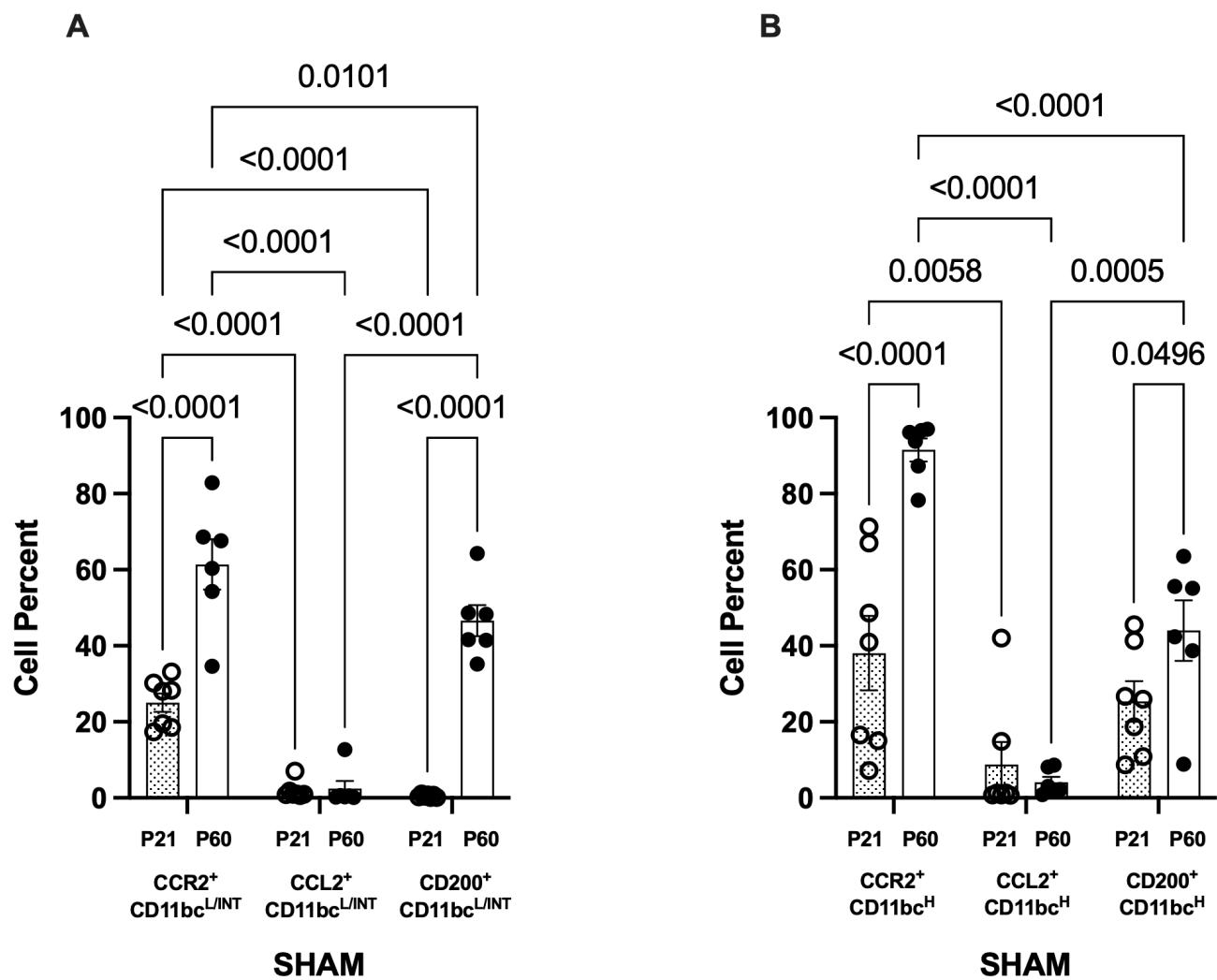


Figure 3

Relative abundance of CCR2⁺, CCL2⁺ and CD200⁺ mononuclear cell subsets in CD11bc^{L/INT} and CD11bc^H cells in sham brains **a.** Proportion of CCR2⁺, CCL2⁺ and CD200⁺ cells within CD11bc^{L/INT} population, **b.** Percentage of CCR2⁺, CCL2⁺ and CD200⁺ cells within CD11bc^H population.

Figure 4

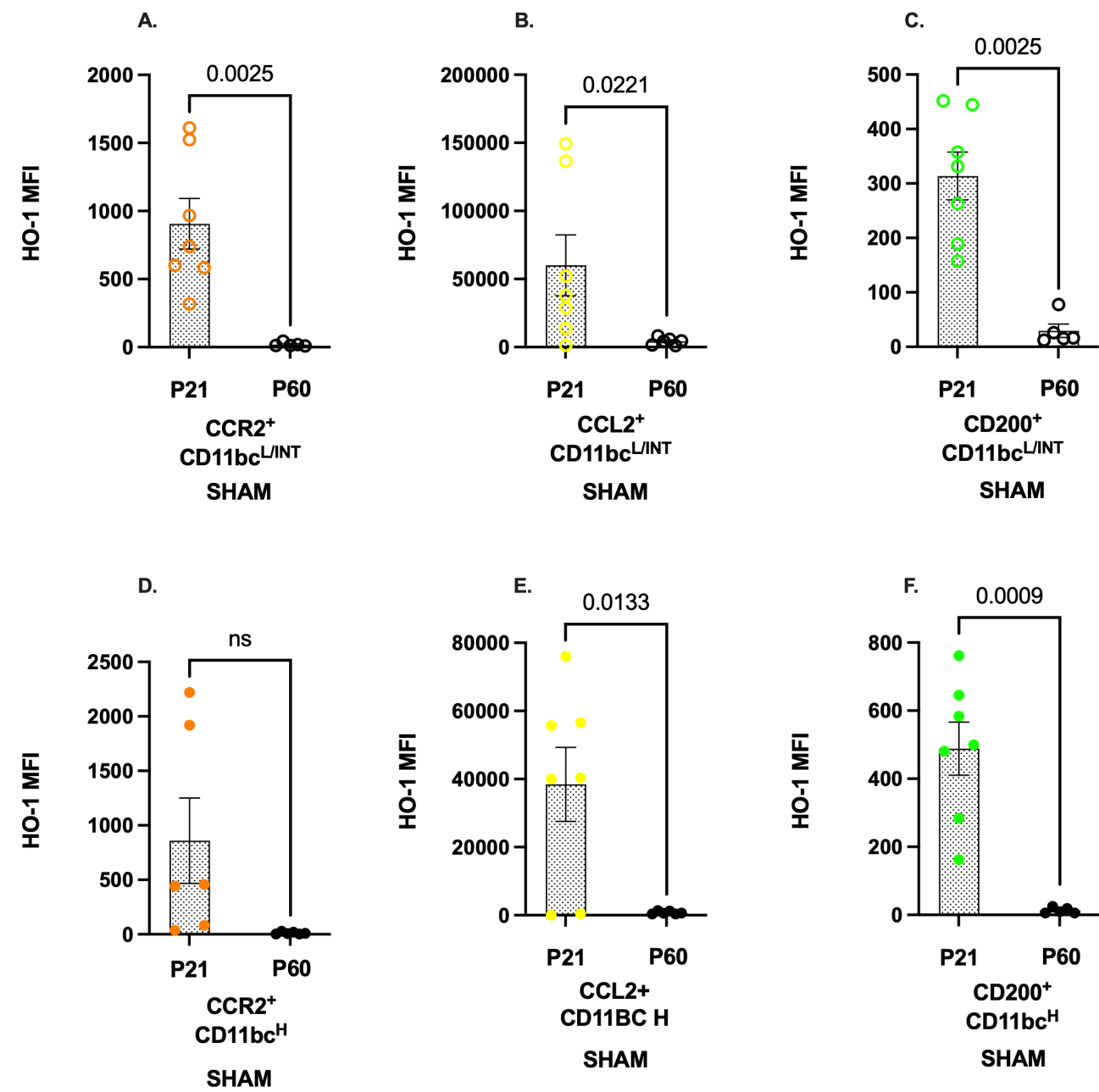


Figure 4

Baseline HO-1 expression trajectory within mononuclear cell subsets in sham brains from P21 to P60. Intracellular HO-1 expression within CD11bc^{L/INT} **a.** CCR2⁺, **b.** CCL2⁺, **c.** CD200⁺ mononuclear cell/macrophage populations in sham brains were higher at P21 compared to P60 (p=0.0025, 0.0221, 0.025, respectively). Intracellular HO-1 expression in **d.** CCR2⁺CD11bc^H subset was similar at P21 and P60 in sham brains, However, intracellular HO-1 expression of mononuclear cell/macrophage population in sham brains within CD11bc^H subset for **e.** CCL2⁺ (0.01), and **f.** CD200⁺ (0.0009) was higher at P21 compared to P60. HO-1 MFI is represented per 100 cell, p<0.05 is significant.

Figure 5

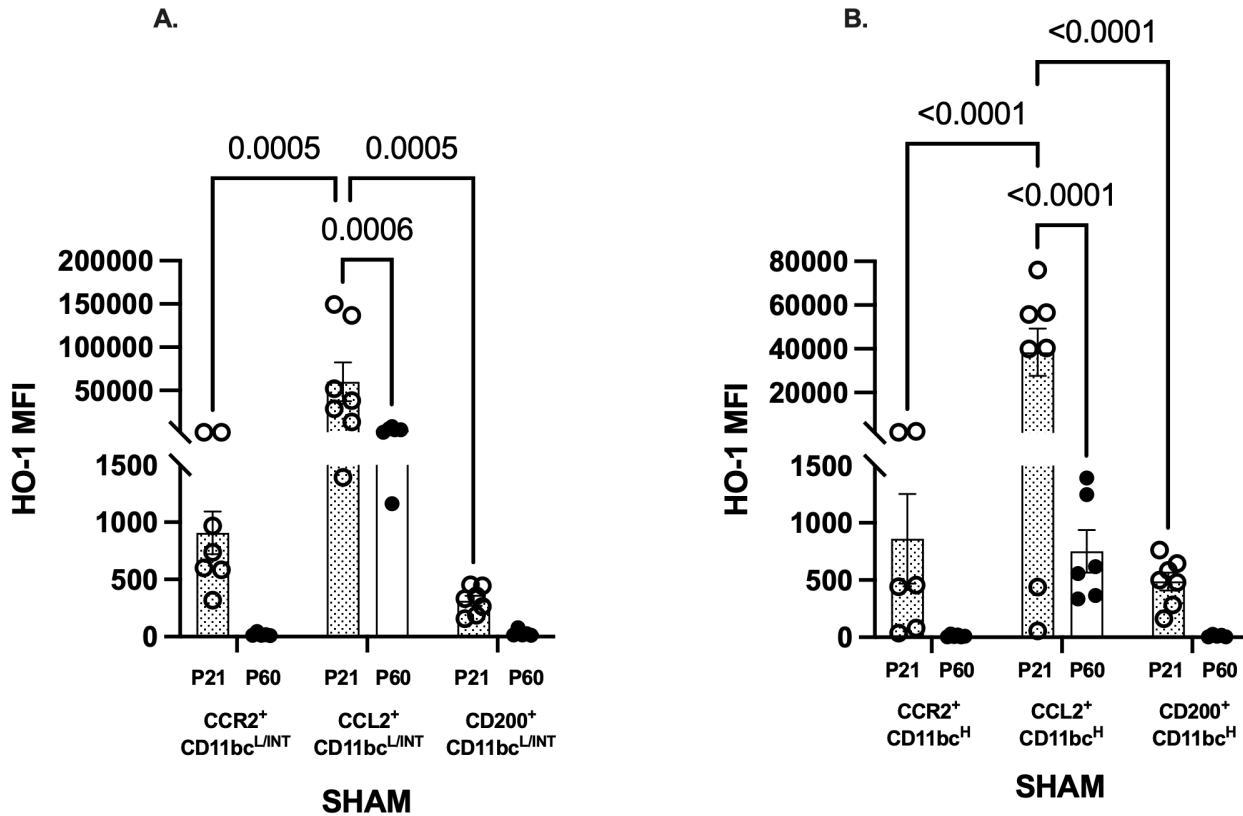


Figure 5

Relative expression of HO-1 in mononuclear cell subsets in sham. Differences in intracellular HO-1 expression at baseline homeostatic state within CD11bc^{L/INT} and CD11bc^H subsets in sham brains are shown. **a.** Intracellular HO-1 expression in CCL2⁺CD11bc^{L/INT} subset was higher than CCR2⁺CD11bc^{L/INT} (p=0.0005) and CD200⁺CD11bc^{L/INT} (p=0.0005) cells at P21. Additionally, HO-1 expression within CCL2⁺CD11bc^{L/INT} subset at P60 was lower compared to P21 (0.0006). **b.** At P60, intracellular HO-1 expression within CCL2⁺CD11bc^H subset was increased compared to CCR2⁺CD11bc^H (p=0.0001) and CD200⁺CD11bc^H (p=0.0001) cells. Likewise, HO-1 expression was lower at P60 compared to P21 for CCL2⁺CD11bc^H subset (0.0001). HO-1 MFI is represented per 100 cell, p<0.05 is significant.

Figure 6

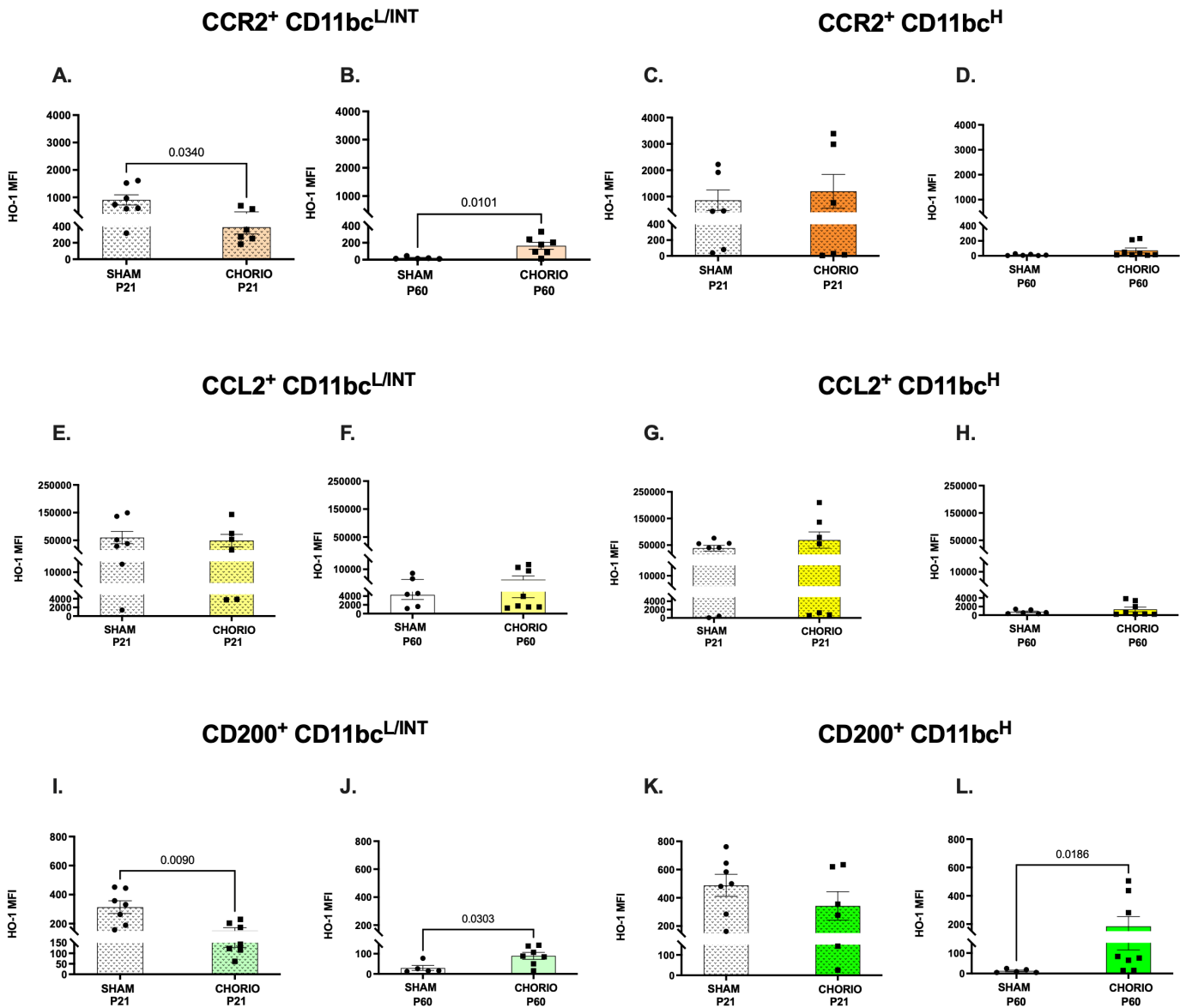


Figure 6

Differential intracellular HO-1 expression in brain mononuclear cell subsets in CHORIO compared to sham. Within CD11bc^{L/INT} mononuclear cell/macrophage cell population intracellular HO-1 expression in CHORIO brains was **a.** decreased in CCR2⁺ cell subset (0.03) at P21 and **b.** increased in CCR2⁺ cell subsets (0.01) at P60, whereas was unchanged in CCL2⁺ cell subset **e.** at P21 and **f.** at P60. Intracellular HO-1 was decreased in CD200⁺ cell subset, **i.** at P21 (0.009) and **j.** increased at P60 (0.03). Within CD11bc^H mononuclear cell populations intracellular HO-1 expression in CHORIO brains was unchanged in CCR2⁺ cell subsets, **c.** at P21 and **d.** at P60; and CCL2⁺ cell subsets **g.** at P21 and **h.** at P60 when compared to sham. **k.** While intracellular HO-1 expression was similar in CD200⁺CD11bc^H subset between sham and CHORIO groups at P21, **i.** at P60, it was increased (0.018) in CHORIO. HO-1 MFI is represented per 100 cell, p<0.05 is significant.

Figure 7

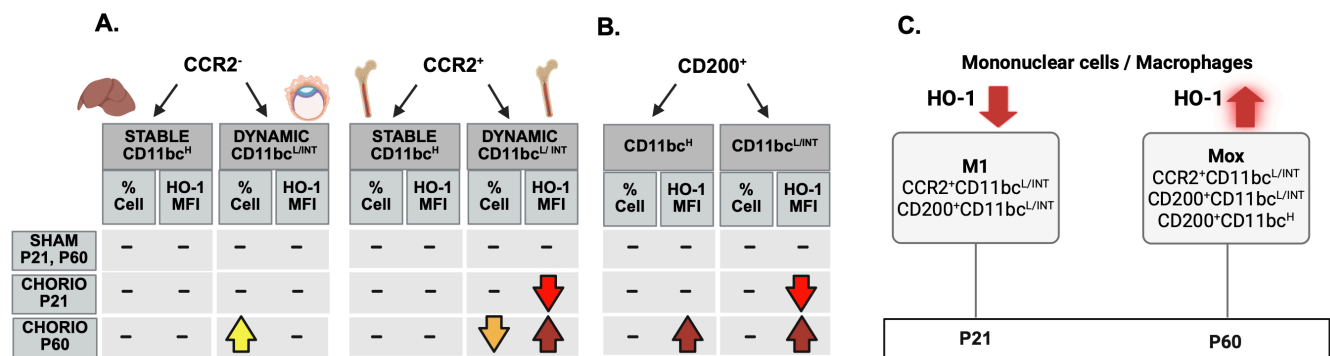


Figure 7

Proposed model summarizing long-term alterations in brain mononuclear cell phenotypes following perinatal CHORIO. HO-1 perturbations are associated with disrupted homeostasis in adult brain mononuclear cells after CHORIO: **a.** CHORIO may disrupt Inflammatory mononuclear cell/macrophage population percentages and HO-1 expressions in the embryonic yolk sac- and bone marrow-derived populations, **b.** Immune inhibitory ligand CD200 positive mononuclear cell/macrophage populations show altered intracellular HO-1 expression after CHORIO, suggesting dysregulation of immune inhibitory signaling, **c.** At P21, CHORIO is associated with predominance of inflammatory CCR2⁺ and CD200⁺ mononuclear cells with decreased HO-1 expression (M1), whereas at P60, oxidative mononuclear cells with increased intracellular HO-1 expression (Mox) predominate. Subpopulations that remain unchanged after CHORIO are labeled as “Stable”, while the subsets that are increased or decreased following CHORIO are labeled as “Dynamic” in the figure. Within CD11bc^{L/INT} population CCR2⁻ cell subset is depicted with yellow, CCR2⁺ cell subset with orange arrows while intracellular HO-1 expression is depicted with the red arrows. Black rectangles denote that cell percentages and HO-1 expression were unchanged.

Supplementary Files

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- [Table1.FinalSciRepMONONUCLEARCELLSUBSETSAug202026.docx](#)
- [Table2.FinalSciRepRATIOSTABLEAug202026.docx](#)
- [SupplementaryFigure1abcdSciRepAug202026.tiff](#)